J. Fish. Sci. Tech. 2(1), 44~51, 1999

JOURNAL OF
FISHERIES SCIENCE
AND TECHNOLOGY

The Growth of Proteolytic Bacteria Immobilized in Capsule Type

Bong-Ho Han, Su-Il Choi[1], Seon-Bong Kim and Sang-Ho Kim*

Dept. of Food Science and Technology, Pukyong National University, Pusan 608-737, Korea
[1]Dept. of Food Technology, Tong Myong College, Pusan 608-740, Korea

(Received December 1998, Accepted May 1999)

Proteolytic bacteria isolated from fermented anchovy *jeotkal* were immobilized in capsule type with 0.8% sodium alginate and $CaCl_2$/carboxymethyl cellulose (CMC). For making the immobilized capsule, the optimal concentration of both $CaCl_2$ and CMC, with respect to the membrane hardness and the growth of proteolytic bacteria in capsule, were 2.0% at following conditions : flow rate of $CaCl_2$/CMC solution and cell suspension were respectively 3.54 mℓ/min and 0.15 mℓ/min when inside diameter of inner and outer capillary tube in immobilizing apparatus were 0.32 mm, 0.74 mm, respectively. The density of proteolytic bacteria in capsule reached maximum, i.e. 10^8~10^9cells /capsule during culture under optimal conditions in TPY broth, and these were 10^2~10^4 times higher than these of before culture. During culture of proteolytic bacteria immobilized in capsule type (PBImC) for 72hrs, few growing cells were lost in the outer medium.

Key words: proteolytic bacteria, immobilized in capsule type

Introduction

Immobilization is a technique to entrap biocatalysts into the gel bead or the hollow sphere membrane by limiting free action of biocatalysts. Because of the merit of stability, reutilization, continiuos process and easy separation of product *etc.*, the immobilized biocatalysts is applied to many study fields, such as fermentation, medical treatment and environment purification area. Studies have carried out continually to search effective methods for immobilization (Jain and Ghose, 1984; Israilides et al., 1989; Yoo, 1993). There are some methods for immobilizing biocatalysts, such as entrapment, attachment and cross-linking *etc.*, especially the entrapment has utilized widely among those methods (Kim, 1995).

Usually, natural or synthetic polymer has been used as the carrier in entrapment. Synthetic polymer has some defects that it may reduce the activity of biocatalysts, since much heat generated during synthesis, and often show poisoning. And it shows that the productivity is relatively low because substrate penetration into the inner gel is difficult (Kuu and Pollack, 1983).

The sodium alginate as natural polymer, has widely used in the food industry, because of their ability to form rapidly spherical gels in the presence of calcium ion and convenience to immobilize biocatalysts. Besides, in the biocatalysts immobilized by sodium alginate, the loss of biocatalysts to the outside was few relatively during reaction because of their stability against acid and heat, and non poisoning (Anita et al., 1992).

In the case of using the immobilized enzyme, the drawback is to need high cost for complex purification of enzyme. There is also drawback on the use of the immobilized microorganism because of the portional loss of viability and activity. But these problem can be solved by the high density cultivation using the immobilized microorganism with high cell density. Immobilization of microorganism was more advantageous than that of enzyme in view of time and cost because the immobilized microorganism has the high recovery of activity (Kim, 1995).

In this study, we investigated that the conditions for making the immobilized capsule when the proteolytic bacteria isolated from the anchovy *jeotkal* fermented for 2 months were immobilized

*To whom correspondence should be addressed.
E-mail : songho@songho.co.kr
Fax : 626-8494. Tel : 620-6418

in hollow-sphere membrane using Ca-alginate membrane, and the optimal conditions for proteolytic bacteria immobilized in capsule type (PBImC).

