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1 **Controlled Enzymatic Synthesis of Polyesters Based on a**
2 **Cellulose-Derived Triol Monomer: A Design of Experiment**
3 **Approach**

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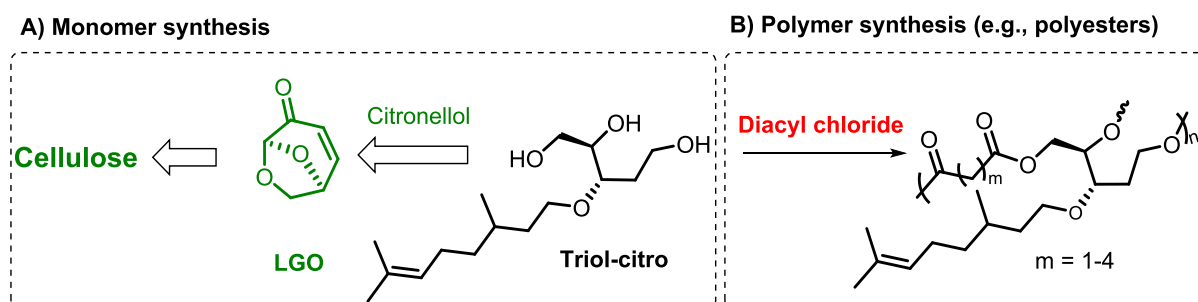
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18 **Abstract**

19 Regioselective enzymatic polycondensation of the bio-based cellulose derived polyol,
20 Triol-citro, and dimethyl adipate using *Candida antarctica* Lipase B (CaLB) was
21 investigated. A Design of Experiment approach with MODDE® Pro 13 was used to
22 determine important factors in the branching behavior of this polymer, and reactant
23 ratio, temperature, reaction time and enzyme wt% were the studied factors.
24 Multifunctional polyesters with pendant hydroxy groups were synthesized and fully
25 characterized using 2D NMR techniques to determine degree of branching.
26 Branching was minimal, with a maximum of 16% observed, and monomer ratio,
27 temperature and reaction time were all determined to be significant factors. In this
28 work, M_n of up to 13 kDa were achieved, while maintaining degree of branching
29 below 15%, resulting in a linear polyester with the potential to be further
30 functionalized.

31 Introduction

32 Since the concept first emerged in the 1990s, the drive to incorporate Green Chemistry into
33 all aspects of the chemical industry has steadily increased. The need for sustainability
34 requires researchers to look away from fossil resources and their growing problematic
35 utilization, and instead turn to biomass feedstock. One platform molecule that has risen to
36 prominence in recent years is levoglucosenone (LGO). It can be synthesized from waste
37 cellulose, and there are many publications focusing on more sustainable production of this
38 chemical.^[1-4] This chiral molecule containing both a cyclic acetal and an α,β -unsaturated
39 ketone has increasingly been used as a starting feedstock for solvents,^[5,6] specialty drugs,^[7,8]
40 and polymers.^[9-12] A recent example involves the development of a green one-pot pathway
41 to synthesise a multifunctional triol monomer (Triol-citro) from LGO.^[13] This monomer
42 features a photocrosslinkable citronellol side group and has been employed in polymer
43 synthesis (Scheme 1.).^[13-15]



44 **Scheme 1. A.** Synthesis of Triol-citro from cellulose and **B.** polymerisation of Triol-citro with diacyl
45 chlorides to form crosslinked polyesters.^[13-15]
46

