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Highly efficient ring-opening polymerization of ε-caprolactone catalyzed by a recombinant *Escherichia coli* whole-cell biocatalyst

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ABSTRACT

This paper discussed the use of a recombinant *Escherichia coli* whole-cell biocatalyst harboring a thermophilic lipase gene from *Fervidobacterium nodosum* in the catalytic synthesis of polyesters. The ring-opening polymerization of ε-caprolactone was used as a model reaction to study the effects of temperature and reaction medium on monomer conversion and the molecular weight of the product. The whole-cell biocatalyst displayed high catalytic activity at high temperatures (70–90 °C), with almost 100% monomer conversion. Meanwhile, high monomer conversion values (>97%) were achieved in both hydrophobic and hydrophilic solvents, with the exception of dichloromethane (85%). Poly(ε-caprolactone) was obtained in 100% monomer conversion, with a number-average molecular weight of 2000 g/mol and a polydispersity index of 1.47 in cyclohexane at 70 °C for 72 h. Furthermore, the whole-cell biocatalyst exhibited excellent operational stability, with monomer conversion values exceeding 90% over the course of 10 batch reactions. To verify the practicality of the procedure, scale-up reaction was also performed with isolated yield and number-average molecular weight of ca. 70% and 2140 g/mol, respectively.

1. Introduction

Aliphatic polyesters are a group of remarkably biocompatible and biodegradable polymers, and are widely used in biomedical applications. For example, they have been employed as resorbable implant materials for tissue engineering and as vehicles for drug delivery [1]. At present, commercially available aliphatic polyesters are mainly synthesized using organometallic catalysts, notably aluminium alkoxides and tin carboxylates [2]. However, the potentially harmful effect of trace metallic residues arising from these catalysts has given rise to some concern in the biomedical field. Over the past two decades, enzymatic polymerization has been extensively developed [3–11], and represents a powerful alternative to toxic, metal-based catalytic systems. Moreover, it offers a new strategy for the production of useful materials including both natural and synthetic polymers that have more well-defined structures than those prepared using conventional chemical catalysts, due to its potential for effective control of enantioselectivity, chemoselectivity and regioselectivity [11].

Previous work has shown that numerous lipases or esterases could be successfully employed as catalysts for polyester synthesis, including porcine pancreatic lipase, *Pseudomonas cepacia* lipase, *Candida antarctica* lipase B, *Humicola insolens* cutinase and *Archaeoglobus fulgidus* esterase [12–16]. Novozym 435, that is *C. antarctica* lipase B immobilized on the macroporous acrylic polymer resin Lewatit OC VOC 1600, has proven to be one of the most useful enzymes, and exhibits extraordinary reactivity towards a wide range of monomers [9]. For example, it has been reported that the polymerization of ε-caprolactone using Novozym 435 in toluene at 70 °C for 4 h produced poly(ε-caprolactone) with an isolated yield of 85% and a number-average molecular weight (*Mn*) of 44,800 g/mol [13]. Recently, a novel cutinase from *H. insolens* was explored with promising polyester synthesis activity, with *Mn* value of the synthesized poly(ε-caprolactone) of 24,900 g/mol at 70 °C for 24 h [15,17]. Despite these impressive results, enzymatic polymerization remains in the research and development stage, in part due to the high cost of producing and immobilizing these lipases. Therefore, the development of a commercially viable biocatalytic system for the green and sustainable synthesis of polyesters on an industrial scale would be of great significance. One potential way to reduce the cost of producing and immobilizing lipases is to use whole-cell biocatalysts, the production of which does not require complex isolation, purification, and immobilization procedures [18]. Recently, Kondo and co-workers constructed

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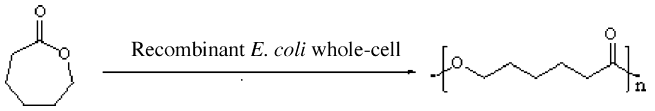

Scheme 1. The ring-opening polymerization of ε-caprolactone catalyzed by the recombinant *E. coli* whole-cell biocatalyst.

a *C. antarctica* lipase B-displaying yeast whole-cell biocatalyst, and successfully applied it in the synthesis of polybutylene adipate, with *Mn* values of 2590–3530 g/mol [19]. Recombinant *Escherichia coli* whole-cell biocatalysts are simpler and potentially more cost-effective than yeast cell-surface display systems, but their use in the synthesis of polyesters has not previously been reported.

