Immobilization of Microorganisms Within Porous Polymeric Capsules

Ryohichi Nagashima,[1,2] Haruko Hirose,[3] Hideto Matsuyama[1]

[1]*Department of Chemical Science and Engineering, Center for Membrane and Film Technology, Kobe University, 1-1 Rokkodai, Nada-ku, Kobe 657-8501, Japan*
[2]*Environmental Technology Research and Development Section, Teijin Entech Co., Ltd., 3-4-1 Minohara, Ibaraki, Osaka 567-0006, Japan*
[3]*Material Analysis Research Laboratories, Teijin Co., Ltd., 4-3-2 Asahigaoka, Hino, Tokyo 191-8512, Japan*

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ABSTRACT: Porous capsules suitable for immobilizing yeast and *Escherichia coli* were prepared by the phase separation of poly(m-phenylene isophthalamide) solution. The microorganisms were coated with starch or dextrin, which was subsequently extracted after capsule formation but served both to position the microorganisms in the capsule pores and to form spaces around the microorganisms. Fermentation experiments were carried out with capsules immobilizing yeast. Yeasts could be cultivated in the capsules, and effective release of CO_2 produced by the fermentation was observed with capsules prepared using polyethylene glycol because of the porous structure at the capsule surfaces. Notably, yeasts could be cultivated at high density even after 1 year. © 2011 Wiley Periodicals, Inc. J Appl Polym Sci 121: 321–326, 2011

Key words: immobilization of microorganism; porous capsule; phase separation; poly(m-phenylene isophthalamide; yeast

INTRODUCTION

Immobilization of biocatalysts such as enzymes and microorganisms has many advantages, such as improvement of reaction efficiency and the recycling of the catalyst after reactions.[1] Significant attention has been paid to the immobilization of microbial cells as a useful catalyst. Immobilized cells can be applied to multistep enzyme reactions for the production of useful materials such as alcohol, organic acids, and amino acids.

Because of the requirements to reduce CO_2 emissions and the increase in crude oil prices, much research has been carried out on the production of biomass ethanol. The establishment of efficient yeast fermentation technology is crucial to the commercialization of ethanol production from biomass. For a commercialized yeast fermentation process, biocatalysts must be immobilized on solid materials. Thus, development of highly efficient immobilization methods has attracted significant attention and many articles have been published in this research field.[2–4]

In general, methods for microbial immobilization can be divided into the following three approaches[1]:

1. The binding method, wherein a microbial organism is physically adsorbed or covalently bonded onto a solid material surface.
2. The crosslinking method, wherein the cell wall or membrane of a microbial organism is strengthen by crosslinking.
3. The encapsulation method, wherein a microbial organism is encapsulated within a polymeric material.

In one example of the encapsulation method, sodium alginate has been widely used as an immobilization material in the laboratory, because it easily forms a gel in the presence of polyvalent metal ions such as calcium.[1,5,6] However, large lattice structures are sometimes formed in this method, which is unsuitable for microbial and enzymatic immobilization.[7]

To solve this problem, there have been reports on the use of a complex calcium gel of alginate and dicarboxy cellulose, which aim to improve the lattice structure.[8] However, gel weakness is still a problem in practice. Junter and Vinet[9] investigated the mechanical strength of alginate gels containing various amounts of yeast cells. The presence of yeast cells in alginate disks led to weakening of the gel structures and this effect increased with the immobilized cell content. Nussinovitch[10] measured the mechanical properties of an

Correspondence to: H. Matsuyama (matuyama@kobe-u.ac.jp).