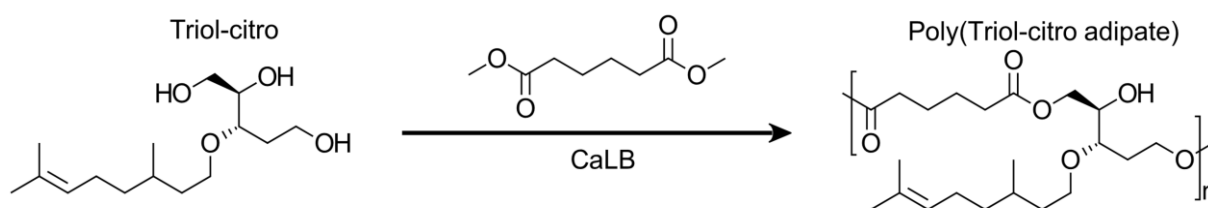
47 Triol compounds are particularly interesting as there is the potential to synthesise polymers
48 with pendant hydroxy groups. Such polymers often have increased hydrophilicity,^[16] which
49 can be useful in drug delivery^[17] or membrane applications.^[18] The presence of reactive
50 hydroxy groups also opens the polymer to functionalisation for further tailoring of their
51 properties. Quaternary ammonium compounds,^[19] proteins^[20] and antibodies,^[21] have all
52 been grafted to a polymer backbone through reaction with an available functional group.
53 Linear polyesters can be converted to crosslinked structures to improve mechanical
54 properties,^[22,23] and these crosslinks can even be reversible to maintain processability.^[24]
55 While functional groups can be introduced at the post-polymerisation stage through surface
56 modification techniques such as enzymatic^[25] or chemical^[26] hydrolysis, as well as physical
57 methods such as plasma treatment,^[27] it is important to note that these methods may lead to
58 a reduced molecular weight polymer. Directly polymerizing a trifunctional monomer
59 eliminates this drawback, but to avoid branching resulting from the reaction of all three
60 hydroxy groups, protecting groups or a selective mode of catalysis must be employed.

61 Lipases are an example of such catalysts, as they catalyze (trans)esterification reactions and
62 often display regioselectivity in their choice of substrate.^[28] Biocatalysts are considered more
63 sustainable compared to more traditional organic catalysts; they are safe, energy efficient
64 and minimize the use of toxic reagents. The enzyme *Candida antarctica* Lipase B (CaLB),
65 due to its *sn*-1,3 regioselectivity, has been used to synthesise polymers based on glycerol,<sup>[29-
66 43]</sup> sorbitol^[36,43-49] and other bio-based triols.^[45,48] By changing different factors and controlling
67 the degree of branching; crosslinked, linear and dendritic polymers can be produced
68 enzymatically.

69 Overall, the controlled synthesis of linear polyesters using triol monomers, with a focus on
70 directing the reaction towards two of the three hydroxy groups to prevent or minimize
71 branching, is of great interest, but most research focuses on glycerol and sugar-derived
72 compounds. The advantage of a monomer such as Triol-citro is twofold. Firstly, the presence
73 of a second (alkene) functional group allows for more than one functionalisation of the
74 polymer. Alternatively, this citronellol side chain can also be photocrosslinked, as shown in
75 Triol-citro based polycarbonates.^[14] Secondly, the synthesis of this molecule is theoretically

76 extremely versatile. The oxa-Michael addition could proceed with a wide range of alkene
 77 alcohols, not only citronellol. There are therefore many potential triol based compounds that
 78 can be synthesized from LGO using this method.

79 Triol-citro based polyesters have been synthesized,^[13] but use of diacyl chloride reagents in
 80 previous approaches resulted in insoluble polymers, due to the formation of
 81 branched/crosslinked structures. In addition, from a green chemistry perspective, diacyl
 82 chloride poses notable hazards due to its highly reactive and corrosive nature. In contrast,
 83 exploring enzymatic approaches, such as utilizing lipase CaLB with diesters, provides a
 84 greener and safer alternative for polymer synthesis. Here, the aim is to use CaLB to
 85 synthesise linear polyesters from the monomers Triol-citro and dimethyl adipate (DMA)
 86 (Scheme 2). A Design of Experiment (DoE) approach was used to investigate the influence
 87 of several factors on polymer molecular weight, conversion, and the degree of branching,
 88 with the intent of producing high molecular weight and preferentially linear polyesters.



89
 90 **Scheme 2.** CaLB-catalyzed polycondensation reaction of Triol-citro and dimethyl adipate to produce
 91 linear polyesters.

92
 93 **Results and Discussion**

94 **Polymer synthesis and characterization**

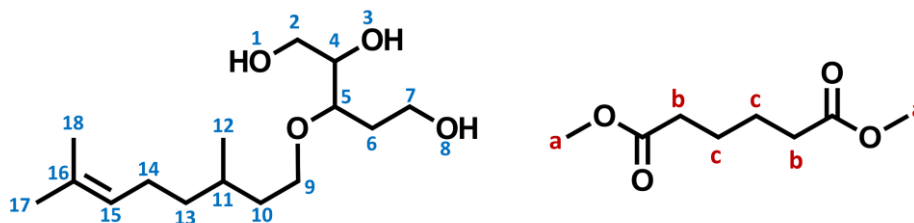
95 In order to identify the factors that affect branching in the enzymatic polycondensation of
 96 polyols, a literature search was conducted. Numerous publications that synthesized
 97 polyesters from polyol monomers were found.^[29,31-44,46-49] Subsequently, temperature,
 98 enzyme wt%, reaction time and monomer ratio were identified as pertinent factors for
 99 investigation in this work (Table 1.). To streamline and optimize the experimental process, a
 100 DoE approach was used.

