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CHITOSAN-CARBOXYMETHYLCELLULOSE HYDROGELS AS SUPPORTS FOR CELL IMMOBILIZATION

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ABSTRACT

The preparation of hydrogels from the polyelectrolyte complexation (PEC) of carboxymethylcellulose (CMC) and hydrogels from the complexation with an additional or simultaneous ionotropic gelation is reported. The reaction yield is high enough and depends strongly on the additional ionotropic gelation (IG). Infrared spectroscopy was used to confirm complexation between the carboxylic (in CMC) and amine (in chitosan) groups. The scanning electron microscope images show the formation of a fibrillar structure with characteristic pore sizes between 0.1 and 1 μm. The swelling capacity, Q, of PEC hydrogels is not high, but the Q value of hydrogels from PEC with an additional IG is high and depends strongly on the pH medium. These hydrogels are mechanically more stable than the PEC hydrogels; their mechanical strength is about 7 times higher than that of PEC hydrogels. The hydrogels were used to immobilize yeast cells with the cell density 1×10^9 cells/mL, about 100 times higher than that in the free-cell culture. Only the hydrogels with

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additional IG were stable enough for continuous fermentation over 10 days. These hydrogels proved to have higher affinity to substrate, which led to higher productivity than the ionotropic gel of CMC and aluminum ion.

INTRODUCTION

Polyionic hydrogels, which obtained by complexation between natural polysaccharides [1–3], have many applications in biotechnology, medicine, cosmetics, and agriculture. Owing to easy, low-cost, and mild formation processes, these hydrogels have been widely used as a support material for the immobilization of enzymes and cells [1, 2, 4, 5]. They were reported to have the following advantages: high permeability to water and substrates, excellent chemical stability and biological compatibility, controllable ion-exchange capacity, and resistance to swelling and shrinking in electrolyte solutions. Nevertheless, the polyionic hydrogels have rather low mechanical strength, and the formation and the use of these hydrogels meets with difficulty, especially in large-scale processes.

A few papers have suggested that a way to overcome this problem is to combine polyelectrolyte complexation (PEC) with simple ionotropic gelation (IG) of a polyelectrolyte substance with inorganic ions, mainly of alginate with calcium ion [3, 4]. In general, the effects of this improvement on the structure and properties of the support materials for immobilization have not yet been made clear.

This paper describes the formation of new supports for cell immobilization, which combine the PEC between carboxymethylcellulose (CMC) and chitosan, and the IG between CMC and aluminum ions. We also discuss the main properties, the structure of the supports, and their ability to immobilize microbial cells.

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EXPERIMENTAL

Materials (Polymers)

A 0.5% (w/v) chitosan–acetate solution was prepared by mixing 5 g of practical-grade chitosan with the degree of deacetylation = 80% with 345 g 0.1 N acetic acid and then stirring for 1 h using a chemical stirrer at room temperature. The chitosan solution was mixed with 650 g 0.1 N sodium acetate solution and stirred further for 30 min.

A 1% (w/v) sodium carboxymethyl cellulose solution was obtained from commercial CMC powder ($M_n \approx 100{,}000$, the degree of substitution ≈ 0.85) dissolved in distilled water. The resultant solutions were sterilized separately by autoclaving at 121°C for 15 min.

Cell Line and Medium

The cells used were yeast cells belonging to *Saccharomyces cerevisiae*: BSRI YB 3-8 (bottom-fermenting brewer's yeast) strain.

The seed culture medium was YEPD (2% bactopeptone, 1% yeast extract, and 2% glucose in distilled water; pH 6.8). The fermentation medium was Henne-

berg Medium with glucose concentration varied from 100 g/L to 200 g/L, and $(NH_4)_3PO_4$ 2 g/L, KH_2PO_4 2 g/L, $MgSO_4 \cdot 7H_2O$ 1 g/L, and $CaCO_3$ 5 g/L.

