Alkaline Protease Production by Immobilized Growing Cells of *Serratia Marcescens* with Interpolymer Complexes of P(TM-*co*-AAm)/PAA

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ABSTRACT: The growing cells of *Serratia marcescens* (SM) were immobilized with the interpolymer complex carrier, which is formed by the cationic polymer, poly(allyltrimethyl ammonium chloride-*co*-acrylamide) [P(TM-*co*-AAm)], and poly(acrylic acid) (PAA). When the association degree of PAA is suitable to the cationic degree of P(TM-*co*-AAm), the effective crosslinking network provides the most favorable circumstances for the cell immobilization. The alkaline protease can be produced by the immobilized SM with high activity. Compared with the free cells, the immobilized SM has higher thermal stability, acid-base stability, operational stability, and storage stability. Under the optimum immobilizing conditions, not only the living cells of SM but also thermophilic *Bacillus firmus* (TBF) were immobilized with the complex of P(TM-*co*-AAm)/PAA. The results show the carrier of P(TM-*co*-AAm)/PAA complex to be superior in properties to the usual carriers, such as Na-alginate and carrageenan.
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Key words: cationic polymer; interpolymer complexes; P(TM-*co*-AAm)/PAA; immobilized cell; alkaline protease

INTRODUCTION

Immobilization of enzymes and cells is a basic bioengineering technique, which locates the free enzymes or cells into a microenvironment with retention of their biological activities.[1–3] Immobilized systems are reusable with long-term stability. Cell immobilization is developed on the basis of enzyme immobilization and is a powerful tool for using enzymes or enzyme systems from eukaryotic or prokaryotic organisms in fermentations. The multienzyme system in the immobilized cells, especially in the immobilized growing cells, facilitates the multienzyme sequence reactions. Production can be carried out in carriers, where cells exist as normal living states, so that the enzyme activities are more stable. The microenvironment created by the matrix and pores of carriers offers protection to entrapped cells. Because cell density is greater, higher substrate concentration may be used, and the quick reaction rate leads to the high productivity. The simplified culture medium and decreased contamination of products enables controlled and continuous production. Many successful examples have been reported.[4–7]

We attempted to immobilize enzymes and cells with interpolymer complexes.[8–10] Interpolymer complexes are formed by the secondary forces between various polymer chains, including hydro-

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gen bonding, coulombic force, Van der Waals force, electron donating and accepting interaction, or hydrophobic interaction in aqueous medium. These associations are sensitive to the surrounding environment and generally accompanied by a change in structures.

We synthesized a new type of cationic polymers, poly(allyltrimethyl ammonium chloride-*co*-acrylamide), [P(TM-*co*-AAm)], and their interpolymer complexes with poly(acrylic acid) (PAA).[11,12] After the low ionic interaction was introduced into the hydrogen bonding complexes, the properties of interpolymer complexes were dramatically improved. The interaction forces between the two polymer chains are pH-dependent and reversible. We reported the application of P(TM-*co*-AAm)/PAA complexes in the immobilization of chymotrypsin.[13] Incorporating relatively small amounts of ion–ion interaction can significantly enhance the thermal stability, storage stability, and the optimum temperature of the solid-phase enzymes. The characteristic pH sensitivity of this kind of complex provides favorable conditions for the enzymatic reactions. In this article, we use the same type of carrier, P(TM-*co*-AAm)/PAA complexes, to immobilize the growing cells of *Serratia marcescens* for the production of alkaline protease. Temperature, pH, and mass transport would be controlled to ensure optimal production and enzyme stability.

EXPERIMENTAL

Materials

PAA with a molecular weight of 6.4×10^4 was prepared by radical polymerization.[14] The cationic polymer P(TM-*co*-AAm) with a molecular weight of 6.8×10^4 and the cationic degree of 11.4% was synthesized by the radical polymerization, which was described in detail.[11] Other reagents (Shanghai Chemicals Co., China) were used as available.

Composition of culture medium (w/v %) were as follows: acidolized casein 0.5%, trypone 2%, yeast extract 0.5%, NaCl 0.5%, K_2HPO_4 0.3%, KH_2PO_4 0.2%, gelatin 1%, H_2O 1000 mL, and pH 7.0.

