Biotechnology and Bioprocess Engineering 22: 89-94 (2017)
DOI 10.1007/s12257-016-0381-4

RESEARCH PAPER

Bacterial Cellulose-chitosan Composite Hydrogel Beads for Enzyme Immobilization

Hyun Jung Kim, Ju Nam Jin, Eunsung Kan, Kwang Jin Kim, and Sang Hyun Lee

Received: 14 June 2016 / Revised: 16 November 2016 / Accepted: 28 December 2016

Abstract In this work, we report the preparation of bacterial cellulose (BC)-chitosan composite hydrogel beads by co-dissolution of BC and chitosan in 1-ethyl-3-methylimidazolium acetate and subsequent reconstitution with distilled water. The BC-chitosan hydrogel beads were used as enzyme supports for immobilizing *Candida rugosa* lipase by physical adsorption and covalent cross-linking. BC-chitosan hydrogel beads immobilized lipase more efficiently than microcrystalline cellulose (MCC)-chitosan hydrogel beads. The amount of protein adsorbed onto BC-chitosan beads was 3.9 times higher than that adsorbed onto MCC-chitosan beads, and the catalytic activity of lipase was 1.9 times higher on the BC-chitosan beads. The lipase showed the highest thermal and operational stability when covalently cross-linked on BC-chitosan hydrogel beads. The half-life time of the lipase cross-linked on BC-chitosan bead at 60°C was 22.7 times higher than that of free lipase. Owing to their inherent biocompatibility and biodegradability, the BC-chitosan composite hydrogel beads described here could be used to immobilize proteins for various biomedical, environmental, and biocatalytic applications.

Keywords: bacterial cellulose, chitosan, hydrogel bead, lipase, immobilization

Hyun Jung Kim, Sang Hyun Lee*
Department of Microbial Engineering, Konkuk University, Seoul 05025, Korea
Tel: +82-2-2049-6269; Fax: +82-2-3437-8360
E-mail: sanghlee@konkuk.ac.kr

Ju Nam Jin
Alvogen Korea, Seoul 07326, Korea

Eunsung Kan
Texas A&M AGRILIFE Research & Extension Center, Texas A&M University, Stephenville, TX 76401, USA
Office of Sponsored Projects, Tarleton State University, Stephenville, TX 76401, USA

Kwang Jin Kim
Urban Agriculture Research Division, National Institute of Horticultural and Herbal Science, Rural Development Administration, Suwon 441-440, Korea

1. Introduction

Some bacteria such as *Gluconacetobacter*, *Agrobacterium, Rhizobium*, *Rhodobacter*, and *Sarcina* can produce extracellular cellulose [1]. Owing to its inherent biocompatibility and biodegradability, bacterial cellulose (BC) has many potential applications in the biocatalytic, biomedical, and pharmaceutical fields [2]. Several enzymes, including glucose oxidase, glutamate decarboxylase, laccase, and urease, have been immobilized on BC membranes [2-5]. The structural features of BC are superior to those of plant cellulose, and BC has unique properties such as high purity, high crystallinity, high degree of polymerization, and remarkable mechanical strength [6]. However, the preparation of BC beads to be used as general enzyme supports is very difficult because BC cannot be dissolved in general solvents because of its many intramolecular hydrogen bonds. Recently, a few examples of the dissolution and regeneration of BC by environmentally friendly ionic liquids (ILs) have been reported. Schlufter *et al*. reported the dissolution, chemical modification, and regeneration of BC by 1-butyl-3-methylimidazolium chloride [7]. Okushita *et al*. investigated the mechanism of BC solubilization by ILs and the characteristics of regenerated BC [8]. These results show that BC can be fabricated into various forms such as beads, films, and fibers by ILs.

