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The use of immobilized *Saccharomyces cerevisiae* on radiation crosslinked acrylamide–maleic acid hydrogel carriers for production of ethyl alcohol

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Abstract

Radiation crosslinked acrylamide/maleic acid (AAm/MA) copolymers were prepared by γ-irradiation. They were used in experiments on swelling, diffusion, and immobilization of yeast cells (*Saccharomyces cerevisiae*) for the production of ethyl alcohol. AAm/MA hydrogels containing different amount of MA, irradiated at different doses, were used for swelling and diffusion studies. The parameters of swelling, diffusional exponents, network constants, diffusion coefficients and percent porosity of the hydrogel/penetrant systems were calculated and evaluated. Yeast cells were immobilized on to the hydrogels by adsorption during multiplication, and ethyl alcohol production by the hydrogels was investigated. Swelling of AAm/MA increased with increase in MA content. Ethyl alcohol production also increased with increasing MA in the hydrogels but decreased with an increase of irradiation dose. © 2002 Published by Elsevier Science Ltd.

Keywords: Acrylamide/maleic acid; Hydrogel; Radiation; Immobilization; *Saccharomyces cerevisiae*; Ethyl alcohol

1. Introduction

In recent years, studies on catalysis by enzymes in whole cells have gained wide attention. The use of immobilized yeast cell systems for the production of ethyl alcohol is an attractive and rapidly expanding research area because of technical and economical advantages compared with the free cell system. For industrial application, further research is needed to obtain new materials. In the conversion of sugars to ethanol, immobilized microbial cell systems offer advantages over cell suspension systems in terms of ethanol productivity and the stability of cell activity. Many support materials for cell immobilization have been reported including calcium alginate, κ-carrageenan gel, polyacrylamide and γ-alumina [1–4].

Yeast cells have been immobilized on hydrogel carriers obtained by chemical initiation [5] or by γ-irradiation [6]. Hydrogels are polymeric networks that absorb and retain water without dissolving. This property makes them interesting materials as carriers for immobilization of bioactive compounds as alternatives to others successfully used [7].

In a previous study, yeast cells were immobilized on to radiation crosslinked 2-hydroxyethylmethacrylate/ acrylamide hydrogels and used for the production of ethyl alcohol [8]. Currently, the immobilization of yeast cells on to AAm/MA hydrogels as a gel matrix and the production of ethyl alcohol were investigated.

2. Materials and methods

Two hydrophilic monomers used in this study, namely, acrylamide (AAm), and maleic acid (MA) were obtained from BDH (Poole, UK) and used as received.

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Yeast extract, peptone, malt extract, glucose, NH_4Cl, NaCl, $CaCl_2$, lactic acid, alcohol dehydrogenase, β-NAD and *Saccharomyces cerevisiae* (Baker's yeast Type I) were obtained from Sigma (St. Louis. MO, USA). Other chemicals were obtained from Merck (Darmstad, Germany). Molasses was a gift from a sugar factory (Kayseri, Turkey).

All solutions and the AAm/MA copolymers were sterilized in a temperature-controlled UV sterilizer at 25 °C for 12 h before using.

2.1. Preparation of radiation crosslinked AAm/MA copolymers

Various amounts of MA (0, 20, 30, 40, 50, 60 mg) were mixed with 1 g of AAm, dissolved in 1 ml distilled water, placed in PVC straws of 3 mm diameter and irradiated to 2.60, 3.73, 4.65, 5.20, and 5.71 kGy in air at ambient temperature in a γ irradiator with the dose rate of 0.72 kGy h^{-1}. The radiation crosslinked AAm/MA copolymers obtained in long cylindrical shapes were cut into pieces of 4–5 mm length, and dried in air at room temperature and then under vacuum in a vacuum oven at 1 mmHg for one week. The preparation and characterization of AAm/MA hydrogels were reported in a previous study [9].

2.2. Swelling and diffusion assays

Radiation crosslinked AAm/MA copolymers were swelled in the nutrient medium of yeast cells at 30 °C to reveal the parameters of swelling and diffusion. Swollen gels removed from the water bath at regular time intervals were dried superficially with filter paper, weighed, and placed in the same bath. The radii of cylindrical swollen gels were measured with a micrometer.

2.3. Preparation of cells

Yeast cells were precultured for 48 h at 28 °C in an aqueous solution containing 1% glucose, 0.1% molasses, 0.5% peptone, 0.3% yeast extract and 0.3% malt extract (pH 4.8).