A previous study from our laboratory focused on cloning a lipase gene, FN1333, from the anaerobic and extremely thermophilic bacterium *Fervidobacterium nodosum* [20]. This gene was then efficiently expressed in *E. coli*, and the lipase's substrate specificity and catalytic mechanism were investigated. It was found that the recombinant enzyme, FNL, was highly thermostable, with an optimum working temperature of 70 °C, and that it could efficiently catalyze the hydrolysis of a broad range of substrates, including *p*-nitrophenyl esters and triacylglycerols. We also found an interesting phenomenon that FNL was over-expressed in an insoluble form, which permitted us to extract and isolate the active enzyme by means of a thermal solubilization procedure [20]. We inferred that it might be bound to or associated with the *E. coli* cell membrane or wall due to its high hydrophobicity. Therefore, this would make the recombinant *E. coli* whole-cell an excellent prospective biocatalyst.

Herein, we described the synthesis of aliphatic polyesters using a recombinant *E. coli* whole-cell biocatalyst harboring the thermophilic lipase gene FN1333 from *F. nodosum*. The ring-opening polymerization of ε-caprolactone was used as a model reaction to study the properties of the recombinant *E. coli* whole-cell biocatalyst (Scheme 1). This work first provided a practical approach that the extraordinary ability of the recombinant FNL whole-cell to catalyze reactions with a high degree of transformation efficiency results in direct synthetic routes to well-defined polymers by reducing the need for traditional approach, which involves tedious purification and immobilization of enzymes.

2. Materials and methods

2.1. Materials

The *E. coli* BL21-CodonPlus (DE3)-RIL strain was purchased from Novagen (Madison, WI). The recombinant *E. coli* BL21 strain, which harbors the thermophilic lipase gene FN1333 from *F. nodosum*, was constructed using the expression vector pET-28a, and stored in our laboratory [20].

ε-Caprolactone and δ-valerolactone were purchased from Fluka Chemical Co. in the highest available purity and used as received. 1,4-Dioxan-2-one was purchased from Daigang Biotech. Co. (Shandong, China). Yeast extract and tryptone were purchased from Oxoid Ltd. Kanamycin and isopropyl β-D-thiogalactopyranoside (IPTG) were purchased from Sigma. Chloroform-d was obtained from Aldrich Chemical Co. 4 Å molecular sieves were purchased from Tianjin Chemical Co. (Tianjin, China), and roasted at 500 °C for 3 h. Organic solvents of analytical grade were purchased from Beijing Chemical Co. (Beijing, China), and dried over 4 Å molecular sieves before use. All other reagents were of the highest reagent grade commercially available, and were used without further purification.

2.2. Preparation of the recombinant E. coli whole-cell biocatalyst

The recombinant *E. coli* BL21 strain, which harbors the FN1333 gene from *F. nodosum*, was cultured in 2YT medium (1% yeast extract, 1.6% tryptone, and 0.5% NaCl) containing kanamycin (50 μg/mL) at 37 °C. When the culture reached an optical density of 1.0 at 600 nm, the induction was carried out by adding IPTG at a final concentration of 1 mM and shaking for an additional 6 h at 37 °C. The cells were harvested by centrifugation at 8000 rpm for 15 min at 4 °C and washed with 50 mM phosphate buffer (pH 8.0). The harvested cells were washed with deionized water three times, lyophilized, and then used as the catalyst.

The *E. coli* BL21-CodonPlus (DE3)-RIL strain, which harbors no target gene, was cultured and treated according to the same protocol save that, in this case, no kanamycin was added to the medium. This strain was employed as a blank.

