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Mechanistic studies on the enzymatic transesterification of polyesters

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Abstract—The effect of solvents on the enzymatic transesterification of aliphatic polyesters has been investigated. It has been shown that the hydrophobicity and the polarity of the solvent have little effect on the reaction, however, the molecular weight of the product depends on the solubility of the product in the medium. Above a certain molecular weight, when the product is no longer soluble in the medium, transesterification does not occur. Deuterium NMR studies have shown that transesterification tends to take place at the ends of the polymer chain rather than at random along the polymer chain.

1. Introduction

In recent years the enzymatic synthesis of polyesters has aroused considerable interest because of the possibility of producing novel polyesters with unusual properties. The early work was done using activated acids and or diols, such as divinyl adipate and halogenated alcohols in non-aqueous solvents,[1] the resulting polyesters were oligomers[2] of relatively low molecular weight of approximately 2000 Da. It was shown subsequently that using the enzyme *Candida antarctica* lipase B supported on acrylic beads (*Novozyme* 435™) it was possible to synthesise much higher molecular weight polyesters in the absence of solvent.[3] A number of workers have also studied the enzymatic catalysis of polyesters by the transesterification of both activated and unactivated diesters with diols[4] and by the ring opening transesterification of lactones.[5]

When the polyesters prepared by enzymatic synthesis in the absence of solvent were studied it was found that they had significantly different properties compared to those prepared by the conventional high temperature process. It was found that not only was it possible to obtain higher molecular weight polymers but they had a lower dispersity, that is a narrower molecular weight distribution than the conventional polyesters with the result that products made from these materials have superior physical properties.[6] These results were explained by the apparent absence of a transesterification reaction during the synthesis.[2] It was subsequently shown that if the reaction was carried out in the presence of toluene then not only was the dispersity higher but that the molecular weight obtainable was reduced. The conclusion being that transesterification occurs in the presence of toluene as solvent causing scission of the growing polymer. This conclusion was confirmed when the synthon, 1,6-hexanedioic acid di(4-hydroxybutyl) ester (BAB) was shown not to react with itself in the absence of solvent, whereas in toluene the enzyme catalysed the transesterification and a number of higher molecular weight oligomers were formed.[7]

Lipase catalysed transesterification reactions in organic media with monoesters are well known and have been used to separate racemic mixtures of alcohols and carboxylic acids or to select a specific ester or alcohol group within a molecule as substrate.[8] Therefore, the original question of why the enzyme appears to catalyse the transesterification of polyesters in some circumstances and not in others becomes more interesting, because one would expect it to catalyse transesterification in all circumstances. It had been considered initially that the enzyme might adopt a different configuration in solvent thereby affecting the reaction and the resulting products. However, it has been shown, using synchrotron CD spectroscopy that no major changes in the structure of the protein take place when used in solvents such as hexane and toluene.[9] The possibility that solvent molecules were being absorbed onto the hydrophobic regions in or around the active site was considered, as even one or two bulky solvent molecules such as toluene absorbed in a critical area could easily affect the rate at which the substrate could diffuse into the active site of the enzyme.

In all our earlier work, the acid-diol esterification reaction

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and any transesterification reaction between ester groups, leading to the formation of the polyester would be taking place simultaneously, with the final composition containing the products of both. Therefore, in order to study the transesterification reaction, it was necessary to develop a method whereby the transesterification reaction could be studied independently of the esterification reaction. If the reaction was being affected by solvent absorbed at the active site, then the effects of the size, shape and physical properties of the solvent on the transesterification reaction were of interest.

2. Results

2.1. Transesterification reactions

The hydroxyl group of the added diol involved in the transesterification reaction causes scission of the high molecular weight polyester, the extent of transesterification being proportional to the reduction in the average molecular weight of the polyester. The results obtained are shown in Table 1.

Table 1. Effect of solvent on the transesterification of polyester

Solvent	M_w	M_n	Dispersity
PTMEG 650	36,000	32,000	1.1
1,4-butanediol	31,000	26,000	1.2
Toluene	5800	2900	2.0
Dioxane	1400	750	1.8

It appears from these results that the amount of transesterification is independent of hydroxyl concentration. The greatest breakdown of the high molecular weight polyester occurred with toluene and dioxane and not with 1,4-butanediol or polytetramethylene ether glycol of molecular weight 650 (PTMEG 650), which have much higher concentrations of hydroxyl groups. This observation appears to contradict the conclusion of Kumar and Gross,[10] who proposed that there is slower transesterification in higher molecular weight polycaprolactones because the higher the molecular weight, the fewer terminal hydroxyls being present to take part in the transesterification reaction.