2.4. Immobilization of yeast cells onto the hydrogels

Dry AAm/MA copolymers weighing 0.1 g were sterilized and then immersed in a mixture of precultured yeast cells (5 mg wet wt.) and nutrient medium (10 ml). The composition of the nutrient medium was of 12% glucose, 1% molasses, 0.15% yeast extract, 0.25% NH_4Cl, 0.1% NaCl, 0.001% $CaCl_2$ and 0.3% lactic acid (pH 4.8). The suspension was incubated in a rotary shaker for 72 h at 30 °C, and the nutrient medium was renewed every 24 h.

2.5. Fermentation and ethyl alcohol analysis

Immobilized yeast cells were immersed in fresh nutrient medium and fermented by incubation at 30 °C under gentle rotary shaking. The concentration of ethyl alcohol produced was determined using alcohol dehydrogenase [10].

3. Results and discussion

3.1. Preparation of gel matrix

When monomers of AAm with or without comonomer are irradiated in water with ionizing rays such as γ-rays, free radicals from water, monomers and comonomer if any are generated. Random reactions of these radicals cause polymerization and formation of a network. When irradiation dose is increased beyond a certain value, the polymer chains crosslink and a gel is obtained [11,12].

It has been reported that acrylamide requires 2.0 kGy of γ-radiation to obtain crosslinked polyacrylamide at ambient temperature [13]. γ-Radiation of 5.2 kGy was chosen for the preparation of the AAm/MA hydrogels. During polymerization and crosslinking reactions, all monomers reacted together by applied γ-radiation. The radiation technique was used for the sterilization of hydrogel systems at the same time. There was no monomer (such as toxic acrylamide) remaining at the end the polymerization and crosslinking reactions, since 2.0 kGy was a sufficient dose for 100% gelation of the acrylamide [13]. In the dry state, hydrogels were hard and glassy, but in the swollen state, gels were soft and easy to handle. On swelling the hydrogels retained their integrity.

3.2. Swelling and diffusion

The percentage of swelling ($S\%$) of the AAm/MA hydrogels in the nutrient medium of the cells was calculated from the following relationship:

$$S\% = \frac{m_t - m_0}{m_0} \times 100 \tag{1}$$

Here m_t is the mass of swollen gel at time t and m_0 is the mass of the dry gel.

The swelling curves of AAm/MA hydrogels in the nutrient medium of the cells are shown in Fig. 1a and b. The values of $S\%$ increased with time but reached a constant value after a certain point (Fig. 1a and b). This value of swelling may be called equilibrium swelling degree ($S_{eq}\%$). The values of $S_{eq}\%$ of AAm/MA hydrogels irradiated to 5.2 kGy are presented in Tables 1 and 2 that show 40 mg MA containing AAm hydrogels irradiated to different doses.
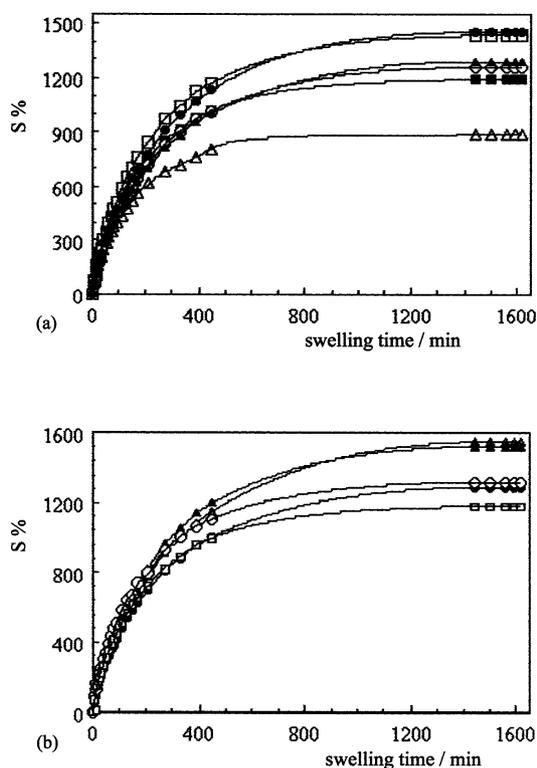
Fig. 1. (a) Change in $S\%$ of AAm/MA hydrogels irradiated at 5.2 kGy with time ($\triangle$, 0 mg MA; $\blacksquare$, 20 mg MA; $\bigcirc$, 30 mg MA; $\blacktriangle$, 40 mg MA; $\square$, 50 mg MA; $\bullet$, 60 mg MA). (b) Change in $S\%$ of 40 mg MA containing AAm/MA hydrogels (irradiation doses: $\square$, 5.71 kGy; $\bullet$, 5.2 kGy; $\bigcirc$, 4.65 kGy; $\blacktriangle$, 3.73 kGy; $\triangle$, 2.6 kGy).

