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Lipase-catalyzed synthesis of aromatic polyesters

XY Wu[1], Y-Y Linko[1], J Seppälä[2], M Leisola[1] and P Linko[1]

[1]*Laboratory of Bioprocess Engineering;* [2]*Laboratory of Polymer Science and Technology, Helsinki University of Technology, FIN-02015 HUT, Finland*

The enzymatic synthesis of aromatic polyesters by direct polyesterification between a diacid and a diol is described. The effects of the type of substrate, type and quantities of lipase, temperature, vacuum, and reaction time on the synthesis of aromatic polyesters were studied in detail. Among three lipases investigated, only Novozym 435 worked well for aromatic polyester synthesis. Temperature and vacuum played an important role in obtaining a high molar mass of the aromatic polyesters. Furthermore, with isophthalic acid and 1,6-hexanediol as substrates, the mass average molar mass of the polyester obtained increased with an increase in the lipase quantity up to 0.375 g (11.7%, w/w of total reactor contents). The mass average molar mass of the polyester was as high as 50 000 g mol^{-1} in 168 h, with a polydispersity of PD $\approx$ 1.4.

Keywords: lipase; enzymatic synthesis; aromatic polyester; diacid; diol; polyesterification

Introduction

There is a growing interest in the biocatalytic production and biosynthesis of polymers with environmentally acceptable properties, such as biodegradability, biocompatibility and chirality. These properties make the polymers suitable for medical and agricultural applications [4,5]. Enzymatic polyesterification of an underivatized diacid and a diol catalyzed by a lipase from *Aspergillus niger* and resulting in a heptamer was first described by Okumura *et al* [21]. Ajima *et al* [1] also reported an attempt of enzyme-catalyzed AB-type polymerization, but only oligomers were obtained. For the first time, a polyester with a relatively high mass average molar mass of 14 900 g mol^{-1} was reported by Wallace and Morrow [22] who used porcine pancreatic lipase to catalyze the polymerization of *bis*(2,2,2-trifluoroethyl) adipate either with 1,4-butanediol or 1,6-hexanediol. Three years later Morrow [18] reported a molar mass of 39 000 g mol^{-1} for the transesterification polymerization of *bis*(3,2,2-trifluoroethyl) glutarate and 1,4-butanediol in anhydrous veratrole in 18 days.

We have previously studied lipase-catalyzed synthesis of aliphatic polyesters [6,11–13,23,24,27]. Among the several commercially available lipases investigated, only powdered (not immobilized) *Rhizomucor miehei* lipase worked well as the biocatalyst in the aliphatic polyester synthesis both by polytransesterification of *bis* (2,2,2-trifluoroethyl) sebacate and direct polyesterifiaction of sebacic acid with 1,4-butanediol. The highest mass average molar mass obtained for the poly(1,4-butyl sebacate) at 45°C was 64 600 g mol^{-1} (DP = 284) and, when reprecipitated from methanol with an 82% yield, the mass average molar mass of the partially purified poly(1,4-butyl sebacate) increased further to 77 400 g mol^{-1} (DP = 340, polydispersity PD = $\overline{M}_w/\overline{M}_n$ = 4.4, and the melting temperature of 66.8°C as determined by DSC), with the maximum molar mass of 131 000 g mol^{-1} (DP = 520). To our knowledge this is the highest molar mass ever reported for lipase-catalyzed polyesterification between a diacid and a diol. The partially purified white solid was shown to be linear poly(1,4-butyl sebacate) by ^{13}C NMR [12].

To our knowledge, there appear to have been few efforts on the synthesis of aromatic polyesters by lipase-catalyzed direct polyesterification. Mezoul *et al* [17] reported the synthesis of aromatic polyesters by enzyme-catalyzed polytransesterification. A linear polyester with a mass average molar mass of 31 700 g mol^{-1} and melting point of 104°C was obtained by polytransesterification of dimethyl isophthalate and 1,6-hexanediol catalyzed by the commercial lipase Novozym, using toluene as a solvent. Methanol produced in the reaction was eliminated by flushing with nitrogen. In the present work the possibility of lipase-catalyzed synthesis of aromatic polyesters by direct polyesterification between a diacid and a diol was investigated, and the effects of different factors, such as the type and quantity of lipase, the type of substrates, temperature, reaction time and vacuum on the polyester synthesis were also studied.