Recently, various cellulose-chitosan composite materials have been developed owing to the ability of ILs to co-

dissolve cellulose and chitosan. For example, Sun *et al*. prepared cellulose-chitosan beads for heavy metal ion adsorption [9]. Park *et al*. prepared cellulose-chitosan microfibers by electrospinning [10]. Stefanescu *et al*. investigated the preparation and characterization of cellulose-chitosan films [11]. Cellulose-chitosan composites can also be used as enzyme supports, because both cellulose and chitosan are biocompatible, and enzymes can be covalently bound to the amino groups of chitosan. Kim *et al*. reported the entrapment of lipase into a cellulose-chitosan hydrogel bead [12]. Peng *et al*. reported the preparation of magnetic cellulose-chitosan composite microspheres and their application in laccase immobilization [13]. Although several plant-based cellulose-chitosan composite materials have been developed, the preparation of BC-chitosan composites by dissolution and regeneration has not been reported. BC-chitosan composite materials may have advantages over plant-based cellulose-chitosan composites, owing to the unique properties of BC.

In this study, BC-chitosan composite hydrogel beads were successfully prepared by co-dissolution of BC and chitosan in 1-ethyl-3-methylimidazolium acetate ([Emim][Ac]), followed by reconstitution with distilled water. The potential applicability of BC-chitosan hydrogel beads as enzyme supports was investigated by immobilizing *Candida rugosa* lipase by physical adsorption and cross-linking. The characteristics of BC-chitosan hydrogel beads were compared with those of microcrystalline cellulose-chitosan hydrogel beads. Here, we studied the lipase loading capacity of BC-chitosan hydrogel beads, and the catalytic activity, thermal stability, and operational stability of lipase immobilized on them.

2. Materials and Methods

2.1. Materials

Microcrystalline cellulose (MCC, product number 435236), chitosan (medium molecular weight, 75-85% deacetylated), [Emim][Ac], *p*-nitrophenyl butyrate, *p*-nitrophenol, monobasic sodium phosphate, dibasic sodium phosphate, 25% glutaraldehyde (GA) solution, and lipase from *C. rugosa* were purchased from Sigma-Aldrich (St. Louis, MO, USA). Yeast extract and peptone were obtained from BD (Franklin Lakes, NJ, USA). Citric acid, acetic acid, glucose, mannitol, ethanol, sodium hydroxide, isopropanol, and acetonitrile were purchased from Samchun Pure Chemical (Gyeonggi-do, South Korea). *Gluconacetobacter xylinus* (KCCM 41431, ATCC 11142) was purchased from Korean Culture Center of Microorganism (Seoul, South Korea). All chemicals used in this study were of analytical grade and were used without further purification.

2.2. Preparation of bacterial cellulose

G. xylinus was cultured in Hestrin-Schramm (HS) medium containing 20 g/L glucose, 5 g/L yeast extract, 5 g/L peptone, 2.7 g/L Na_2HPO_4, and 1.15 g/L citric acid. The pH of the culture medium was adjusted to 5.5 by addition of NaOH. All the cells were precultured in test tubes for 3 days, and then inoculated into 90-mm petri dishes containing 30 mL of HS media. The cultures were incubated for 7 days at 30°C under static conditions. The resulting BC hydrogel pellicle was boiled with 4% NaOH for 30 min, and then neutralized with repeated washes in 1% acetic acid [2]. The purified BC hydrogel pellicle was immersed in distilled water for 1 day, and then vacuum dried at 60°C for 2 days. The dried BC film was ground with a homogenizer and stored at room temperature until needed.

2.3. Preparation of cellulose-chitosan hydrogel beads with [Emim][Ac]

To prepare BC-chitosan hydrogel beads, 1% (w/w) chitosan was dissolved in 5 mL of [Emim][Ac] with stirring at 75°C for 3 h. After a clear chitosan solution was obtained, 3% BC powder was added to the chitosan solution and the mixture was stirred at 110°C for an additional 5 h. The transparent solution containing BC and chitosan was dried under vacuum at 60°C for 1 h to remove air bubbles. One milliliter of BC-chitosan solution was added drop-by-drop, using a 1-mL syringe with a 26-gauge needle and a syringe pump (LSP01-2A; Longer Pump, China) into 1 L of distilled water at a rate of 50 μL/min, with vigorous stirring. The hydrogel beads were then cured in distilled water for 1 h. A washing procedure was repeated three times using distilled water, and the absence of [Emim][Ac] was confirmed by measuring the optical density of the washing solution at 211 nm. The resulting BC-chitosan hydrogel beads were stored in 0.1 M phosphate buffer (pH 7.0) at 4°C until needed.