101 **Table 1.** Factors that have been shown to affect branching and the observed effect in the enzymatic
 102 polycondensation of polyols in previous literature.

Factor	Effect on branching
Temperature	Increased or decreased branching depending on solvent. ^[29] Increased branching with increasing temperature. ^[32]
Enzyme wt%	Increased enzyme wt% results in increased branching. ^[29,31]
Reaction time	Increased reaction time results in increased branching. ^[31,39] Increased reaction time increases molecular weight and has no effect on branching. ^[32]
Monomer ratio	Increasing the amount of polyol monomer results in both increased branching ^[31] , and decreased branching. ^[29] Excess (other) monomer results in increased branching. ^[29,35,40]

103 A central composite design model generated 27 experiments (N1 – N27), seen in Table S1.
 104 The polymers produced pre-washing were liquids of varying viscosity and degrees of
 105 precipitation (see table S2). Conversions and molecular weights were obtained directly from
 106 unwashed sample and were determined using ¹H-NMR and GPC analysis, respectively, with
 107 Equation (1) used to calculate conversion.

$$\text{Conv. (\%)} = 100 - \left(\left(\frac{(I_a/N_a)}{(I_a/N_a) + (I_b/N_b)} \right) \times 100 \right) \quad (1)$$



108 **Figure 1.** Monomers with labels pertaining to equations (1) and (2).
 109

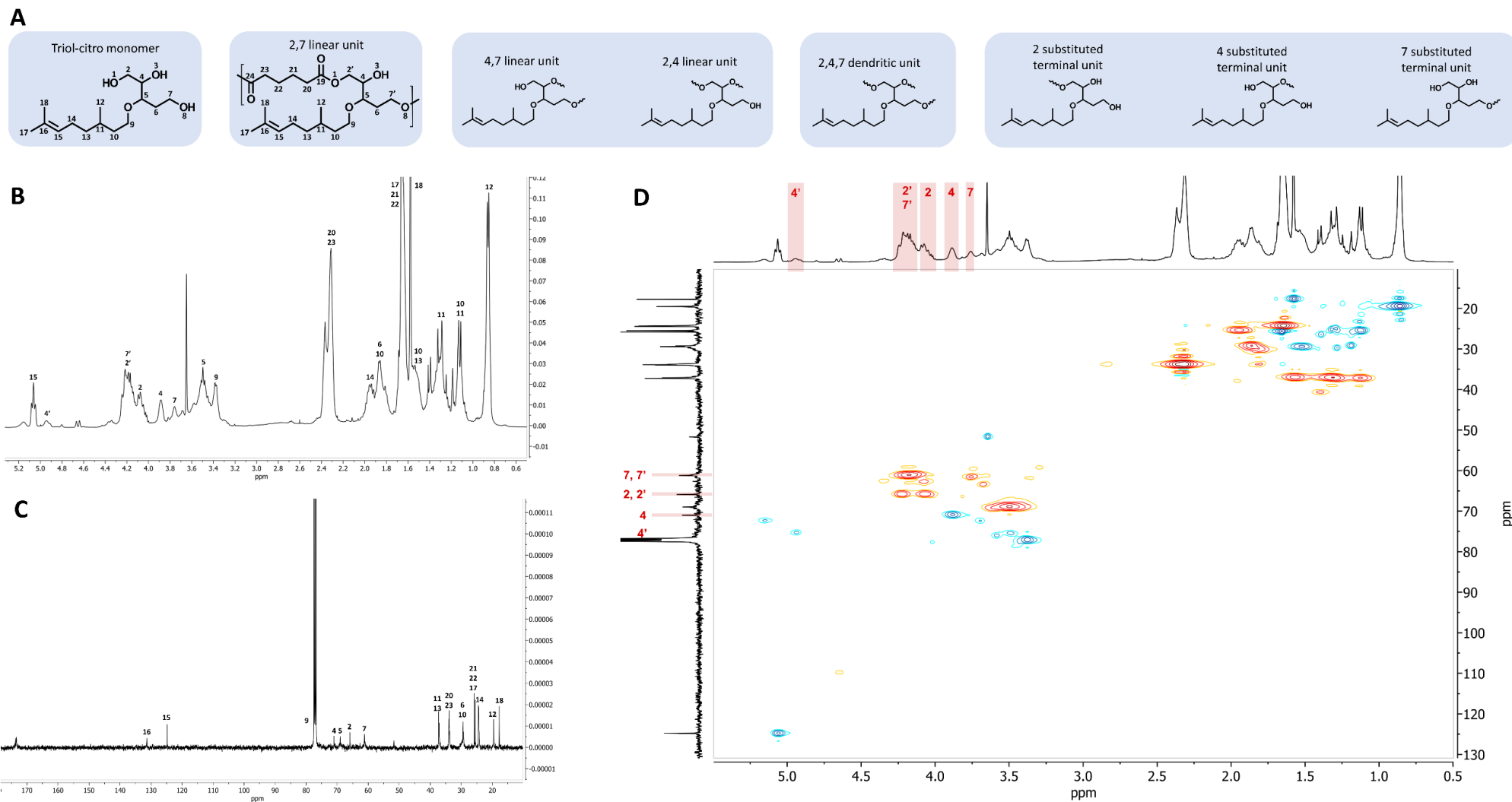
110 Determining the degree of branching proved to be challenging. ¹H-NMR analysis clearly
 111 showed the presence of unreacted monomer in the obtained products, as along with other
 112 impurities (Figures S1 – S27). Attempts were made to wash the crude polymer with MeOH,
 113 however a significant portion of the polymer mass consisted of short chain oligomers that
 114 were also soluble in MeOH at this concentration. For example, N6 was washed with 2 x 5 mL
 115 MeOH and the mass of the polymer obtained was insufficient for ¹³C-NMR and any 2D
 116 techniques.

