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Immobilization of *Saccharomyces cerevisiae* on to radiation crosslinked HEMA/AAm hydrogels for production of ethyl alcohol

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Abstract

Radiation crosslinked 2-hydroxyethyl methacrylate/acrylamide (HEMA/AAm) copolymers prepared by γ-irradiation were used in experiments on swelling, diffusion, immobilization of yeast cells (*Saccharomyces cerevisiae*) and production of ethyl alcohol. HEMA/AAm hydrogels irradiated at 3 kGy were used for swelling and diffusion studies in water and the nutrient medium of the cells. The parameters of equilibrium swelling, maximum swelling, initial swelling rate, diffusional exponent, network constant and diffusion coefficient of the hydrogel/penetrant systems were calculated and evaluated. All parameters for the hydrogel/water system were higher than that of the hydrogel/nutrient medium systems. Yeast cells (*Saccharomyces cerevisiae)* were immobilized on to the hydrogels by adsorption during multiplication and ethyl alcohol production of the hydrogels was investigated. Swelling of HEMA increased with the addition of AAm and ethyl alcohol production increased with increasing AAm in the hydrogels.

Keywords: 2-Hydroxyethyl methacrylate/acrylamide; Hydrogel; Radiation; Immobilization; *Saccharomyces cerevisiae*; Ethyl alcohol

1. Introduction

Biocatalysts, enzymes and cells, are often immobilized in matrices in order to protect them from environmental stresses such as pH, temperature, salts, solvents, self-destruction, inhibitors, and poisons. Immobilization also permits easier handling and recovery of the biocatalyst [1]. A wide variety of materials, are currently used as supports for cell immobilization. Many gel-like materials are used as carriers and these may be based on unmodified and modified natural (alginate, carrageenan, agar, gelatine, chitin, chitosan, etc.) [2–4] or synthetic (polyacrylamide, polyacrylate, polyurethane, epoxy resin etc.) precursors [5–8]. Advantages of whole cell systems include their ability to catalyze the synthesis of various useful and complicated chemicals using multi-enzyme steps, and regeneration activity to prolong their catalytic life [9].

The use of immobilized yeast cell systems for alcoholic fermentation is an attractive and rapidly expanding research area because of the additional technical and economical advantages compared with the free cell system [10]. Yeast cells have been reported to be immobilized on hydrogel carriers obtained by chemical initiation [7,11] or by γ-irradiation [5].

Hydrogels are polymeric networks, which 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. Radiation crosslinked polymerization enables hydrogels in a wide variety of forms to be easily prepared for application both in biomedicine and biotechnology [12].

In our previous articles, radiation induced acrylamide-based hydrogels [13] have been studied in adsorption of proteins, pharmaceuticals, cationic dyes and heavy metal ions [14–23], swelling behavior in solutions of amino acids [24,25], biocompatibility of human sera and rat tissue [26–28]. Currently, the immobilization of

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yeast cells onto the radiation crosslinked HEMA/AAm copolymers as a gel matrix and the production of ethyl alcohol were investigated.

2. Materials and methods

2.1. Materials

Two hydrophilic monomers used in this study, namely, 2-hydroxyethyl methacrylate (HEMA) and acrylamide (AAm) were obtained from BDH (Poole, UK).

Yeast extract, pepton, malt extract, glucose, NH_4Cl, NaCl, $CaCl_2$, lactic acid, alcohol dehydrogenase, β-NAD and *Saccharomyces cerevisiae* were obtained from Sigma (St. Louis. MO, USA). Other chemicals were obtained from Merck (Darmstatd, Germany). Molasses was a gift from a Sugar Factory (Kayseri, Turkey).

All solutions and the radiation crosslinked HEMA/AAm copolymers were sterilized before use.

2.2. Preparation of radiation crosslinked HEMA/AAm copolymers

Five grams of AAm and various amounts of HEMA (0, 1, 2, 3, and 5 ml) or 5 ml HEMA were mixed in distilled water (5 ml for homopolymers, and 6, 7, 8, and 10 ml for copolymers), and placed in PVC straws of 3-mm diameter and irradiated. A dose of 3 kGy at air temperature in a γ irradiator was applied at a fixed rate of 6 kGy h^{-1}. The radiation crosslinked HEMA/AAm copolymers obtained in long cylindrical shapes were cut into pieces of 4–5 mm long, dried in air and under vacuum.