It had been found by Harffey[11] that the addition of as little as 6% w/w of toluene to the reaction medium gave the same result as when the reaction was carried out in toluene as the reaction solvent. The possibility that toluene was being absorbed from the medium onto the hydrophobic areas of the protein, thereby affecting the mechanism of the reaction, was considered. Therefore, the effect of low concentrations of toluene in 1,4-butanediol on the degree of transesterification was determined. The results are shown in Table 2.

Table 2. Effect of toluene concentration on transesterification

Principal solvent	[Toluene] (mM)	M_w	M_n	Dispersity
1,4-BD	0.11 (0.001%)	26,000	14,000	1.9
1,4-BD	1.1 (0.01%)	28,000	19,000	1.5
1,4-BD	67 (0.625%)	27,000	17,000	1.6
1,4-BD	200 (1.87%)	28,000	18,000	1.6

The differences between these results are not considered to be significant. It appears that small additions of solvent do not affect the degree of transesterification. It is unlikely, therefore, that absorption of solvent into the hydrophobic areas of the enzyme takes place, as it would be expected that the hydrophobic attraction of the lipase would extract toluene from such a polar medium as 1,4-butanediol, even at these low concentrations.

The effect of the shape of the solvent molecule was then investigated. The standard transesterification experiment was carried out with a number of solvents of different shapes and hydrophobicity. The results are as shown in Table 3 below.

Table 3. Effect of solvent configuration on transesterification

Solvent	M_w	M_n	Dispersity	$C \log P$
Toluene control	5800	2900	2.0	2.7
n-Butylbenzene	5200	2800	1.9	4.0
iso-Butylbenzene	5900	3000	2.0	4.0
tert-Butylbenzene	5900	2900	2.0	4.1
4-Chlorotoluene	5900	2900	2.0	3.3
Hexane	18,000	8600	2.0	3.8

The GPC profiles for all the experiments listed in Table 3 were essentially the same for all the aromatic solvents.

It is evident from these results that the degree of transesterification as measured by chain scission is not affected by the geometry of the solvent molecule. The differences seen in the experiments with the aromatic solvents are not considered significant; the only significant difference being between the aromatic solvents and the aliphatic hexane. It does not appear, however, that the hydrophobicity as measured by $C \log P$ is the cause of the difference; as there is significant transesterification at a $C \log P$ of -0.4 in dioxane and also at $C \log P$ of 4.1 in *tert*-butylbenzene.

The transesterification reaction was repeated using solvents that were substantially more polar in order to determine if the reaction is affected by the polarity of the solvent. Propylene carbonate, triethylene glycol methyl ether and tetraethylene glycol methyl ether were chosen as the added solvent. In all cases extensive transesterification took place and the molecular weight fell to around 3000 Da as measured by GPC. There did not appear to be any significant difference in the amount of transesterification in relation to the polarity of the solvent.

The only two media in which transesterification does not occur are 1,4-butanediol and polytetramethylene ether glycol both of these are very poor solvents for the high molecular weight polyester. It would appear that the transesterification reaction takes place in any solvent in which the higher molecular weight polyester is soluble.

2.2. NMR studies

In order to elucidate the mechanism, the transesterification reaction was studied using deuterium NMR. The insertion of deuterated 1,4-butanediol into the polyester being followed

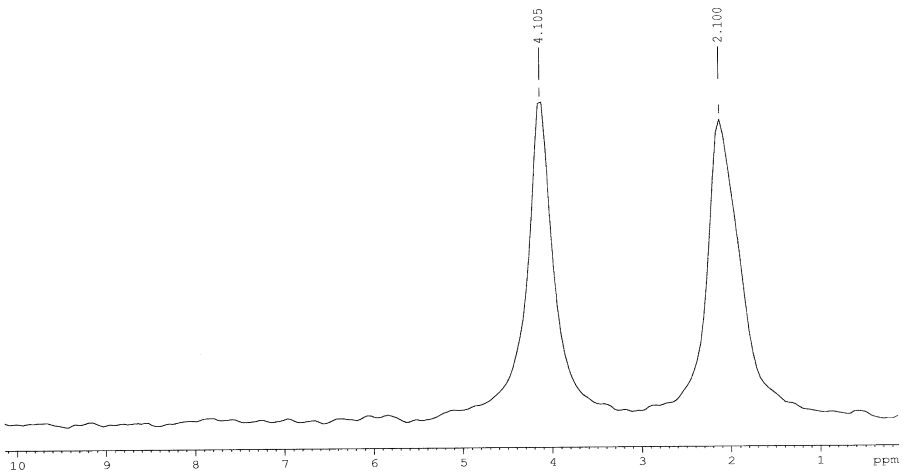

Figure 1. ^{2}H NMR spectrum of deuterated 1,4-butanediol.