Materials and Methods

Immobilization of proteolytic bacteria : Five kinds of proteolytic bacteria which isolated from anchovy *jeotkal* were in previous study (Kim et al., 1999), such as *Staphylococcus* sp.-1, *Staphylococcus* sp.-2, *Bacillus* sp., *Photobacterium* sp. and *Volcaniella* sp., were cultivated in TPY broth (tryptone 0.5%, peptone 0.5%, yeast extract 0.3%, Cha et al., 1988), and those cell densities were adjusted up to about 10^7 cells/mℓ. In order to immobilize proteolytic bacteria in capsule type, the mixture of carboxymethyl cellulose (CMC), $CaCl_2$ and the culture medium were dropped continually into the stirred 0.8% sodium alginate solution through inner (inside diameter : 0.32 mm) and outer capillary tube (inside diameter : 0.74 mm) in immobilizing apparatus with 3.54 mℓ/min and 0.15 mℓ/min, respectively, as shown in Fig. 1 (Kim et al., 1997). To stabilize capsule membrane, the capsules, which were made spherically in sodium alginate solution, were soaked in the sterilized 2% $CaCl_2$ solution until used. The hardness of capsule was measured by Rheometer (Compac-100, Sun Scientific Co., Japan). The shape of the immobilized capsule was photographed by Micro hi-scope system (Hirox Co. Ltd., Japan), and the thickness of capsule membrane, inside diameter of inner and outer capillary tube in immobilizing apparatus was measured by microscope (Biophot type 114, Nicon Co. Ltd., Japan) and electronic digital calipers (Whirt Worth Co. Ltd., USA), respectively.

Culture of PBImC : PBImC was cultivated in shaking incubator (HB-201SF, Han Baek Scientific Co., Korea) controlled to 40℃, 200rpm. During PBImC culture, the same amount of PBImC was taken up every 2 hrs. Those were inserted into the sterilized 0.1 M phosphate buffer (pH 7.0) and stayed for 4~5 days at 4℃ for dissolving capsule membrane. After dissolving a capsule membrane, densities of proteolytic bacteria were measured by the method of A.P.H.A (1970).

Results and Discussion

Formation of the immobilized capsule : As shown in Fig. 2, the sphere at the edge portion of

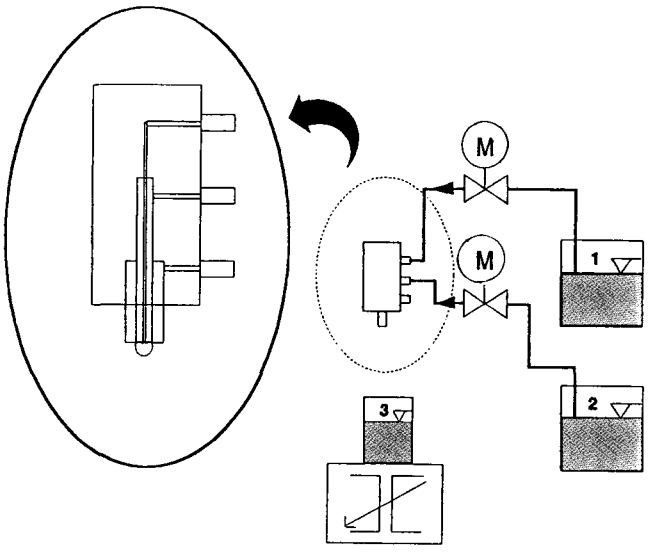

Fig. 1. Schematic diagram of immobilizing apparatus.
1. CMC/$CaCl_2$ solution, 2. Biocatalysts, 3. Sodium alginate solution

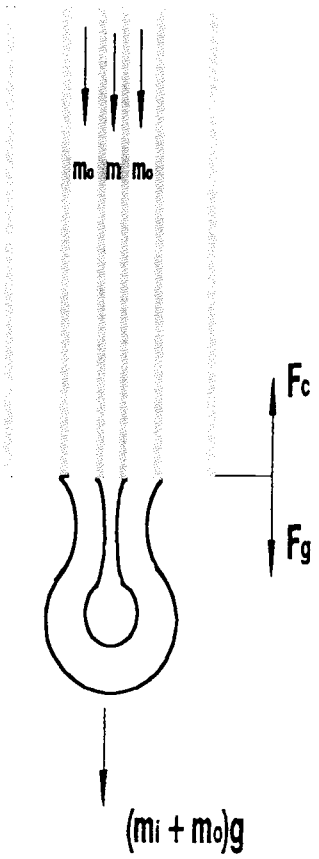

Fig. 2. Schematic diagram of the formation of sphere at the end of capillary tubes in immobilizing apparatus.