2.3. Swelling and diffusion assays

Radiation crosslinked HEMA/AAm copolymers were swelled in distilled water and nutrient medium of yeast cells at 30 °C to determine the parameters of swelling and diffusion. Swollen gels removed from the water bath at regular 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.

2.4. Preparation of cells

Saccharomyces cerevisiae was used in this study. The 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).

2.5. Immobilization of yeast cells on to the hydrogels

Dry radiation crosslinked HEMA/AAm copolymers (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 latter being 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, the nutrient medium being renewed every 24 h.

2.6. 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 [29].

3. Results and discussion

3.1. Preparation of gel matrix

Preparation of 2-hydroxyethyl methacrylate, 2-hydroxyethyl methacrylate/acrylamide and acrylamide hydrogels employed ionizing radiation [30–33].

Ionizing radiation such as cobalt-60 gamma radiation is very useful in producing polymers from monomeric units and in modifying the properties of pre-existing polymers. Ionizing radiation provides a clean method for the production and modification of polymers. No chemicals or catalysts have to be added to the reaction matrix. The polymerization is achieved by free radicals (occasionally ions) created in the material and therefore no chemicals or catalysts remain in the material after radiation [31].

When monomers of HEMA and AAm in aqueous solutions have been irradiated with ionization rays such as γ-rays, free radicals are generated in the aqueous solutions. Random reactions of these radicals with monomers lead to the formation of copolymers of HEMA/AAm. When the irradiation dose was increased beyond a certain value, the polymer chains crosslink and then a gel is obtained. A schematic presentation of possible copolymerization and crosslinking reaction mechanisms between HEMA and AAm monomers is shown in Fig. 1.

It is reported that complete gelation of acrylamide has been achieved using 2.00 kGy of γ rays irradiation at ambient temperature [33]. γ-ray irradiation doses (3.00 kGy) were therefore used for preparation of the hydrogels.

The radiation technique is a sterilization technique used in many applications. During polymerization and

crosslinking reactions, all monomers react together with applied γ rays irradiation. This process is used for sterilization of hydrogel systems at the same time. It can be said that there is no monomer (such as toxic monomers) at the end of the copolymerization and crosslinking reaction between HEMA and AAm, because 2.00 kGy is a sufficient dose for gelation [33].

3.2. Swelling and diffusion

The swelling of the HEMA, HEMA/AAm and AAm hydrogels in distilled water and the nutrient medium of the cells were calculated from the following relationship:

$$S = \frac{m_t - m_o}{m_o} \quad (1)$$

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

The swelling curves of the hydrogel in distilled water and the nutrient medium of the cells are shown in Fig. 2a,b for the HEMA/AAm hydrogels in water and the nutrient medium, respectively.

Swelling increased with time but reached a constant value after a certain point (Fig. 2a,b). This value of swelling may be called equilibrium swelling (S_{eq}). The values of S_{eq} of the hydrogels are presented in Table 1.

For non-ionic hydrogels such as HEMA and AAm, swelling is controlled by the hydrophilicity of the monomers or polymers. The hydrophilicity of AAm is higher than that of HEMA. As shown in Fig. 2a,b, and Table 1, the values of the equilibrium swelling of the radiation crosslinked HEMA homopolymer ($S_{eq} = 0.34$ and 0.54 g (g gel)$^{-1}$) were approximately 20-fold lower than that of the radiation crosslinked AAm homopolymer ($S_{eq} = 9.25$ and 10.0 g (g gel) $^{-1}$). On the other hand, the equilibrium swelling of HEMA hydrogel increased with the addition of AAm monomer. At the same time, the equilibrium swelling of the hydrogels increased with an increase of AAm content in the HEMA/AAm hydrogels (Table 1).