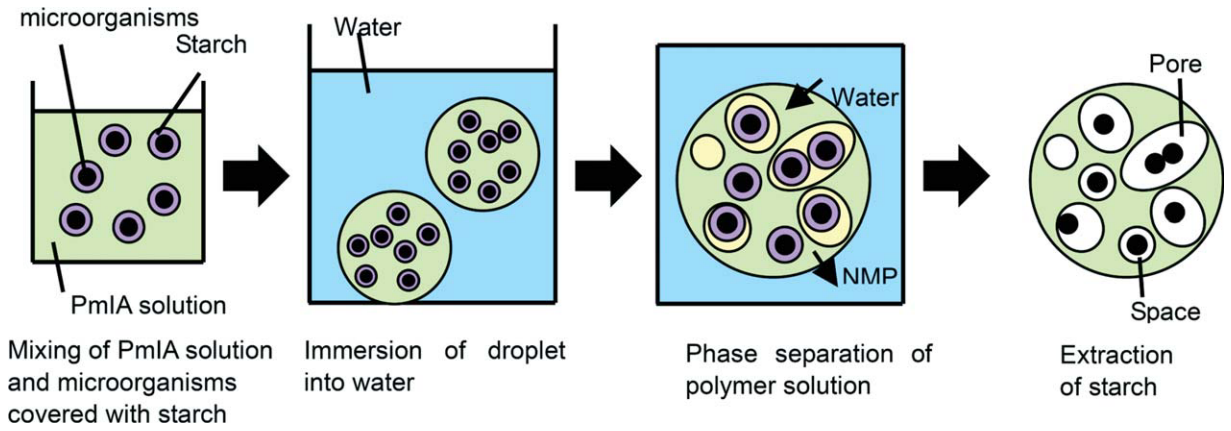

Figure 1 Procedure for the formation of porous capsules containing microorganisms. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

alginate gel immobilizing fungal spores. A simple mathematical model was presented to describe the relative stress.

Lin et al.[11] investigated the development of anionic polyurethane (APU), which has excellent mechanical properties for the immobilization of yeast (*Saccharomyces cerevisiae*). APU gel beads could be formed simply by dropping calcium chloride into an emulsion of APU. The problem with this method is that calcium chloride is corrosive and therefore equipment and piping must be constructed from expensive materials. This leads to the risk of higher materials costs for plant construction, when applied on an industrial scale.

Yeast encapsulation in a stable, semipermeable microcapsule, prepared by interfacial polymerization between 1,6 hexanediamine and poly(allylamine) crosslinked with diacid chlorides, has also been reported.[12] The size and distribution of the cells within the capsules were investigated by a combination of confocal laser and transmission electron microscopy (TEM). Interfacial polymerization enables the preparation of thin, mechanically stable capsules but may suffer drawbacks at industrial scale because of the complexity and expense of the preparation process.

In previous work, we prepared porous particles that included adsorbents.[13] A new method was proposed for the formation of space around these adsorbents by coating them with starch and effective adsorption was shown to result from this space formation.

In this work, yeast and *Escherichia coli* were immobilized within porous polymer capsules, located mainly inside the pores. A fermentation experiment was carried out using these immobilized yeast microcapsules.

EXPERIMENTAL

Materials

Poly(m-phenylene isophthalamide) (PmIA) was supplied by Teijin Entech (Osaka, Japan). *N*-Methyl-2-pyrrolidone (NMP), polyethylene glycol (PEG) with molecular weight of 600, sucrose, agar, and potassium permanganate were purchased from Wako Pure Chemical Industries (Tokyo, Japan). Starch, dextrin (Amycol 3L), and anionic surfactant (Hiroshima, Japan) (emal 0) were purchased (Osaka, Japan) from Daiso Sangyo, Nippon Chemical Starch, and Kao (Tokyo, Japan), respectively. Yeast (Nissin super camellia dry yeast) was purchased from Nissin Foods. *E. coli* was supplied by Teijin Entech.

Procedure for making porous PmIA capsules containing microorganisms with effective space

A schematic of the procedure is shown in Figure 1. First, polymer solution was prepared by dissolving PmIA in NMP. The solution was mixed with microorganisms covered with starch. Droplets of this solution were immersed in the precipitation solution (water). In this situation, the inflow of water and outflow of NMP occurred simultaneously within the droplet. Because water is an antisolvent for the polymer, phase separation of the polymer solution was induced, resulting in the formation of a porous structure in the polymer material.[14] Microorganisms inside the capsule existed in both the pore region and the polymer region. However, because the yeast surface was covered with hydrophilic starch, the yeast was likely to exist in the more hydrophilic solvent phase formed after phase separation. Because the solvent phase finally formed the pores, yeast cells were mainly located inside the pores; where the cells existed within the polymer region, a space was formed between the microorganisms and the polymer after starch extraction.

Preparation of immobilized yeast capsules

The PmIA concentration in NMP solution was fixed at 6 wt %. Starch and dry yeast were mixed in the weight ratio 2:1 to prepare a homogeneous paste.