Table 1. Influence of CMC/$CaCl_2$ concentration on the formation of the immobilized capsule

CMC/ $CaCl_2$[a] Concentration (%)	Viscosities[b] of carrier solution (cp)	Flow rates of CMC/$CaCl_2$ (mℓ/min)	Sphere numbers of PB culture medium-carrier mixture[c] (spheres/min)	Formation of immobilized capsule[d]
2.0	20.8	4.1	151 ± 4.2	−
3.0	67.8	4.0	150 ± 3.6	+
4.0	209.1	3.9	152 ± 0.8	+
5.0	297.6	3.8	153 ± 1.4	+
6.0	390.5	3.8	149 ± 1.3	+
8.0	−[e]	3.7	130 ± 1.1	±

a : The mixed ratio of CMC and $CaCl_2$ was 1 : 1,
b : Viscosity was measured in a rotational viscometer at 25℃ and 1.5 rpm, using the SC-18 spindle (cylinder type),
c : Sphere numbers ; Numbers sphere formed from proteolytic bacteria culture medium-carrier (CMC/$CaCl_2$) mixture at the end of capillary tubes of the immobilizing apparatus,
d : + ; Forming capsule, − ; Not forming capsule, ± ; Ratio of forming capsule was about 50%
e : Viscosity could not measured at condition of b,
Flow rate of proteolytic bacteria culture medium : 0.15 mℓ/min, Repeat runs of measurement : 5 times.

inner and outer capillary tube is formed by droping into the 0.8% sodium alginate when the force, F_g is higher than the capillary attraction F_c (Cho, 1994; Song and Park, 1995; Kim, 1994).

If the formed sphere hangs on with maintaining a contact angle 0 between the edge portion of the outer or inner capillary tubes and the formed sphere, the gravity of sphere F_g ($g \cdot cm/s^2$) and the capillary attraction F_c (dyne) can be represented as $F_g = F_c$. This equation can be replaced as follows,

$$m \cdot g = 2 \cdot \pi \cdot r \cdot \gamma \quad (1)$$

where m, g, r and γ are weight of sphere (g), surface tension (dyne/cm), acceleration of gravity (cm/s^2) and outer radius of capillary tube (cm), respectively.

After sphere is dropped into the sodium alginate solution, outer calcium alginate membrane, one of the hollow-sphere gel membrane type, is formed by the combination of a Ca^{2+} ion in the $CaCl_2$ of sphere and a -COOH radical in sodium alginate. Thus, the immobilized capsule is formed (Cho, 19 94). However, if perfectly spherical capsule is not formed or tails are created around capsule space by some mistakes during immobilizing proteolytic bacteria in capsule type, these abnormal capsules can be broken or cracked during cultivation, which result in the loss of proteolytic bacteria on outside of capsule. Therefore, it is important to make spherical capsule perfectly with respect to the immobilizing of proteolytic bacteria in capsule type (Chung and Park, 1995).

To determine optimal conditions for making spherical capsule perfectly, when supply rate of culture medium into the immobilizing apparatus were adjusted 0.15 mℓ/min, changes of carrier viscosities and numbers of sphere, which was formed by proteolytic bacteria culture medium/ mixture of carrier on the edge of the outer and inner capillary tubes in immobilizing apparatus, were measured with different concentration of carrier, as shown in Table 1.

When the concentration of CMC and $CaCl_2$ were below 2%, it is suggested that supply rate of CMC /$CaCl_2$ solution was kept up regularly with 3.9~4.1 mℓ/min because the viscosities of carrier were low relatively as below 209.1cp.

Thus numbers of sphere of proteolytic bacteria culture medium/ (CMC/$CaCl_2$) mixture, which were formed with breaking equilibrium of $F_g = F_c$, were regular. Numbers of the immobilized capsule in 0.8 % sodium alginate solution were similar to numbers of those sphere. However the immobilized capsule was not formed at 1.0% of CMC and $CaCl_2$. It could be considered that this reason was caused the immobilized capsule formed in 0.8% sodium alginate was affected from the concentration of Ca^{2+} ion. Supply rate of carrier tended to slow by increasing carrier viscosities at the concentrations of CMC and $CaCl_2$ exceeding 2%. Numbers of sphere of proteolytic bacteria culture medium/ (CMC/$CaCl_2$) mixture and the immobilized capsule formed in 0.8 % sodium alginate solution also tended to decrease comparing with the concentration of both CMC and $CaCl_2$ below 2%.

From the equation (1), surface tension is represented as written below (2) :

$$\gamma=(r \cdot \rho \cdot g \cdot \ell)/2 \quad (2)$$

where ρ and ℓ are density of carrier/biocatalysts mixture (g/cm^3) and length of capillary tube (cm), respectively. Capillary attraction, F_c, can be replaced as the equation (3) from the equation (1) :

$$F_c=2 \cdot \gamma/r=\rho \cdot g \cdot h \quad (3)$$

Therefore it was considered that the surface tension in the equation (2) and the capillary attraction in the equation (3) might have been decreased due to the increased total density of carrier/biocatalysts mixture, ρ, when the concentration of CMC/$CaCl_2$ mixture were increased under the condition that proteolytic bacteria with fixed concentration was supplied to immobilizing apparatus. For this reason, it was considered that numbers of sphere formed at the edge of capillary tubes might be decreased somewhat when the concentration of both CMC/$CaCl_2$, carrier has been exceeded 2.0%.