Materials and methods

Materials

Immobilized lipases (E.C. 3.1.1.3, triacylglycerol hydrolase) Novozym®435, immobilized on a macroporous acrylic resin of phenolic type (*Candida antarctica* lipase expressed in *Aspergillus oryzae*; hydrolytic activity 20 U g^{-1}; ester synthesis activity 7000 PLU g^{-1}; water 1.4%), and Lipozyme IM, immobilized on porous, weakly acidic anion exchange resin (*Rhizomucor miehei* lipase expressed in *Aspergillus oryzae*; hydrolytic activity 200 U g^{-1}; water 3.4%) were kind gifts from Novo Nordisk A/S (Bagsvaerd, Denmark). *Rhizomucor miehei* lipase (hydrolytic activity 6000 U g^{-1}; ester synthesis activity 92% yield of *n*-butyl oleate in 10 h, 37°C; water 8.7%) was purchased from Biocatalyst (Pontypridd, Wales, UK). All lipases were used without further purification and within the time frame rec-

Correspondence: Dr Y-Y Linko, Laboratory of Bioprocess Engineering, Helsinki University of Technology, PO Box 6100, FIN-02015 HUT, Finland
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ommended by the manufacturer. Moisture content of the enzyme preparations was determined by drying *ca* 2 g samples overnight at 105°C.

Terephthalic acid (1,4-benzene dicarboxylic acid) was obtained from Fluka Chemie AG (Buchs, Switzerland) and isophthalic acid (1,3-benzene dicarboxylic acid) from Riedel-deHaen AG (Seelze, Germany). 1,4-Butanediol and diphenyl ether were purchased from Aldrich-Chemie AG (Steinheim, Germany) and 1,6-hexanediol from Merck (Darmstadt, Germany). Diphenyl ether (b.p. 259°C at 1 kPa and 66.1°C at 130 Pa; log $P = 4.9$) was stored over a 4-Å (0.4-nm) molecular sieve. All other chemicals used in the present work were of the highest commercially available purity.

Synthesis of aromatic polyester

To a reaction mixture containing 1.5 mmol diacid and 1.5 mmol diol in 2.25 ml diphenyl ether in a 5-ml round bottom flask equipped with a condenser, lipase was added. Diphenyl ether was chosen as the solvent because of previous experience [13,23]. The reactions were carried out at 60°C (except when otherwise stated), and the reaction mixtures were magnetically stirred at 600 rpm using a multireactor system immersed in a temperature-controlled oil batch. A reduced pressure of 2.6 kPa was exerted for 10 min both after 5 h and 10 h of the reaction. After 22 h, the pressure was further decreased to 20–60 Pa until the reaction was stopped by removing the reactor from the magnetic stirring plate. The product was extracted three times with chloroform, lipase was filtered off, and chloroform was removed by distillation at 40–50°C under reduced pressure. A blank test without lipase was also carried out for each experiment.

Scaling-up of the aromatic polyester synthesis was carried out up to 30 mmol of isophthalic acid and 30 mmol 1,6-hexanediol as substrates and 7.5 g (11.7%, w/w) of Novozym 435 as biocatalyst in 45 ml of diphenyl ether. The reaction was carried out at 60°C for 168 h, under conditions as described above. The weight percentage (w/w) of the lipase was expressed as a percentage of the total reaction mixture.

Time course of poly(1,6-hexanediyl isophthalate) synthesis

The time course of poly(1,6-hexanediyl isophthalate) synthesis was carried out using 0.375 g (11.7%, w/w) of Novozym 435 as biocatalyst, at 60°C. Duplicate samples, each with the total content of a reaction flask, were taken at certain time intervals and the mean values were reported.