TABLE I
Weight Ratios of PmIA, Dextrin, Yeast, and PEG in Polymer Solutions

Capsule	PmIA	Dextrin	Yeast	PEG
1	1	2	4	5
2	1	2	4	13
3	1	2	4	20
4	1	2	4	30

This paste was then added to the PmIA solution, giving a PmIA to yeast weight ratio of 1 : 2, and the solution was mixed with a stirring rod.

The polymer solution was then added to the precipitation solution (1 wt % aqueous anionic surfactant solution) using a needleless microsyringe (NIPRO, 1 mL type) at a rate of 5 drops per minute, which gave a spherical body with a diameter of about 5 mm. The resultant capsules were immersed in water overnight to extract the starch.

Preparation of immobilized *E. coli* capsules

A paste was prepared by adding an *E. coli* colony grown in nutrient agar to starch. PmIA solution (5 wt %) and *E. coli* paste (9.1 wt %) were mixed at a PmIA to *E. coli* weight ratio of 10 : 1. The remaining procedure for preparation of immobilized *E. coli* capsules was the same as that described above.

Alcoholic fermentation experiment using immobilized yeast capsules

Preparation of capsules

In this case, dextrin was used instead of starch. Yeast was added to an aqueous dextrin solution prepared at a dextrin to water weight ratio of 2 : 1 and the solution was mixed into a paste. The yeast to dextrin weight ratio was kept at 2 : 1 in the paste and this was then added to a 5-wt % PmIA solution. The final weight ratio of PmIA, dextrin, and yeast was 1 : 2 : 4 in the dope solution. This polymer solution was dropped into the precipitation solution as described above by the needleless microsyringe to form immobilized yeast capsules with diameters of about 5 mm.

In addition to these capsules, the porous capsules were also prepared by adding PEG with molecular weight of 600 into the polymer solution as a pore forming material. Various amounts of PEG were added to the 5 wt % PmIA solution and the remainder of the preparation procedure was the same as that described above. Finally, four kinds of polymer solutions were prepared. The weight ratios of PmIA, dextrin, yeast, and PEG, based on the PmIA weight, are listed in Table I.

Fermentation experiments

The fermentation experiments were carried out using immobilized yeast capsules prepared with and without PEG. For experiments using capsules