Carrier concentration : In order to entrap biocatalysts in the hollow-sphere gel membrane for making PBImC, it was important to consider the capsule size, physical strength, and substrate transfer through the membrane, i.e. mass transfer, etc. (Kim et al., 1989; Chun et al., 1996).

Staphylococcus sp.-2 among the proteolytic bacteria in previous study (Kim et al., 1998) was immobilized in capsule type with CMC/$CaCl_2$ concentration, and the capsule hardness, membrane thickness and growth of proteolytic bacteria in capsule were investigated. The capsule hardness and diameter were increased with increasing CMC/$CaCl_2$ concentration, but there were few differences at over 2% of CMC, $CaCl_2$ concentration as shown in Table 2. Capsule hardness and diameter might be increased with increasing of CMC/$CaCl_2$ concentration, since Ca^{2+} ion concentration, which combined with -COOH in alginate, has been increased with increasing concentration of CMC/$CaCl_2$, and thickness of calcium alginate membrane was increased. Capsule hardness and diameter increased hardly over 2% CMC and 2% $CaCl_2$ because membrane has been formed enough. Similar to these results, Cho (1994) reported that membrane thickness of capsule increased much more with 112~939 μm, according to increasing $CaCl_2$ concentration in the range of 0.5~4.0% $CaCl_2$ and 1.5% CMC.

$CaCl_2$ concentration was changed and mixed with 2.0% CMC for immobilizing. The changes of the immobilized *Staphylococcus* sp.-2 growth in capsule after culture for 24hrs and thickness of capsule membrane with $CaCl_2$ concentration are shown in Table 3. The thickness of capsule membranes were increased with increasing of $CaCl_2$ concentration. *Staphylococcus* sp.-2 growth in capsule was decreased with increasing membrane thickness. Mass transfer rate of nutrition component through the membrane might be slow due to increasing thickness of capsule membrane. As similar this result, Kim et al. (1997) reported that hydrolysis ratio shown few difference over 2% CMC and $CaCl_2$ in the study on the minced anchovy hydrolysis

Table 2. Influence of CMC/$CaCl_2$ concentration on the hardness of immobilized capsule membrane and diameter of the capsule

CMC/$CaCl_2$ concentration (%)[a]	Hardness (×10 dyne/cm^2)	Capsule diameter (mm)
2.0	15.56 ± 2.52	3.24 ± 0.08
3.0	20.78 ± 2.87	3.67 ± 0.08
4.0	24.72 ± 4.63	4.05 ± 0.04
5.0	24.80 ± 4.08	4.16 ± 0.08
6.0	26.98 ± 2.73	4.26 ± 0.05

a : The mixed ratio of CMC and $CaCl_2$ was 1 : 1, Hardness measuring conditions : Maximum weight ; 10 kg, Distance ; 4.2 mm, Velocity ; 60 mm/min, Repeat runs of measurement : 5 times.

Table 3. Influence of $CaCl_2$ concentration on the membrane thickness of the immobilized capsule and bacterial growth in capsule

$CaCl_2$ concentration (%)[a]	Membrane thickness (μm)[b]	Concentration of *Staphylococcus* sp. (cells/capsule)[c]
1.0	115.40 ± 0.01	—[d]
1.5	192.30 ± 0.13	2×10^8
2.0	230.80 ± 0.22	9×10^7
2.5	269.20 ± 0.33	7×10^7
3.0	307.70 ± 0.14	1×10^7
3.5	346.10 ± 0.23	3×10^6

a : $CaCl_2$ mixed with 2.0% CMC ; the mixed ratio was 1 : 1,
b : Repeat runs of measurement : 5 times,
c : Concentrations of cells measured after 24 hr shaking incubation at 40℃,
d : Capsule membrane was broken during culture
Repeat runs of the measurement of membrane thickness : 5 times.

using commercial enzyme immobilized with CMC and $CaCl_2$. Cho et al. (1989) was also recognized as the similar trend. According to the study, the growth rate of yeast, which was immobilized in alginate gel by using alginate, was decreased over 2.5% alginate. On the culture of *Staphylococcus* sp.-2 immobilized in capsule type, the capsule immobilized with 1% $CaCl_2$ was broken all during culture for 24 hours. 1.5% $CaCl_2$ was shown that the growth of *Staphylococcus* sp.-2 was fast whereas the hardness of capsule membrane became weak.