To prepare MCC-chitosan hydrogel beads, 1% (w/w) chitosan was dissolved in 5 mL of [Emim][Ac] with stirring at 75°C for 3 h. After the clear chitosan solution was obtained, 4% MCC was added to chitosan solution, and the mixture was stirred at 75°C for an additional 2 h. After the transparent MCC-chitosan solution was obtained, the procedure described above for preparing BC-chitosan hydrogel beads was followed to prepare MCC-chitosan hydrogel beads.

2.4. Activation of cellulose-chitosan hydrogel beads for lipase immobilization

The amino groups of chitosan in the cellulose-chitosan beads were converted to aldehyde groups by GA treatment in order to immobilize lipase molecules by cross-linking.

Fifty cellulose-chitosan hydrogel beads were incubated in 5 mL of 0.025% (v/v) GA solution with stirring at 25°C. After incubation for 1 h, the beads were filtered and washed with distilled water to completely remove unreacted GA. The beads were considered free of GA when the absorbance of washing solution at 280 nm was less than 0.01. The activated cellulose-chitosan hydrogel beads were stored in 0.1 M phosphate buffer (pH 7.0) at 4°C until needed.

2.5. Immobilization of lipase on cellulose-chitosan hydrogel beads

To prepare the lipase solution, 10 mg of enzyme was added to 10 mL of 0.01 M sodium phosphate buffer (pH 7.0) and mixed for 1 min. After centrifugation for 15 min at 8,000 rpm, the supernatant was used for immobilization experiments. To physically adsorb lipase onto cellulose-chitosan hydrogel beads, 10 beads were added to 1 mL of lipase solution (120 μg protein per mL) and incubated at 25°C with shaking at 120 rpm. After incubation for 5 h, the beads were filtered to remove unbound enzyme and washed three times with 0.1 M sodium phosphate buffer (pH 7.0) for 5 min at 25°C to remove weakly adsorbed lipase. The filtrate and washing solutions were collected for protein measurement. The amount of protein adsorbed on the beads was indirectly estimated by calculating the difference between the initial protein content in the enzyme solution and the total protein content in the filtrate and washing solutions. The protein content in the solutions was measured using a Micro BCA™ protein Assay Kit (Thermo Scientific, USA) according to a standard protocol.

To immobilize lipase on cellulose-chitosan hydrogel beads by cross-linking, GA-activated cellulose-chitosan hydrogel beads were added to 1 mL of lipase solution. The same procedure described for the physical adsorption of lipase onto cellulose-chitosan hydrogel beads was then followed.

2.6. Bead characterization

The wet size of the cellulose-chitosan hydrogel beads was measured using a digital caliper (Fuso, Japan). Ten beads were used to calculate the average bead size. The diameter of each bead was measured at three different angles, and the measurements were then averaged. The dry weight of hydrogel beads was measured after drying at 60°C for 12 h. For the analysis of cellulose-chitosan bead surface, the beads were frozen overnight at -70°C and then dried at -80°C under vacuum for 12 h. All freeze-dried samples were sputter-coated with gold prior to observation under a scanning electron microscope (JSM 6308; JEOL; Japan).

