脂肪酶催化缩聚法合成可完全降解的聚羟基丙酸酯

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摘要: 以 3-羟基丙酸甲酯为聚合单体, 建立了以固定化脂肪酶 Novozym 435 为催化剂的酶催化缩聚反应体系, 合成可完全降解的高分子聚酯聚羟基丙酸酯, 考察了反应条件和介质对反应性能的影响, 结果表明, 纯度大于 95% 的单体即可在温和条件下合成聚羟基羧酸酯; 降低反应压力可有效提升产物产率和分子量. 通过选择合适的有机溶剂介质和表面活性剂, 可使产物分子量提升至 13000 (M_w) 以上. 脂肪酶催化剂重复利用能力优异, 经 6 批次反应后, 其相对活性保持在 95% 以上.
关键词: 聚羟基羧酸酯; 聚合; 酶催化; 脂肪酶; 3-羟基丙酸甲酯
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Synthesis of Polyhydroxyalkanoates by Polymerization of Methyl 3-Hydroxypropionate in the Catalysis of Lipase

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Abstract: The polymerization of methyl 3-hydroxypropionate as monomer catalyzed by lipase was selected as a model system in this study. The monomer with the purity around 95% could be polymerized to polyhydroxyalkanoates in the catalysis of Novozym 435. Decreasing the reaction pressure would result in the increase of product yield and high molecular weight. By choosing appropriate organic solvents, surfactants and reaction pressure, the molecular weight of polyhydroxy propionate polyester could be controlled from 1800 to 13000 (M_w value). The reusing ability of enzymatic catalyst was comparatively good. The relative activity could be maintained above 95% after 6 repeated batches reaction.
Key words: polyhydroxyalkanoates; polymerization; enzyme catalysis; lipase; methyl 3-hydroxypropionate

Polyhydroxyalkanoates (PHAs) have been demonstrated to be a family of biopolymers with good biodegradability and biocompatibility. Due to the biological degradability and the extraordinary properties similar to those of common thermoplastics or elastomers, PHAs have several very useful potential applications, especially in medicine and agriculture [1–3]. Natural biodegradable polyesters are widespread in many kinds of microbial cells. More than 300 different microorganisms are known to synthesize and accumulate PHAs in their cells. Bacterial fermentation of PHAs has become an environment-friendly and efficient method, by which some commercial products of PHAs have

been obtained successfully [4–6]. But there are some limitations in the traditional fermentation process of PHAs. Firstly, the special unbalanced nutrient conditions needed by PHAs synthesis will lead to slow cell growth and low product yield. Secondly, the carbon sources used in fermentation for synthesizing PHAs are comparatively expensive. Thirdly, the separation and purification of PHAs in cells are complex and expensive processes [7]. These limitations lead to the high PHAs price compared with petrochemical-based polymers. The commercial applications of PHAs are limited due to the cost [8].

The enzyme-catalyzed polymerization has been regarded as a new environment-friendly synthetic process for polymeric materials, providing some good examples of "green polymer chemistry" [9]. The polyesters can be synthesized through the ring-opening polymerization, polycondensation, and transesterification catalyzed by enzyme [10–13]. The enzyme-catalyzed polymerization has many distinct advantages, such as mild reaction conditions, excellent recycle ability and high region-selectivity [14]. The enzyme-catalyzed polymerization does not require the exclusion of water or air. Enzymes can also be used in bulk, organic media, and at various interfaces, which give them an extra edge over the use of organo-metallic catalysts [15–18]. Most enzyme-catalyzed polymerization of PHAs are about ring-opening reactions of lactones [19]. The reported linear hydroxyester monomers of polycondensation included hydroxybutyrate, hydroxypentanoate, and hydroxyhexanoate [20–22]. In these reports, the molecular weight was relatively low and the monomer conversion was inefficient. The reactions were conducted commonly in the organic solvents or ionic liquids. The byproducts of macrolactones were produced with linear polyesters. Although some good examples of the enzyme-catalyzed polymerization of PHAs were well known, the polymerization of the linear chemical monomer, polyhydroxypropionate, catalyzed by enzyme has not been reported [23].