For non-ionic hydrogels such as AAm, swelling is controlled by the hydrophilicity of the monomers or polymers. The hydrophilicity of AAm/MA is higher than AAm due to ionization of carboxylic group from MA. As shown in Fig. 1a and Table 1, the values of the equilibrium swelling degrees of the radiation crosslinked AAm were lower than those of the radiation crosslinked AAm/MA hydrogels. The equilibrium swelling of AAm hydrogels increased with the addition of MA monomer. At the same time, the equilibrium swelling of the hydrogels decreased with an increase of adsorbed irradiation dose (Fig. 1b and Table 2).

Analysis of the mechanisms of water diffusion in swellable polymeric systems has received considerable attention in recent years because of the important applications of swellable polymers in the biomedical, pharmaceutical, environmental, and agricultural fields. The following equation was used to determine the nature of diffusion of water and nutrient medium into the hydrogels [14,15]:

$$F = kt^n \tag{2}$$

In this equation F denotes the amount of penetrant (i.e. nutrient medium) fraction at time t; k is a constant incorporating the characteristics of the polymeric network system and the penetrant; and n is the diffusional exponent, which is indicative of the transport mechanism. This equation was applied to the initial stage of swellings. Fickian diffusion and Case II transport are defined by n equal to 1/2 and 1, respectively. Anomalous transport behaviour (non-Fickian diffusion) is defined by values of n between 0.5 and 1 [14]. This equation was applied to the initial stages of swellings, and plots of ln F versus ln t shown in Fig. 2. The values of the exponents n and k were calculated from the slope and intercept of the lines, respectively, and are presented in Tables 1 and 2.

The values of n were found to be between 0.50 and 1 (Tables 1 and 2), and hence the diffusion of the fluids into AAm/MA hydrogels was taken to be *non-Fickian* in character. This is generally explained as being a consequence of the slow relaxation rate of the hydrogel [14].

The study of diffusion phenomena in hydrogels and fluids is of value in that it clarifies polymer behaviour. For hydrogel characterization, the diffusion coefficient (D) of the cylindrical AAm/MA hydrogels was calculated from the following relationship:

$$F = 4\left[\frac{D}{\pi l^2}\right]^{1/2} t^{1/2} \tag{3}$$

Here D (in $cm^2 \, s^{-1}$) is the apparent diffusion coefficient for the transport of the penetrant into the gel, t (in s) is the time and l is the radius of cylindrical polymer sample. For the hydrogels, the graphs of F versus $t^{1/2}$

Table 1
The variation in the equilibrium swelling degree and diffusion parameters of AAm/MA hydrogels with MA content in the nutrient medium (total dose: 5.2 kGy)

MA content (mg)	Equilibrium swelling, $S_{eq}\%$	Diffusional exponent, n	Network parameter, $k \times 10^2$	Diffusion coefficient, $D \times 10^7$ ($cm^2 \, s^{-1}$)	Porosity, $P\%$
0.00	885	0.60	2.84	6.97	92.02
20.0	1190	0.66	1.95	4.72	93.94
30.0	1255	0.64	1.97	5.69	94.25
40.0	1290	0.66	1.70	6.10	94.40
50.0	1435	0.65	1.90	6.62	94.92
60.0	1455	0.65	1.75	5.63	95.00

Table 2
The variation in the equilibrium swelling degree and diffusion parameters of AAm/MA hydrogels with irradiation dose in the nutrient medium (MA content: 40 mg)

Dose (kGy)	Equilibrium swelling, $S_{eq}\%$	Diffussional exponent, n	Network parameter, $k \times 10^2$	Diffusion coefficient, $D \times 10^7$ ($cm^2 \, s^{-1}$)	Porosity, $P\%$
2.60	15.48	0.65	1.59	5.44	95.29
3.73	15.22	0.68	1.53	5.90	95.21
4.65	13.19	0.63	2.26	6.87	94.51
5.20	12.90	0.66	1.70	6.10	94.40
5.71	11.78	0.65	2.07	5.76	93.90

are plotted and are shown in Fig. 3. The values of the diffusion coefficients of AAm/MA hydrogels are listed in Tables 1 and 2. The values of the diffusion coefficient varied from 4.72×10^{-7} to 6.97×10^{-7} $cm^2 \, s^{-1}$. This result is parallel to the swelling result of the hydrogels.