Determination of lipase activity

Lipolytic (hydrolytic) activity was determined according to Sigma Technical Bulletin No. 800 using 50% olive oil emulsion (Sigma, St Louis, MO, USA) as substrate at 37°C and pH 7.0 for 30 min [25]. Free fatty acids liberated were titrated with 0.05 M sodium hydroxide using phenolphthalein as the indicator. One unit of lipase activity was defined as the amount of enzyme which catalyzes the release of one μmole of fatty acid per minute at 37°C and pH 7.0.

Synthetic activity was determined either as described previously [7,10,25] and reported as a yield (%) of *n*-butyl oleate at a certain time or as described by Novo Nordisk A/S [19] and reported as propyl laurate units (PLU mg^{-1}). One PLU was defined as the amount of enzyme that catalyzes the synthesis of one μmole of propyl laurate from lauric acid and 1-propanol per minute at 60°C. Activities were given as the average of duplicate determinations.

Molar mass measurement

Mass average molar mass of the polyesters obtained was determined by gel permeation chromatography (GPC), with three serially-connected Styragel columns (100 Å, 500 Å and 10^4 Å, Waters, Millford, MA, USA), using a HP 1047 A refractive index detector (Hewlett-Packard, CA, USA). Eleven different polystyrene standards with known molar masses ranging from 162 g mol^{-1} to 370 000 g mol^{-1} (Polymer Laboratories, Church Stretton, UK) were used to construct a calibration curve. TC*SEC software and the Turbochrom Chromatography System from Perkin Elmer (Norwalk, CT, USA) were used for data analyses. Samples were dissolved in tetrahydrofuran in a concentration of *ca* 0.05 g ml^{-1}, filtered through a 0.5-μm Millex-LCR_4 disposable filter (Millipore, Bedford, UK) or 0.45-μm GHP Acrodisc syringe filter (Gelman Sciences, MI, USA). The filtrate (50 μl) was injected for GPC analysis. Tetrahydrofuran was used as the mobile phase at a flow rate of 0.8 ml min^{-1}, at ambient temperature, for 1 h. The average molar mass of the polyester was estimated by the constructed calibration curve.

Statistical analysis

A standard deviation was calculated from the molar masses obtained from nineteen parallel experiments from six different reaction batches, using 1.5 mmol of isophthalic acid and 1.5 mmol of 1,6-hexanediol as substrates and 0.25 g Novozym 435 as biocatalyst, at 60°C, 168 h using the Regression Analysis Tool of Microsoft Excel.

Melting point measurement

Melting point of the aromatic polyester was determined by differential scanning calorimeter (DSC), using PL Thermal Science DCS equipment (Reometric Scientific, UK). Nitrogen was used as the sweeping gas. A 4- to 8-mg sample was heated and cooled at a rate of 10°C min^{-1}, and the heating and cooling cycle was repeated twice. The scanning temperature range was from −20 to 180°C.

Results and discussion

Type of substrates

The effect of different substrates on the synthesis of aromatic polyesters was investigated with Novozym 435 that is recommended as biocatalyst for ester synthesis [20], and the results are illustrated in Table 1. The relative position of the two functional carboxylic groups on the benzene ring was a critical factor for the lipase-catalyzed reaction. No polyester was observed using terephthalic acid (1,4-benzene dicarboxylic acid, *p*-phthalic acid) as the diacid. However, a polyester with a high molar mass was obtained with isophthalic acid (1,3-benzene dicarboxylic acid, *m*-phthalic acid). This phenomenon suggested that the lipase

Table 1 Effect of different substrates on aromatic polyester synthesis using Novozym 435 as biocatalyst

Substrates		Molar mass (g mol^{-1}) at		
Diacid	Diol	45°C	60°C	70°C
Terephthalic acid	1,4-butanediol	nd	nd	nd
Terephthalic acid	1,6-hexanediol	nd	nd	nd
Isophthalic acid	1,4-butanediol	1100	2300	nd
Isophthalic acid	1,6-hexanediol	2000	38000	55000

nd, not detected.