Microorganisms

Serratia marcescens N12 (SM) and thermophilic *Bacillus firmus* (TBF) (Institute of Microbiology, Academic Sinica, China) were cultured aerobically in tryptone yeast medium at pH 7.0 and 3.0, respectively, by using a rotating shaker for 24 h at 28°C. The products were washed down with saline water. After centrifugation at 6000 rpm for 20 min, the cell pellets were separated and suspended in saline water.

Immobilization of SM with the Complex of P(TM-*co*-AAm)/PAA

Ten milliliters of 10% P(TM-*co*-AAm) aqueous solution and 10 mL 10% PAA aqueous solution were sterilized at 50°C/5 mmHg for 20 min. After cooling to room temperature, the solution of P(TM-*co*-AAm) was mixed with the SM spore suspension for 20 min, and the mixture contained approximately 10^8 spores/mL. Then, the PAA solution was added dropwise into the gently stirred mixture of P(TM-*co*-AAm) and cells. After stirring for 10 min, the mixture was undisturbed for 20 min. The precipitate was separated, washed with the sterile saline water, and then dried to the constant weight.

Semicontinuous Batch Fermentation

The immobilized SM was used in the repeated replacement fermentation. After removing the cultivated medium, the immobilized cells were washed with sterile water, and 50 mL of fresh culture medium was added for the next cycle of fermentation. This procedure was subsequently repeated and the culture medium was replaced every 24 h.

Determination of Free Cell Concentration in the Medium

The concentrations of free cells were determined by turbidimetry. The optical densities (OD) at 600 nm of diluted culture samples were measured by UV-Vis spectrophotometer.

Assay of Alkaline Protease Activity

The measurements of enzyme activities were carried out as in reference 15. One milliliter of diluted inoculum and l mL 20% casein solution were mixed after preincubation in the water bath with constant temperature of 40°C for 10 min. The stopwatch was started. After the mixture solution was kept at 35°C for 20 min, 3 mL of 0.4*M* trichloroacetic acid was added with shaking. The reaction solution was filtered after standing at room temperature for 15 min. Five milliliters of

Table I Effect of Concentration of Individual Components on Protease Activity and Carrier Stability

$C_{P(TM\text{-}co\text{-}AAm)}$ or C_{PAA} (w/w %)	Stability of Carrier Granules	Concentration of Spores in the Inoculum (OD_{600})	Protease Activity in the Inoculum (u)
5	Attrited after 4 cycles	2.8	304.8
10	Stable over 6 cycles	1.4	442.1
15	Stable over 6 cycles	0.9	298.9

0.4*M* Na_2CO_3 and 1 mL Folin reagent were added to 1 mL filtrate. The mixture was incubated at 40°C for 30 min, and the absorbance at 680 nm was measured on UV-Vis spectrophotometer (UVIDEC-32.0, Shimadzu, Japan).

The activity of alkaline protease was expressed in units (u). One unit of alkaline protease was defined as the amount of tyrosine (μg) produced by the enzymatic hydrolysis of casein per minute per milliliter of alkaline protease at 40°C and pH 11. The relative activity was calculated by the ratio of the activity of alkaline protease produced by immobilized cells over the activity of alkaline protease produced by free cells.

RESULTS AND DISCUSSION

Immobilization of SM with the Complexes of P(TM-*co*-AAm)/PAA

There exists an optimum living environment for a specific kind of cells. It requires that the carriers for the immobilization have the adaptable pore capacity and pore diameter to avoid the leakage of cells and good transport property to ensure the supply of substrates and rejection of metabolites as well.

The polymer complexes of P(TM-*co*-AAm)/PAA is a porous hydrophilic gel. Such a complex network can envelope cells, but its suitable pores enable the effective mass transport of substrates, products, and oxygen, and so on, to form the favorable microenvironment for the living cells. The interpolymer complexes have higher elasticity and strength because of the crosslinking between the polymer chains. The network can be occupied by water with various forms: bound water, oriented water, and free water. When the concentration of the components of the complex is lower, the network structure with the lower crosslinking points is easy of the higher cell leakage because of bigger pore sizes. When the concentration of components is higher, the fine and close porous structure blocks the permeation of the substrates and products. The lower efficiency of mass transport brings about the lower enzyme product activity.

