Immobilization of *Saccharomyces cerevisiae* on to acrylamide–sodium acrylate hydrogels for production of ethyl alcohol

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Abstract

Acrylamide/sodium acrylate (AAm/SA) copolymers, prepared by using various crosslinkers, were used in experiments on swelling, diffusion, immobilization of yeast cells (*Saccharomyces cerevisiae*) and production of ethyl alcohol. AAm/SA hydrogels were used for swelling and diffusion studies in 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. Yeast cells were immobilized onto the hydrogels by adsorption method during multiplication and ethyl alcohol production of the hydrogels was investigated. Swelling of AAm increased with the addition of SA and ethyl alcohol production increased with increasing SA in the hydrogels. The best system for immobilization is found to be AAm/SA hydrogels containing *N,N′*-methylenebisacrylamide as crosslinker.
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1. Introduction

In the conversion of sugars to ethanol, immobilized microbial cell systems offer advantages over cell suspension systems in terms of ethanol production 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. The use of immobilized yeast cell system for alcoholic fermentation is an attractive and rapidly expanding research area because of its additional technical and economical advantages compared with the free cell system [1–5].

Yeast cells have been reported to be immobilized on hydrogel carriers obtained by chemical initiation [6] or by γ-irradiation [4]. 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 materials [7].

In our previous study, yeast cells were immobilized on to radiation synthesized HEMA/AAm and AAm/MA hydrogels and, used for the production of ethyl alcohol [8,9]. Currently, the immobilization of yeast cells onto acrylamide/sodium acrylate (AAm/SA) copolymers as a gel matrix and the production of ethyl alcohol were investigated.

2. Materials and methods

Two hydrophilic monomers used in this study, namely, AAm, and SA were obtained from BDH (Poole, UK). Ammonium persulphate as initiator were obtained from Merck (Darmstadt, Germany). Trimethylolpropan triacrylate (T), 1,4-butandiol dimethacrylate (B), ethylene glycol dimethacrylate (E), *N,N′*-methylenebisacrylamide (N) as crosslinkers, *N,N,N′,N′*-tetramethylenediamine (TEMED) as catalyst, 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). Molasses was a gift from a Sugar Factory (Kayseri, Turkey). All chemicals were used as received.

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All solutions and the AAm/SA copolymers were sterilized before using. The concentrations of all used solutions were in percent mass/volume.

2.1. Preparation of AAm/SA copolymers

AAm/SA hydrogels were prepared by free-radical crosslinking copolymerization of AAm and SA with a small amount of different type crosslinkers in aqueous solution. SA was used as the ionizable comonomer. Ammonium persulphate and TEMED were the initiator and the accelerator, respectively [10].

To obtain AAm/SA hydrogels, 1 g of AAm, various amounts of SA (0, 10, 20, 30, and 40 mg), and 0.25 ml ammonium persulphate (1%), 0.15 ml TEMED (1%) and a crosslinker (1% B or E or N or T) were mixed in distilled water and placed in PVC straws of 3 mm diameter. A gel formed after 2 h of reaction at ambient temperature. After 24 h, the hydrogel rods containing different types of crosslinkers and various amounts of SA, were cut into 4–5 mm in length and washed with distilled water and dried in air and vacuum.

2.2. Swelling and diffusion assays

AAm/SA copolymers containing different types of crosslinkers and various amounts of SA were swelled in nutrient medium of yeast cells at 30 °C to discover 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 by a micrometer.

2.3. Preparation of cells

In this study, *S. cerevisiae* was used. 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).

2.4. Immobilization of yeast cells onto the hydrogels

Total 0.1 g weighing dry AAm/SA copolymers were sterilized and then immersed in a mixture of precultured yeast cells (5 mg wet weight) 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 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 [11].