(Novozym 435) was somewhat selective for the 1,3-position of the two functional carboxylic groups. No polyesterification took place in the absence of lipase. The direct conventional synthesis of AA/BB-type polyesters would require more severe conditions such as the heating of the mixture of a dicarboxylic acid and a diol to above 150°C under inert gas, preferably followed by a second stage at 270–280°C at a reduced pressure [16].

These results are in agreement with those described by Mezoul *et al* [17], who attempted to prepare aromatic polyesters by enzymatic polytransesterification by using dimethyl esters of *o*-phthalic acid, terephthalic acid or isophthalic acid and 1,6-hexanediol as substrates. They carried out the reaction in toluene, also using Novozym as the biocatalyst. No product was found with dimethyl phthalate. Dimethyl terephthalate gave only a number average molar mass of 1860 g mol^{-1} and weight average molar mass of 2790 g mol^{-1}, whereas dimethyl isophthlate resulted in a number average molar mass of 6100 g mol^{-1} and a relatively high weight average molar mass of 31700 g mol^{-1}. No polymerization was observed with dimethyl phthalate, which was believed to be due to the ortho-position of the two methylated carboxylic groups. To explain the reason why Novozym 435 works well with isophthalic acid, and poorly with terephthalic acid needs further work applying molecular modeling.

In addition, the type of diol was also very important in order to obtain a high molar mass polyester. We have previously demonstrated the effect of different chain lengths of the diol on the synthesis of aliphatic polyesters. The molar mass of the polyester was found to increase with the chain length of the diol up to the carbon number of 5, with the 1,5-pentanediol giving the best result [24]. In the present case, the molar mass of the obtained polyester was only *ca* 2000 g mol^{-1} (PD = 1.6) with 1,4-butanediol and isophthalic acid, but as high as 38000 g mol^{-1} (PD = 1.5) with 1,6-hexanediol and isophthalic acid, under otherwise similar reaction conditions. The standard deviation derived from 19 tests from six different batches under the same reaction conditions (60°C, 0.25 g of Novozym 435) was *ca* ± 10000 g mol^{-1}, with the obtained range of the mass average molar mass from 28000 g mol^{-1} to 48000 g mol^{-1}. Although the standard deviation was high, this could be expected in the relatively small scale under conditions in which mixing with the immobilized lipase presents difficulties.

Effect of temperature

For investigating the effect of temperature, 0.25 g (8.1%, w/w) of Novozym 435 was used as the biocatalyst at temperatures varying from 45 to 70°C. The results are given in Figure 1, which shows that a relatively steep and nearly linear increase in the molar mass was observed. The mass average molar mass obtained at 45°C was only *ca* 2300 g mol^{-1} (PD = 1.6), and increased to as high as *ca* 55000 g mol^{-1} (PD = 1.3) at 70°C. A similar behavior has also been shown in the synthesis of saturated polyesters [11]. An increase in the molar mass from 1200 g mol^{-1} to 4600 g mol^{-1} by raising the temperature from 25 to 69°C for the enzymatic polymerization of methyl 6-hydroxyhexanoate has been described by Knani *et al* [8]. Consequently, temperature plays an important role in the enzymatic synthesis of polyesters. However, in the present case, the product yield obtained at 70°C was only *ca* 30% (w/w, based on the total weight of substrates) after three subsequent precipitations by methanol, while it was *ca* 50% (w/w) at 60°C. Obviously, lipase was partly inactivated at 70°C during the relatively long reaction time. Novozyme 435 has a relatively high ester synthetic activity up to 70°C under typical operating conditions, but the stability of the enzyme decreases rapidly at temperatures above 60°C and the enzyme is recommended to be used within the temperature range of 40 to 60°C [19,20]. Further, increasing evaporation of the solvent at temperatures above 70°C at pressures below 130 Pa made it impractical to run experiments at higher temperatures. Consequently, 70°C was chosen as the maximum experimental temperature, and 60°C was selected as a standard temperature for further studies.