In this work, the immobilized cells were prepared at pH 3 by using complexes of P(TM-*co*-AAm)/PAA with the different concentrations of individual components: 5, 10, and 15%. The comparisons of the immobilization yield of SM and the activity of enzyme produced by the immobilized SM are listed in Table I.

When choosing the carrier component concentration of 15%, the component solutions are too viscous to be mixed well with cell suspensions, whereas the component solutions with concentration of 10% are relatively viscous but may be mixed homogeneously with cell suspension. As shown in Table I, the concentration of 10% of the individual component of complex, P(TM-*co*-AAm) or PAA, is suitable for the immobilization of SM, at which concentration the enzyme product alkaline protease has highest activity. The resulting material has sufficient porosity to allow the transport of substrate in and product out while retaining the cells.

When the concentration of complex components, P(TM-*co*-AAm) or PAA, is 10%, varying the pH of the component solution, the pH effect of the immobilization system on the alkaline protease production of the immobilized SM is shown in Figure 1.

The activity of alkaline protease produced by the immobilized SM is very low at the lower pH procession. The strong acidic environment probably produces a toxic effect on the living cells of SM. It is very interesting that the cell leakage of the immobilized SM is lowest, but protease activity is highest at pH 3. When pH deviates from this optimum point, the free cell concentration increases and the enzyme activity decreases. As pH is at 3, the association degree of PAA is suitable to the cationic degree of P(TM-*co*-AAm).[12] The sim-

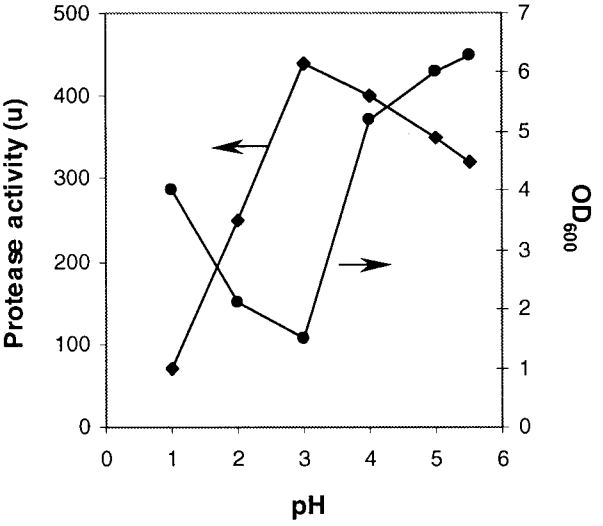


Figure 1 Alkaline protease activity and free cell concentration (OD at 600 nm) of the immobilized SM at different pHs during immobilization, $C_{P(TM\text{-}co\text{-}AAm)} = C_{PAA} = 10\%$.

ilar extended states of macromolecular chains leads to the effective formation of intermolecular crosslinking network structure for the cell immobilization with the lowest cell leakage and highest protease activity (Scheme 1).

In addition, the electrostatic interaction plays an important role in the immobilization process. Microbial cell surfaces are predominantly negatively charged at physiological pH.[16] Living cells of SM could be first partially captured by the cationic polymer P(TM-*co*-AAm) via electrostatic interaction and then entrapped by the complex of P(TM-*co*-AAm)/PAA.

Production of Alkaline Protease by the Immobilized SM

The immobilized SM, prepared at pH 3, and 10% of the carrier concentration [P(TM-*co*-AAm) or PAA], is compared with the free SM by the batch fermentation under the same conditions. It was found that spore concentration in the medium of free SM fermentation is about 80% higher than that in the culture medium of immobilized SM fermentation. It indicates that the complex carrier of P(TM-*co*-AAm)/PAA has the appropriate distributions of pore capacity and pore diameter, which effectively ensure the SM retention and the higher protease activity (442.1 u).

It is understandable that the protease activity of free SM fermentation (487.2 u) is slightly higher than that of the immobilized SM fermentation (442.1 u). It is unavoidable for the mass transport process to be somewhat blocked after the immobilization of cell by solid support. The diffusion and providence of oxygen and nourishing substances cannot reach the equilibrium promptly, and the release of protease to the medium is also partially limited. However, the immobilized cells are superior in their operational simplicity and capability of repeated use.