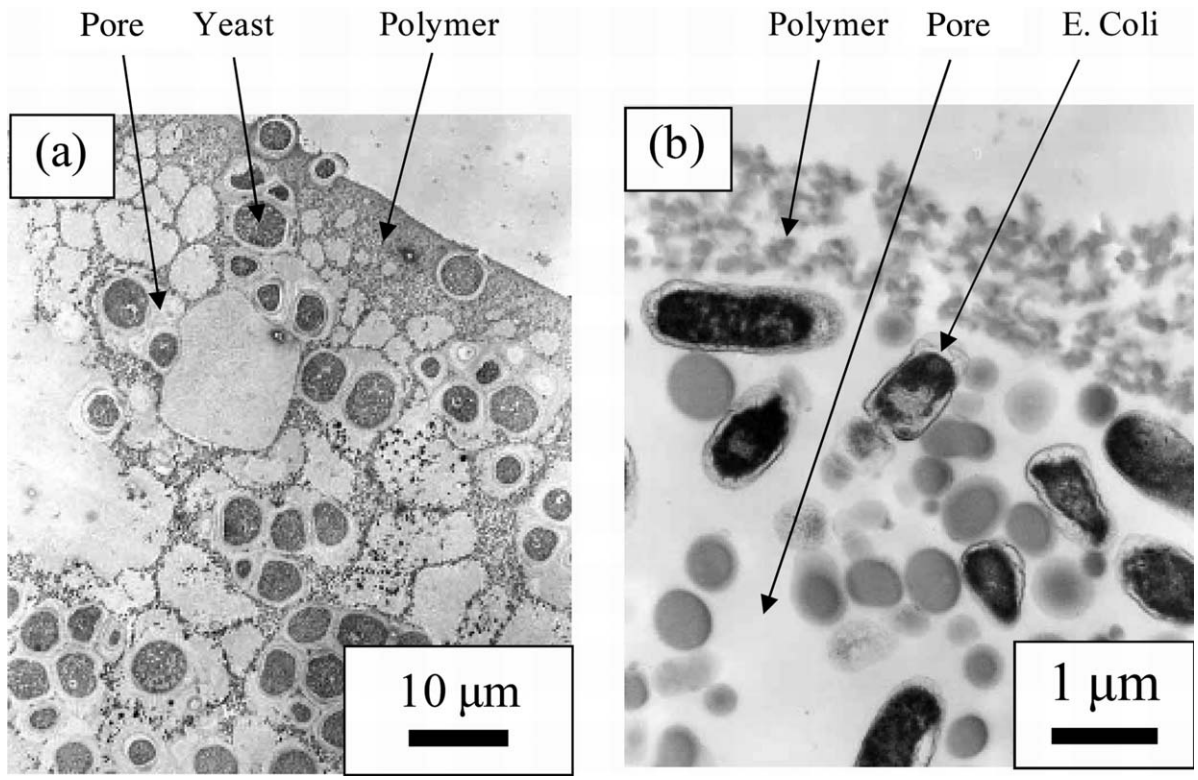

Figure 2 TEM images of the prepared capsules: (a) immobilized yeast capsule; (b) immobilized *E. coli* capsule.

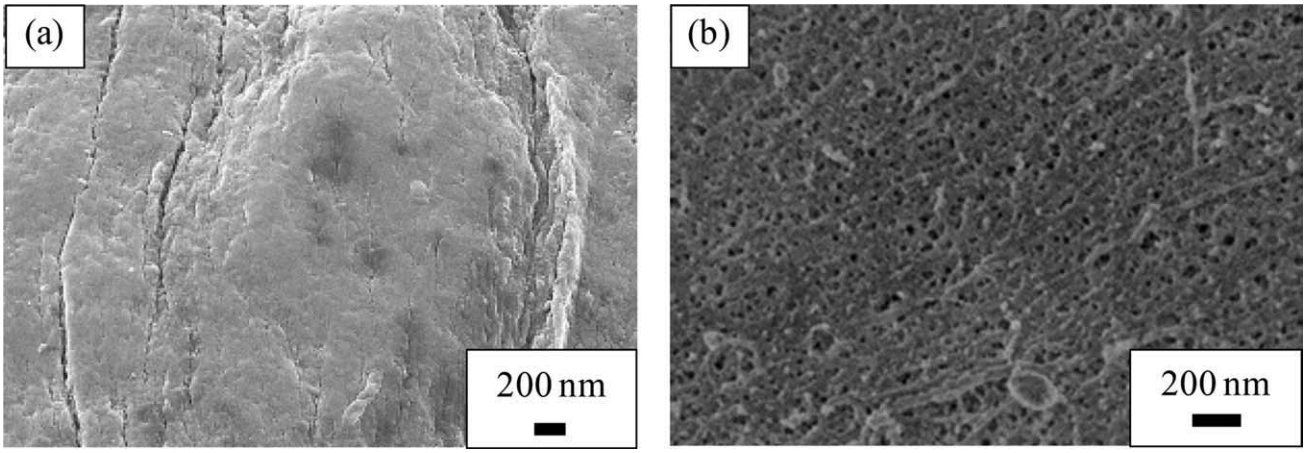

Figure 3 SEM images of prepared capsule surfaces: (a) capsule prepared without PEG; (b) capsule prepared with PEG (Capsule 2).

prepared without PEG, 15 capsules were placed in a 50-mL glass syringe, and the top plugged by a piston. Ten milliliters of a 5-wt % sucrose solution was then added to the syringe, and it was placed in a water bath controlled at 40°C for 90 min.

In the case of capsules prepared with PEG, capsules containing 1 g yeast and 10 mL of 5 wt % aqueous sucrose solution were poured into the syringe and the syringe was placed in a water bath controlled at 40°C. The amount of CO_2 released by the fermentation was measured by the movement of the piston.

Yeast culture test

Capsule 2, prepared under the conditions shown in Table I, was placed into an incubator at 30°C and cultured at a shaking speed of 112 rpm in YPD medium that had been sterilized in an autoclave at 121°C for 15 min. The capsules were collected after 3 and 24 h, and respective capsule cross sections were observed by a optical microscope (Nikon, OPTIPHOT).

For checking the yeast growth over longer periods, capsules prepared by the procedure described in Preparation of Immobilized Yeast Capsules section were stored in a sample bottle without medium, at room temperature, for 1 year. A cross section of the capsule was observed by TEM using the procedure described below.