by observing the downfield shift due to the presence of adjacent ester carbonyl groups.

A 1% solution of d^{8}-1,4-butanediol in 1,4-butanediol was prepared and the ^{2}H NMR spectrum obtained (Fig. 1).

The two peaks of the deuterated diol were at 4.11 ppm, corresponding to the 1,1′- and 4,4′-deuteriums and 2.14 ppm, corresponding to the 2,2′- and 3,3′-deuteriums. The signal-to-noise ratio was 150:1, therefore we were confident that using this method we would be able to see whether transesterification had taken place, by looking for the insertion of the deuterated 1,4-butanediol into the polyester.

In order to maximise the visibility of the deuterated diol in the polyester, the concentration of the deuterated diol was increased to 10%. Samples were taken at 2, 5 and 21 h and the ^{2}H NMR spectra obtained, no difference could be seen in any of the spectra, thus indicating that in the 1,4-butanediol medium no observable transesterification had taken place. After 24 h a very small peak at 4.68 ppm was starting to show, indicating that a small amount of deuterated diol had been incorporated into the polyester. After 48 h a somewhat larger peak was observed at 4.68 ppm, which indicated that transesterification does take place in 1,4-butanediol, but that it is very slow.

This experiment was repeated after adding 6 ml of toluene and stirring at 60 °C a ^{2}H NMR spectrum with excellent signal-to-noise ratio (2000 scans) was obtained. After 24 h, there was a small but distinct peak at 4.68 ppm due to the increased chemical shift when one end of the diol is incorporated into an ester group and the peak at 2.12 ppm was starting to split, with a pronounced shoulder at 2.21 ppm, as a result of the d^{8}-1,4-butanediol now forming a significant part of the ester groups. This indicated that the d^{8}-1,4-butanediol had been incorporated into the polyester and that transesterification had occurred (see Fig. 2A).

After 48 h it could be seen that both of the deuterium resonances had split and the new peaks had moved downfield (see Fig. 2B). The $OC^{2}H_{2}$ peak had moved in its entirety to 4.73 ppm, leaving only a small peak at 4.18 ppm. The $C^{2}H_{2}-C^{2}H_{2}$ peak had also moved downfield to 2.33 ppm, which proved that there had been significant incorporation of the deuterated diol into the polyester.

The initial experiments without added toluene were repeated using a polyhexane adipate polyester of 2000 Da. The sample taken at 24 h showed that the $HO-C^{2}H_{2}$ peak at 4.11 ppm had split equally with the $COO-C^{2}H_{2}$ peak at 4.68 ppm. Similarly, the diol $C^{2}H_{2}-C^{2}H_{2}$ peak at 2.21 ppm had also split equally with the polyester $C^{2}H_{2}-C^{2}H_{2}$ peak at 2.12 ppm. This showed that after 24 h, significant chain scission caused by transesterification with the added diol had taken place and after 48 h even more transesterification was found.

The polyester with the lowest and most uniform molecular weight is the simple oligomer 1,6-hexanedioic acid di(4-hydroxybutyl) ester (BAB). In order to see if this was also susceptible to transesterification in the absence of solvent, a sample of BAB was prepared starting from 6-carboxy-11-hydroxy-7-oxaundecanoic acid (AB) synthesised by the method of Harffey.[11] AB was reacted with a two-fold molar excess of 1,4-butanediol using *Novozyme* 435 as catalyst. After 24 h ^{1}H NMR spectroscopy showed that 100% conversion to BAB had taken place. The enzyme and solvent were removed and 10% deuterated diol and *Novozyme* 435 were added and the mixture heated at 60 °C for 24 h. ^{2}H NMR spectroscopy on the purified sample showed a very small, deuterated ester peak at 4.68 ppm, this indicated that some but not very much transesterification had occurred.