Preparation of Hydrogels

Samples of the chitosan–CMC hydrogel was prepared as spherical capsules (samples A) in the following way: 20 mL of the CMC solution were added dropwise, with a syringe, into 40 mL of the chitosan solution previously degassed. Spherical capsules formed under mild agitation at room temperature. After formation was completed, the capsules were washed by rapidly diluting the suspension with twice its volume of phosphate-buffered saline (PBS). The capsules were allowed to settle and the supernatant was removed by aspiration. After the wash was repeated, the capsules were rinsed in a 0.01% CMC solution in PBS and then resuspended in PBS. This treatment served to stabilize the external capsule surfaces and prevented intercapsule adhesion.

Samples B were obtained by rinsing the chitosan–CMC capsules collected after the formation of capsule membranes in a solution of 5% (w/v) $Al_2(SO_4)_3$ for 30 min. Degree of the gelation between CMC and aluminum ion can be controlled by changing the rinsing time.

Samples C were obtained by simultaneous reactions of CMC with chitosan and Al^{3+}. The droplets of the CMC solution were introduced into a solution containing a part of the 0.5% w/v chitosan solution. The ratio of the chitosan solution to the $Al_2(SO_4)_3$ solution was varied (samples C1: chitosan/$Al_2(SO_4)_3$ = 10/1, sample C2: chitosan/$Al_2(SO_4)_3$ = 2/1, sample C3: chitosan/$Al_2(SO_4)_3$ = 1/1).

The immobilization of yeast cells was carried out by adding a cell mass collected from culture medium to the CMC solution. The cell–CMC suspension was agitated for 10 min at 10°C to obtain a uniform suspension containing 1×10^9 cells/mL. Then this suspension was continually treated in the three above-mentioned ways, like the CMC solution.

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Mechanical Strength

All of the cell-immobilized beads obtained by these formulations were subjected to strength tests. The mechanical strengths were determined by measuring the force required to rupture a 1 mm diameter bead using uniaxial compression between parallel plates.

Swelling Property

The swelling of samples was followed by immersion in buffer solution (0.1 M) at pH = 4.0, 5.0 (acetate buffer) and pH = 7.2, 9.0 (phosphate buffer). The degree of swelling, α, of the samples was determined by weighing the samples after wiping at various time intervals, and was defined as:

$$\alpha = \frac{(\text{weight of hydrated gel} - \text{weight of dry gel})}{(\text{weight of dry gel})} \times 100 \quad (1)$$

The swelling capacity, Q, was determined:

$$Q = \text{(weight of gel at maximum swelling)/(weight of dry gel)} \quad (2)$$

Fermentation Experience

All the samples of the cell-immobilized beads were used in a continuous fermentation process employed in a multistage rhomboid bioreactor MBR 051 (Tokyo Rikakikai Co. Ltd., Tokyo, Japan). The immobilized yeast cells were packed into the bioreactor containing 300 mL of the fermentation medium with various glucose concentrations. The medium was continuously fed into the lower unit of the bioreactor and fermented at 15°C. The exhaust gas and the effluent solution were removed and the effluent solution was measured by gas chromatography using a Perkin-Elmer model 1B chromatograph.

RESULTS AND DISCUSSION

Preparation and Reaction Yield

Table 1 presents the preparation of hydrogels and the yield of the formation reactions, in which the CMC/chitosan ratio is 1/1.

The reaction yields were determined using the following relationship:

$$\text{Yield} = \frac{\text{(weight of dry gel)}}{\text{(weight of chitosan)} + \text{(weight of CMC)}} \times 100 \quad (3)$$

The yield of the complexation between CMC and chitosan was not high; it might be that the CMC/chitosan ratio was not proper for the reaction (sample A). The appearance of aluminum ion in the additional ionotropic gelation between CMC and $Al_2(SO_4)_3$ increased the yield (sample B). But in the simultaneous reaction of CMC with chitosan and $Al_2(SO_4)_3$, when the amount of $Al_2(SO_4)_3$ was far greater than that of chitosan (about 10 times), as indicated in sample C3, the yield was greatly decreased. It can be deduced that the rate of the reaction between CMC and