3.3. Porosity

The percentage porosity ($P\%$) of the hydrogel is one of the important parameters of the crosslinked networks. The porosity of the hydrogels can be calculated from the following equation:

$$P\% = \frac{V_d}{1 - V_d} \times 100 \qquad (4)$$

here V_d is volume ratio of fluid imbibed to the gel phase in equilibrium state.

The values of porosity of radiation induced AAm/MA hydrogels in the nutrient medium are shown in Tables 1 and 2. As can be seen from these tables, $P\%$ increased with the MA content of hydrogels and decreased with the extent of adsorbed dose. The change in the $P\%$ of studied hydrogels in this work complies with the swelling experiments.

3.4. Immobilization and ethyl alcohol production

For production of ethyl alcohol, *S. cerevisiae* was immobilized onto radiation induced AAm/MA hydrogels. These copolymers were successively used in the production of ethyl alcohol in the nutrient medium of the cells.

Concentrations of ethyl alcohol versus incubation time were plotted and are shown in Fig. 4a and b. The production of ethyl alcohol increased with time but reached a constant value after 40–80 min. The amount of ethyl alcohol produced increased with an increase in the MA content in the hydrogels but decreased with irradiation dose.

For production of ethyl alcohol by immobilized cells, the following second-order kinetics relationship can be written:

$$\frac{t}{C} = Q + Wt \qquad (5)$$

where C is the concentration of ethyl alcohol at time t, $W = 1/C_{max}$ is the inverse of the maximum or equilibrium concentration of the producing ethyl alcohol, $Q = 1/(dC/dt)_0$, is the reciprocal of the initial ethyl alcohol production rate (r_P) of the gel [8].

Fig. 5 shows the linear regression of the production of ethyl alcohol obtained with Eq. (5). The values of initial production rate and maximum concentration of ethyl alcohol were calculated from the slope and the intersections of the lines and are presented in Tables 3 and 4.

Tables 3 and 4 show that the parameters of the maximum concentration of the ethyl alcohol produced and the initial ethyl alcohol production rate of the radiation induced hydrogels increased with an increase in MA content in the AAm/MA hydrogels and decreased with irradiation doses. These results are parallel to the results of the equilibrium swelling and $P\%$ of the hydrogels.

3.5. Correlation between production of ethyl alcohol by immobilized yeast cells and properties of AAm/MA hydrogels

Ethyl alcohol production by fermentation is possible using free or immobilized cells. The use of immobilized
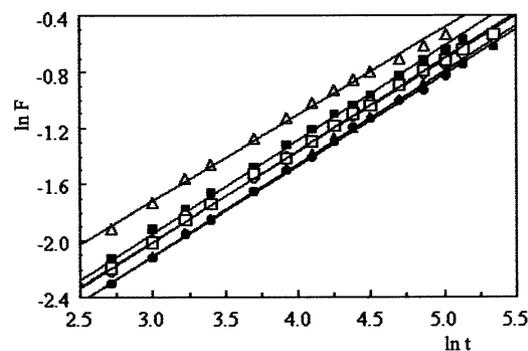
Fig. 2. ln F vs. ln t of AAm/MA hydrogel irradiated to 5.2 kGy ($\triangle$, 0 mg MA; $\blacksquare$, 20 mg MA; $\bigcirc$, 30 mg MA; $\blacktriangle$, 40 mg MA; $\square$, 50 mg MA; $\bullet$, 60 mg MA).
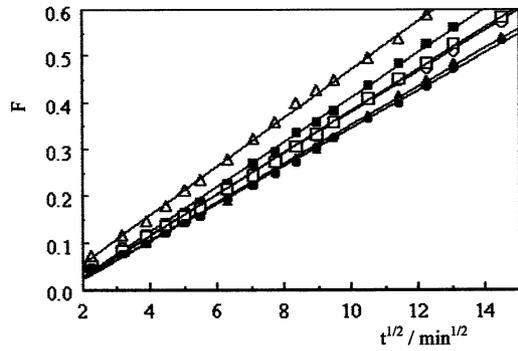
Fig. 3. F vs. $t^{1/2}$ of AAm/MA hydrogel irradiated to 5.2 kGy ($\triangle$, 0 mg MA; $\blacksquare$, 20 mg MA; $\bigcirc$, 30 mg MA; $\blacktriangle$, 40 mg MA; $\square$, 50 mg MA; $\bullet$, 60 mg MA).