2.6. Operational stability of immobilized cells

At the end of experiments of fermentations, the AAm/SA hydrogels containing 30 mg SA and NNMBA were separated from fermentation nutrient medium. After the hydrogels were washed with nutrient medium, fermentation was repeated every 90 min. The concentration of produced ethyl alcohol was determined in every sample.

3. Results and discussion

3.1. Swelling and diffusion

The swelling degree of the AAm/SA hydrogels in the nutrient medium of the cells was calculated from the following relationship:

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

where 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 the AAm/SA hydrogels in the nutrient medium of the cells are shown in Fig. 1a and b. Swelling increased with time and reached a constant value after a certain point (Fig. 1a and b). This value of swelling degree may be called equilibrium swelling degree (S_{eq}). The values of S_{eq} of the hydrogels are presented in Tables 1 and 2.

As can be seen from Fig. 1a and Table 1, the biggest swelling degree of various crosslinker containing AAm/SA hydrogel was the one containing N crosslinker. In order

Table 1
The variation of the values of swelling degree and diffusion parameters of the AAm/SA hydrogels with the different type of crosslinker (amount of SA 30 mg)

Crosslinkers	S_{eq}	n	$k \times 10^2$	$D \times 10^7$ ($cm^2 s^{-1}$)	P (%)
N	16.72	0.68	1.01	5.49	95.62
E	13.78	0.69	1.89	14.35	94.73
B	12.15	0.63	2.50	11.74	94.07
T	8.44	0.58	3.74	14.40	91.68

Table 2
The variation of the values of swelling degree and diffusion parameters of the in AAm/SA hydrogels with SA content (crosslinker: N)

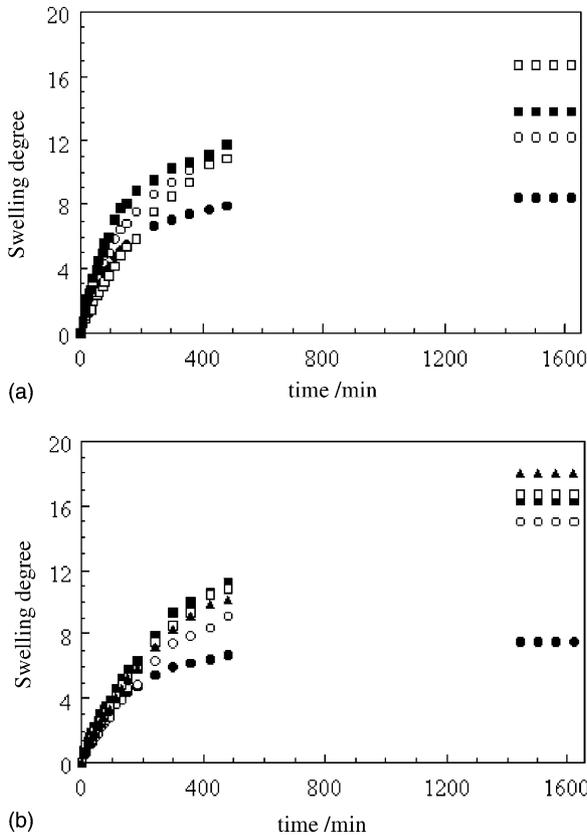| SA content (mg)	S_{eq}	n	$k \times 10^2$	$D \times 10^7$ ($cm^2 s^{-1}$)	P (%)
0	7.59	0.59	3.14	9.42	90.84
10	15.06	0.68	0.92	4.23	95.16
20	16.30	0.67	1.18	5.92	95.51
30	16.72	0.68	1.04	5.49	95.62
40	18.04	0.69	0.88	5.29	95.93

Fig. 1. (a) The change in the swelling degree of 30 mg SA containing AAm/SA hydrogels with various crosslinkers with time, (●) T; (○) B; (■) E; (□) N. (b) The change in the swelling degree of different amount of SA containing and N crosslinked AAm/SA hydrogels with time, (●) 0 mg SA; (○) 10 mg SA; (■) 20 mg SA; (□) 30 mg SA; (▲) 40 mg SA.

to investigate the effect of SA content of AAm/SA hydrogels on the swelling behavior in the nutrient media, the N crosslinked AAm/SA hydrogels has been chosen due to their higher swelling degrees when compared to other crosslinkers.