Effect of type and quantity of lipase

Of several different commercially available crude lipases such as the lipases from *Candida rugosa*, *Pseudomonas fluorescens*, *Rhizomucor miehei* and porcine pancreas, only the lipase from *R. miehei* works well in the synthesis of high molar mass aliphatic polyesters [7,23,26]. Apparently the three-dimensional structure of the *R. miehei* lipase active site favors the enzyme substrate complex formation in polyesterification [6]. Therefore, this lipase was chosen for the synthesis of the aromatic polyester from isophthalic acid and 1,6-hexanediol. Surprisingly, neither the commercial immobilized lipase Lipozyme IM nor the solid *R.*

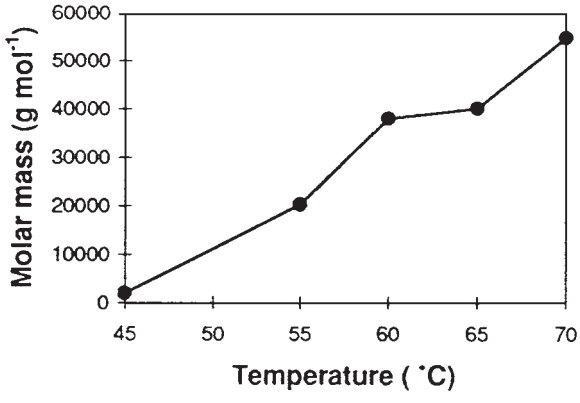

Figure 1 Effect of temperature on lipase-catalyzed synthesis of poly(1,6-hexanediyl isophthalate) (0.25 g, 8.1%, w/w, of Novozym 435; 168 h).

miehei lipase powder used in the present work catalyzed the synthesis of aromatic polyesters beyond the oligomer stage, although the quantity (and total activity) of the *R. miehei* lipase powder was three-fold of that used successfully in the aliphatic polyester synthesis [27]. Consequently, of the three lipase preparations tested, only Novozym 435 was a relatively efficient biocatalyst under the reaction conditions used. Lipases possess regioselectivity, stereoselectivity and chemoselectivity [15], and for a specific application a careful screening of the lipases is recommended [14,25].

Quantities of Novozym 435 up to 0.5 g (15%, w/w) were used in the synthesis of poly(1,6-hexanediyl isophthalate). The results are illustrated in Figure 2. In our previous work, the investigation of the effect of different quantities of *R. miehei* lipase on the synthesis of aliphatic polyesters showed that the highest molar mass was obtained by only 0.125 g of non-immobilized *R. miehei* lipase powder (protein content: 23%, w/w) [26,27]. In the present work, a considerably higher quantity of immobilized lipase Novozym 435 was required for aromatic polyester synthesis, suggesting that the biocatalytic synthesis of aromatic polyesters is considerably more difficult than the synthesis of aliphatic polyesters. However, because different non-immobilized or immobilized powdered lipases were used in the previous [27] and present investigations, the results obtained concerning the amount of enzyme cannot be directly compared. In the present work, with 0.125 g (4.2%, w/w) of Novozyme 435, a mass average molar mass of only *ca* 3000 g mol^{-1} (PD = 1.2) was obtained in 168 h, and 0.375 g (11.7%, w/w) of the enzyme was needed to obtain a molar mass of *ca* 50000 g mol^{-1} (PD = 1.4) of poly(1,6-hexanediyl isophthalate). Nevertheless, a further increase in the lipase quantity up to 0.5 g (15%, w/w) resulted in a slight decrease in the molar mass as shown in Figure 2, apparently due to the poor mixing or diffusion limitations in such a highly viscous system. No polymer synthesis was observed when no lipase was added to the reaction system.

Time course of synthesis of poly(1,6-hexanediyl isophthalate)

A study of the time course gives important information about the governing strategy for the polyester synthesis reaction. The time courses of the synthesis on poly(1,6-hexanediyl isophthalate) with and without vacuum are shown in Figure 3. Vacuum was important in aromatic polyester synthesis in order to obtain a high average molar mass. With vacuum, the highest molar mass of the polyester obtained was *ca* 50000 g mol^{-1} (PD = 1.4), while without vacuum a molar mass of only *ca* 16000 g mol^{-1} (PD = 1.3) was reached. This is in a good agreement with the results of others in that the control of the thermodynamic equilibrium of the reaction system for example by a reduced pressure [11,13,17,23], molecular sieve [2] or flushing by nitrogen [9] is essential in the enzyme-catalyzed esterifications. For example, although a certain amount of water is necessary for the enzyme to function, excess water may result in reverse hydrolysis.