117 Subsequently, three experiments (N28 – N30) were performed in a longer timeframe in an
 118 attempt to increase molecular weight and induce some branching of the polymer, as it has
 119 done for other polyol based polyesters in the literature.^[31,39] The conditions of experiment
 120 N15 (1:1 monomer ratio, 20 wt% enzyme, 90 °C, 24 h) were selected as this was the highest
 121 molecular weight polymer produced, with the reaction time extended from 24 h to 96 h.
 122 Immediately after the workup, it was evident that the polymer had branched/crosslinked
 123 structures to some extent as experiment N28 was mostly insoluble in tetrahydrofuran (THF).
 124 Chloroform (CHCl₃) dissolved a larger proportion, leaving a small amount of insoluble residue
 125 in the glassware. N29 and N30 were soluble in THF and dimethyl sulfoxide (DMSO) which is
 126 attributed to the fact that these polymers were synthesized using a different batch of Triol-
 127 citro monomer which was flash purified using a slightly different method (see ESI). NMR
 128 analysis for unwashed N28 was performed in CDCl₃, which produced spectra of sufficient
 129 resolution so as to assign peaks. The remaining amount of N28 was washed (2 x 1 mL), and
 130 an attempt was made to dissolve it in DMSO-*d*₆ and CDCl₃ for analysis, which was
 131 unsuccessful. This is likely due to the washing step having removed a lot of the short linear
 132 chains and increasing the proportion of branched/crosslinked structures present.

133 Observing the ¹H-NMR spectrum for unwashed N28 (Figure 2.), there is a considerable
 134 decrease in signal intensity from 3.2 – 3.6 ppm and the formation of new signals from 3.7 –
 135 5.0 ppm are observed when compared to that of the Triol-citro monomer. These new signals
 136 relate to the formation of ester groups, and the corresponding shift of the signals. To
 137 definitively assign signals in the ¹H-NMR spectrum, ¹³C-NMR analysis was also performed,
 138 as well as HSQC, HMBC and COSY (Figure 2. and Figures S28 – 29). From the HSQC,
 139 HMBC and COSY, the majority of peaks could be definitively assigned to protons in the
 140 polymer structure. It was determined that Triol-citro protons 2, 4 and 7 (Figure 2.A.), if
 141 reacted, will show a shift towards a higher ppm on the ¹H-NMR spectra, but not necessarily
 142 in the ¹³C-NMR one. Reacted forms of 2, 4 and 7 are noted as 2', 4' and 7'. When reacted,
 143 proton 2 shows a shift from 4.07 ppm to 4.22 ppm on ¹H, while the ¹³C peak stays at 66 ppm.
 144 Proton 7 shows a shift from 3.76 ppm to 4.22 ppm on ¹H, and the ¹³C stays at 61 ppm.
 145 However, proton 4 shows a shift from 3.76 ppm to 4.94 ppm on ¹H spectra, and also shows a