2.7. Determination of lipase activity

The hydrolytic activity of lipase was determined using a spectrophotometric assay. Free or immobilized lipase was placed in a 50 mL Falcon tube containing 10.5 mL of 0.1 M phosphate buffer (pH 7.0). The reaction was initiated by the addition of 0.5 mL of a substrate solution prepared by dissolving 10 mM *p*-nitrophenyl butyrate in isopropanol, and was sustained at 25°C in a water bath with agitation at 150 rpm. Periodically, 200 μL aliquots were removed, diluted with 800 μL of acetonitrile, and centrifuged to obtain the supernatant. The activity was expressed as the initial rate and determined by measuring the increase in the absorbance at 400 nm caused by *p*-nitrophenol produced during the lipase-catalyzed hydrolysis of *p*-nitrophenyl butyrate. The initial rate measurements were carried out in triplicate [12].

To measure the operational stability of the immobilized lipases, the hydrogel beads containing lipase were recovered after the reaction. The recovered beads were washed with 0.1 M phosphate buffer (pH 7.0) and the residual activity of lipase was determined by the same assay. For residual activity calculations, the initial rate of lipase was considered 100%, and subsequent measurements were expressed as percentages of the initial rate.

2.8. Thermal stability of lipase immobilized on cellulose-chitosan hydrogel beads

To measure the thermal stability of the immobilized lipase, hydrogel beads were placed in a 50-mL Falcon tube containing 1 mL of 0.1 M phosphate buffer (pH 7.0). The beads were incubated at 60°C for a determined period and then cooled in cold water for 10 min. Subsequently, the remaining activity of lipase was determined by measuring the hydrolysis of *p*-nitrophenyl butyrate as described.

3. Results and Discussion

3.1. Preparation of cellulose-chitosan hydrogel beads

To prepare BC-chitosan hydrogel beads, 3% BC and 1% chitosan were co-dissolved in [Emim][Ac]. The resulting BC-chitosan solution yielded rigid BC-chitosan hydrogel beads with uniform shapes. A BC-chitosan solution containing 4% BC and 1% chitosan was also tested, but was too viscous and could not be used to fabricate hydrogel beads with uniform shapes. Initially, MCC-chitosan hydrogel beads were prepared with 3% MCC and 1% chitosan to act as controls, however at least 4% MCC was needed to obtain rigid MCC-chitosan hydrogel beads. Therefore, the characteristics of BC-chitosan hydrogel beads prepared with 3% BC and 1% chitosan were compared with those of MCC-chitosan hydrogel beads prepared with 4% MCC and 1% chitosan. The wet bead sizes of BC-chitosan and MCC-chitosan hydrogel beads

Table 1. Characteristics of lipases immobilized on cellulose-chitosan hydrogel beads

Immobilized lipase	Wet bead size (mm)	Dried bead weight (mg/bead)	Loaded protein (μg/bead)	Activity ($\times 10^{-3}$ μmol/min per bead)	Specific activity (μmol/min per mg protein)	Haft-life time at 60°C (h)
Free lipase			0.46[a]	6.3 ± 0.5	13.7 ± 1.1	0.3
BC1 (Physically adsorbed on BC-chitosan bead)	2.0 ± 0.2	0.25	6.32	23.8 ± 1.1	3.8 ± 0.2	0.8
BC2 (Cross-linked on BC-chitosan bead)	2.0 ± 0.2	0.25	6.76	16.7 ± 1.1	2.5 ± 0.2	6.8
MC1 (Physically adsorbed on MCC-chitosan bead)	2.6 ± 0.2	0.47	1.63	12.4 ± 0.8	7.6 ± 0.2	1.1
MC2 (Cross-linked on MCC-chitosan bead)	2.6 ± 0.2	0.47	2.64	9.5± 1.2	3.6 ± 0.4	1.5

[a]Amount (μg) of free lipase used to determine activity.

were 2.0 and 2.6 mm, respectively (Table 1). The size variations in BC-chitosan and MCC-chitosan hydrogel beads were less than 10%. The dried weights of BC-chitosan and MCC-chitosan bead were 0.25 mg and 0.47 mg per bead, respectively (Table 1). The larger wet bead size and higher dried weight of MCC-chitosan beads may have resulted from the use of a higher MCC content in the fabrication solution.