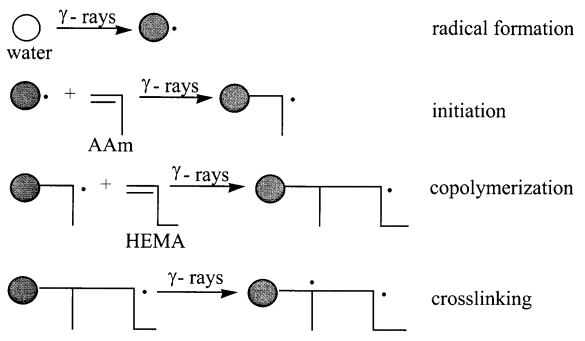

Fig. 1. The possible copolymerization mechanism of HEMA with AAm.

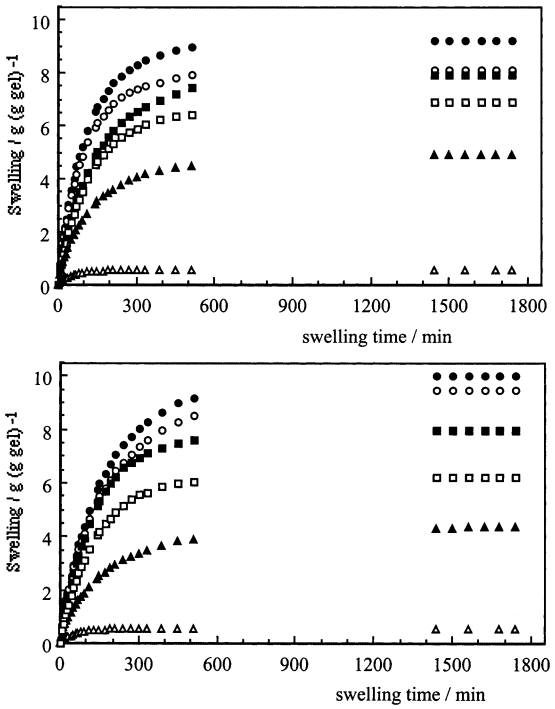

Fig. 2. (a) Swelling curves of HEMA/AAm hydrogels in water: ●, 100% AAm; ○, 89.58% AAm; ■, 81.09% AAm, □, 74.17% AAm; ▲, 63.18% AAm, △, 0% AAm. (b) Swelling curves of HEMA/AAm hydrogels in the nutrient medium: ●, 100% AAm; ○, 89.58% AAm; ■, 81.09% AAm, □, 74.17% AAm; ▲, 63.18% AAm, △, 0% AAm.

Table 1 shows that the values of equilibrium swelling of the radiation crosslinked HEMA/AAm hydrogels were between 0.54 and 9.25 g (g gel)$^{-1}$ in distilled water, while the values of the hydrogels were between 0.34 and 10.0 g (g gel)$^{-1}$ in the nutrient medium of the cells. Environmental conditions such as pH, ionic strength, and composition of the surrounding fluids affected the swelling nature of the hydrogels. Thus, the surrounding fluids formed the difference in the swelling degrees of the hydrogels.

For extensive swelling of polymers, the following relationship can be written [34];

$$\frac{t}{S} = A + Bt \quad (2)$$

Where S is degree of swelling at time t, $B = 1/S_{max}$ is the inverse of the maximum or equilibrium swelling, $A = 1/(dS/dt)_o$, is the reciprocal of the initial swelling rate (r_S) of the gel. The relationship represents second order kinetics.

Fig. 3. shows the linear regression of the swelling curves obtained by means of Eq. (2). The initial swelling rate and the values of maximum swelling of the hydrogels were calculated from the slope an intersection of the lines and, are presented in Table 1. The values of maximum swelling and initial swelling rate of the hydrogels suggest similar swelling behavior (Table 1).