whole cells in industrial processes has attracted considerable attention during the past few years due to advantages over traditional processes [16,17]. Immobilization is the restriction of cell mobility within a defined space. Immobilization provides high cell concentrations and cell reuse.
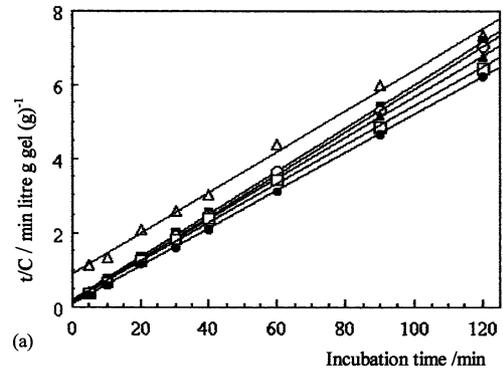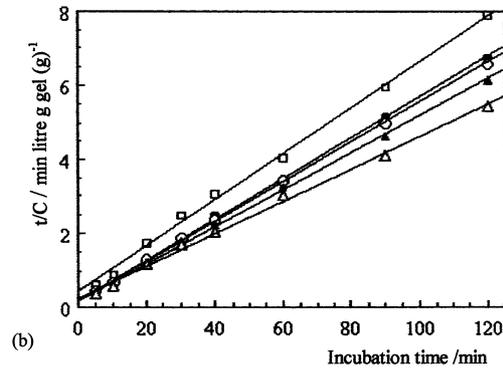
The values of maximum production of ethyl alcohol
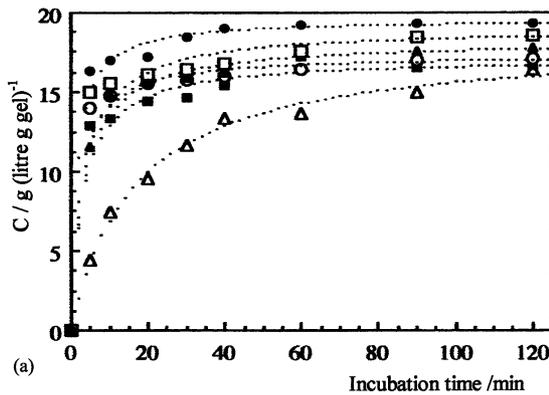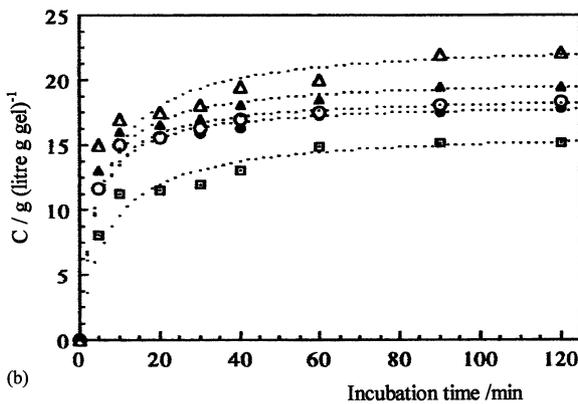
Fig. 4. (a) Change in the concentration of produced ethyl alcohol with MA content ($\triangle$, 0 mg MA; $\blacksquare$, 20 mg MA; $\bigcirc$, 30 mg MA; $\blacktriangle$, 40 mg MA; $\square$, 50 mg MA; $\bullet$, 60 mg MA). (b) Change in the concentration of produced ethyl alcohol with irradiation dose (irradiation doses: $\square$, 5.71 kGy; $\bullet$, 5.2 kGy; $\bigcirc$, 4.65 kGy; $\blacktriangle$, 3.73 kGy; $\triangle$, 2.6 kGy).