The enzymatic synthesis of polyesters from hydroxyester was proposed to proceed in three parts [19]: initial rapid transesterification (Scheme 1), followed by intermediate polymerization with a significant molecular weight increase, and finally slow polymerization reaching the maximum of the molecular weight. The polyester would also undergo degradation via alcoholysis.
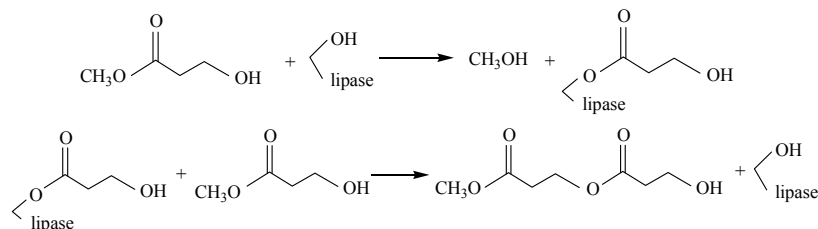
In this article, we report the polymerization of methyl 3-hydroxypropionate as monomer catalyzed by lipase (Scheme 2). The product of polyhydroxypropionate polyester (the simplest type of PHAs) was obtained under mild conditions. Compared with the microorganism fermentation, it was a more convenient and inexpensive process. The simple purification of products and using synthetic chemical monomer (methyl 3-hydroxypropionate could be obtained by a simple chemical synthesis process with low costs in our laboratory) could reduce the costs of the polymerization obviously. The enzyme-catalyst could also be used repeatedly. Furthermore, the molecular weight of polyester could be controlled by changing the reaction conditions, which was unavailable in the traditional microorganism fermentation of PHAs.
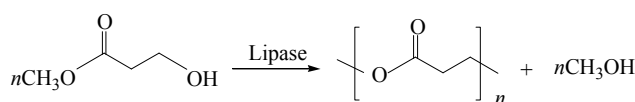
1　Experimental

1.1　Enzymes and chemicals

Four lipases, Novozym 435 (lipase acrylic resin from *Candida antarctica*, 10 units/mg solid), PPL (lipase from porcine pancreas, 55 units/mg solid), CRL (lipase from *Candida rugosa*, 1140 units/mg solid), and L-PS (Amano lipase PS from *Burkholderia cepacia*, 30 units/mg solid), were purchased from Sigma and used without treatment.

Methyl 3-hydroxypropionate with above 95% purity was obtained in our laboratory by methoxycarbonylation of propylene oxide [24–25], which was the most simple and economic synthetic method in this field. The synthetic

Scheme 1.　Mechanism of step-growth polymerization in the catalysis of lipase.

Methyl 3-hydroxypropionate　　　　Polyhydroxy propionate polyester

Scheme 2.　Polymerization of methyl 3-hydroxypropionate in the catalysis of lipase.

product could be used directly in the enzyme-catalyzed polymerization. The purification of monomer was not needed, which could reduce greatly the costs of polymeric substrates. On the other hand, it was very difficult to complete polymerization by monomer with such low purity in the catalysis of organo- metallic catalysts.

All other chemicals and reagents were of analytical grade.

1.2   General procedure for the lipase-catalyzed reaction

The enzyme-catalyzed reaction was performed in a solvent-free system by mixing the substrate of methyl 3-hydroxypropionate (100 mmol) and lipase (preset concentration, wt%) in a round-bottom flask (50 ml). The flask was then placed into a constant temperature oil bath on a magnetic stirrer at 1000 r/min. Vacuum was applied to facilitate removal of methanol (byproduct in the polymerization). Since methyl 3-hydroxypropionate was relatively volatile, the loss of monomer under pressure should be considered. Thus, a cold trap was used for recovering substrate. After reaction, the products and unreacted monomers were extracted with chloroform. The mixture was stirred for 10 min, and the undissolved lipase was filtrated. The methanol at 4 °C was dropped into the chloroform phase, and then the white solid products of polyhydroxy propionate were precipitated by cold methanol. The polyester was separated by filtration, washed twice with methanol, and dried at 60 °C for 2 h. At the same time, the lipase could be reused.