2.3. Ring-opening polymerization of lactones

A quantity of the lyophilized cells (typically 50–150 mg) was dried in a desiccator overnight, and then transferred to a dried screw-capped vial containing 200 μL ε-caprolactone and 600 μL organic solvent (no solvent addition for solvent-free system). For the ring-opening polymerization of δ-valerolactone and 1,4-dioxan-2-one, the amounts added were 200 μL and 102 mg (1 mmol), respectively. Ethylbenzene (50 μL) was added as an internal standard for the quantification of monomer conversion by gas chromatography (GC). The vial was then sealed and placed into a thermostatic reactor, and the reaction mixture was stirred at 180 rpm at the appropriate temperature. After a specified time, the reaction was terminated by adding dichloromethane, and the biocatalyst was removed by filtration. The biocatalyst was washed several times with dichloromethane, and the combined filtrate and washings were collected and concentrated by rotary evaporation. The crude product thus obtained was precipitated in methanol at −20 °C, and the cloudy solution was centrifuged (8000 rpm, 15 min, 4 °C). The ensuing white precipitate was collected and dried in a vacuum oven, and then characterized by ^{1}H nuclear magnetic resonance (NMR) and gel permeation chromatography (GPC).

2.4. Structural characterization

The ^{1}H NMR spectra were recorded on an AVANCE DMX 500 spectrometer in chloroform-d. For ^{1}H NMR spectra, chemical shifts were reported in parts-per-million (ppm) relative to the tetramethylsilane peak (0.00 ppm) which served as an internal reference.

2.5. Determination of monomer conversion

Monomer conversion values were determined by GC using a Shimadzu 2014 gas chromatograph equipped with an Rtx-1 capillary column (30 m × 0.25 mm × 0.25 μm) and a hydrogen flame ionization detector. Nitrogen was used as the carrier gas at an average velocity of 31 cm/s. The injection volume was 1.0 μL. The temperatures of the injection pool and the detector were set at 200 °C and 240 °C, respectively. Following injection, the column temperature was held at 70 °C for 2 min, and then programmed to rise at 10 °C/min to a final temperature of 140 °C, which was maintained for 2 min.

2.6. Determination of molecular weight and polydispersity index (PDI)

The *Mn*, weight-average molecular weight (*Mw*) and PDI (*Mw*/*Mn*) values of the polymers obtained were determined by GPC. Analyses were carried out using a Shimadzu HPLC system equipped with a refractive index detector and Shim-pack GPC-804 and GPC-8025 ultrastyragel columns in series. Tetrahydrofuran (THF) was used as the eluent, at a flow rate of 1.0 mL/min. The sample concentration and injection volume were 0.3% (w/v) and 20 μL, respectively. The GPC system was calibrated using polystyrene standards of narrow molecular weight distribution.

2.7. Scale-up reaction of the ring-opening polymerization of ε-caprolactone

To verify the practicality of the procedure, a scale-up reaction for the ring-opening polymerization of ε-caprolactone was performed. The reaction mixture was composed of 10 mL ε-caprolactone, 30 mL toluene and 2.5 g recombinant *E. coli* whole-cell biocatalyst. The reaction was carried out at 70 °C with shaking at 180 rpm for 72 h. The monomer conversion, isolated yield, *Mn* and PDI values were then determined.

3. Results and discussion

3.1. Structural characterization of polyesters

To begin with, we investigated the ability of the recombinant *E. coli* whole-cell biocatalyst to affect the synthesis of poly(ε-caprolactone) under solvent-free conditions. The biocatalyst (50 mg) was stirred with ε-caprolactone (200 μL) at 70 °C for 72 h, affording the desired polymer in an isolated yield of 76%. The ^{1}H NMR spectrum of the materials thus obtained was shown in Fig. 1 and contained numerous peaks that unambiguously identified the product: 1.39 (H_c, m, J = 7.88, 7.60 Hz); 1.66 (H_b and H_d, m, J = 7.20, 7.46, 4.04 Hz); 2.31 (H_a, t, J = 7.30 Hz); 3.65 (H_f, t, J = 6.47 Hz); and 4.06 (H_e, t, J = 6.54 Hz). The small triplets at 4.16 ppm were attributed to the –CH_2O– of the cyclic polymers and dimmer, which was likely to be present in trace quantities, as was the case with