TABLE 1. Preparation of Hydrogels and Yield of the Formation Reactions

Method of preparation	Sample	Yield (%)
1. Polyelectrolyte complexation (PEC)		
CMC + chitosan	A	69.35
2. Additional ionotrpic gelation (PEC + IG)		
Sample A + $Al_2(SO_4)_3$	B	86.60
3. Simultaneous PEC and IG (PEC/IG)		
CMC + chitosan + $Al_2(SO_4)_3$	C	
Chitosan/$Al_2(SO_4)_3$ = 10/1	C1	78.65
Chitosan/$Al_2(SO_4)_3$ = 2/1	C2	85.50
Chitosan/$Al_2(SO_4)_3$ = 1/1	C3	73.28

Note. Concentration of CMC solution: 1%; concentration of chitosan solution: 0.5%; concentration of $Al_2(SO_4)_3$ solution: 5%.

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Al^{3+} was higher than the rate of the reaction between CMC and chitosan, so in that case most of CMC molecules reacted with Al^{3+} ions. On the contrary, when the amount of $Al_2(SO_4)_3$ decreased as in sample C1, the reaction between CMC and chitosan was dominant.

It is shown in Table 1 that the sample C2 and the sample B have the same yield; they may have the same structure and properties.

Structure of Hydrogels

Figure 1 shows the infrared (IR) spectra of chitosan, carboxymethylcellulose natrium salt, and CMC–chitosan polyelectrolyte complex. The chitosan spectrum has the characteristic bands at 1653 cm^{-1} (amide I), 1597 cm^{-1} (amide II), and three bands at 1419, 1377, and 1319 cm^{-1}.

The characteristic bands for the CMC natrium salt are at 1597, 1417, and 1327 cm^{-1}.

The CMC–chitosan PEC spectrum has all the characteristic bands of both chitosan and CMC; there is only a small difference in the position of the peaks (1633 and 1593 cm^{-1}) and in the shape of the peaks (1417, 1379 and 1323 cm^{-1}).

The scanning electron micrographs (SEM) of the PEC hydrogel shown in Fig. 2 explain that the gels are porous [Fig. 2(a)] and that the formation of fibrillar structures occurred [Fig. 2(b)] as in Ref. 1.

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Swelling

The swelling of the hydrogels is relatively high and depends strongly on the pH of the medium (Fig. 3). Figures 3(a)–(d) describe the swelling properties of the hydrogel samples (A, B, C1, C2) in different buffer solutions. The swelling capacity, Q, can be observed to increase with an increase in pH, possibly due to the formation of carboxylic anions in the polymer network, leading to the development of strong electrostatic forces contributing to the network expansion. Similar results have been reported previously [6].

Sample A from CMC–chitosan hydrogels without additional or simultaneous ionotropic gelation had a distinctly lower swelling capacity, which was equivalent to the value of xanthan–chitosan hydrogels in Ref. 1. On the other hand, the samples B, C2, C3 obtained from the polyelectrolyte complexation combined with ionotropic gelation have high swelling capacity, Q, and swelled with similar profiles. Sample C3 had a slightly higher swelling capacity, perhaps due to the denser network.

This result conveys the higher resistance to swelling of the polyelectrolyte complex compared to a typical ionotropic gel.

Mechanical Strength

The rupture strengths of the prepared hydrogels are shown in Fig. 4. It can be noted that the ionotropic gelation contributes to the increase of the rupture strength (samples B, C2, C3). The samples B, C2, C3 exhibited good strengths which are 7–10 times higher than the value of the simple polyelectrolyte complex (sample A). The sample C1 with a little ionotropic gelation (see Table 1) has only a little higher