Fig. 1b, and Table 2 show that the values of S_{eq} increased with an increase of SA content in the AAm/SA hydrogels.

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 [12,13]:

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

where F is the amount of penetrant fraction at time t; k a constant incorporating the characteristics of the polymeric network system and the penetrant; n is the diffusional exponent, which is 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 1, respectively. Anomalous transport behavior
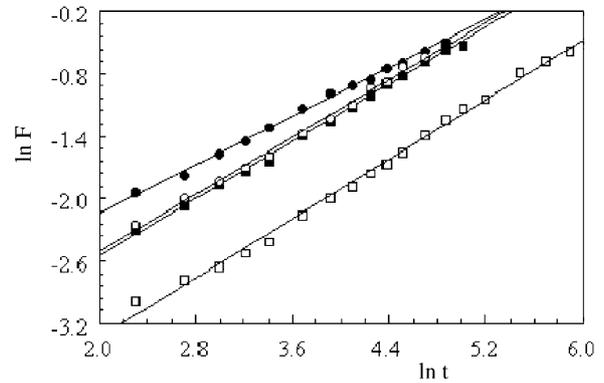

Fig. 2. ln F vs. ln t curves of 30 mg SA containing hydrogels with various crosslinkers, (●) T; (○) B; (■) E; (□) N.

(non-Fickian diffusion) is defined by values of n between 1/2 and 1 [13]. This equation was applied to the initial stages of swelling, and plots of ln F versus ln t shown in Fig. 2. The exponents n and k values were calculated from the slope and intercept of the lines, respectively, and are presented in Tables 1 and 2.

The values of n were generally found to be between 0.50 and 1 (Tables 1 and 2), and hence the diffusion of the fluids into AAm/SA 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 [13].

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 AAm/SA hydrogels [12]. The diffusion coefficients (D) of the cylindrical AAm/SA hydrogels were calculated from the following relationship:

$$F = 4\left(\frac{Dt}{\pi l^2}\right)^{1/2} \tag{3}$$

where D (cm^2 s^{-1}) is the apparent diffusion coefficient for the transport of the penetrant into the gel; t (s) the time; and l is the radius of cylindrical polymer sample. For the hydrogels, F versus $t^{1/2}$ graphs were plotted and, are shown in Fig. 3. The values of the diffusion coefficients of AAm/SA hydrogels are listed in Tables 1 and 2. The values of the diffusion coefficient varied from 5.49×10^{-7} to 14.40×10^{-7} cm^2 s^{-1}. This result is in accordance with the swelling result of the hydrogels.

3.2. Porosity

The porosity of the hydrogel is one of the important parameters of the crosslinked networks. The porosity percent of the hydrogels can be calculated from the following equation:

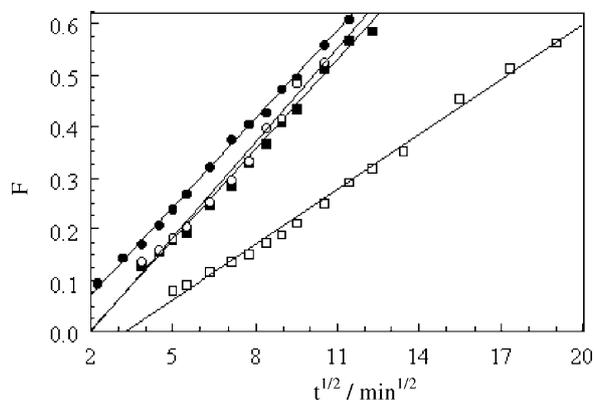$$P(\%) = \frac{V_d}{1 - V_d} \times 100 \tag{4}$$

Fig. 3. F vs. $t^{1/2}$ curves of 30 mg SA containing hydrogels with various crosslinkers, (●) T; (○) B; (■) E; (□) N.