The yield of polyhydroxy propionate polyester was calculated by $\text{Yield} = \dfrac{\text{obtained product (g)}}{\text{theoretical procuct (g)}} \times 100\%$, where the

theoretical product amount (g) $= \dfrac{7.2 \times \text{substrate amount (g)}}{10.4}$.

The polymerization product was confirmed by NMR. The spectra were recorded on a Varian INOVA spectrometer in CDCl$_3$ at 30 °C (^{1}H NMR 400 MHz, ^{13}C NMR 100 MHz).

The molecular weight and its distribution were determined by the GPC system (Styragel HT 3, 5, 6 E in series using HPLC (Waters 2695, USA)/RID (Waters 2414, USA)). Polystyrene was used as a standard reference (solvent, 0.1 mg/ml chloroform).

The monomer concentration was analyzed by an Agilent 6820 gas chromatograph equipped with a flame ionization detector and a SE-54 capillary column (50 m × 0.32 mm × 2.00 mm).

1.3   Water activity

Lipase and substrates were absorbed or equilibrated with solid sorbent or saturated salt solutions at 25 °C in separate closed containers [26]. The solid sorbent and salts used

were molecular sieve 3A ($a_w < 0.01$), LiBr ($a_w = 0.06$), LiCl ($a_w = 0.11$), MgCl$_2$ ($a_w = 0.33$), Mg(NO$_3$)$_2$ ($a_w = 0.54$), and NaCl ($a_w = 0.75$). Absorption or equilibration was performed for at least 48 h.

2   Results and discussion

2.1   Enzyme selection

We compared the lipase-catalyzed polymerization of methyl 3-hydroxypropionate by using four different lipases with 1%–10% dosage (relative to monomer). The results showed that L-PS and CRL failed to catalyze the polymerization in the selected temperature range from 30 to 100 °C. The PPL (10 wt% catalyst: monomers; 55 units/mg) catalyzed the reaction with 7.3% product yield at 60 °C. Novozym 435 performed excellent catalyst activity, because 85.1% product yield could be achieved by using smaller amount (3 wt% catalyst: monomers; 10 units/mg) of the enzyme-catalyst.

The white polyhydroxypropionate polyester was obtained under mild conditions by using Novozym 435 as catalyst. The polymer dissolved in some organic solvents such as chloroform and dichloromethane, but could not dissolve in methanol, ethanol or water. The melting point of polymer was 52.8 °C, M_w value was about 2300 and polydispersity (M_w/M_n) was 1.07.

2.2   Optimization of polymeric reaction conditions

2.2.1   Effect of catalyst dosage

The selected catalyst dosages were 1 wt%, 3 wt%, 5 wt%, 10 wt% (relative to monomer) in this study. The results showed that high conversion ratio was obtained by increasing the catalyst dosage. But the change was not obviously when enzyme dosage was increased from 3 wt% to 10 wt%. The molecular weight was not affected by the different catalyst dosages (Table 1). Considering costs, 3 wt% was chosen as the optimum catalyst dosage in the latter studies.

2.2.2   Effect of monomer purity

The monomer with 95% purity was obtained by meth-

Table 1   Effect of Novozym 435 catalyst dosage

Catalyst dosage (wt%)	M_w	M_w/M_n	Yield (%)
0	—	—	0
1	2159	1.05	67.5
3	2286	1.07	85.1
5	2423	1.08	86.2
10	2072	1.06	88.1

Reaction conditions: 55 °C, 30 h, solvent-free, 80 kPa.

oxycarbonylation of propylene oxide. They could be used directly in the polymeric reaction with good results. The impurity in monomer didn't result in inactivation of enzyme catalyst. Further purification of monomer would improve product yield slightly, but the optimal reaction time would be shortened from 30 to 23 h when using monomer with 99.5% purity. There was no obvious effect on molecular weight by using high purity monomer (Table 2).

