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ORIGINAL PAPER

Preparation of polymeric microcapsules enclosing microbial cells by radical suspension polymerization via water-in-oil-in-water emulsion

Takayuki Takei · Kaoru Ikeda · Hiroyuki Ijima · Koei Kawakami · Masahiro Yoshida · Yasuo Hatate

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Abstract Polymeric microcapsules enclosing *Saccharomyces cerevisiae* were prepared by radical suspension polymerization via water-in-oil-in-water emulsion. Trimethylolpropane trimethacrylate and 2,2′-azobis(4-methoxy-2,4-dimethylvaleronitrile) were used as monomer and radical initiator, respectively. A culture medium with suspended yeast cells, monomer solution with the dissolved radical initiator, and poly(vinyl alcohol) aqueous solution were used as inner aqueous phase, oil phase, and outer aqueous phase, respectively. The influence of microcapsule preparation parameters on the viability of encapsulated cells and encapsulation efficiency was investigated. The radical polymerization process did not cause significant damage to encapsulated yeast cells. Decreased weight ratio of aqueous phase to oil phase resulted in increased encapsulation efficiency of the cells. The diameter of the microcapsules could be controlled by varying the agitation rate.

Keywords Microcapsules · Microbial cell · Radical polymerization · Water-in-oil-in-water emulsion · Bioengineering

Introduction

Immobilization of microbial cells has many advantages over free cell formulations, including relative ease of product separation, reusability, increased cell densities, protection from abiotic stresses resulting from the inhibitory effect of toxic

T. Takei (✉) · K. Ikeda · H. Ijima · K. Kawakami
Department of Chemical Engineering, Graduate School of Engineering, Kyushu University,
744 Motooka, Nishi-ku, Fukuoka 819-0395, Japan
e-mail: takei@chem-eng.kyushu-u.ac.jp

M. Yoshida · Y. Hatate
Department of Chemical Engineering, Graduate School of Engineering, Kagoshima University,
Kagoshima 890-0065, Japan

compounds, and reduced susceptibility to contamination by foreign microorganisms [1–4]. For these reasons, immobilized cells have been utilized for production of useful compounds such as alcohols, amino acids, organic acids, and antibiotics, and for degradation of wastewater pollutants [3, 5].

Many techniques for immobilization of microbial cells have been reported [6]. Encapsulation in spherical polymer supports is one of the most frequently used techniques for cell immobilization, and hydrogels such as calcium alginate [3], poly(vinyl alcohol) (PVA) [7], and κ-carrageenan [2] have been widely utilized as support materials. The disadvantages of microcapsules are their susceptibility to disruption and leakage of encapsulated cells during long-term cultivation, due to low stability and mechanical strength of the hydrogel materials. To provide the desired stability and mechanical strength, solid synthetic polymer resins are more promising than hydrogels as microcapsule materials.

Previously, we prepared solid microcapsules composed of poly(methyl methacrylate) (PMMA) for microbial cell immobilization [8]. The microcapsules were fabricated by a solvent evaporation method via emulsion, and had much higher stability and mechanical strength than hydrogel capsules. Briefly, sodium alginate solution with suspended denitrifying bacteria was mixed with a dichloromethane (DCM) solution of PMMA to form a water-in-oil (W/O) emulsion. The emulsion was dispersed in outer aqueous solution to form water-in-oil-in water (W/O/W) emulsion. Then, DCM was evaporated under reduced pressure, resulting in cell-enclosing PMMA microcapsules.

The purpose of the present study was to prepare solid polymeric microcapsules enclosing microbial cells by radical suspension polymerization via W/O/W emulsion to avoid the use of harmful organic solvents such as DCM (octanol–water partition coefficient, log $P_{ow} = 1.25$). To the best of our knowledge, there have been no reports concerning solid polymeric microcapsules enclosing microbial cells prepared by radical suspension polymerization. We selected trimethylolpropane trimethacrylate (TRIM) as monomer for the base material of the microcapsules (Fig. 1). The monomer, which is in liquid form at ordinary temperature and pressure, has low polarity (log $P_{ow} = 4.39$) compared to other widely used monomer such as methyl methacrylate (log $P_{ow} = 1.38$). It has been reported that toxicity of low polarity materials is lower than high polarity ones [9]. It indicates that the monomer is much less toxic to microbial cells compared to DCM used in previous method, resulting in high viability of encapsulated cells. Additionally,