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

The values of porosity percent of crosslinked AAm/SA hydrogels in the nutrient medium are shown in Tables 1 and 2. Tables 1 and 2 show that similar results, and comply with the swelling behaviors of AAm/SA hydrogels with change in the SA content.

3.3. Immobilization and ethyl alcohol production

To produce ethyl alcohol, *S. cerevisiae* was immobilized onto AAm/SA hydrogels.

The concentrations of ethyl alcohol versus incubation time graphs were plotted and, are shown in Fig. 4a and b. Production of ethyl alcohol increased with time but reached a constant value after 40–80 min. As shown in Fig 4a, ethyl alcohol production amount with crosslinkers varying in the
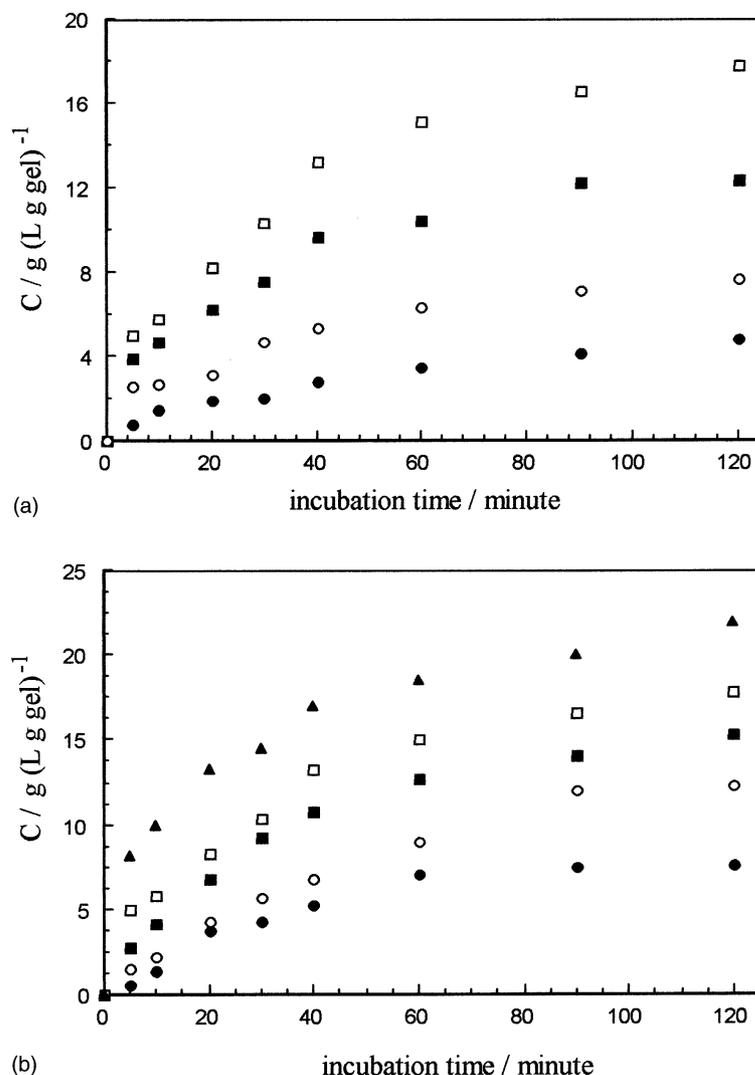

(a)

(b)

Fig. 4. (a) Ethyl alcohol production curves of immobilized *S. cerevisiae* in different SA containing hydrogels with various crosslinkers, (●) T; (○) B; (■) E; (□) N. (b) Ethyl alcohol production curves of immobilized *S. cerevisiae* in the different amount of SA containing and N crosslinked AAm/SA hydrogels, (●) 0 mg SA, (○) 10 mg SA; (■) 20 mg SA; (□) 30 mg SA, (▲) 40 mg SA.

order of N > E > B > T. At Fig. 4b, it is shown that the production of ethyl alcohol increased with the increase in SA content in the AAm/SA hydrogels. The results are also in an agreement with the swelling behaviors of AAm/SA hydrogels (Fig. 1a and b).