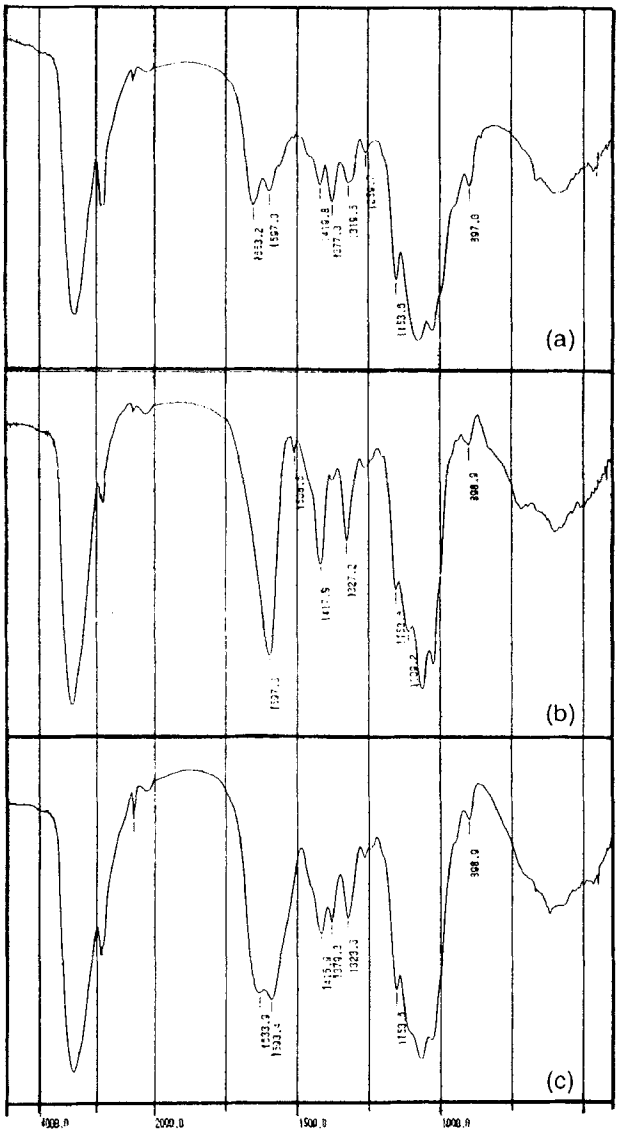

FIG. 1. Infrared spectra of (a) chitosan; (b) CMC-natrium salt; (c) CMC-chitosan polyelectrolyte complex.

rupture strength than the sample A, which is hydrogel without ionotropic gelation.

The data of the rupture strength also prove the above assumptions that samples B and C2 have similar structures and that sample C1 has a structure like sample A.

We can see that the swelling capacity and also the rupture strength of the hydrogels depend on the network formation, which is ionotropic gelation here.

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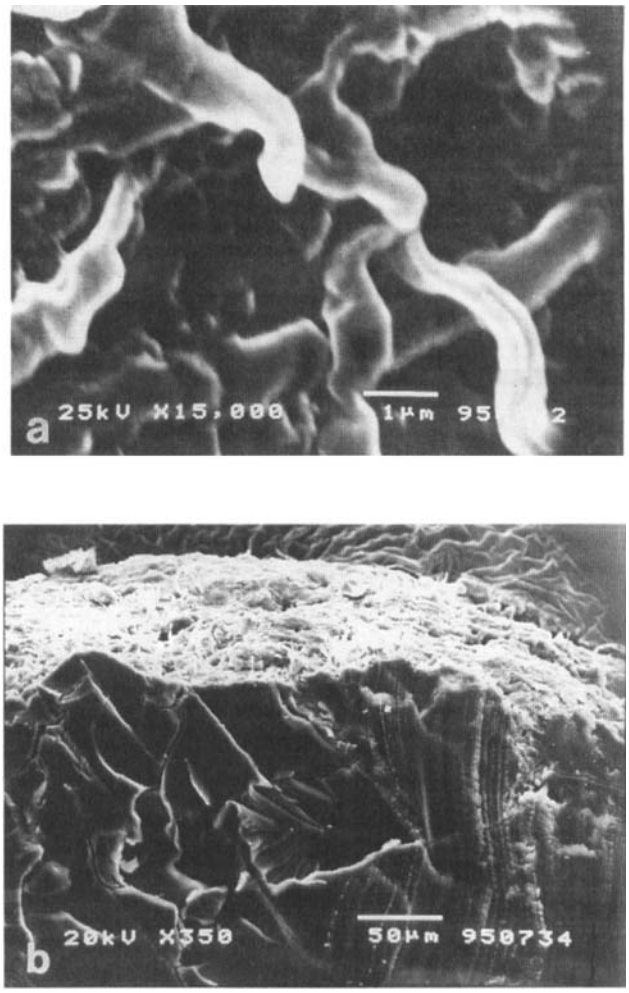

FIG. 2. Scanning electron microscope (SEM) images of CMC-chitosan polyelectrolyte complex combined with ionotropic gelation: (a) image of internal section (above); (b) image of external surface (under).