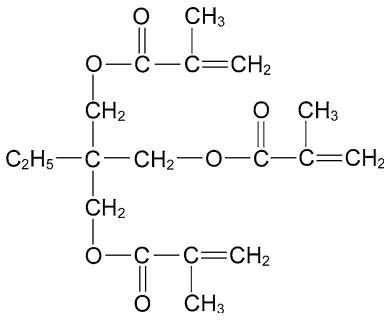

Fig. 1 Chemical structure of TRIM

avoiding usage of the harmful solvent such as DCM is beneficial from the viewpoint of green chemistry. To obtain basic experimental knowledge essential for preparing the microcapsules enclosing microbial cells by radical suspension polymerization, we investigated the effects of preparation parameters on cell viability, and entrapment efficiency of the cells. *Saccharomyces cerevisiae* cells were utilized due to their frequent use for cell encapsulation.

Experimental

Materials and cell culture

Trimethylolpropane trimethacrylate monomer, 2,2′-azobis(4-methoxy-2,4-dimethylvaleronitrile) as radical initiator, and PVA (completely hydrolyzed, molecular weight: 40,000–48,000 g/mol) were purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan). *Saccharomyces cerevisiae* NBRC 0216 was obtained from The NITE Biological Resource Center (NBRC, Chiba, Japan). The yeast cells were maintained in 108 medium (10 g of glucose, 5 g of peptone, 3 g of yeast extract, and 3 g of malt extract in 1 L distilled water).

Preparation of microcapsules

The preparation apparatus was a 1-L round-bottomed glass-jacketed vessel equipped with a mechanical stirrer. The stirrer was fitted with a 77-mm crescent Teflon-coated blade. A culture medium suspending yeast cells (cell density: 5.8×10^8–9.4×10^8 cells/mL; viability: 44.0–99.3%), the inner aqueous phase, was dispersed in TRIM with 1% (w/w) dissolved radical initiator, the oil phase, for 30 s using a homogenizer (20,000 rpm, Polytron PT 1600E; Kinematica Inc., Lucerne, Switzerland) to form W/O emulsion. We confirmed that the homogenization process hardly cause cell damage. The total weight of inner aqueous phase and oil phase was fixed at 6.0 g. The emulsion was then dispersed in 300 mL of 1% (w/v) PVA solution, the outer aqueous phase, in the glass-jacketed vessel using a mechanical stirrer at 35 °C, for formation of W/O/W emulsion. Agitation was continued in a nitrogen atmosphere for several hours. The mixture was filtered and the resultant microcapsules were collected. The agitation rate was fixed during the microcapsule preparation process. The total cell number (CN) of yeast including dead and viable cells (abbreviated to "total CN") in the inner aqueous phase was determined using a hemacytometer. The viable CN in the phase was determined as the number of colony forming units (cfu) on an agar medium (108 medium containing 1.5% agar). The viability of yeast cells in the phase was determined as [(viable CN)/(total CN)] $\times$ 100.

Determination of monomer conversions

Monomer conversion was determined with a gravimetric method [10]. Microcapsules were prepared at the weight ratio inner aqueous phase:oil phase = 1:5 without yeast cells. The agitation rate was fixed at 200 rpm, and the agitation time after

addition of W/O emulsion to the outer aqueous phase was varied from 1 to 8 h. One milliliter of 1% (w/v) hydroquinone solution, a radical inhibitor, was then added to the outer aqueous solution. Collected microcapsules were suspended in a large amount of 99% (v/v) ethanol solution at 4 °C and gently stirred for 30 min to remove unreacted monomer from the microcapsules. Each sample was then vacuum dried. Monomer conversion was calculated as [(weight of dried microcapsules)/(total weight of monomer + initiator in oil phase)] × 100. We ignored the weight of medium components in the inner aqueous phase in the above calculation because the weight was very small compared with those of monomer and initiator.