Table 2 Effect of monomer purity

Monomer purity (%)	M_w	Yield (%)	Optimal reaction time (h)
95	2286	85.1	30
98	2235	85.9	28
99.5	2329	86.8	23

Reaction conditions: 3 wt% Novozym 435, 55 ℃, solvent-free, 80 kPa.

2.2.3 Effect of reaction temperature

The polymeric reactions were performed at 35, 45, 50, 55, 60, 65, and 75 ℃, respectively. Figure 1 shows the product yields at different temperature in the range of 35–75 ℃. When the temperature increased from 35 to 55 ℃, the product yields increased obviously. But a distinct decrease appeared at higher temperature. The optimum reaction temperature was 55 ℃ and the product yield achieved 85.1% after 30 h polymerization. As a well-known lipase catalyst, Novozym 435 had shown good stability when the reaction temperature was above 55 ℃ in many reports [19,21,27]. The enzyme activity would not be affected in the selected temperature range. So the change of enzyme activity was not the major reason for the product yields decreasing with the temperature increasing. Because of the volatility, more and more substrates lost under the pressure with increasing the temperature, which should be the reason for the product yields decreasing.

There was no significant change of molecular weight when the temperature enhanced from 35 to 55 ℃. However,
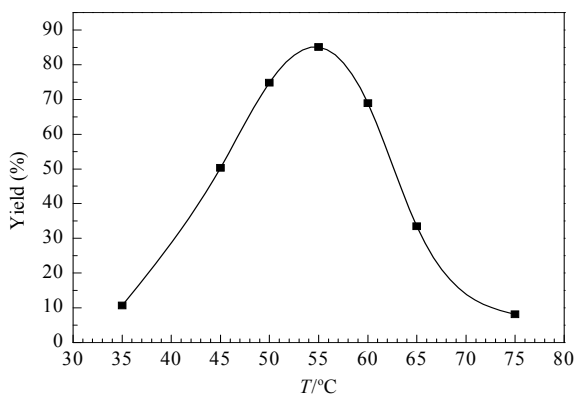

Fig. 1. Effect of reaction temperature on product yield. Reaction conditions: 3 wt% Novozym 435, 30 h, solvent-free, 80 kPa.

a small increase (from M_w 2300 to 4800) was observed when the temperature reached 70 ℃. The polydispersity (M_w/M_n) of polymer increased simultaneously from 1.07 to 1.30 with the temperature increasing from 55 to 70 ℃. It was above 1.40 when the temperature was further increased.

2.2.4 Effect of water activity

Water activity has been recognized as a key parameter which determines the enzymatic activity. The physical properties of the enzymes depend on the hydration state of the proteins, which is influenced by the water activity.

The water activity of polymeric reaction of methyl 3-hydroxypropionate was controlled in the range from 0.01 to 0.75. The effect of water activity was shown in Fig. 2.
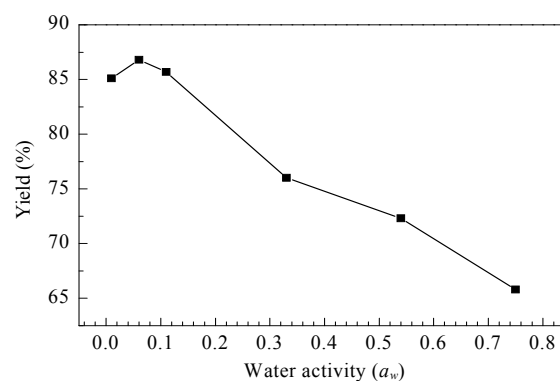

Fig. 2. Effect of water activity on product yield. Reaction conditions: 3 wt% Novozym 435, 55 ℃, 30 h, solvent-free, 80 kPa.

Most enzymes were known to require high water activity to obtain high activity. However, some lipases showed a different behavior [26]. They were relatively active at low water activity and some even showed an optimal activity in the region below 0.3. It was showed that lipases were different from other enzymes in the respect of the water requirement. From Fig. 2, the product yields were similar at water activities between 0.01 and 0.11, but it decreased as the water activity increased. The possible reasons were: (1) Excessive water molecule accumulated on the surface of lipase with increasing the water concentration in the system, which might prevent hydrophobic groups of monomer from approaching enzyme molecule; (2) High water concentration would also lead to degradation of short-chain oligomer, which was not helpful to obtain high molecular weight polymer. So the water activity in this study was controlled at the lowest value (< 0.01) by preequilibrating of molecular sieve 3A.