Fermentation

The fermentation experiments were carried out to test the role of the support materials in immobilized cell activity. Samples A, B, C2 have the same cell density (1×10^9 cells/mL hydrogel). Immobilized cells in ionotropic gel beads of CMC and aluminum ion with the equal cell concentrations were taken as a control. To some extent, the cells can be regarded as a complex enzyme system catalyzing the conversion of glucose into ethanol. So we can use the Michaelis-Menten model to analyze the kinetic data of the fermentation reactions.

A reformulation of the Michaelis-Menten equation by Lineweaver and Burk

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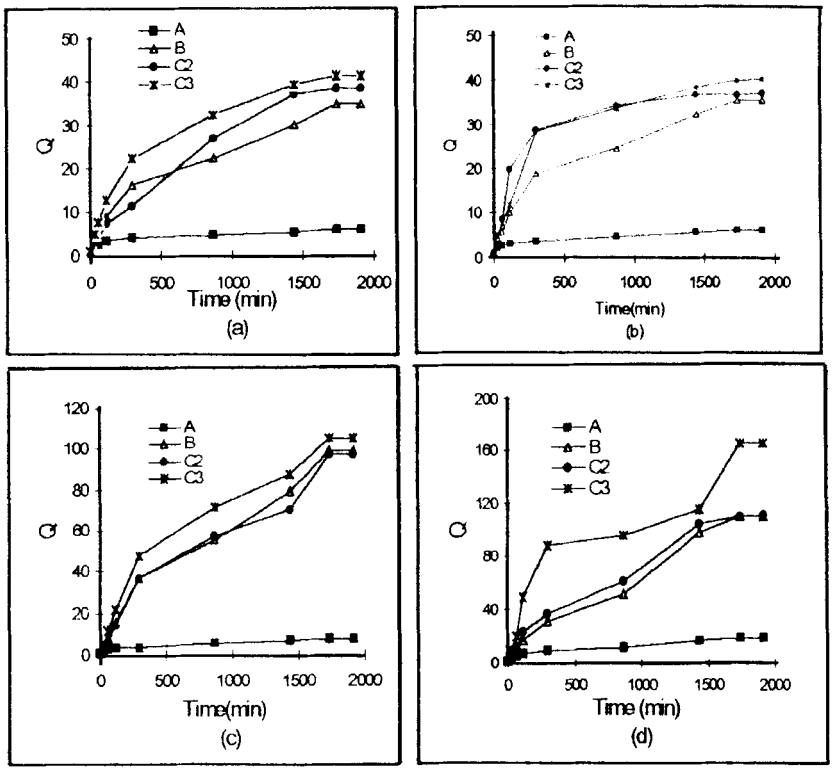

FIG. 3. Variation of swelling capacity Q as a function of time in different buffer solutions: (a) pH = 4.0; (b) pH = 5.0; (c) pH = 7.2; (d) pH = 9.0.

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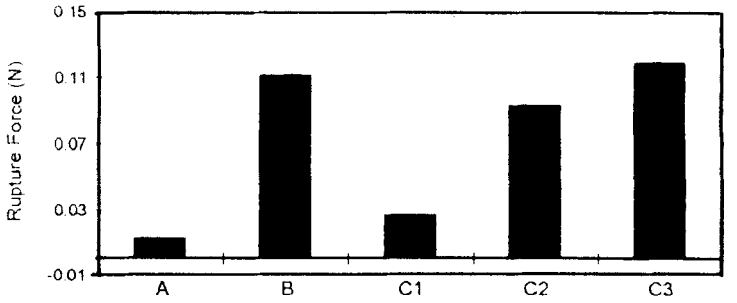

FIG. 4. Rupture strength of the prepared hydrogels: A, polyelectrolyte complex; B, with additional ionotropic gelation; C1, C2, C3, simultaneous PEC and IG with increasing amounts of $Al_2(SO_2)_3$.