Determination of encapsulation efficiency of yeast cells

After microcapsule preparation, total CN of yeast cells in the outer aqueous solution was measured using a hemacytometer. Encapsulation efficiency of the cells in microcapsules was calculated as [1 − (total CN in outer aqueous solution after polymerization)/(total CN in inner aqueous phase before polymerization)] × 100.

Determination of undamaged percentage of encapsulated yeast cells

A portion (approximately 2 g) of collected microcapsules was carefully ground and suspended in 20 mL saline. The suspension was gently stirred for 30 min at 4 °C to release yeast cells from crushed microcapsules. The viable CN of the cells in microcapsules was calculated on the basis of that in the saline, which was determined using an agar medium as described above. The viability of encapsulated cells was determined as {(viable CN in microcapsules)/[(total CN in inner aqueous phase before polymerization) − (total CN in outer aqueous phase after polymerization)]} × 100. The undamaged percentage of the cells after polymerization was calculated as [(viability of encapsulated cells)/(viability of the cells in inner aqueous phase before polymerization)] × 100.

Observation by scanning electron microscopy

Microcapsules were sputter-coated with gold and their morphology observed by scanning electron microscopy (SEM; SS-550; Shimadzu, Kyoto, Japan) at the Center of Advanced Instrumental Analysis, Kyushu University.

Statistical analysis

Data are presented as mean ± standard deviation and compared using one-way analysis of variance (ANOVA).

Results and discussion

In this study, we aimed to prepare solid polymeric microcapsules enclosing microbial cells by radical suspension polymerization via W/O/W emulsion.

Although 2,2′-azobisisobutyronitrile (AIBN, 10 h half-life decomposition temperature in toluene (10 HDT): 65 °C) and 2,2′-azobis(2,4-dimethylvaleronitrile) (ADVN, 10 HDT: 51 °C) are commonly used as a radical initiators in polymerization reactions, they require an elevated temperature for generation of radicals (generally >50 °C) [10, 11]. Those conditions are unsuitable for encapsulation of microbial cells because such high temperatures cause serious cell damage or extinction of the cells except for heat-resistant microorganisms. It has been reported that a similar azonitrile compound, 2,2′-azobis-(2,4-dimethyl-4-methoxyvaleronitrile), generates an adequate amount of radical species for radical polymerization at room temperature or below, because the half life data of this compound (10 HDT: 30 °C) indicated a more unstable species than AIBN and ADVN [12]. Thus, we selected the material as a radical initiator for preparation of microcapsules enclosing microbial cells.

We first investigated the polymerization time that achieved high monomer conversion without using yeast cells, because the conversion closely correlates with mechanical strength of the resultant microcapsules. Figure 2 shows the relationship between monomer conversion and polymerization time. The conversion at 4 h polymerization time was 82.6% and the value hardly increased at longer times, which indicates that a polymerization time of 4 h was sufficient for polymerization of the monomer in this suspension polymerization system. The diameters of the microcapsules prepared under each condition were approximately 450 μm. Examination using SEM revealed that the microcapsules had a multihollow-type inner structure (data not shown). Microcapsules with yeast cells had similar inner structure to those without the cells (Fig. 3a, c). Encapsulation efficiencies at reaction times 2, 4, and 8 h were almost the same, and the values were approximately 60% (Fig. 4). Although undamaged cell percentage decreased with increase in polymerization time, the value at 4 h reaction was more than 30%. We confirmed that mechanical stirring did not cause cell damage (data not shown). The