Capsule observation

The capsule surfaces were observed by scanning electron microscopy (SEM, Hitachi, S-5200) and cross sections of the capsules were observed by TEM (TOPCON, LEM-2000). For TEM observation, the capsules were molded by an aqueous potassium permanganate (1 wt %) solution and cut by a ultramicrotome (Leica, Ultracut S) to a thickness of 50 nm.

RESULTS AND DISCUSSION

Preparation of yeast and immobilized *E. coli* capsules

Figure 2(a) shows a TEM image of an immobilized yeast capsule. Yeasts mainly existed inside the pores formed by the phase separation. The polymer solution was dropped into a water bath and outflow of solvent from the polymer solution and inflow of water occurred, resulting in phase separation of the polymer solution and pore formation inside the capsules. The yeast surfaces were covered with hydrophilic starch and therefore, as described above, yeasts mainly existed inside the pores. A careful observation of Figure 2(a) shows that some yeasts existed in the polymer phase. However, spaces were formed around yeasts because starch covering the

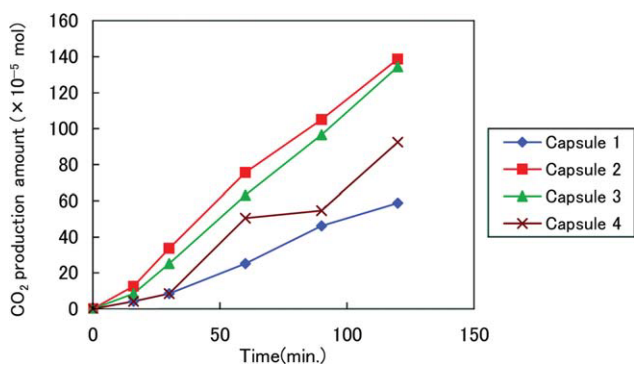

Figure 4 Time-courses of CO_2 evolution from prepared capsules. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

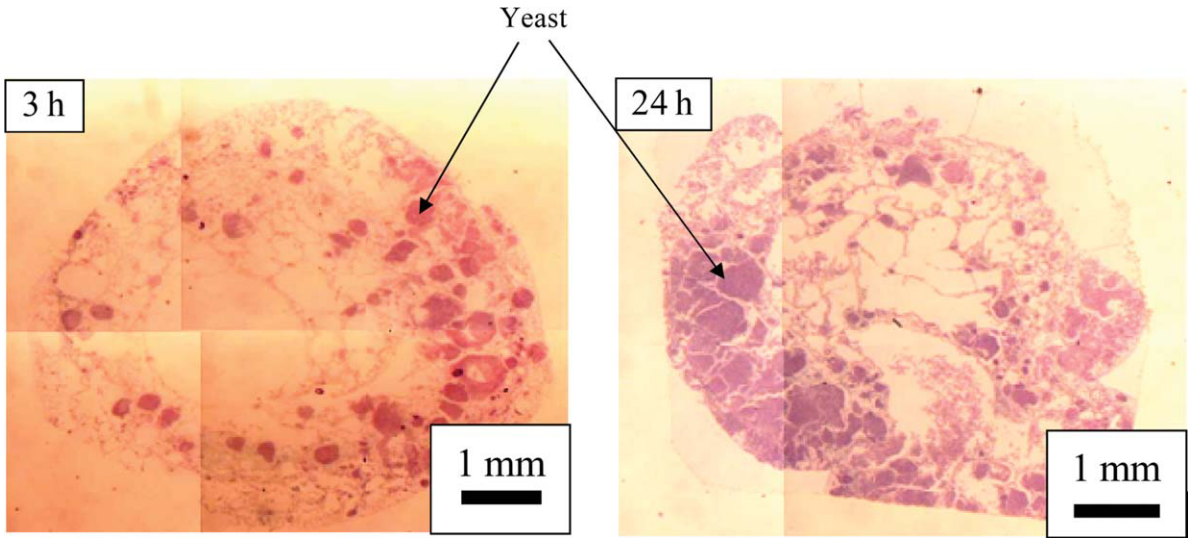

Figure 5 Cross-sections of immobilized yeast capsules after cultivation. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

yeast surface was extracted after capsule preparation so that the cells did not come into direct contact with polymer. If the yeast surface were covered by polymer, the fermentation ability of the yeast would be decreased, due to a decrease in its effective cell surface area. Therefore, the yeast cells within the capsules prepared in this work are expected to be suitable for fermentation.