From above results, the optimal concentration of both $CaCl_2$ and CMC for immobilizing proteolytic bacteria were 2.0%.

Supply rate of carrier : When the carrier of the mixed of 2% CMC and 2% $CaCl_2$ was supplied to immobilizing apparatus with different supply rate, numbers of sphere of proteolytic bacteria culture medium/ (CMC/$CaCl_2$) solution, which were dropped into 0.8% sodium alginate solution, formation ratio of perfect spherical capsule and diameter of capsule after forming capsule are shown in Table 4. The supply rate of proteolytic bacteria culture medium was adjusted to 0.15 mℓ/min.

Supply rate of carrier with 3.54 mℓ/min might have been most advantageous respect to forming ratio of capsule, because formation ratio of perfect spherical capsule was the most highest as 99.8%. The formation ratio of perfect spherical capsule became lower gradually when supply rate of carrier was slower or faster than that of 3.54 mℓ/min. But the immobilized capsule sizes were shown few differences even if supply rate of carrier has been changed. Therefore, the supply rate of proteolytic bacteria culture medium and CMC/$CaCl_2$ solution respect to capsule hardness and bacterial growth in capsule were 0.15 mℓ/min and 3.54 mℓ/min, respectively when inside diameters of inner and outer capillary tube were 0.32 mm and 0.74 mm, respectively.

Table 4. Influence of flow rate of CMC/$CaCl_2$ on the formation of perfectly spherical capsule

Flow rate of CMC/$CaCl_2$[a] (mℓ/min)	Sphere numbers of PB culture medium-carrier mixture[b] (spheres/min)	Ratio of capsule formation (%)[c]	Capsule diameter (mm)
1.08	57	0	–[d]
2.20	85	30.35 ± 1.60	0.39 ± 0.06
2.76	121	57.52 ± 1.25	0.39 ± 0.05
3.54	150	99.80 ± 0.31	0.39 ± 0.08
4.20	185	85.63 ± 1.55	0.40 ± 0.07
4.98	210	65.42 ± 3.66	0.41 ± 0.04
5.50	250	0	–

a : The mixed ratio of CMC and $CaCl_2$ was 1 : 1,
b : Sphere numbers ; Numbers sphere formed from proteolytic bacteria culture medium-carrier (CMC/$CaCl_2$) mixture at the end of capillary tubes of the immobilizing apparatus,
c : Formation ratio of perfectly spherical capsule,
d : Capsule not formed Repeat runs of measurement : 5 times.

High density culture of proteolytic bacteria immobilized in capsule type : In order to increase productivity on fermentation process, it is recognized that the utilization of high density culture cell is effective. But high density culture cell was shown some drawback in view of time and cost because of the complication of process and equipment, impossibility of repeat run (Chun et al., 1996). But when the immobilized cell was used in fermentation process, the cell could be cultivated with high density in the immobilized capsule or bead (Yoshioka et al., 1990; Kiy et al., 1991), and cell viability was kept relatively high even though cell was cultivated for a long time (Kim et al.,1993). The cell immobilized in capsule type also showed high productivity because of lowere loss of cell in capsule outer (Lancy and Tuovinen, 1984). Therefore, PBImC cultured to high density was used for anchovy hydrolysis. PBImC using apparatus Fig. 1 was cultivated on the TPY broth with 2% NaCl at pH 7.0 and 40℃ for 72 hrs and the changes of proteolytic bacteria concentration in capsule are shown in Fig. 3.

In previous study (Kim et al., 1999), where non-immobilized proteolytic bacteria of free cell was cultivated on the TPY broth with 2% NaCl at pH 7.0 and 40℃, the maximum cell densities reached up to $6\times10^7\sim1.1\times10^8$ cells/mℓ after culture for 20 hours respect to kinds of proteolytic bacteria (Kim et al., 1998). When the culture medium of proteolytic bacteria, which was cultivated up to $6\times10^7\sim1.1\times10^8$ cells/mℓ with kinds of bacteria, was supplied into immobilizing apparatus with 0.15 mℓ/min. Those supply rates were practically became $9\times10^6\sim1.7\times10^7$ cells/min. Because the capsule forming rate was almost 150 capsules/min, the proteolytic bacteria densities in one capsule just after immobilizing were about $6\times10^4\sim1.1\times10^5$ cells/capsule. When PBImC cultivated in TPY broth, *Staphylococcus* sp.-2 could be cultivated to 10^9 cells/capsule range after 24hrs culture. *Staphylococcus* sp.-1, *Bacillus* sp.,