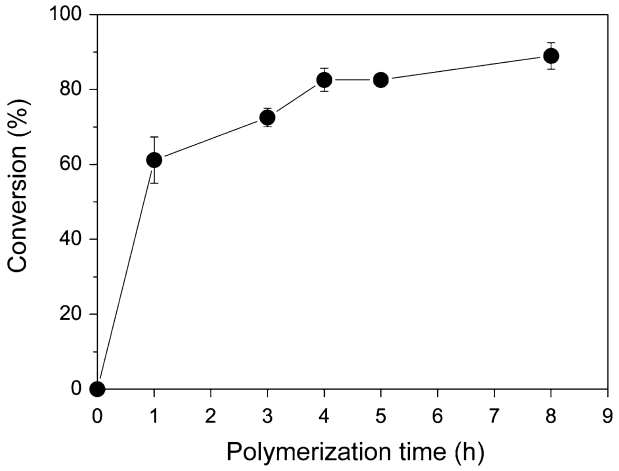

Fig. 2 Dependence of monomer conversion of microcapsules on polymerization time

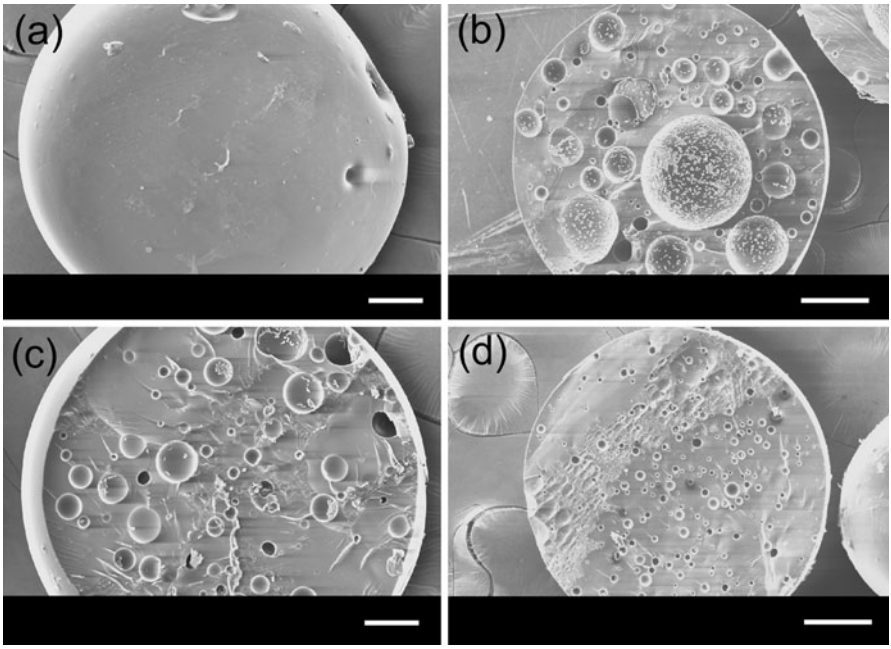

Fig. 3 **a** Surface morphology of microcapsules enclosing yeast cells prepared with polymerization time 4 h and weight ratio inner aqueous phase:oil phase = 1:5, **b–d** cross-sections of the microcapsules prepared with polymerization time 4 h and weight ratios **b** 1:2; **c** 1:5; **d** 1:20, *Bars*: **a**, **c** 100 μm; **b**, **d** 200 μm

reason of decrease of the percentage with increase in polymerization time might be due to depletion of nutrient in inner aqueous solution associated with microbial metabolism. These observations indicate that the polymerization process did not cause significant damage to the encapsulated yeast cells.

Subsequently, we investigated the effects of preparation parameters, such as weight ratio of inner aqueous phase to oil phase and agitation rate, on the microcapsule characteristics. The inner aqueous phase:oil phase weight ratio was varied from 1:2 to 1:20 with agitation rate and polymerization time fixed at 200 rpm and 4 h, respectively. The pore size inside the microcapsules increased with increase in the weight ratio (Fig. 3b–d) due to coalescence of inner aqueous phase droplets during the polymerization process. The encapsulation efficiency at ratio 1:2 was lower than at other ratios ($p < 0.05$, Fig. 5). We speculate that the low encapsulation efficiency at the ratio 1:2 was due to decreased stability of W/O/W emulsion associated with increased size of inner aqueous phase droplets in the oil phase, as shown in Fig. 3b [13]. Very little difference in undamaged percentage of encapsulated yeast cells was found among the various conditions that were used.