3.2. Immobilization of lipase on cellulose-chitosan hydrogel beads

Candida rugosa lipase was immobilized on BC-chitosan and MCC-chitosan hydrogel beads by physical adsorption and covalent cross-linking using GA (Table 1). The activity of lipase physically adsorbed on BC-chitosan hydrogel beads (BC1) was 1.9 times higher than the activity of lipase physically adsorbed on MCC-chitosan hydrogel beads (MC1). The higher activity of BC1 may be a result of greater protein loading on BC-chitosan hydrogel beads than on MCC-chitosan hydrogel beads. The total amount of protein loaded per bead was 3.9 times higher on BC-chitosan hydrogel beads (6.32 μg) than on MCC-chitosan hydrogel beads (1.63 μg), despite the smaller size of the BC-chitosan beads. The BC-chitosan beads adsorbed 25.3 μg protein per mg dried bead (BC1), while the MCC-chitosan beads adsorbed 3.5 μg protein per mg dried bead (MC1). Based on the dry weights of the beads, the adsorbed protein content on BC-chitosan beads was 7.2 times higher than that on MCC-chitosan beads. The higher protein loading on BC-chitosan beads may be a result of more hydrogen bonds between the BC and the lipase, since there is a much higher degree of polymerization and internal hydrogen bonds in BC than MCC [14]. The specific activity of BC1 was 2 times lower than that of

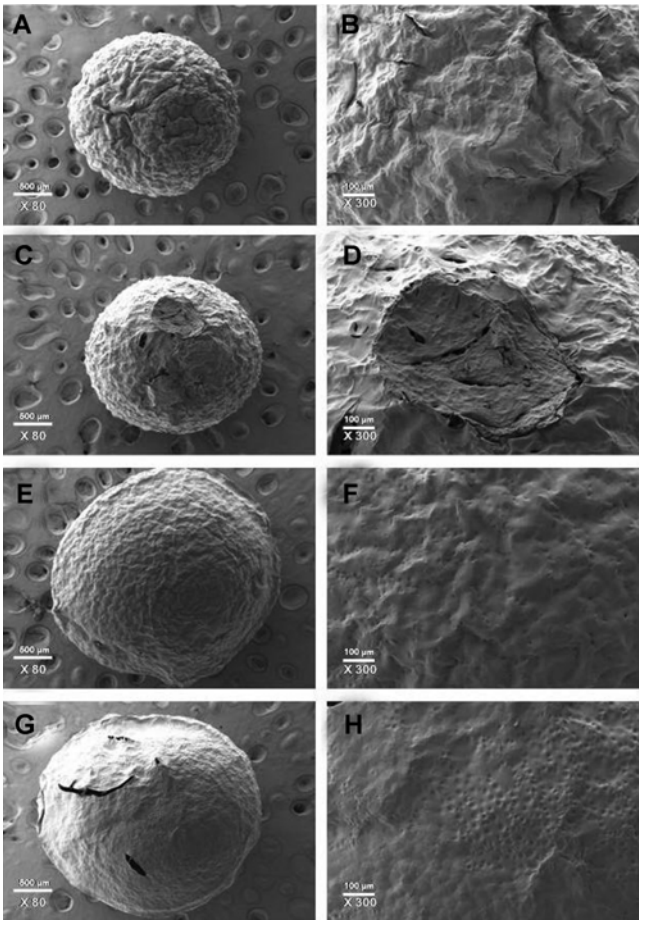

Fig. 1. Structures of cellulose-chitosan hydrogel beads. Scanning electron microscopy images of lipase-coated cellulose-chitosan beads (A, C, E, G) and their surfaces (B, D, F, H) are shown. (A, B) Images of BC-chitosan hydrogel beads with adsorbed lipases (BC1), and (C, D) cross-linked lipases (BC2) are shown. (E, F) Images of MCC-chitosan hydrogel beads with adsorbed lipases (MC1), and (G, H) cross-linked lipases (MC2) are shown.