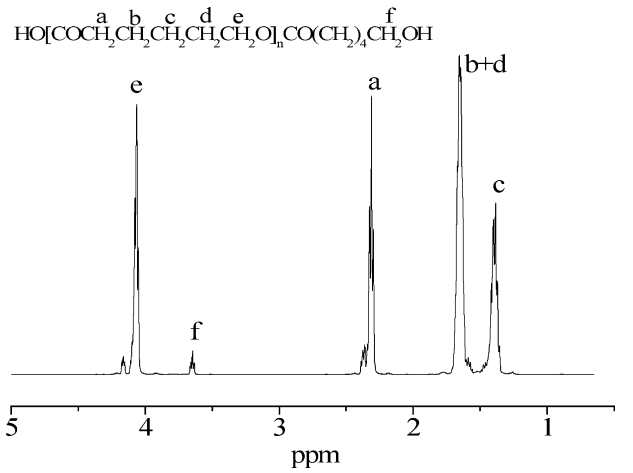

Fig. 1. ^{1}H NMR spectrum of the product synthesized by ring-opening polymerization of ε-caprolactone using the recombinant *E. coli* whole-cell biocatalyst at 70 °C for 72 h under solvent-free conditions.

the poly(ε-caprolactone) synthesized using *C. antarctica* lipase B and *A. fulgidus* esterase [16,21]. The triplets at 2.31 and 2.37 ppm also suggested the presence of a diverse mixture of linear and cyclic polymers. By calculating the areas of peaks e and f, the molecular weight of the poly(ε-caprolactone) obtained was determined to be 1890 g/mol.

The ^{1}H NMR spectra confirmed the structures of poly(δ-valerolactone) and poly(1,4-dioxan-2-one) as follows: poly(δ-valerolactone) 1.68 (–$CH_2CH_2CH_2CH_2O$–), 2.35 (–$COCH_2$–), 3.64 (–CH_2OH end group), 4.09 (–OCH_2–); poly(1,4-dioxan-2-one) 3.80 (–OCH_2CH_2OCO–), 4.36 (–OCH_2CH_2OCO–), 4.19 (–$COCH_2O$–).

3.2. The effect of temperature on monomer conversion and Mn

The stability of a biocatalyst is particularly important if it is to be used in industrial chemical synthesis [22]. To examine the thermal stability of the recombinant *E. coli* whole-cell biocatalyst, 72 h polymerization reactions were conducted at different temperatures in toluene, and the monomer conversion and *Mn* values were determined in each case. As can be seen in Table 1, the recombinant *E. coli* biocatalyst could catalyze the ring-opening polymerization of these three lactones, with monomer conversion values of 97% and 65% for δ-valerolactone and 1,4-dioxan-2-one, respectively. For the ring-opening polymerization of ε-caprolactone, no product was obtained in the blank experiment, but complete consumption of the monomer was observed in all of the other reactions. For comparative purposes, an additional experiment using 50 mg of purified FNL in place of the whole-cell biocatalyst was conducted, with a reaction temperature of 70 °C and a reaction time of 72 h. In this case, the monomer conversion only reached 80% (unpublished data). It was assumed that in the whole-cell system, the lipase was associated with the cell membrane. That being the case, the superior catalytic activity of the whole-cell biocatalyst as compared to the purified lipase might be due to the beneficial effect of the hydrophobic cell membrane on the stability and catalytic activity of the recombinant enzyme. The *Mn* and PDI values were in the range of 1190–1310 g/mol and 1.24–1.29, respectively, and exhibited little dependence on the concentration of the biocatalyst or the reaction temperature. Thus, the synthesized polymers are of low molecular weight and narrow molecular weight distribution, similar to *A. fulgidus* esterase [16], and may be of wide utility, for example as the soft block in a thermoplastic elastomer or in the construction of controlled release drug carriers, particularly for localized drug delivery.