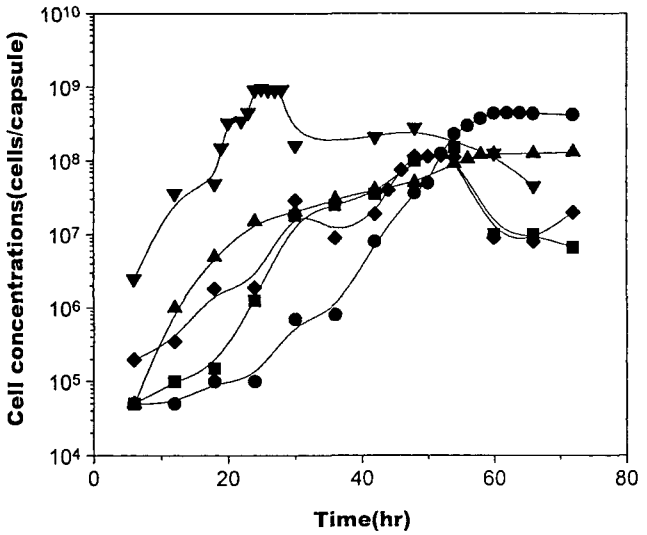

Fig. 3. Growth of the proteolytic bacteria in capsule during shaking culture in TPY broth at 40℃, pH 7.0.
−■− : *Staphylococcus* sp.-1,
−●− : *Photobacterium* sp,
−▲− : *Volcaniella* sp,
−▼− : *Staphylococcus* sp.-2,
−◆− : *Bacillus* sp.

Photobacterium sp., and *Volcaniella* sp. could be cultivated to 10^8 cells/capsule range after 54 and 60 hrs culture, respectively. It was convinced that PBImC could be cultivated to 900~1,700 times or 9,000~17,000 times higher than those obtained in the free cell culture.

Similar to the above results, Kiy and Tiedtke (1991) reported that a cell density in hollow sphere reached from 2.1×10^5 cells/mℓ to 0.9×10^7 cells/mℓ when bacteria encapsulated in hollow Ca-alginate sphere was cultivated for 2 days. Yoshioka et al. (1990) reported that the maximum cell density of mammalian cell in capsule encapsulated with chitosan/CMC reached 1×10^7 cells/mℓ, 10 times higher than that obtained in the free cell culture. Yu et al. (1997) also reported that human hepatoma cell densities in capsule encapsulated with chitosan /alginate reached from 10^5 cells/mℓ to 10^7 cells/mℓ.

Fig. 4 showed photographs of the immobilized *Staphylococcus* sp.-2 capsules before and after culture in TPY broth for 24hrs, which is magnified with 40 times on microscope. The outside of capsule composed with many layers and cell densities in capsule after 24hrs culture was increased clearly comparing with that before culture. Since it was reported generally that pore size of calcium alginate membrane was very smaller than that of bacteria diameter (Kiy and Tiedtke, 1991), it was considered that few *Staphylococcus* sp.-2 were lost through capsule membrane.

The entrapment for high density cell culture can be divided mainly 2 kinds of methods. One is bead type of the mixture of carrier and cell, and the other is capsule type of entrapped cell in hollow-sphere gel membrane. During the immobilized cell culture, it is important to minimize the cell loss to out of capsule or bead and growth at outer medium, *i.e.* outgrowing cell. During culture of the cell immobilized bead type mixing carrier and cell, it is possible to get outgrowing cell occurred by the leakage of attached cell on the bead surface or