146 shift in the ^{13}C spectra, from 71 ppm to around 77 ppm. It is difficult to determine exactly
147 where the ^{13}C peak falls for 4', as peaks in this region overlap with the CDCl_3 signals; both
148 signals for carbons 4' and 9 are expected to fall in this area. $\text{DMSO-}d_6$ was therefore used as
149 an NMR solvent for samples N29 and N30.

150 All ^1H spectra have the peak characteristic of the methyl groups of DMA (3.56 ppm in DMSO-
151 d_6), meaning unreacted Triol-citro will also likely be present. Furthermore, many experiments
152 were run with an excess of Triol-citro (Table S1), and this unreacted monomer is expected to
153 contribute significantly to the signals for protons 2 and 7. While substituted terminal units
154 may also be responsible for 2', 4' and 7' proton signals, the presence of the 4 substituted
155 terminal unit (as shown in Figure 2.A.) was deemed improbable due to enzyme
156 regioselectivity. In previous works investigating CaLB synthesized glycerol-based polyesters,
157 glycerol units that had reacted only on the secondary hydroxy were a very small proportion of
158 the polymer^[29,31], and in some cases not observed at all.^[31]



159

160 **Figure 2.A.** Labelled Triol-citro and possible linear, dendritic and terminal structures of the Triol-citro unit within the polyester, with atom labelling of the 2,7 linear unit, **B.** ^1H -
 161 NMR spectra of N28 in CDCl_3 with peaks fully assigned, **C.** ^{13}C -NMR spectra of N28 in CDCl_3 with peaks fully assigned, and **D.** HSQC spectra of N28 in CDCl_3 with peaks for
 162 2, 2', 4, 4', 7 and 7' highlighted and labelled in red. Peaks labelled with ' are from the reacted form of that atom.

163 Unwashed N29 and N30 were not characterized, instead they were washed with MeOH,
 164 which produced sufficient polymer for comprehensive characterization using NMR. DMSO-*d*₆
 165 was used, resulting in spectra with noticeably fewer impurity peaks and the absence of a
 166 sharp peak at 3.56 ppm attributed to the methyl ester group of DMA. Although terminal group
 167 analysis of this polymer would be of interest due to its post-polymerisation functionalisation
 168 potential, this is not possible due to overlapping peaks in this region. Similar to the ¹H spectra
 169 of N28, a peak was observed at 4.93 ppm, attributed to 4' (Figure S32). The ¹³C-NMR
 170 spectra no longer had obscured peaks in the range of 75 – 80 ppm (Figure S31). The
 171 positioning of peaks in DMSO-*d*₆ spectra differed from CDCl₃ spectra slightly, which can be
 172 seen when comparing Figure 2.B. and Figure S32. DMSO-*d*₆ was therefore used as a
 173 solvent for ¹H-NMR analysis to determine branching of N1 – N27. All 27 spectra can be
 174 found in the ESI.

175 Upon observing N1 – N27, a peak at 4.93 – 4.94 ppm is noticeable in the majority of ¹H
 176 spectra. In many spectra, this peak clearly overlaps with an impurity peak (see Figures S1 –
 177 27), consequently, selected samples underwent washing in an attempt to eliminate this
 178 issue. Polymers with several different molecular weights were selected (N13, N9 and N27)
 179 and approx. 100 mg were washed with 2 x 1 mL MeOH. The solubility of the short chain
 180 oligomers in MeOH is evident, as these 2 washes resulted in up to 96% mass loss (Table
 181 S3). After washing, a white powder was obtained; a contrast to the samples before washing,
 182 which were viscous liquids. These samples proved insoluble in DMSO-*d*₆, suggesting that
 183 this solid fraction is the part of the polymer that has crosslinked or branched. This is
 184 supported by the fact that N2, a polymer lacking a peak at 4.93 ppm, was fully soluble in 1
 185 mL of MeOH. Degree of branching was therefore calculated with the spectra of the
 186 unwashed polymer using equation (2); with ' denoting the reacted form of the atom (Figure
 187 1.), and integrals normalized at the CH₃ peak (0.82 ppm).