The size of the cell-enclosing microcapsules significantly influences their performance such as production rate of useful compound [14] and their handling ability. The agitation rate is the main factor that affects the size, so it is important for controlling the size of the microcapsules to obtain information on the

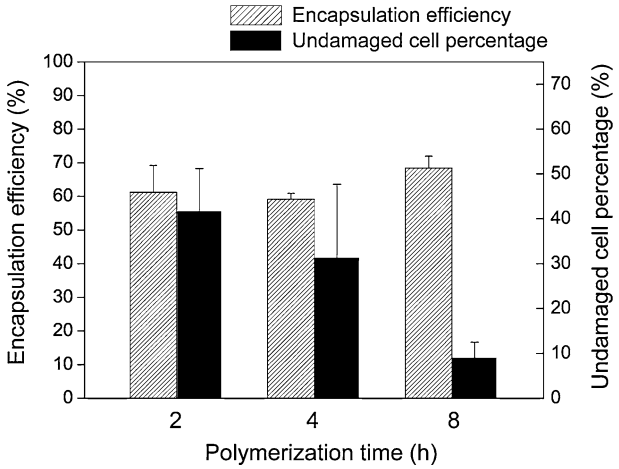

Fig. 4 Encapsulation efficiency and undamaged percentage of yeast cells as a function of polymerization time

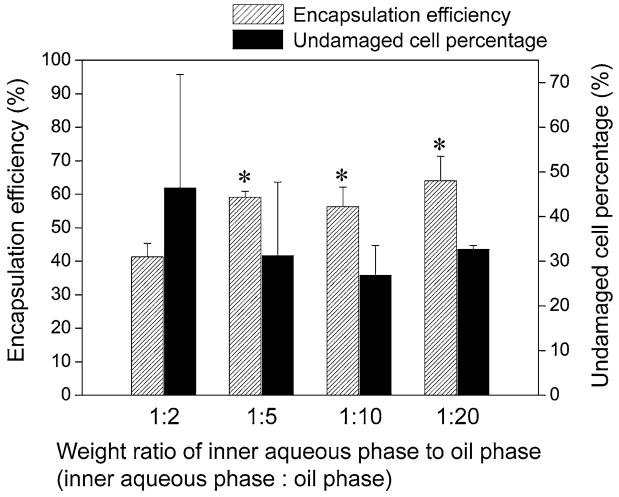

Fig. 5 Encapsulation efficiency and undamaged percentage of yeast cells as a function of weight ratio of inner aqueous phase to oil phase, * $p < 0.05$ versus weight ratio 1:2

relationship between agitation rate and microcapsule size. Thus the agitation rate was varied from 150 to 300 rpm with the weight ratio and polymerization time fixed at 1:5 and 4 h, respectively. The diameter of microcapsules prepared at 150 rpm was 1050 ± 453 μm (Fig. 6). The diameter decreased as the agitation rate increased, and became 216 ± 102 μm at 300 rpm. The agitation rate had little influence on encapsulation efficiency and undamaged percentage of yeast cells (Fig. 7).

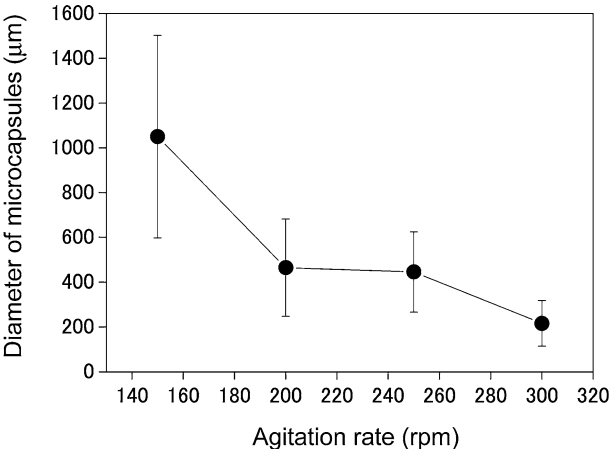

Fig. 6 Diameter of microcapsules as a function of agitation rate

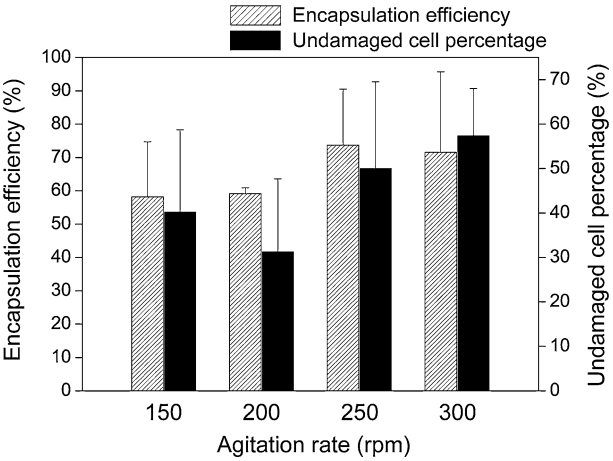

Fig. 7 Encapsulation efficiency and undamaged percentage of yeast cells as a function of agitation rate