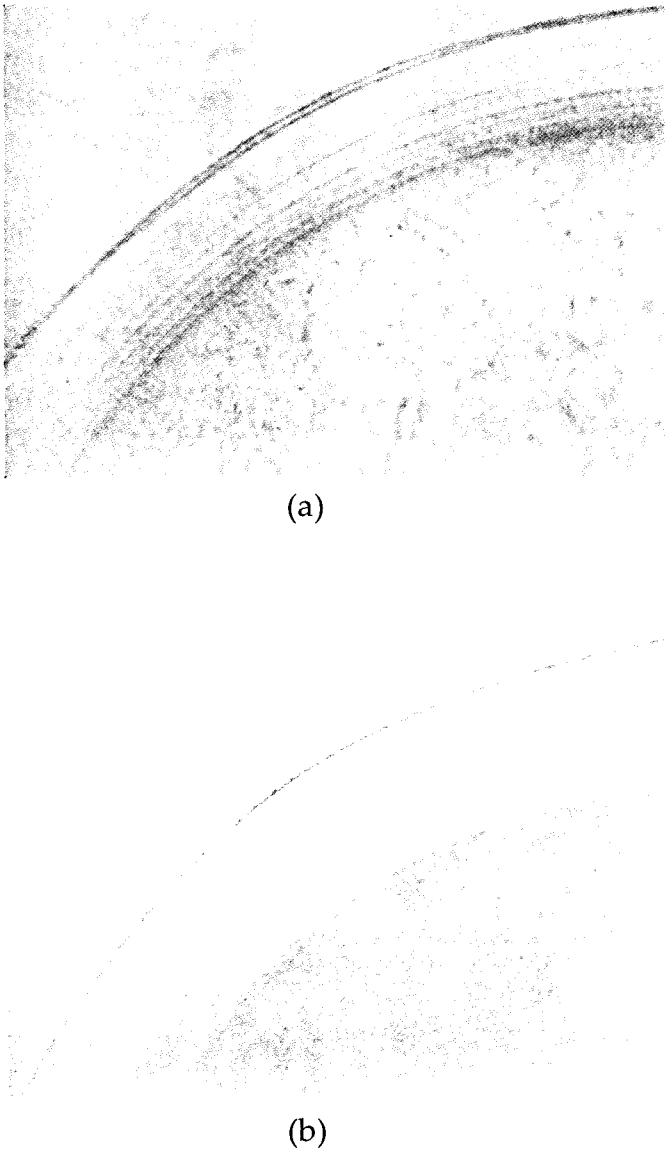

Fig. 4. Photographs of PBImC.
(a) before culture, (b) after 24hrs incubation in TPY broth

growing cell in the inner of bead during culture (Chung and Park, 1995). Because of these drawback, it was reported that capsule type was more effective than bead type respect to loss cell during culture. Roca et al. (1996) reported that the loss ratios of cell were increased slowly with elapsing culture time when *Saccahromyces cerevisiae* was immobilized in bead type for production of xylitol for 15 days.

Kim et al. (1996) also reported that amount of the lost cell was shown 40% difference with the kinds of the immobilized carrier during culture on the study for ethanol production with yeast immobilized in bead type. On the other hand, Pandya and Knorr (1991) reported that no outgrowing cell was shown in outer medium when the cell immobilized in capsule type with chitosan for 2 weeks.

To investigate outgrowing cell during culture in this study, cell densities in outer medium were detected during PBImC culture for 72 hrs. Few cell in outer medium during culture were detected. From these results, repeat run as well as withdrawing of all proteolytic bacteria after hydrolysis was possible, because few proteolytic bacteria were lost to outer medium by using of PBImC in anchovy hydrolysis.

Conclusion

In order to utilize the immobilized proteolytic bacteria for the rapid anchovy hydrolysis, optimal conditions for the immobilizing and cultivation of proteolytic bacteria were investigated. The optimal concentration of both $CaCl_2$/CMC was 2% for making the immobilized capsule. Proteolytic bacteria in immobilized capsule could be cultivated $10^2 \sim 10^4$ times higher than free cell at optimal condition. During culture for 72 hours in TPY broth, few proteolytic bacteria in the immobilized capsule were lost out of capsule. From the above results, it was considered that PBImC could be cultivated with high density compared with that of free cell, and repeat run of it was possible for anchovy hydrolysis.

Acknowledgements

This paper was devoted to the late Prof. Dr. Bong-Ho Han for praising his academic achivement.

References

Anita, M., S. Ivar and G.S. Bræk. 1992. Alginate as immobilization material : III. Diffusional properties. Biotechnol. and Bioeng., 39, 186~194.

A.P.H.A. 1970. Recommended procedure for the bacteriological examination of sea water and shellfish 3rd Ed., Am. Publ. Health Assoc. Inc., New York, 17~24.

Cha, Y.J., E.H. Lee, K.H. Lee and D.S. Chang. 1988. Characterization of the strong proteolytic bacteria isolated from low salt fermented anchovy and of protease produced by that strain. J. Kor. Fish Soc., 21 (2), 71~79.