A second-order equation based on the production of ethyl alcohol may be expressed in the form:

$$\frac{\mathrm{d}S}{\mathrm{d}t} = k(C_{max} - C)^2 \tag{5}$$

where C is the concentration of produced ethyl alcohol at time t; k the rate constant of second-order production of ethyl alcohol; and C_{max} is the maximum or equilibrium concentration of the produced ethyl alcohol. After definite integration by applying the initial conditions $C = 0$ at $t = 0$ and $C = C$ at $t = t$, Eq. (5) becomes:

$$\frac{t}{C} = Q + Wt \tag{6}$$

where $W = 1/C_{max}$ is the inverse of the maximum or equilibrium concentration of the produced ethyl alcohol, $Q = 1/(\mathrm{d}C/\mathrm{d}t)_0$ is the reciprocal of the initial ethyl alcohol production rate (r_p) or $1/kC_{max}^2$ of the gel.
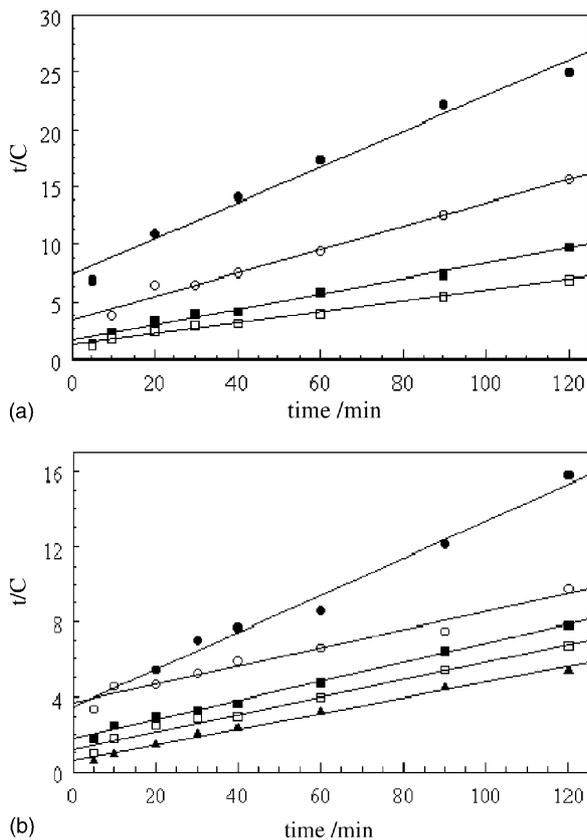

(a)

Fig. 5. (a) Ethyl alcohol production kinetics curves of immobilized *S. cerevisiae* in different amount of SA containing hydrogels with various crosslinkers, (●) T; (○) B; (■) E; (□) N. (b) Ethyl alcohol production kinetics curves of immobilized *S. cerevisiae* in the different amount of SA containing and N crosslinked AAm/SA hydrogels, (●) 0 mg SA; (○) 10 mg SA; (■) 20 mg SA; (□) 30 mg SA; (▲) 40 mg SA.

Table 3
The variation of the values of ethyl alcohol production parameters of the AAm/SA hydrogels with the various crosslinker and SA content

	C_{max} (g (1 g gel)$^{-1}$)	r_p (g (1 g gel h)$^{-1}$)
Crosslinker		
N	21.5	49.2
E	14.5	40.2
B	9.8	17.4
T	6.4	8.4
SA content (mg)		
0	10.1	17.4
10	20.4	16.2
20	20.0	34.2
30	21.5	49.2
40	23.8	33.6

If second-order kinetics are applicable, the plot of t/C against t of Eq. (6) should give a linear relationship, from which C_{max}, and r_p can be determined from the slope and intercept of the plot and there is no need to know any parameter beforehand.