Conclusion

We prepared solid polymeric microcapsules enclosing yeast cells by radical suspension polymerization via W/O/W emulsion. The polymerization process did not cause significant damage to encapsulated yeast cells. Decrease in the weight ratio of aqueous phase suspending the cells to oil phase resulted in increased encapsulation efficiency of the cells. The diameter of the microcapsules could be controlled by varying the agitation rate.

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References

1. Moslemy P, Guiot SR, Neufeld RJ (2002) Production of size-controlled gellan gum microbeads encapsulating gasoline-degrading bacteria. Enzyme Microb Technol 30:10–18
2. Buyukgungor H (1992) Stability of *Lactobacillus bulgaricus* immobilized in kappa-carrageenan gels. J Chem Technol Biotechnol 53:173–175
3. Talebnia F, Niklasson C, Taherzadeh MJ (2005) Ethanol production from glucose and dilute-acid hydrolyzates by encapsulated *S. cerevisiae*. Biotechnol Bioeng 90:345–353
4. Uemura Y, Hamakawa N, Yoshizawa H, Ando H, Ijichi K, Hatate Y (2000) Effect of calcium alginate coating on the performance of immobilized yeast cells in calcium alginate beads. Chem Eng Commun 177:1–14
5. Bowen GD, Rovira AD (1999) The rhizosphere and its management to improve plant growth. Adv Agron 66:1–102
6. Cassidy MB, Lee H, Trevors JT (1996) Environmental applications of immobilized microbial cells: a review. J Ind Microbiol 16:79–101
7. Guobin S, Jianmin X, Chen G, Huizhou L, Jiayong C (2005) Biodesulfurization using *Pseudomonas delafieldii* in magnetic polyvinyl alcohol beads. Lett Appl Microbiol 40:30–36
8. Tenokuchi D, Yoshida M, Hatanaka C, Toorisaka E, Hatate Y (2006) Core-shell microbioreactor microencapsulated denitrifying bacteria for nitrate-nitrogen treatment. Polym Bull 56:275–284
9. Laane C, Boeren S, Vos K, Veeger C (1987) Rules for optimization of biocatalysis in organic-solvents. Biotechnol Bioeng 30:81–87
10. Kim JW, Ko JY, Jun JB, Chang IS, Kang HH, Suh KD (2003) Multihollow polymer microcapsules by water-in-oil-in-water emulsion polymerization: morphological study and entrapment characteristics. Colloid Polym Sci 281:157–163
11. Ho CH, Chen SA, Amiridis MD, VanZee JW (1997) Dispersion polymerization of styrene in alcohol media: effect of initiator concentration, solvent polarity, and temperature on the rate of polymerization. J Polym Sci Pol Chem 35:2907–2915
12. Kita Y, Sano A, Yamaguchi T, Oka M, Gotanda K, Matsugi M (1997) Additions of malononitrile radicals to alkenes under mild conditions using 2,2′-azobis-(2,4-dimethyl-4-methoxyvaleronitrile) (V-70) as an initiator. Tetrahedron Lett 38:3549–3552
13. Takei T, Yoshida M, Hatate Y, Shiomori K, Kiyoyama S (2008) Lactic acid bacteria-enclosing poly(ε-caprolactone) microcapsules as soil bio-amendment. J Biosci Bioeng 106:268–272
14. Naoshima Y, Nakamura A, Nishiyama T, Haramaki T, Mende M, Munakata Y (1989) Asymmetric bioreduction of ethyl 3-oxobutanoate by immobilized baker's yeast entrapped in calcium alginate beads. Application of the immobilized biocatalyst to the synthesis of (5z,13s)-5-tetradecen-13-olide, aggregation pheromone of cryptolestes grain beetle. Chem Lett 6:1023–1026