MC1. The lower specific activity of BC1 may be caused by steric hindrance of the lipase resulting from the higher density of protein loading on BC-chitosan beads [15]. The lipases were covalently bound to the amino groups of the chitosan in the cellulose-chitosan beads by Schiff base linkage using GA activation. The activity of lipase cross-linked on BC-chitosan hydrogel beads (BC2) was 1.8 times higher than the activity of lipase cross-linked on MCC-chitosan hydrogel beads (MC2). The amount of protein covalently bound to BC-chitosan beads (BC2) was 2.6 times higher than that bound to MCC-chitosan beads (MC2). The specific activity of MC2 was 1.4 times higher than that of BC2. Overall, these results show that BC-chitosan hydrogel beads can immobilize greater amounts of lipase, yielding higher lipase activity than MCC-chitosan hydrogel beads.

Fig. 1 shows scanning electron microscopy (SEM) images of cellulose-chitosan beads coated with lipase. The BC-chitosan beads maintained their regular spherical shapes after freeze-drying, and their surface morphologies and sizes were not changed by the lipase cross-linking process. The MCC-chitosan beads showed partly microporous structures. Although the microporous structures of MCC-chitosan beads may have been advantageous for protein adsorption, the protein content adsorbed onto the BC-chitosan beads was much higher. This suggests that the higher protein adsorption capacity of BC-chitosan beads may be due to enhanced chemical interactions between BC and lipase.

3.3. Stability of lipase immobilized on cellulose-chitosan hydrogel beads

The thermal stability of lipase immobilized on cellulose-chitosan hydrogel beads was measured at 60°C (Fig. 2). The thermal stability of the lipase was slightly enhanced upon adsorption on BC-chitosan or MCC-chitosan beads, compared with the thermal stability of free lipase. Cross-linked lipases (BC2 and MC2) showed higher stabilities than adsorbed lipases (BC1 and MC1). The lipase cross-linked on BC-chitosan hydrogel beads (BC2) showed the highest thermal stability. The residual activity of BC2 was 76% after incubation for 30 min, while free lipase retained only 43% of the initial activity. The residual activity of BC2 was 44% after incubation for 10 h, whereas that of free lipase was 15%. The half-life times of free and immobilized lipases were also measured under incubation at 60°C (Table 1). The half-life time of lipase adsorbed on cellulose-chitosan beads was 2.7 ~ 3.7 times higher than that of free lipase. The half-life time of MC2 was 5.0 times higher than that of free lipase. Interestingly, the half-life time of lipase was 22.7 times longer when cross-linked to BC-chitosan beads (BC2) than that when free in solution. These results show that BC enhances the thermal stability of lipase, presumably because of the greater number of interactions between BC and lipase than between MCC and lipase. These interactions may be a consequence of the more hydrophilic nature of BC, which has more internal hydrogen bonds than MCC, but additional studies will be

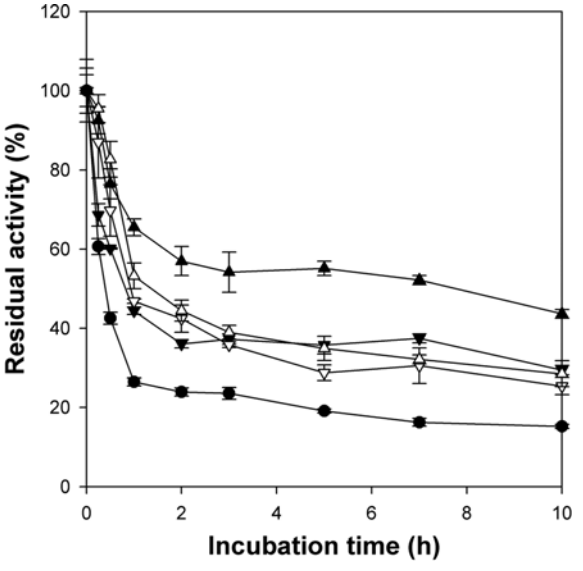

Fig. 2. Thermal stability of free and immobilized lipase. Graph shows the residual activity as a percentage of the initial activity versus the time of incubation at 60°C for lipase: Free lipase (●); lipase adsorbed on BC-chitosan beads (BC1; ▼); lipase cross-linked on BC-chitosan beads (BC2; ▲); lipase adsorbed on MCC-chitosan beads (MC1; ▽); and lipase cross-linked on MCC-chitosan beads (MC2; △).