Fig. 5. (a) Ethyl alcohol production kinetics of immobilized *S. cerevisiae* in the AAm/MA hydrogels ($\triangle$, 0 mg MA; $\blacksquare$, 20 mg MA; $\bigcirc$, 30 mg MA; $\blacktriangle$, 40 mg MA; $\square$, 50 mg MA; $\bullet$, 60 mg MA). (b) Ethyl alcohol production kinetics of immobilized *S. cerevisiae* in the AAm/MA hydrogels (irradiation doses: $\square$, 5.71 kGy; $\bullet$, 5.2 kGy; $\bigcirc$, 4.65 kGy; $\blacktriangle$, 3.73 kGy; $\triangle$, 2.6 kGy).

by immobilized yeast cells onto AAm/MA hydrogels against the content of MA in the hydrogels and absorbed dose are illustrated and presented in Fig. 6a and b, respectively. When the content of MA in the hydrogel increased the production of ethyl alcohol also increased. On the other hand, an increase in irradiation dose decreased the production of ethyl alcohol (Fig. 6b). These results show that ethyl alcohol production was strongly dependent on the equilibrium swelling of the hydrogels (i.e. MA content in the hydrogel and irradiation dose).

Table 3
The variation of the values of ethyl alcohol production parameters of AAm/MA hydrogels with MA content (total dose: 5.2 kGy)

MA content (mg)	Maximum concentration, C_{max} (g (l g gel)$^{-1}$)	Initial production rate, r_P (g(l g gel h)$^{-1}$)
0.00	17.00	66.37
20.0	17.15	311.59
30.0	17.35	312.00
40.0	18.21	313.15
50.0	18.92	341.22
60.0	19.60	730.70

Table 4
The variation of the values of ethyl alcohol production parameters of AAm/MA hydrogels with irradiation doses (MA content: 40 mg)

Irradiation dose (kGy)	Maximum concentration, C_{max} (g(l g gel)$^{-1}$)	Initial production rate, r_P (g(l g gel h)$^{-1}$)
2.60	22.10	300.00
3.73	20.10	309.06
4.65	18.76	304.29
5.20	18.21	313.15
5.71	16.07	142.02

Tables 3 and 4 show that the values of initial production rate were similar to the results of the swelling behaviour of AAm/MA hydrogels with changes in the amount of MA content and irradiation dose. Thus, the production process with AAm/MA hydrogels is faster than AAm hydrogel.

The yeast cells were adsorbed and became immobilized. At the early stage of immobilization the AAm/MA hydrogels swelled with the nutrient medium of cells as much as possible. This nutrient medium inside of the hydrogels must be replaced with yeast cells during the immobilization process. The hydrophilic groups of the hydrogels were increased with the increasing amount of MA content. Thus, the swelling of the hydrogels was increased. The higher swelling of the hydrogels permitted the presence of more nutrient medium and cells inside of the hydrogel. Some yeast cells adsorbed onto the surface of the hydrogel and the adsorbed yeast cells infiltrated into the hydrogel through the small pores. Than the yeast cells inside the hydrogel multiplied. The increase in the volume caused by the multiplying yeast cells resulted in the extension of the pores in the hydrogels. In this way the production of ethyl alcohol were increased. The elasticity of hydrogel network gives great advantages for the extent of immobilized yeast cell and for the production of ethyl alcohol.

In general, it can be said that hydrogels could also be used for the immobilization of other cells due to their soft and elastic nature for the production of some bioactive species.
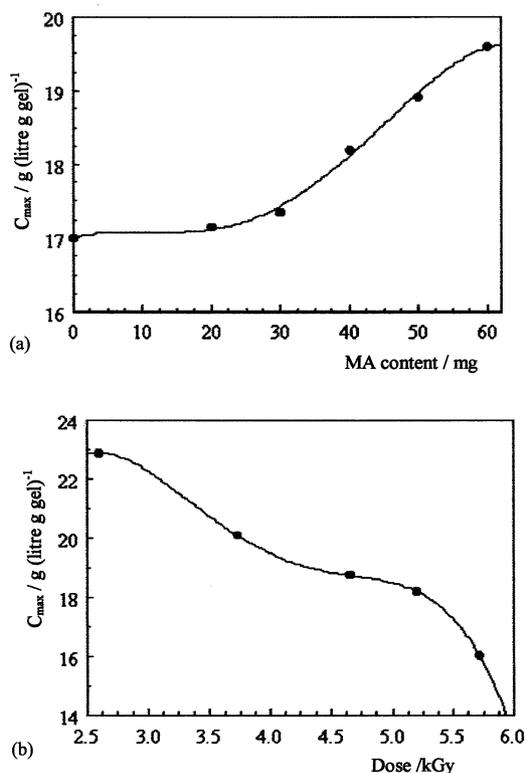
Fig. 6. (a) Change in the capacity of produced ethyl alcohol with MA content of AAm/MA hydrogels. (b) Change in the capacity of produced ethyl alcohol with irradiation dose.

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