$$\text{Branching (\%)} = \left(\frac{(I_{4'}/N_{4'})}{(I_{4'}/N_{4'}) + (I_{2'+7'}/N_{2'+7'})} \right) \times 100 \quad (2)$$

188

189 The results for those polymers discussed in the text are summarized in Table 2., and a more
 190 complete table can be found in the ESI (Table S1). Overall, conversion was consistently high
 191 (always >68%), and degree of branching never exceeded 16%, but there was significant
 192 variability in molecular weight. Higher molecular weight polymers also exhibited considerably
 193 greater dispersity (*D*), with a highest observed *D* of 37.3 (polymer N30). Such high
 194 dispersities are unusual for polycondensations conducted in bulk,^[50] especially for reactions
 195 N29 and N30, which were run for 96h. This can likely be attributed to a combination of
 196 molecular weight and branching behavior of these polymers, as branched structures exhibit a
 197 much larger diversity in shape compared to linear structures. Once a structure has branched,
 198 it has more sites where polycondensation can occur, further increasing molecular weight in
 199 the branched chains. There are many examples in the literature of highly disperse branched
 200 polymers to the extent that many researchers focus on limiting *D* in these structures.^[51,52]

201 **Table 2.** Effect of several reaction conditions (highlighted in grey) on the conversion, molecular weight
 202 and degree of branching (DB) observed in the synthesis of poly(Triol-citro adipate).

Reaction	Triol/DMA [moles]	Enzyme [wt%]	Temp. [°C]	Reaction time [h]	Conv. [%] ^[a]	M _n [Da] ^[b]	M _w [Da] ^[b]	<i>D</i> ^[b]	DP ^[b]	DB [%] ^[a]
N2	2	5	40	6	68	2,058	4,792	2.33	5.8	0
N9	1	5	40	24	88	735	869	1.18	2.1	13
N13	1	5	90	24	92	960	1,335	1.39	2.7	12
N15	1	20	90	24	92	2,789	8,044	2.88	7.8	16

N27	1.5	12.5	65	18	93	1,161	2,130	1.84	3.3	9
N28	1	20	90	96	92	-	-	-	-	13
N29	1	20	90	96	100 ^[c]	13,300	157,400	11.82	37.4	13
N30	1	20	90	96	100 ^[c]	13,000	484,500	37.3	36.4	12

^[a] Determined through ¹H NMR.

^[b] Determined through GPC analysis (All chromatograms available in ESI, Figures S35 – 63). N28 was not analyzed due to lack of sample so is marked with -

^[c] Calculated after washing with MeOH to remove DMA.

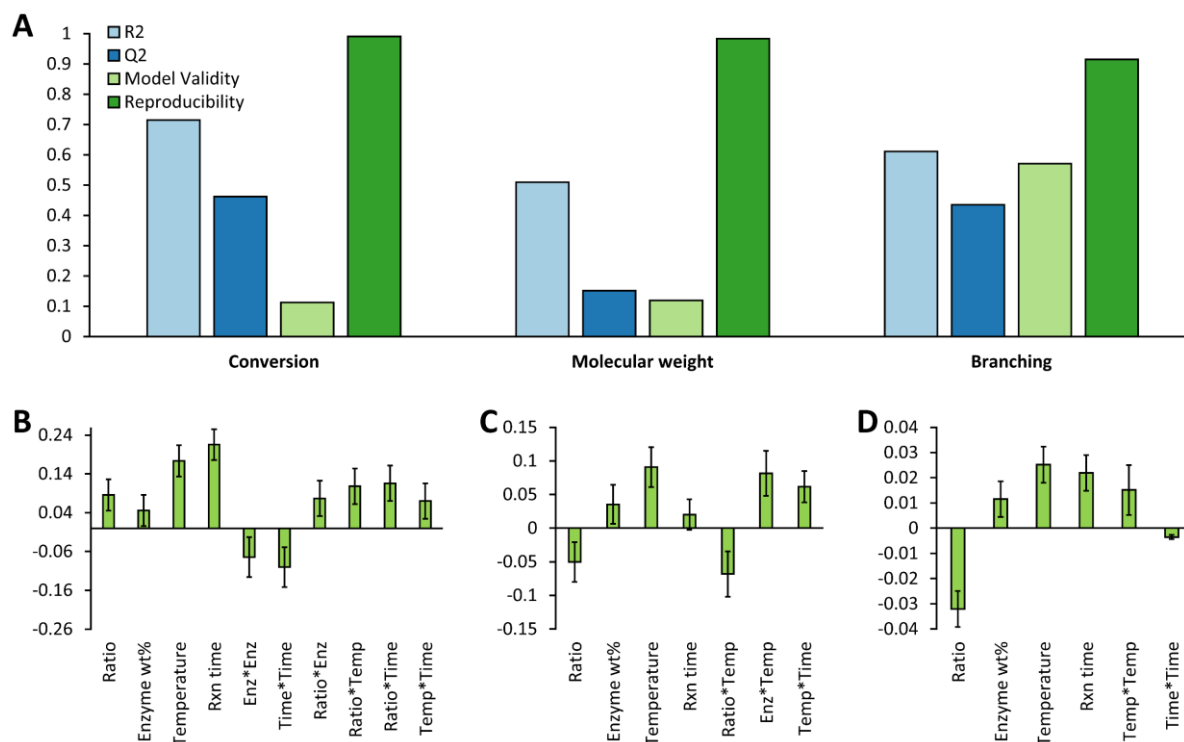
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204 To gain a better understanding of factors that significantly affected these parameters, as well
205 as any interaction effects, the results were analyzed using DoE software.