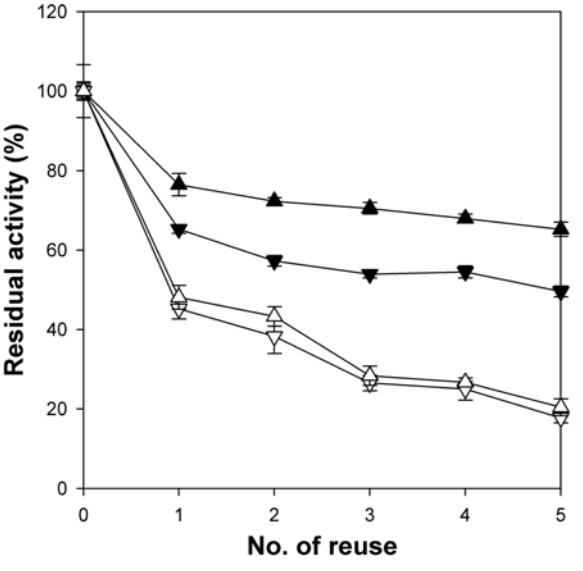

Fig. 3. Operational stability of free and immobilized lipase. Graph shows the mean residual lipase activity as a percentage of the initial activity (0 cycle) versus the number of subsequent reaction cycles performed for lipases: Lipase adsorbed on BC-chitosan beads (BC1; ▼); lipase cross-linked on BC-chitosan beads (BC2; ▲); lipase adsorbed on MCC-chitosan beads (MC1; ▽); and lipase cross-linked on MCC-chitosan beads (MC2; △).

needed to validate this hypothesis.

The residual activities of the immobilized lipases after repeated rounds of *p*-nitrophenyl butyrate reactions with intermittent rinses (operational stabilities) were also measured (Fig. 3). The activity of lipase cross-linked to BC-chitosan beads (BC2) was decreased to 76% after the first reaction, but decreased only slightly following subsequent reactions. The residual activity of BC2 was 65% of the initial activity after five reaction cycles. The highest operational stability across all cycles was seen for BC2, followed by that for BC1, then MC2, and lastly MC1, which had the lowest operational stability. Lipase immobilized on BC-chitosan beads showed much higher stability than lipase immobilized on MCC-chitosan beads. As expected, cross-linked lipase showed higher operational stability than adsorbed lipase for both bead types. When the operational and thermal stabilities are considered together, lipase cross-linked on BC-chitosan beads shows much higher stability than lipase cross-linked on MCC-chitosan beads. These results clearly show that the surface environment of a BC-chitosan hydrogel bead is more favorable for lipase immobilization than that of an MCC-chitosan hydrogel bead.

4. Conclusion

In this work, BC-chitosan composite hydrogel beads were simply prepared by co-dissolution of BC and chitosan in [Emim][Ac] and reconstitution with water. The BC-chitosan hydrogel beads were successfully used as enzyme supports to immobilize *C. rugosa* lipase, which was easily cross-linked to the amino groups of chitosan by GA treatment. BC-chitosan hydrogel beads immobilized greater amounts of lipase, yielding greater catalytic activity, and better enzyme stability than MCC-chitosan hydrogel beads. These features may be attributed to the biocompatible structure of BC, which has many internal hydrogen bonds and a high degree of polymerization. Overall, BC-chitosan composite hydrogel beads are biocompatible and biodegradable supports, with many potential biomedical, environmental, and biocatalytic applications.

Acknowledgement

This study was supported by Konkuk University in 2014.