Table 2
Monomer conversion and product *Mn* values for the ring-opening polymerization of ε-caprolactone catalyzed by the recombinant *E. coli* whole-cell biocatalyst in various organic solvents at 70 °C for 72 h.

Solvent	log *P*	Monomer conversion[a] (%)	*Mn*[b] (g/mol)	PDI[b]
Dioxane	−1.10	100	710	1.17
Acetone	−0.23	98	740	1.19
Tetrahydrofuran	0.49	100	830	1.16
Dichloromethane	0.93	85	860	1.16
Chloroform	2.00	99	800	1.17
Toluene	2.50	100	1200	1.25
Cyclohexane	3.09	98	2000	1.47
n-Hexane	3.50	100	1950	1.44
Solvent-free	–	97	1950	1.46

[a] Monomer conversion values were determined by GC.
[b] *Mn* and PDI values were determined by GPC in THF, using polystyrene standards.

3.3. The effect of reaction medium on monomer conversion and Mn

When biocatalytic reactions are conducted in non-aqueous medium, the nature of the solvent employed plays a crucial role in determining the stability of the biocatalyst and in the partitioning of substrates and products between the solvent and the biocatalyst [3,13,23]. To evaluate the activity and stability of the recombinant *E. coli* whole-cell biocatalyst in various organic media, a series of polymerization experiments in solvents with different log *P* values were conducted. These experiments were conduced at 70 °C, with a reaction time of 72 h; the observed monomer conversion and *Mn* values were summarized in Table 2. High monomer conversion values (>97%) were achieved in both hydrophobic (toluene, cyclohexane and n-hexane) and relatively hydrophilic (dioxane and acetone) solvents, although the conversion in dichloromethane was relatively low (85%). Enzymatic polyester synthesis generally proceeds with high monomer conversion only in hydrophobic solvents [13]; thus, the recombinant *E. coli* whole-cell biocata-

Table 1
Monomer conversion and product *Mn* values for the ring-opening polymerization of lactones catalyzed by the recombinant *E. coli* whole-cell biocatalyst[a].

Monomers	Amount of biocatalyst (mg)	Temperature (°C)	Monomer conversion[c] (%)	*Mn*[d] (g/mol)	PDI[d]
δ-Valerolactone	50	70	97	1020	1.22
1,4-Dioxan-2-one	50	70	65	910	1.09
ε-Caprolactone	50	70	100	1200	1.25
ε-Caprolactone	100	70	100	1220	1.27
ε-Caprolactone	150	70	100	1310	1.29
ε-Caprolactone	50	80	100	1250	1.24
ε-Caprolactone	50	90	100	1190	1.24
Blank[b]	50	70	No product		

[a] Reaction conditions: 200 μL ε-caprolactone (or 200 μL δ-valerolactone or 102 mg 1,4-dioxan-2-one) and 600 μL toluene, 72 h, no vacuum.
[b] In the blank reaction, ring-opening polymerization of ε-caprolactone was performed using the *E. coli* BL21-CodonPlus (DE3)-RIL strain harboring no target gene FN1333.
[c] Monomer conversion values were determined by GC.
[d] *Mn* and PDI values were determined by GPC in THF, using polystyrene standards.

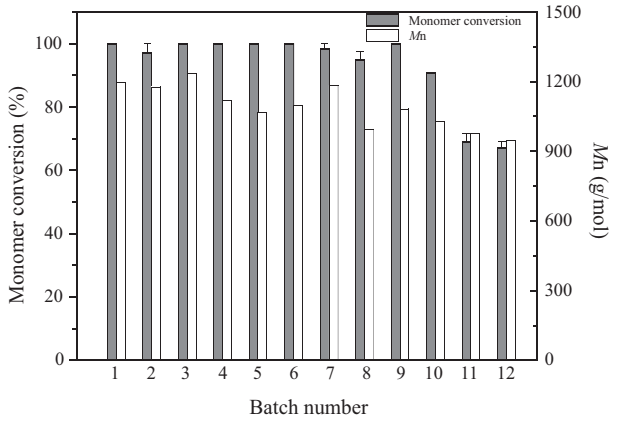

Fig. 2. Monomer conversion and *Mn* values for a series of 12 consecutive batch reactions conducted using a single 50 mg sample of the recombinant *E. coli* whole-cell biocatalyst; on completion of one reaction, the catalyst was washed and recycled for use in the next. Reactions were conducted at 70 °C for 72 h each, using 200 μL of ε-caprolactone and 600 μL of toluene each time.