Figures 2 and 3 show the properties of alkaline protease production by free SM fermentation and immobilized SM fermentation at different pHs and temperatures. We can see that the optimum pH range of the immobilized SM gets wider than that of the free cells. The immobilized cells remain high protease activity during pH 5–10, but a narrower pH range, pH 7–9, is achieved for the free cells. Temperature has a similar effect on the protease activity. The immobilized SM has a wider optimum temperature range of 22–35°C than the free one (25–33°C). This implies that the

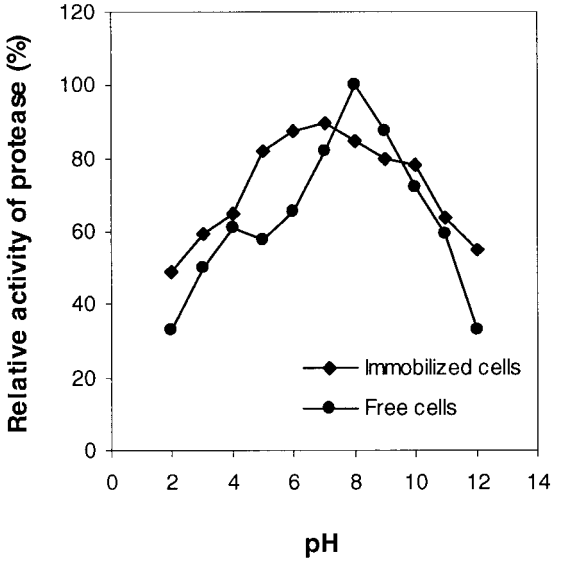
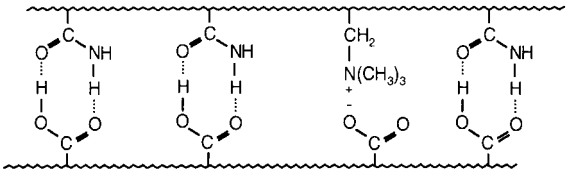


Figure 2 Relative activity of alkaline protease at different pHs of the inoculum (the highest activity of free enzyme is taken as 100%).



Scheme 1 Interaction between polymer chains.

complex carriers of P(TM-*co*-AAm)/PAA have good biocompatibility with SM. The hydrogel of interpolymer complexes possess some properties similar to the cellular organization, promoting the ability of protease production as well as protecting the microbial, which results in its higher stability. We have observed this phenomenon in the process of immobilizing chymotrypsin with the same interpolymer complex carrier.[13]

Repeated use is an important characteristic of immobilized cells. To this end, we investigated the stability of the immobilized SM during semicontinuous batch fermentation. The results are shown in Figure 4. After 18 batches of the semicontinuous fermentation, which lasted 18 days, only ~ 20% of protease activity decreased. The carrier remained the initial morphology without solution and breakage, which manifested good properties of repeated use.

In addition, we studied the storage stability of immobilized cells during the process of semicontinuous batch fermentation. The freshly prepared immobilized SM were put in the refrigerator for 2 days, two batches were fermented and then were left in the refrigerator for another 3 days; protease activity remained the initial value, which indicated good storage stability.

Under the optimum conditions for immobilization (i.e., 10% of the complex concentration and pH 3), TBF was immobilized with the same complex carrier of P(TM-*co*-AAm)/PAA. Its protease activity was shown as 420 u, 90% relative activity to free cells, which means that the higher protease activity is maintained after immobilization. It suggests that the complex carriers of P(TM-*co*-AAm)/PAA are of universal significance in the immobilizing enzymes and cells.