206 DoE analysis

207 The raw data was analyzed using MODDE®. Data for N28 – N30 were not included as N28
208 had a considerable amount of insoluble residue which could not be tested and N29 and N30
209 were washed with methanol, removing a significant amount of short chain oligomers. This
210 would affect the degree of branching and molecular weight respectively. Data for molecular
211 weight and conversion required a transformation to obtain a normal data distribution; a
212 logarithm (Log(10Log(Y))) and negative logarithm (-10Log(1-Y)) transformation respectively.
213 Selection of significant terms for the coefficients plot was performed using the auto tune
214 function.

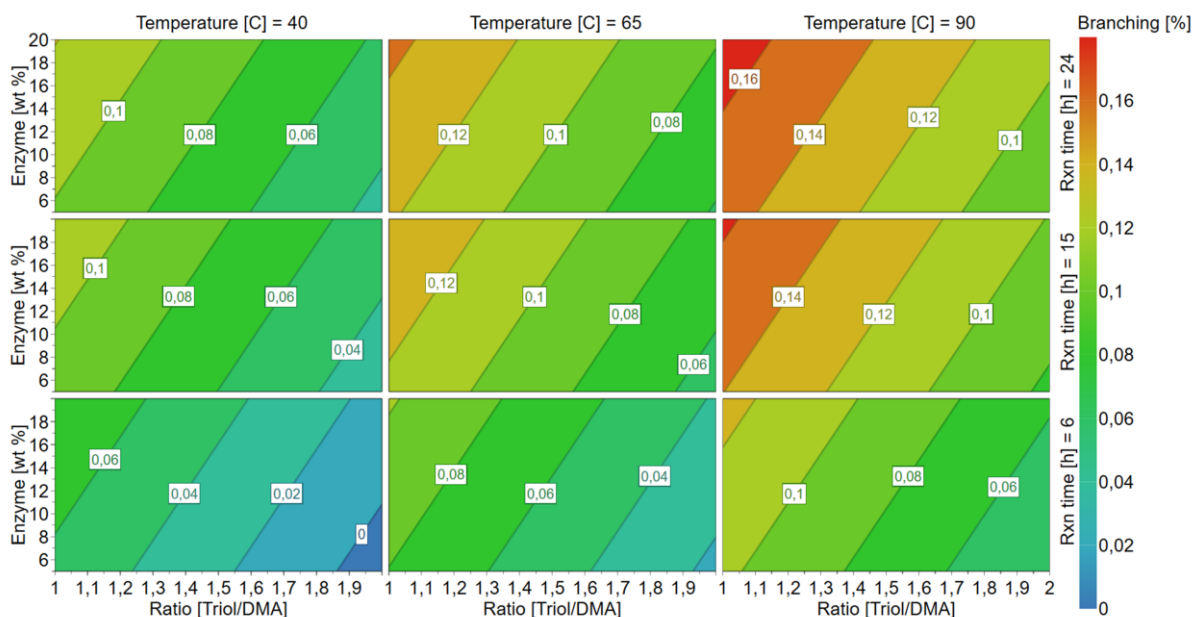
215 The summary of fits for each factor tested can be seen in Figure 3.A., where a value of 1 is
216 optimal. The model for the branching factor is statistically significant, with a $R^2 > 0.5$, a $Q^2 >$
217 0.1 and a difference of < 0.3 between the two parameters. The conversion and molecular
218 weight factors, however, have a low validity and a large difference between the R^2 and Q^2
219 parameters. R^2 is a measure of how well the model fits to the original data, while Q^2 shows
220 how well this model can be used to predict future datasets. Although Q^2 for molecular weight
221 and conversion is above 0.1, the rather low model validity indicates statistically significant
222 issues for both conversion and molecular weight. Nevertheless, the main objective of the
223 DoE was to maximize the limitation of the polymer branching, and the model for this
224 response was both valid and reproducible.