Cho, M.G. 1994. Verfahrenstechnische Auslegung einer Apparatur zur Herstellung mikroverkapselter Biokatalysatren mit getrennter Zuführung von Katalysatörl sung und Kapselgrundsubstanz. Fortschritt Berichte VDI, Reihe 17, Biotechnik, Nr. 108.

Chun, G.T., T.H. Lee and Y.K. Chang. 1996. Determination of optimal bead size calculating effectiveness factors in cyclosporin a fermentation by immobilized cells. Kor. J. Biotechnol. Bioeng., 11 (1), 30~36.

Chung, S.H. and J.K. Park. 1995. Application of the immobilized technique of whole cell. Biothechnol. news, 2 (4), 339~347.

Israilides, C.J., A.N.C. Weir and A.T. Bull. 1989. Effect of antibiotics on lysine production in free and immobilized cells of *Bacillus subtilis*. Appl. Microbiol. Biotechnol., 32, 134~136.

Jain, D. and T.K. Ghose. 1984. Cellobiose hydrolysis using Pichia etchellsii cells immobilized in calcium alginate. *Biotechnol.* and *Bioeng.*, 26. 340~346.

Jang, G., C.R. Kim and Y.K. Lee. 1990. Studies on the immobilization of β-galactosidase from Bacillus subtilis. Kor. J. Food Sci. Technol., 22, 426.

Kim, E.Y., S.W. Kim and K. Kim. 1993. Ethanol production by a new method of alginate-immobilization. Kor. J. Appl. Microbiol. Biothechnol., 21 (4), 373~380.

Kim, K., Y.I. Sunwoo and S.C. Park. 1989. Effective diffusivity of substrate of an immobilized microorganism in Ca-alginate gel. Kor. J. Biotechnol. Bioeng., 4 (2), 110~117.

Kim, S.H., J.Y. Park, K.T. Kim, Y.S. Chung and B.H. Han. 1997. Fish meat hydrolysis using commercial proteases immobilized with alginate in capsule and bead type. Kor. J. *Biotechnol. Bioeng.*, 12 (5), 501~508.

Kim, S.H. Y.M. Kim, H.K. Seong, S.I. Choi, S.B. Kim and B.H. Han. 1999. The growth of proteolytic bacteria immobilization in capsule type. J. Fish. Sci. Tech., 2 (1), 36~43.

Kim, S.W. 1995. Production of various metabolism using immobilizing technique. *Biothechnol.* news, 2 (3), 229~241.

Kim, S.W., E.Y. Kim and Y.G. Hong. 1996. Development of alginate-celite immobilization technique for the improvement of ethanol productivity. Kor. J. Biotechnol. Bioeng., 11 (1), 77~85.

Kiy, T. and A. Tiedtke. 1991. Lysosomal enzymes produced by immobilized *Tetrahymena thermophila*. App. Microbiol. Biotechnol., 35, 14~18.

Kuu, W.Y. and J.A. Pollack. 1983. Improving immobilized biocatalysts by gel phase polymerization. Biotechnol. and Bioeng., 25, 1995~2006.

Lancy, E.D. and O.H. Tuovinen. 1984. Ferrous ion oxidation by Thiobacillus ferrooxidans immobilized in calcium alginate. App. Microbiol. Biotechnol., 20, 94~99.

Pandya, Y., and D. Knorr. 1991. Diffusion characteristics and properties of chitosan coacervate capsules. Process Biochemistry, 26, 75~81.

Roca, E., M. Meinander and B. Hahn-Hägerdal. 1996. Xylitol production by immobilized recombinant *Saccharomyces cerevisiae* in a continuous packed-bed bioreactor. Biotechnol. and Bioeng., 51, 317~326.

Yoshioka, T., R. Hirano, T. Shioya and M. Kako. 1990. Encapsulation of mammalian cell with chitosan-CMC capsule. Biotechnol. and Bioeng., 35. 66~72.

Yoo, I.S. 1993. Comparison of immobilization techniques using Phanerochaete chrysosporium for the treatment of pulp waste effluent. Kor. J. Biotechnol. Bioeng., 8 (4), 351.

Yu, S.H., J.H. Son and S.K. Kim. 1997. The growth of encapsulated human hepatoma. Kor. J. Biotechnol. Bioeng., 12 (4), 410~413.

Song, J.C. and H.J. Park. 1995. Phisical properties of Food. Ulsan Univ. Pub., p. 55~60.

Kim, B.H. 1994. Physiocochemistry dictionary. Minister of education pub., p. 1409~1410.