References

1. Iguchi, M., S. Yamanaka, and A. Budhiono (2000) Bacterial cellulose-a masterpiece of nature's arts. *J. Mater. Sci.* 35: 261-270.
2. Kim, Y. H., S. Park, K. Won, H. J. Kim, and S. H. Lee (2013) Bacterial cellulose-carbon nanotube composite as a biocompatible electrode for the direct electron transfer of glucose oxidase. *J. Chem. Technol. Biotechnol.* 88: 1067-1070.
3. Akduman, B., M. Uygun, E. P. Coban, D. A. Uygun, H. Biyik, and S. Akgol (2013) Reversible immobilization of urease by using bacterial cellulose nanofibers. *Appl. Biochem. Biotechnol.* 171: 2285-2294.
4. Frazao, C. J. R., N. H. C. Silva, C. S. R. Freire, A. J. D. Silvestre, A. M. R. B. Xavier, and A. P. M. Tavares (2014) Bacterial cellulose as carrier for immobilization of laccase: Optimization and characterization. *Eng. Life Sci.* 14: 500-508.
5. Yao, W. Y., X. Wu, J. Zhu, B. Sun, and C. Miller (2013) *In vitro* enzymatic conversion of γ-aminobutyric acid immobilization of glutamate decarboxylase with bacterial cellulose membrane and non-linear model establishment. *Enz. Microb. Technol.* 52: 258-264.
6. Shah, N., M. Ul-Islam, W. A. Khattak, and J. K. Park (2013) Overview of bacterial cellulose composites: A multipurpose advanced material. *Carbohydr. Polym.* 98: 1585-1598.
7. Schlufter, K., H. P. Schmauder, S. Dorn, and T. Heinze (2006) Efficient homogeneous chemical modification of bacterial cellulose in the ionic liquid 1-N-butyl-3-methylimidazolium chloride. *Macromol. Rapid Commun.* 27: 1670-1676.
8. Okushita, K., K. E. Chikayama, and J. Kikuchi (2012) Solubilization mechanism and characterization of the structural change of bacterial cellulose in regenerated states through ionic liquid treatment. *Biomacromol.* 13: 1323-1330.
9. Sun, X., B. Peng, Y. Ji, J. Chen, and D. Li (2009) Chitosan(chitin)/cellulose composite biosorbents prepared using ionic liquid for heavy metal ions adsorption. *AIChE J.* 5: 2062-2069.
10. Park, T. J., Y. J. Jung, S. W. Choi, H. Park, H. Kim, E. Kim, S. H. Lee, and J. H. Kim (2011) Native chitosan/cellulose composite fibers from an ionic liquid *via* electrospinning. *Macromol. Res.* 19: 213-215.
11. Stefanescu, C., H. W. Daly, and I. I. Negulescu (2012) Biocomposite films prepared from ionic liquid solutions of chitosan and cellulose. *Carbohydr. Polym.* 87: 435-443.
12. Kim, M. H., S. An, K. Won, H. J. Kim, and S. H. Lee (2012) Entrapment of enzymes into cellulose-biopolymer composite hydrogel beads using biocompatible ionic liquid. *J. Mol. Catal. B: Enz.* 75: 68-72.
13. Peng, S., H. C. Meng, L. Zhou, and J. Chang (2014) Synthesis of novel magnetic cellulose-chitosan composite microspheres and their application in laccase immobilization. *J. Nanosci. Nanotechnol.* 14: 7010-7014.
14. Shanshan, G., W. Jianqing, and J. Zhengwei (2012) Preparation of cellulose films from solution of bacterial cellulose in NMMO. *Carbohydr. Polym.* 87: 1020-1025.
15. Zhang, D. H., L. X. Yuwen, C. Li, and Y. Q. Li (2012) Effect of poly(vinyl acetate-acrylamide) microspheres properties and steric hindrance on the immobilization of *Candida rugosa* lipase. *Bioresour. Technol.* 124: 233-236.