2.2.5 Effect of reaction time

Figure 3 shows that the curve of the monomer conversion

was similar with that of the product yield. The initial reaction rate measured was 12.1 mmol/(h·g). Under the optimum condition (55 ℃, 3 wt% Novozym 435, 80 kPa), the product yield achieved at 80% during the first 26 h. After that, the product yield increased slowly with prolonging the reaction time. At the time of 30 h, the product yield was improved to 85.1%. Then, the reaction reached equilibrium and the product yield remained almost unchanged. So the optimal polymeric reaction time was 30 h.
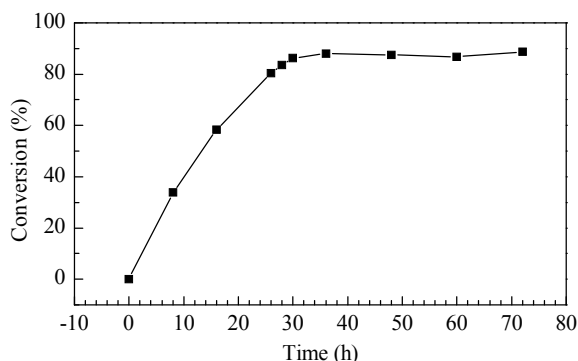

Fig. 3. Monomer conversion as a function of reaction time. Reaction conditions: 3 wt% Novozym 435, 55 ℃, solvent-free, 80 kPa.

2.3 Control of the molecular weight

2.3.1 Effect of reaction pressure

In the most cases of enzyme-catalyzed polymerization, in order to obtain the high molecular weight products as well as the high product yield, high vacuum was employed to remove the byproducts, such as water and alcohol. The reaction pressure had great effect on the enzyme-catalyzed polymerization of methyl 3-hydroxypropionate. In the reaction, the presence of methanol as byproduct was harmful for the polymerization. Under the normal pressure, the polymerization would not be completed because of the chemical equilibrium of the reaction. Methanol could be eliminated absolutely by increasing the vacuum of the system, which would shift the chemical equilibrium towards polymerization. Thus, the preceding polymeric reactions were conducted under the pressure of 80 kPa.

High vacuum was also helpful for the molecular weight and the product yield of polyhydroxy propionate polyester. Table 3 shows that the product yield and the molecular weight were improved with decreasing the pressure from 101.3 to 19.6 kPa. The molecular weight of polymer achieved about M_w 13100 and the product yield was 89.1% under the low pressure of 19.6 kPa.

2.3.2 Effect of organic solvent system

Using free-solvent system in polymeric reaction had

Table 3 Effect of reaction pressure on the polymerization

p/kPa	M_w	M_w/M_n	Yield (%)
101.3	2135	1.10	76.1
80	2286	1.07	85.1
40	6834	1.34	88.2
19.6	13128	1.43	89.1

Reaction conditions: 3 wt% Novozym 435, 55 ℃, 30 h, solvent-free.

many advantages, such as rapid reaction rate, convenient products separation, and enzyme activity preservation. But the viscosity of oligomers increased rapidly with the reaction processed in the free-solvent system. The speed and intensity of the stirrer were decreased by gel or soft solid oligomers, which was not benefit to the normal polymeric reaction. In this study, the stirrer was stopped because of the abundant sticky solid oligomers with low molecular weight. To improve the polymerization, we chose some different organic solvents to decrease the oligomers viscosity and make sure the reaction could continue.

The solvent polarity and the substrate solubility often showed great influence on enzyme activity. Table 4 shows that some organic solvents didn't promote polymerization of methyl 3-hydroxypropionate. Because of inhibiting effect of enzyme activity, the product yield decreased obviously in high polar organic solvent systems. But some organic solvents with high value of hydrophobic parameter (Log$P$) were benefit to molecular weight. For example, the M_w value enhanced from 2300 to 3300 and 3500 in the diphenyl ether and isooctane system, respectively.