Table 1
The values of swelling parameters of the hydrogels

Medium	Water			Nutrient medium		
AAm mol%	S_{eq} g (g gel)$^{-1}$	S_{max} g (g gel)$^{-1}$	r_s g (g gel h)$^{-1}$	S_{eq} g (g gel)$^{-1}$	S_{max} g (g gel)$^{-1}$	r_s g (g gel h)$^{-1}$
0.00	0.54	0.55	1.46	0.34	0.35	1.09
63.18	4.92	5.22	3.33	4.36	4.67	2.38
74.17	6.93	7.33	4.95	6.25	6.60	4.74
81.09	7.95	8.45	5.20	7.97	8.44	5.81
89.58	8.14	8.47	8.54	9.49	10.19	5.27
100.0	9.25	9.67	8.54	10.00	10.74	5.66

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 [35,36]:

$$F = kt^n \quad (3)$$

In this equation F denotes the amount of penetrant fraction at time t; k is a constant incorporating the characteristics of the polymeric network system and the penetrant; n is the diffusional exponent, which is the indicative of the transport mechanism. This equation was applied to the initial stage of swelling. Fickian diffusion and Case II transport are defined by n equal to 1/2 and n equal to 1, respectively. Anomalous transport behavior (non-Fickian diffusion) is defined by values of n between 1/2 and 1 [36]. This equation was applied to the initial stages of swelling, and plots of ln F versus ln t are shown in Fig. 4. The values of n and k were calculated from the slope and intercept of the lines, respectively, and are presented in Table 2.

n was generally found to be between 0.50 and 1 (Table 2), and hence the diffusion of the fluids in to HEMA/AAm hydrogels was taken to be of *non-Fickian* character. This is generally explained as being a consequence of the slow relaxation rate of the hydrogel [36].

The study of diffusion phenomena in hydrogels and fluids is of value in that it clarifies polymer behavior. For hydrogel characterization, the diffusion coefficient D can be calculated by various methods. The short time approximation method was used for calculation of diffusion coefficients of HEMA/AAm hydrogels [35]. The short time approximation is valid for the first 60% of the swelling. The diffusion coefficients D of the cylindrical HEMA/AAm hydrogels were calculated from the following relationships:

$$F = 4\left[\frac{Dt}{\pi l^2}\right]^{1/2} - \pi\left[\frac{Dt}{\pi l^2}\right] - \frac{\pi}{3}\left[\frac{Dt}{\pi l^2}\right]^{3/2} + \ldots \quad (4)$$

where D is in cm^2 s^{-1}, t in s and l is the radius of cylindrical polymer sample. A graphical comparison of Eqs. (3) and (4) shows the semi-empirical Eq. (3) with $n = 0.5$ and $k = 4(D/\pi r^2)^{1/2}$. For the hydrogels, F versus $t^{1/2}$ graphs were plotted and, are shown in Fig. 5. The values of the diffusion coefficients of HEMA/AAm hydrogels are listed in Table 2. The values of the diffusion coefficient varied from 5.6×10^{-7} to 17.8×10^{-7} cm^2 s^{-1}. This result is parallel to the swelling result of the hydrogels.

3.3. Immobilization and ethyl alcohol production

For production of ethyl alcohol, *Saccharomyces cerevisiae* was immobilized on to radiation induced HEMA/AAm hydrogels. The *Saccharomyces cerevisiae* immobilized radiation crosslinked HEMA/AAm copolymers produced ethyl alcohol in the nutrient medium of the cells, but HEMA homopolymer did not. So, HEMA/AAm hydrogels were selected for production of the ethyl alcohol.

The concentrations of ethyl alcohol versus incubation time graphs were plotted and are shown in Fig. 6. Production of ethyl alcohol increased with time but reached a constant value after 40–80 min. On the other hand, the amount of ethyl alcohol produced increased with an increase in the AAm content in the hydrogels.

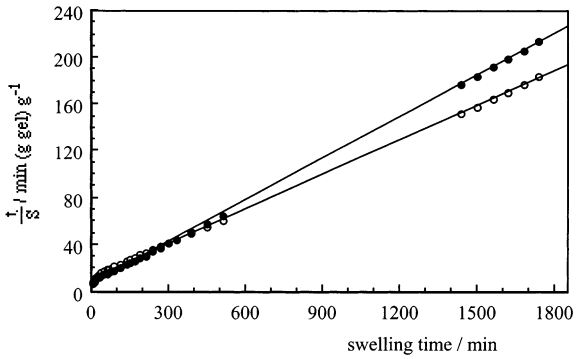

Fig. 3. Swelling kinetics curves of HEMA/AAm hydrogel containing 89.58% AAm: ●, water; ○, nutrient medium.