A TEM image of immobilized *E. coli* capsules is shown in Figure 2(b). *E. coli* also existed in the pore region. The pores in the polymer wall are much smaller than the size of *E. coli*, so *E. coli* cannot leak out through the polymer wall and, therefore, sufficient *E. coli* immobilization in the capsules was obtained.

Fermentation results

During the fermentation experiments, about half of the capsules floated on the solution surface after 90 min for capsules prepared without PEG. This occurred because the CO_2 produced during fermentation could not be released from the capsules, due to the dense wall surfaces. Indeed, no gas bubbles were observed around the capsules during the experiment. Figure 3(a) shows the dense surface structure of this capsule.

In contrast, CO_2 was released from capsules prepared with PEG during fermentation experiments. The time-courses of CO_2 production are shown in Figure 4 for various capsules. It can be seen that the maximum amount of CO_2 was produced from capsules 2 and 3. The surface structure of Capsule 2 is shown in Figure 3(b). Numerous pores were formed at the capsule surface, which enabled the effective CO_2 release. CO_2 production decreased in the case of Capsule 4 prepared with highest amount of PEG. This is probably because the pores at the surface were too large allowing some yeast to leak out from the capsules.

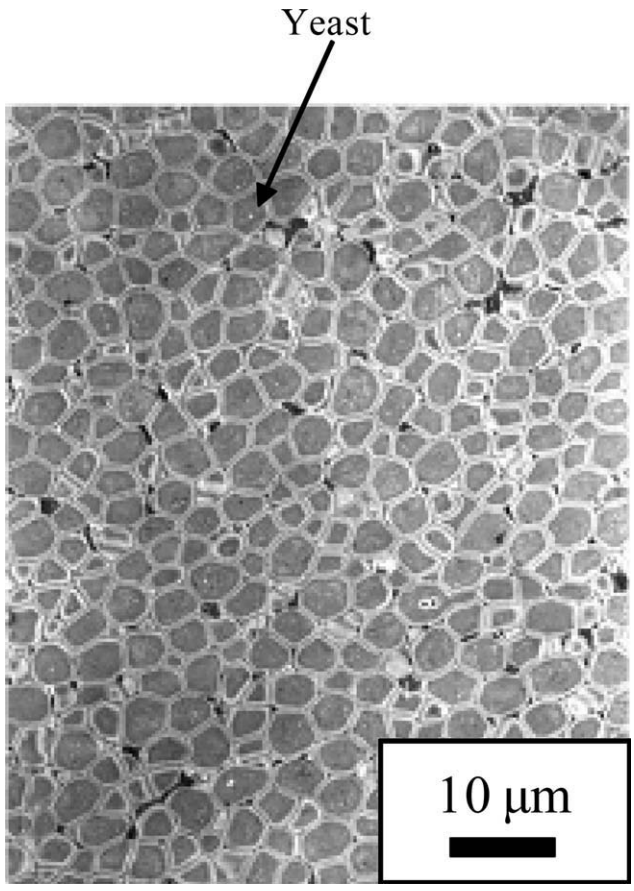

Figure 6 Inside of the yeast-immobilized capsule kept in bottle for 1 year.

Yeast cultivation

Yeast cultivation results are shown in Figure 5, with optical micrographs of cross sections shown for capsule Type 2 cultivated for 3 and 24 h. It can be clearly seen that yeast cell numbers increased greatly after 24 h. Thus, effective yeast cultivation in these porous capsules was proven to be possible.

As mentioned above, the capsule shown in Figure 2(a) was kept in a sample bottle for 1 year. The condition inside the capsule at the end of this time is shown in Figure 6. Yeast cells were packed densely and contacted each other inside the capsule. This indicates that high density cultivation is possible by this yeast-immobilization method.

CONCLUSIONS

Yeast and *E. coli* were immobilized in porous capsules prepared by polymer phase separation. It was confirmed that microorganisms existed within the capsule without contacting the polymer wall. Results of fermentation experiments showed that effective production and release of CO_2 could be accomplished through use of capsules with porous structures at the surfaces. The capsules were also suitable for yeast cultivation, whereby, after 1 year, yeasts were cultivated at high density within the capsule.

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