Table 4 Effect of organic solvent system on polymerization

Solvent	Log$P$ o/w	M_w	M_w/M_n	Yield (%)
Solvent-free	–	2286	1.07	85.1
Tetrahydrofuran	0.46	2135	1.12	49.2
Cyclohexane	3.4	1992	1.04	75.0
Chloroform	1.94	2090	1.05	53.7
Toluene	2.69	2644	1.07	78.3
1,4-Dioxane	−0.42	2269	1.11	57.7
Diphenyl ether	4.2	3287	1.12	82.6
Isooctane	4.5	3532	1.09	83.9

Reaction conditions: 3 wt% Novozym 435, 55 ℃, 30 h, 80 kPa, different organic solvents (equal volume with substrate).

The inhibiting effect of solvent polarity on enzyme activity was attributed to the solvent's ability to trap enzyme from the essential water layer, or to penetrate into the water layer, then break the interaction between water and enzyme molecule. Therefore, the inhibiting effect on enzyme activity was unobvious when the solvent was highly hydrophobic. The more hydrophobic of the solvent, the larger the hydrophobic parameter (log$P$) was. The Log$P$ values of the selected organic solvents were in the range from −0.42 to 4.5. The molecular weight of the products was increased

when LogP was more than 4.0. The product yield was maintained above 80% in the same solvent systems. But the product yield decreased obviously with increasing the hydrophilic of the solvents, especially when logP was less than 2.0. The combined water molecule could be separated from the enzyme surface by hydrophilic solvents, which resulted in the inactivation of enzyme. The enzyme molecular conformation would also be changed by the interaction between enzyme protein and the intensive polar solvents, which was harmful for the contact between enzyme active centers and substrates. In the polymeric reaction system of methyl 3-hydroxypropionate, it was concluded that polymer chain-increasing was enhanced in hydrophobic solvents with high LogP value (＞4.0).

2.3.3　Effect of surfactants

The surfactants were very important for adjusting lipase activity and molecular weight. Adding appropriate surfactants would create enormous water-oil interface which was needed in lipase catalysis. The results in Table 5 shows that the constricted lipase conformation was expanded by adding the right surfactant with concentration of 30 mg/ml into the reaction system, which improved the degree of polymerization. Two surfactants (Triton X-100 and Triton X-114) had notable effect on the molecular weight of the products. The M_w value reached 9800 by using Triton X-100 as surfactant.

Table 5　Effect of surfactants on polymerization

Surfactant	M_w	M_w/M_n	Yield (%)
Triton X-114	4528	1.46	84.2
Triton X-100	9858	1.65	86.8
Tween 40	2743	1.16	83.1
Tween 80	1776	1.04	81.7
None	2286	1.07	85.1

Reaction conditions: 3 wt% Novozym 435, 55 ℃, 30 h, solvent-free, 80 kPa, 30 mg/ml surfactants.

2.4　GPC monitoring of molecular weight

Figure 4 displays GPC traces of the products within reaction time of 8, 16, 24, and 30 h. The data from this analysis allowed the following discussion about the chain growth as a function of time. Compared the peak shapes of 8 and 30 h, we can conclude that the chain buildup was very rapidly. The short-chain oligomer with low molecular weight had begun to build at 8 h, and the peak shapes became better with the reaction process, which showed that the short chains could be polymerized continuously to the long chain polymer. Thus, an uninterrupted increasing of molecular weight was observed. The white solid oligomers of M_w = 2300 with perfect polydispersity (M_w/M_n) 1.07 were ob-