Samples from the experiments above were analysed by GPC to see if the effect of the transesterification could be determined. Most interestingly, the GPC of the high molecular weight polyester transesterified with deuterated 1,4-butanediol in the absence of toluene showed that the peak molecular weight, M_{w} 36,000, had declined very little but a number of low molecular weight oligomers had appeared (Fig. 3), whereas the 2000 M_{w} polyester had reduced to 980 M_{w} in the same time.

This result shows that the scission is not taking place at random along the polyester backbone, but rather that it takes

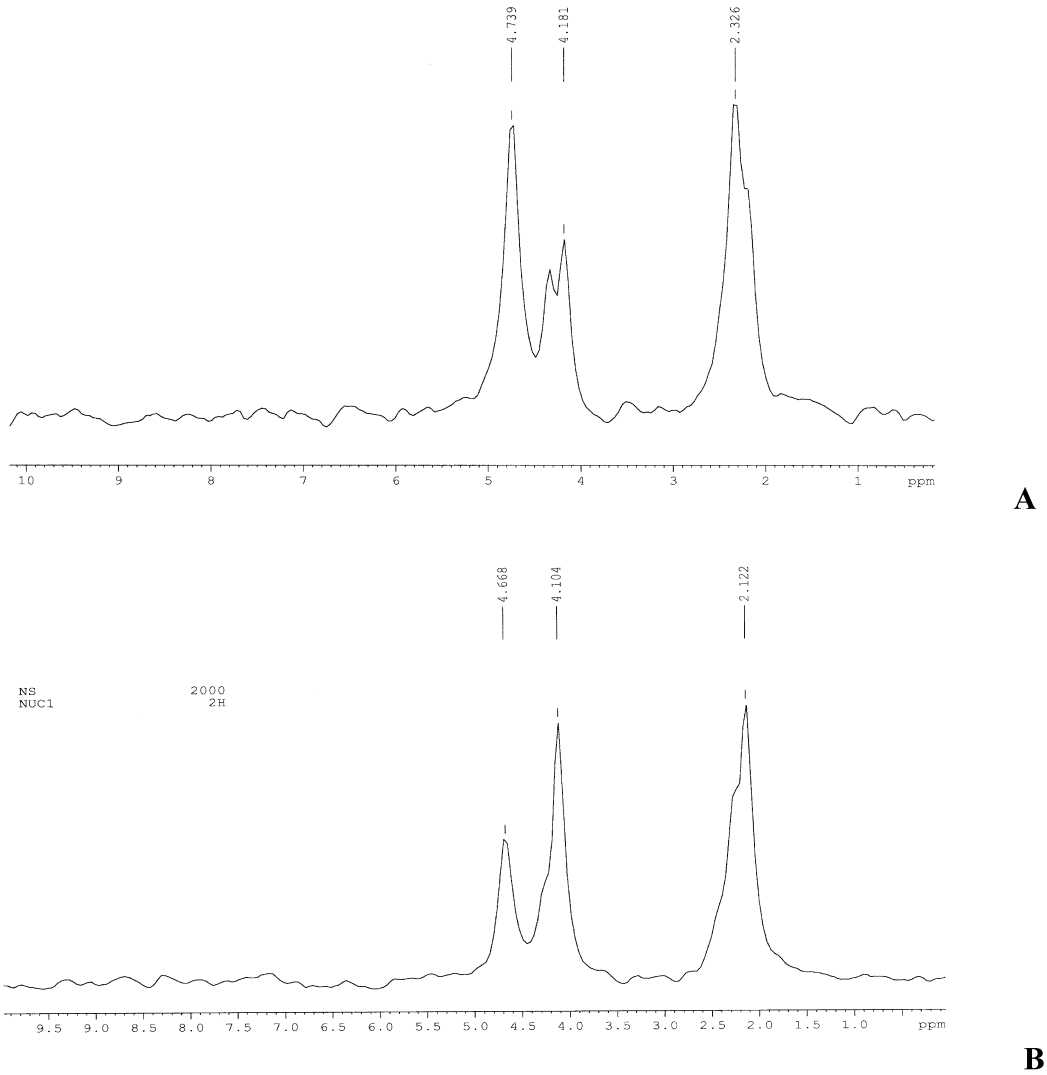

Figure 2. ^{2}H NMR spectrum of polyester+$^{2}H_8$-1,4-butanediol+toluene A after 24 h and B after 48 h.

place at the ester groups near to the end of the polymer chain. This is unlikely to be due to any property of the enzyme because each ester group and its environs are identical and any could fit into the pocket of the enzyme. It is more likely to be a property of the polyester. It is possible that it forms a tight coil in these media and it is only the ends of the chain that are available to the enzyme.