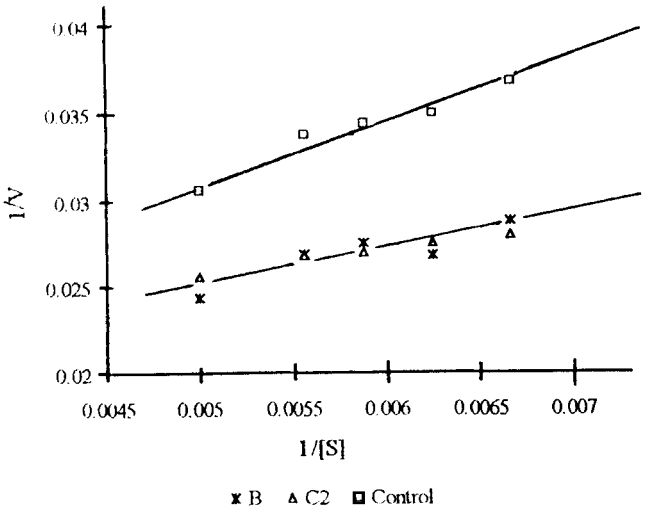

FIG. 5. Lineweaver–Burk plot of kinetic data in the fermentation experiments. V, reaction velocity (gEtOH/h·g wet catalyst); [S], substrate concentration (g/L); B, C2, polyelectrolyte complex with additional and simultaneous ionotropic gelation; control, ionotropic gel.

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predicts a direct linear relationship between the reciprocal of the reaction velocity, V, and the reciprocal of the substrate concentration [S]:

$$1/V = (1/V_{max}) + (K_m/V_{max} \times 1/S)$$

where V_{max} is maximum reaction velocity, and K_m is the Michaelis constant.

Figure 5 illustrates plots of V^{-1} versus $[S]^{-1}$ for the samples. The fermentation reactions using samples B, C2, and control were stable for 10 days; but in the case of samples A, the capsules ruptured after a few hours and the data of this process were not counted.

The slope of the line is K_{max}/V_{max} and the y intercept is $1/V_{max}$. The results confirm the prediction that samples B and C2 have the same structure. From Fig. 5, it is clear that these samples have values of K_m lower than that of the control sample. So they have higher affinity to substrate than the control sample.

The higher permeability to substrate of the sample B and C2 is probably due to the more "open" gel structure and the polyelectrolyte complex membrane.

CONCLUSION

Hydrogels from chitosan and carboxymethylcellulose (CMC) mixtures can be obtained by simple complexation in the aqueous phase. In order to improve the swelling capacity and the mechanical strength of the gels, polyelectrolyte complexation between chitosan and CMC has been carried out with an additional or simultaneous ionotropic gelation. The yields of complexed chitosan vary from about 70%

to 85% depending on the presence of the ionotropic gelation and the amount of $Al_2(SO_4)_3$.

Structural analysis of the gels indicates that the gels are porous, and that the formation of fibrillar structures with characteristic pore dimensions between 10^{-7} and 10^{-6} m (0.1 to 1 μm) occurred.

The large swelling capacity of the hydrogel depends strongly on medium pH, and PEC between chitosan and CMC with an additional or simultaneous ionotropic gelation are hard and stable enough for their applications.

The hydrogels have been used as supports for yeast cell immobilization in ethanol production. The polyionic hydrogel with the additional ionotropic gel has shown an improvement as compared with two normal kinds of support: polyelectrolyte complex capsule and gel bead. It has higher mechanical strength than the capsule and higher permeability to substrate than the gel bead. So it can run in continuous fermentation for a long time and give high productivity (41.03 g EtOH/h·g wet catalyst at the concentration of 200 g glucose/L).

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