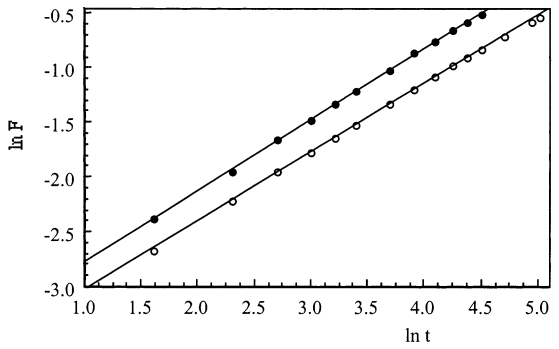

Fig. 4. ln F–ln t curves of HEMA/AAm hydrogel containing 89.58% AAm: ●, water; ○, nutrient medium.

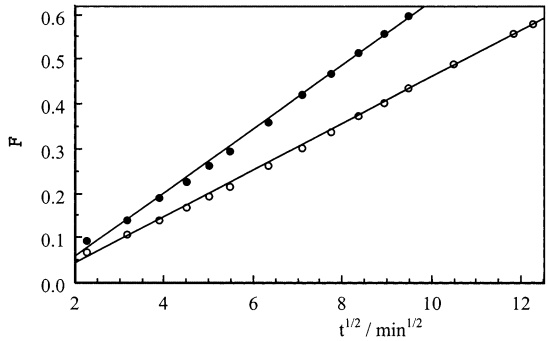

Fig. 5. F–vt curves of HEMA/AAm hydrogels containing 89.58% AAm: ●, water; ○, nutrient medium.

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

$$\frac{t}{C} = Q + Wt \tag{5}$$

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, $Q = 1/(dC/dt)_o$, is the reciprocal of the initial ethyl alcohol production rate (r_P) of the gel.

Fig. 7 shows the linear regression of the production of ethyl alcohol curves obtained by means of Eq. (5). The values of initial production rate and maximum concentration of ethyl alcohol were calculated from the slope an intersection of the lines and, are presented in Table 3.

Table 3 shows that the parameters of the maximum concentration of the producing ethyl alcohol and the initial ethyl alcohol production rate of the radiation induced hydrogels increased with the increase in the AAm content in the HEMA/AAm hydrogels. These results are parallel to the results of the equilibrium swelling of the hydrogels.

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

The maximum production of ethyl alcohol by yeast cells immobilized with various HEMA/AAm hydrogels was plotted as the equilibrium swelling of the hydrogels in the nutrient medium (Fig. 8). When the values of equilibrium swelling of the hydrogels increased, the production of ethyl alcohol also increased. As shown in Table 1, the swelling of the hydrogels in water and the nutrient medium of the cells increased with an increase in AAm content in the hydrogels.

Xin et al. investigated the effect of water content of radiation induced acrylic and methacrylic esters hydrogels on the ethanol productivity with the present method of study. They reported that both requirements of high water content, or swellability, and of a high porosity of the polymer matrices have to be met to increase the extent of yeast cell immobilization [5].

These results shows that, ethyl alcohol production is strongly dependent on the equilibrium swelling of the hydrogel.