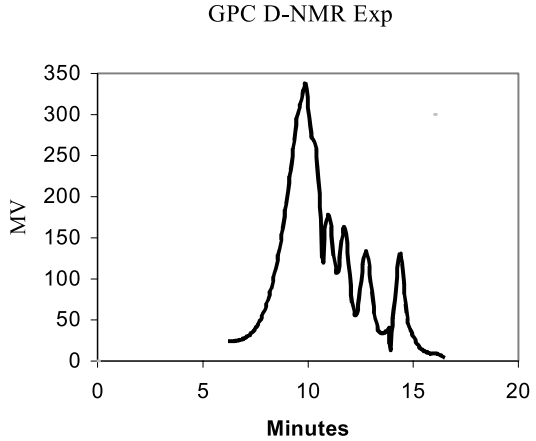

Figure 3. High molecular weight polyester after transesterification with deuterated 1,4-butanediol.

It appears from these results, that the rate of transesterification reaction, measured by the amount of insertion of $^{2}H_8$-1,4-butanediol into the polyester, whilst occurring in 1,4-butanediol is very much faster in toluene. The amount of insertion into the high molecular weight polyester and the 2000 molecular weight polyester appear to be approximately the same, however, the results of the transesterification are quite different. In the case of the high molecular weight polyester there is nominal reduction in the molecular weight as the chain scission only occurs at the ends of the molecule. In the 2000 Da polyester the chain scission occurs at random along the chain with the result that there is a significant reduction in molecular weight.

The transesterification of the oligomer BAB appears to be quite rapid, however, BAB cannot polymerise in the presence of excess diol, therefore the deuterated diol can only react at one of its two hydroxyls and this is reflected in the relative size of the peak shifted downfield.

The conclusion from these experiments seemed to be that

the reason for the lack of transesterification in the absence of solvent is the insolubility of the high molecular weight polyester in 1,4-butanediol or PTMEG 650. It is proposed that in the absence of solvent the growing polymer chain is held in the proximity of the enzyme active site by hydrogen bonding to the surface of the enzyme, a mechanism known in the synthesis of biopolymers such as RNA.[12] It is only when the polymer is in solution that the visceral ester groups are available to the enzyme for transesterification to take place. It might be that as the polyester reaches a critical molecular weight it starts to drop out of solution in the diol, thus limiting the transesterification reaction.

Modelling using Sybyl 6.0 and Sculpt 3.0 produced a very similar result, in both cases the growing polyester partially wrapped around the surface of the enzyme, bound by multiple hydrogen bonds to the surface of the enzyme. The non-bound part of the polymer chain adopted a tightly coiled configuration held together by intramolecular hydrogen bonds. If this is an accurate simulation then in a medium in which the polyester is not soluble it is only at or near to the ends of the molecule that the ester groups are available for transesterification. This would explain the fact that in the absence of solvent much higher molecular weight products are obtained, there being no transesterification to cause scission of the growing polymer.

3. Conclusions

Transesterification reactions catalysed by *Novozyme* 435 have been carried out between high molecular weight polyester and the diols 1,4-butanediol, 1,6-hexanediol and PTMEG 650 in the presence of a number of different solvents. It appears that the transesterification reaction depends predominantly on the solubility of the polyester in the medium and that excess hydroxyl content does not influence the transesterification if the polyester is not soluble in the diol. The hydrophobicity, polarity and shape of the solvent molecules do not influence the nature of the transesterification reaction. It appears that unless the polyester is soluble in the medium the growth of the polymer is not limited by the transesterification reaction and high molecular weight polyester is formed. The limited amount of transesterification occurs preferentially at the ends of the polymer rather than at random along the chain.