Fig. 5 shows the linear regression of the production of ethyl alcohol curves obtained by means of Eq. (6). 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.

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

Table 3 shows that, the parameters of the maximum concentration of the producing ethyl alcohol and the initial ethyl alcohol production rate of the crosslinked hydrogels are increased with the order of N > E > B > T crosslinkers, and in increasing amount of the SA content in the AAm/SA hydrogels. These results are parallel to the results of the swelling curves of the hydrogels. The maximum amount of ethyl alcohol produced with crosslinkers in order of N > E > B > T and with the increase in the amount of SA content of AAm/SA hydrogel. From these figures, it can be deduced that N crosslinker provides maximum swelling resulting in maximum amount on immobilization of *S. cerevisiae*. Hence, the production of ethyl alcohol is greater than the other used crosslinkers. Some explanation is valid for SA content of AAm/SA hydrogels.

3.5. Operational stability

To investigate the operational stability of immobilized cells, repetitive use of fermentation experiments showed lightly decreases in amount of produced ethyl alcohol with operation number the same AAm/SA hydrogel containing 30 mg SA and N (Fig. 6).
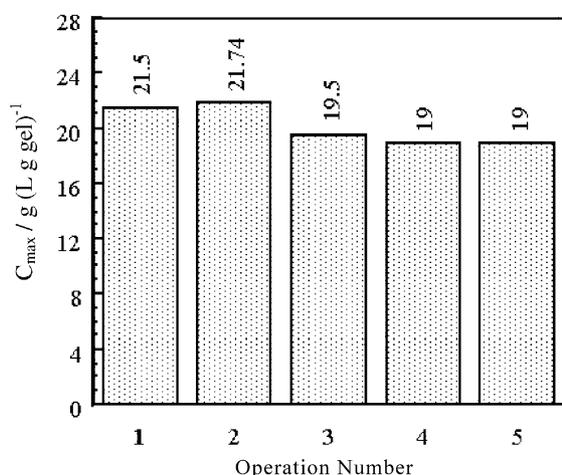
Fig. 6. The change in the amount of produced ethyl alcohol with operation number (reuse).

4. Conclusions

Ethyl alcohol production by fermentation is possible by 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 [14,15]. Immobilization is the restriction of cell mobility within a defined space. Immobilization provides high cell concentrations and cell reuse.

With the study it is aimed to find the best carrier for immobilization of yeast cells and production of ethyl alcohol by these cells. For this purpose, the AAm/SA hydrogels containing various amount of SA with the different swelling degree were produced by using different crosslinker. The hydrogels were immersed into the nutrient medium containing yeast cells. The yeast cells were adsorbed and immobilized. At the early stage of immobilization the AAm/SA 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 SA 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. However, the crosslinker has great effect on pore size. For instance, T has three functional groups and cause more tight network structure resulting in decrease in the pore size of AAm/SA hydrogels. Consequently, the swelling is low as well as the immobilization of yeast cells. Other used crosslinkers E and B make the pore size between N and T. And these was

reflected on swelling behavior and immobilization of yeast cell at same magnitude which caused in the amount of produced ethyl alcohol in between N and T used crosslinkers. For SA content of AAm/SA hydrogel same rationalization is valid. Thus, the higher the amount of SA content of AAm/SA hydrogels, the higher the immobilization of yeast cell and the higher the amount of produced ethyl alcohol.

To sum up, these results show that using different crosslinker crosslinked AAm/SA hydrogels can be used as support materials for production of ethyl alcohol by immobilization of yeast cells. These systems could be useful model for other biochemical reactions with yeast cells.

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