lyst tolerated a broader range of organic solvents than enzymes. Unlike the situation with monomer conversion, reactions run in hydrophobic solvents afforded higher *Mn* values than those run in hydrophilic solvents, with the highest *Mn* value (2000 g/mol) being obtained in cyclohexane. Similar solvent effects have been reported in studies of enzymatic polymerization [3], and can be explained by the fact that hydrophobic solvents tend to favor the retention of essential water molecules (i.e. the water of hydration) on the enzyme's surface, which helps it maintain its catalytic conformation and activity. Notably, higher PDI values were also obtained in hydrophobic solvents. In addition to catalyzing polymerization, lipases and esterases could also cleave the ester bonds of polyesters, thus catalyzing the degradation, inter-transesterification and intra-transesterification of the polymer chain [20,24,25]. The use of hydrophobic solvents resulted in improved degree of polymerization, but also might lead to increased rates of hydrolysis and transesterification; this in turn resulted in a polymer with a broader molecular weight distribution. In addition, the recombinant *E. coli* whole-cell biocatalyst worked efficiently under solvent-free conditions, affording a high monomer conversion (97%) and *Mn* (1950 g/mol as determined by GPC, or 1890 g/mol as determined by ^{1}H NMR analysis).

3.4. Operational stability

To test the operational stability of the recombinant *E. coli* whole-cell biocatalyst towards repeated use, a series of consecutive ring-opening polymerization reactions was performed in toluene at 70 °C for 72 h using a single sample of the biocatalyst. After each reaction, the whole-cell biocatalyst was recovered by filtration, washed with dichloromethane to remove residual substrate and product, and then reused in the next batch reaction in the series. These washes reduced the tendency of the cells to clump together, which would decrease the efficiency of mass transport during the reaction; washing thus enhanced the catalyst's recyclability. As shown in Fig. 2, high monomer conversions (>90%) were obtained in each of the first ten batch reactions. Although the efficiency of monomer conversion declined slightly in the last two reactions, all of the reactions in this series afforded comparable *Mn* values (950–1230 g/mol). Thus, the recombinant *E. coli* whole-cell biocatalyst exhibited excellent operational stability and might offer dramatically improved cost-effectiveness relative to conventional enzymatic approaches in industrial settings.

3.5. Scale-up reaction of the ring-opening polymerization of ε-caprolactone

To verify the practicality of the protocol, recombinant *E. coli* whole-cell biocatalyst catalyzing the ring-opening polymerization of ε-caprolactone was performed in toluene at 70 °C for 72 h. The monomer conversion and isolated yield were measured to be 100% and ca. 70%, respectively. The polymer was obtained with *Mn* value of 2140 g/mol and PDI value of 1.21. Thus, the high isolated yield and low molecular weight distribution obtained using recombinant *E. coli* whole-cell biocatalyst suggested that the procedure would be very useful for preparing the polyesters of great biodegradability and biocompatibility.

4. Conclusion

In conclusion, a novel recombinant *E. coli* whole-cell biocatalyst harboring a thermophilic lipase gene from *F. nodosum* has been shown to be an excellent catalyst for the synthesis of poly(ε-caprolactone). This biocatalyst offered significant advantages over the conventional enzymatic process due to its high catalytic efficiency, low cost of production, and excellent operational stability. The ability to synthesize polymers as potential drug carriers using the novel recombinant *E. coli* whole-cell are highly promising for the mild, metal-free synthesis of polyesters and enhances its widespread application as an important strategy.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.procbio.2010.09.020.

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