4. Experimental

4.1. Synthesis of polyhexane adipate polyester

R_1-[$OCH_2(CH_2)_4CH_2OCO(CH_2)_4COO]_n$-$R_2$

A high molecular weight polyhexane adipate polyester was synthesised using *Novozyme* 435 as the catalyst. A 1:1 molar ratio of 1,6-hexanediol and adipic acid was stirred at 60 °C for 1 h, 0.5% w/w *Novozyme* 435 added and a vacuum of 100 mb applied for 24 h. The vacuum was increased to 10 mb and the stirring and heating continued for a further 24 h at which point the vacuum was increased to 3 mb for a further 24 h. When the polyester had reached a molecular weight of 36,000 as measured by GPC the reaction was stopped and the acid number and hydroxyl number determined by titration. The bound enzyme was filtered off and the residual enzyme deactivated by heating the polyester at 200 °C for 15 min. This polyester had a M_n of 17,500, a M_w of 37,000 and dispersity 1.9. The acid number was measured as 2 mg KOH g^{-1} and the hydroxyl number was 13 mg KOH g^{-1}. This polyester sample became the standard for all our subsequent transesterification experiments. The molecular weights for this and all subsequent reaction products were determined by using a Waters HPLC with a model 510 pump, model 410 refractive index detector and the Waters 717 autosampler. The column was packed with Polymer Labs. 1000 Å polystyrene copolymer packing and the eluent used was THF stabilised with 250 ppm of butylated hydroxytoluene (BHT) at a flow rate of 1 ml min^{-1}. The sample concentration for all experiments was 0.5% w/vol with an injection volume of 40 μl. The data were analysed using the Millennium 32 GPC software. The GPC trace for the polyhexane adipate polyester is shown in Figure 4.

4.2. Procedure for transesterification

In order to study the effects of different solvents on the transesterification reaction a standard transesterification reaction was developed.

2 g of high molecular weight polyester plus 8 ml of solvent, 0.1 g of 1,6-hexanediol and 0.1 g of *Novozyme* 435 were added to a stirred cell reactor and heated at 60 °C for 24 h. The reaction was then stopped by filtering off the bound enzyme and cooling rapidly to 20 °C. In order to simulate the conditions that had existed in earlier syntheses, the transesterification was carried out in the presence of the solvents toluene and dioxane in the presence of 1,4-butanediol and polytetramethylene ether glycol (PTMEG) 650 to simulate the excess monomer and oligomer that is present in the early stages of the solvent free polymerisation. *n*-butylbenzene, *iso*-butylbenzene, *tert*-butylbenzene, 4-chlorotoluene, hexane, propylene carbonate, triethyleneglycol methyl ether and tetraethyleneglycol methyl ether were used to investigate the effect of hydrophobicity, polarity and size of the solvent molecule on the transesterification

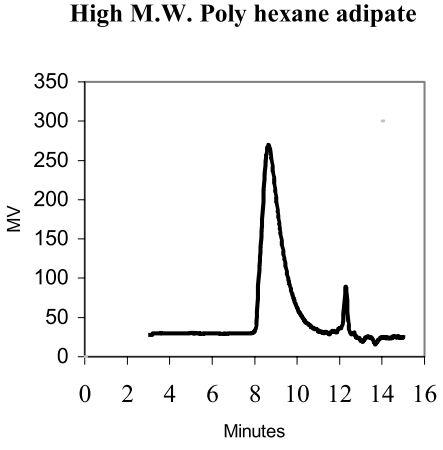

Figure 4. GPC of the high molecular weight polyhexane adipate used as substrate for transesterification.

reaction. All materials were purchased from Sigma-Aldrich and were standard reagent grade purity.

4.3. NMR studies

The toluene and 1,4-butanediol experiments were repeated using 1,1′,2,2′,3,3′,4,4′-octadeutero-1,4-butanediol in place of the 1,6-hexanediol, as the transesterification agent. A 10% solution of the d^8-1,4-butanediol in 1,4-butanediol was added to 5 g of the polyester, and 0.1 g of *Novozyme* 435. The mixture was stirred in a cell reactor at 60 °C and sampled at 24 and 48 h. Filtering off the *Novozyme* stopped the reaction and the residual 1,4-butanediol was stripped off using a Kugelrohr evaporator prior to observing the NMR spectra.

4.4. Molecular modelling

The molecular modelling was done using both Sculpt 3.0[13] and Sybyl 6.0[14] on a SGI Octane UNIX work station. The enzyme was modelled using the pdb file 1tca. A 2000 Da polybutane adipate polyester was constructed and docked manually to the Ser 105 residue of the active site. The Sculpt model was energy minimised using MM3 with both van der Waals and electrostatic interactions. The Kollman all Atom forcefield was used for the Sybyl model, which was then energy minimised using the Powell method within the Sybyl program.

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