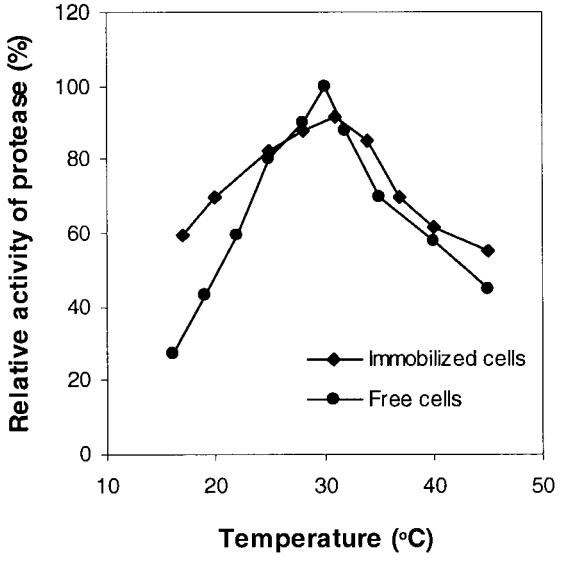


Figure 3 Relative activity of alkaline protease at different fermenting temperatures (the highest activity of free enzyme is taken as 100%).

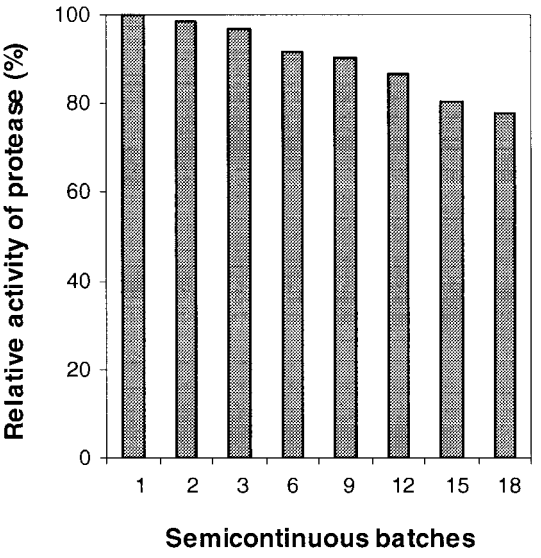


Figure 4 Relative activity of alkaline protease in the semicontinuous batch fermentation of immobilized SM (the highest activity of free enzyme is taken as 100%).

To further illustrate the characteristics of complexes as the carrier for cell immobilization, we compared the properties of various carriers, carrageenan, Na-alginate, and complex of P(TM-*co*-AAm)/PAA.

Table II shows that the complex carrier of P(TM-*co*-AAm)/PAA has good mechanical properties, chemical stability, microorganism compatibility, and storage stability. When it is used as the carrier for TBF, the immobilized TBF has high production of protease, good operational stability, and good resistance to the inorganic salts in the culture medium, which is conspicuously superior to a traditional carrier, such as Na-alginate and carrageenan. The complex carrier of P(TM-*co*-AAm)/PAA can satisfy the basic requirement of practical use and can be used in common for immobilizing enzymes and cells.

CONCLUSION

The growing cells of SM were immobilized with the interpolymer complex carriers on the basis of cationic polymers, P(TM-*co*-AAm) and PAA. The

Table II Properties of Various Carriers for Immobilization of TBF

Properties	P(TM-*co*-AAm)/PAA	Na-alginate	Carrageenan
Tensile strength (MPa)	0.7	0.3	0.1
Elongation at break (%)	140	70	20
Optimum content of network (%)	10	4	6
Optimum quantity of immobilized cell (%)	8	4	6
Leakage of free cell (OD_{600})	1.4	3.8	1.6
Relative activity of protease (%)	90	75	80
Resistance to the salts in the medium	Stable	Broken by PO_4^{3-}	Dissolved by NaCl
Operational stability (Decrease in relative activity of protease after 18 batches, %)	20	40	50
Tolerance to pH		All superior to the free cells	
Tolerance to temperature		All superior to the free cells	

alkaline protease was produced by the immobilized SM with higher relative activity. The immobilized SM has a wider optimum pH and temperature range than the free cell. The immobilized SM has good properties in repeated use and storage. Under the optimum immobilizing conditions, the living cells of TBF were immobilized with the same carrier of P(TM-*co*-AAm)/PAA and compared with the usual carriers, Na-alginate and carrageenan. It suggests the complex of P(TM-*co*-AAm)/PAA is a novel, superior, and universally applicable carrier for the immobilization of enzymes and cells. It opens up a new field for the application of ion-containing polymers.

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