Table 2
The values of diffusion parameters of the hydrogels

Medium	Water			Nutrient medium		
AAm/mol%	$k \times 10^2$	n	$D \times 10^6$/cm^2 s^{-1}	$k \times 10^2$	n	$D \times 10^6$/cm^2 s^{-1}
0.00	7.88	0.51	0.68	10.59	0.46	0.56
63.18	2.64	0.65	0.88	2.62	0.62	0.66
74.17	2.28	0.69	1.28	2.53	0.66	0.92
81.09	2.52	0.65	1.08	2.36	0.68	1.22
89.58	3.21	0.65	1.78	2.57	0.63	0.95
100.0	2.82	0.67	1.61	2.27	0.65	1.04

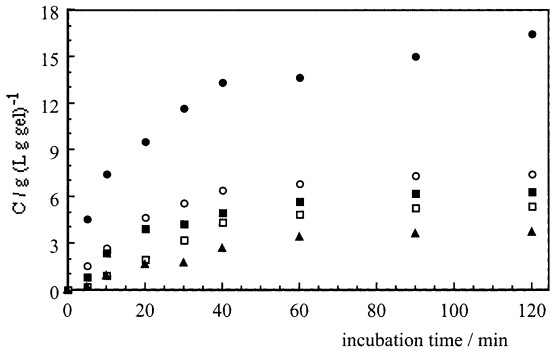

Fig. 6. Ethyl alcohol production curves of immobilized *S. cerevisiae* in the HEMA/AAm hydrogels: ●, 100% AAm; ○, 89.58% AAm; ■, 81.09% AAm, □, 74.17% AAm; ▲, 63.18% AAm.

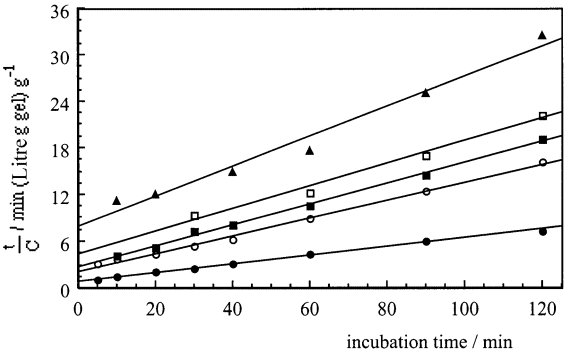

Fig. 7. Ethyl alcohol production kinetics curves of immobilized *S. cerevisiae* in the HEMA/AAm hydrogels: ●, 100% AAm; ○, 89.58% AAm; ■, 81.09% AAm, □, 74.17% AAm; ▲, 63.18% AAm.

4. Conclusions

Ethyl alcohol production by fermentation is possible using free or immobilized cells. The use of immobilized whole cells in industrial processes has attracted considerable attention during the past few years due to advantages over traditional processes [37,38]. Immobilization is the restriction of cell mobility within a defined space. Immobilization provides high cell concentrations and cell reuse.

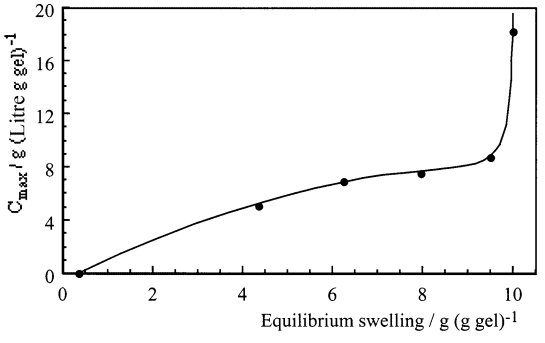

Fig. 8. The relationship between equilibrium swelling and C_{max} of HEMA/AAm hydrogels.

Table 3
The values of ethyl alcohol production parameters of the hydrogels

AAm/mol%	C_{max}/g (l g jel)$^{-1}$	r_P/g (l g jel h)$^{-1}$
0.00	0.000	0.00
63.18	5.146	7.68
74.17	6.932	13.68
81.09	7.455	22.20
89.58	8.755	27.90
100.0	18.201	66.36

The aim of this study was to investigate the best carrier for immobilization of yeast cells and production of ethyl alcohol by these cells. For this purpose, HEMA/AAm hydrogels containing various amounts of AAm with different degrees of swelling were produced by radiation-induced polymerization. Hydrogels were immersed into the nutrient medium containing yeast cells. The yeast cells were adsorbed and became immobilized. At the early stage of immobilization the HEMA/AAm 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 increased with an increase in AAm 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. Then 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 was increased.

Acknowledgements

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