tained at 30 h. The homopolymer (M_w = 5200) of oligomer (M_w = 2300) was obtained after 10 h by lipase-catalyzed polymerization when the reaction temperature was increased to 70 ℃.
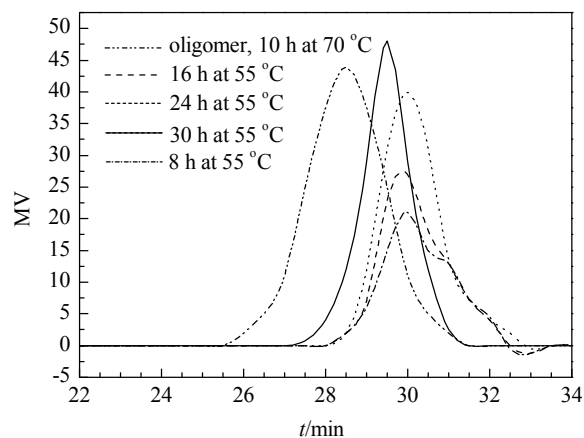

Fig. 4.　GPC traces of polymeric products.

The results of GPC traces indicated that the lipase-catalyzed polycondensation of methyl 3-hydroxypropionate should occur by a step-growth mechanism. Our results were consistent with the step-growth kinetics to propagation. Furthermore, both polymerization and alcoholysis reaction occurred simultaneously, although the equilibrium was shifted towards polymer formation by removing methanol under vacuum. Hence, the more details about the polymerization mechanisms needed to be studied further.

2.5　Reuse of the catalyst and preparative scale experiment

Due to the insolubility in the reaction system, the biocatalyst lipase could be recovered conveniently for reuse. Novozym 435 was stable in the reaction. It was used repeatedly by filtrating and washing. The repeated reactions were carried out under the same optimal reaction conditions. Figure 5 shows that the Novozym 435 was very stable and its relative activity kept above 95% throughout 6 batches reactions.

To show the applicability of the lipase-catalyzed polymerization process, a preparative scale experiment with 0.5 mol substrate (about 52 g) was performed. The experiment was carried out under the optimum reaction conditions (3 wt% catalyst; 55 ℃; 40 kPa; solvent-free reaction system; impeller stirrer at 800 r/min; 48 h reaction time). The polyhydroxypropionate product with 82.5% yield was obtained with high molecular weight M_w 9100, which was probably due to the great intensity of impeller stirrer. The polymeric process was prolonged by stirring sticky solid oligomers
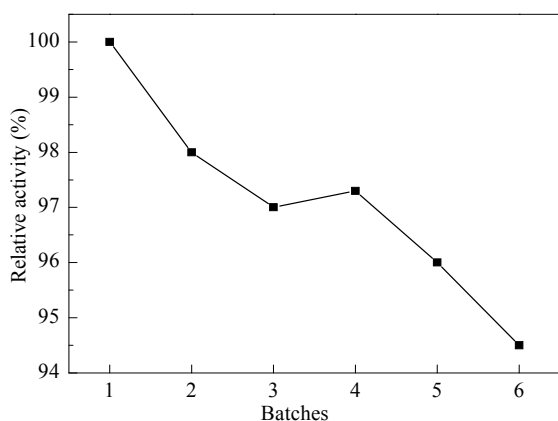
Fig. 5. Reuse of the catalyst in the polymerization. Reaction conditions: 55 ºC, 30 h, solvent-free, 80 kPa.

using impeller stirrer. The degree of polymerization and the molecular weight would be improved by continuous homopolymerization of oligomers. The preparative scale experiment showed good possibility and applicability in the further amplification of production.

3 Conclusions

We developed a convenient enzyme-catalyzed polymerization route for synthesizing polyhydroxypropionate, which was different from the traditional biosynthesis route. The polyester could be obtained directly by enzyme-catalyzed polycondensation using inexpensive chemical monomers, which would decrease the production costs efficiently. Compared with the traditional microorganism fermentation process, the products with high purity could be separated conveniently from the enzyme-catalyzed reaction system. In the polymeric reaction, the lipase of Novozym 435 acted as an efficient biocatalyst with high activity and stability. By optimizing reaction conditions, the product yield could be improved and the molecular weight could be controlled, which were crucial for the polymerization. In conclusion, the enzyme-catalyzed polymerization using chemical monomers was a novel method, by which the polymer of PHAs could be obtained conveniently in a mild environment-friendly process.

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