The proper reaction time is also important in order to obtain a high molar mass of the product (Figure 3). During the first 48–96 h, the molar mass of the polyester increased rapidly from *ca* 3000 g mol^{-1} (PD = 1.2) to *ca* 40000 g mol^{-1} (PD = 1.5). Thereafter, the molar mass continued to increase to a maximum of *ca* 50000 g mol^{-1} at 168 h. According to Chaudhary *et al* [3], the achievable molar mass in polyester synthesis might also be limited by a combination of lipase instability, the equilibrium position for the reaction and a change in the enzyme specificity during the reaction.

Furthermore, a mass average molar mass of poly(1,6-hexanediyl isophthalate) of *ca* 40000 g mol^{-1} (PD = 1.6) was obtained from 20-fold scaling-up of the starting materials in 168 h at 60°C, by using 11.7% (w/w) of Novozym 435 as biocatalyst. The melting point of the obtained poly(1,6-hexanediyl isophthalate) ranged from 60 to 90°C from batch to batch, probably because of the dispersity or complexity of the reaction and/or variations in the molar mass distribution of the polymer. Conventional chemical polymerization methods suffer from the relatively harsh reaction conditions required. The advantage of biocatalysis, compared with conventional chemical methods, is the relatively low temperature and pressure, which would markedly reduce the most undesirable side-reactions. Further work is going on to improve the properties of the polyesters obtained, especially in further increasing the polymer molar

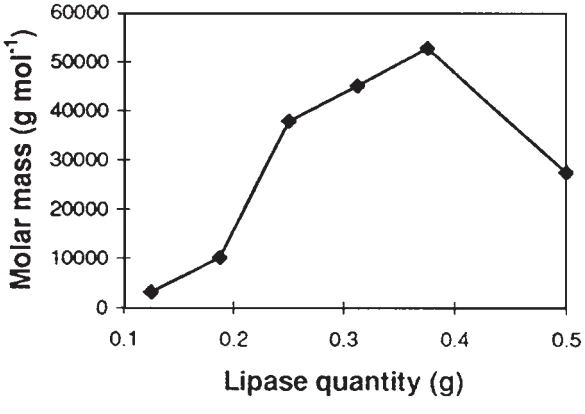

Figure 2 Effect of lipase bulk quantity on lipase catalyzed synthesis of poly(1,6-hexanediyl isophthalate) (Novozym 435, ester synthesis activity 7000 PLU g^{-1}; 60°C; 168 h).

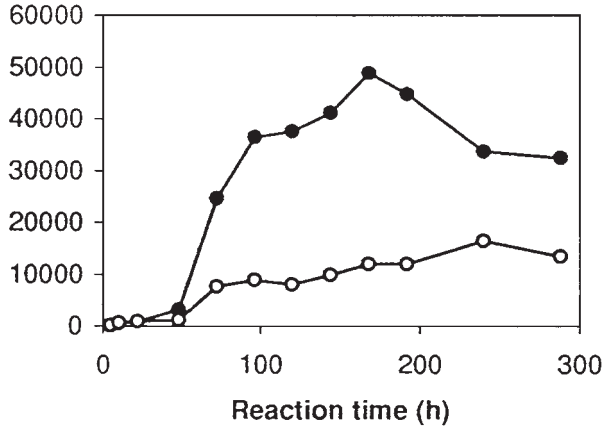

Figure 3 Time courses of the synthesis of poly(1,6-hexanediyl isophthalate) with (•) and without (○) vacuum (0.375 g, 11.7%, w/w, of Novozym 435; 60°C.)

mass, in obtaining a product with a higher melting point, and in further optimizing the reaction conditions.

Acknowledgements

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