225
 226 **Figure 3.A.** Summary of fit for all responses showing the degree of fit (R^2), prediction power (Q^2)
 227 model validity and reproducibility, and the coefficients plot for **B.** conversion, **C.** molecular weight and
 228 **D.** branching.

229 From the graph (Figure 3.C.), although there are many factors that look to have an effect on
 230 the molecular weight, the p values (Table S4) show that only temperature is significant (at a
 231 confidence value of 0.95). Temperature is well known to affect molecular weight in enzymatic
 232 polycondensations; by increasing reaction temperature higher molecular weights are
 233 achieved. This has been observed for many different polymer systems,^[53,54] although
 234 temperature is limited by the thermostability of the enzyme. Interaction effects for
 235 temperature are also significant; namely Enz*Temp and Temp*Time. Mahapatro *et al.* also
 236 observed some interaction between reaction time and temperature, as no significant effect of
 237 temperature on M_n was observed up to 24h, but at 48h there was a significant difference in
 238 the M_n between 65 and 90 °C.^[53] This should be further investigated, as GPC analysis of N29
 239 and N30, which were run for 96h, gave much higher molecular weights.

240 At a 0.95 confidence, temperature and reaction time are significant factors for the
 241 conversion, which agrees with established literature. The monomer ratio is significant only
 242 when it interacts with either temperature or time (Ratio*Temp and Ratio*Time). Conversion
 243 did not differ greatly between conditions, ranging from $\geq 68\%$ up to 99%, with the lowest
 244 conversion obtained at 40 °C run for 6h. Enzyme wt% seems to have little effect on the
 245 conversion, indeed, with all other conditions kept the same, an increase from 1 wt% to 25
 246 wt% of enzyme actually resulted in a decrease in conversion (from 93% to 90%) seen in
 247 Table S1.



248 **Figure 4.** 4D contour response plot showing the effect of monomer ratio, enzyme wt%, temperature
 249 and reaction time on the degree of polymer branching.
 250

251 Ratio, temperature, reaction time and Time*Time are all significant factors ($p = 0.95$) that
 252 determine the degree of branching (Figure 3.D.). The predictive contour plot in Figure 4.
 253 gives an overview of this, and it can clearly be seen that a higher temperature, lower ratio
 254 and a longer reaction time are associated with a higher degree of branching, while enzyme
 255 wt% is much less significant. Increasing amounts of Triol-citro, increasing temperature and
 256 increasing reaction time all result in more branched structures. The most branched structure
 257 obtained (N15) had a 16% degree of branching, was run at the highest temperature (90 °C),
 258 had the longest reaction time (24h) and had a 1:1 ratio of triol to diester.

259 The fact that enzyme wt% is an insignificant parameter in predicting degree of branching
 260 supports the hypothesis that branching is mainly due to acyl migration rather than enzymatic
 261 coupling on the secondary hydroxy group. This phenomenon is well studied in
 262 carbohydrates, and is an intramolecular reaction that allows the exchange of ester groups
 263 among adjacent hydroxy groups,^[55] independent of the enzyme.^[56] This reaction has been
 264 shown to be affected by temperature, reaction time, reaction solvent, material used to
 265 immobilize the enzyme, pH and acyl species.^[57] Temperature is particularly significant, both
 266 in literature and in this work, which is to be expected as this reaction is a thermodynamic
 267 process.^[58]

268 Perin *et al.*, determined that in the enzymatic synthesis of esterification of poly(glycerol
 269 sebacate), the esterification of primary hydroxy groups was mainly due to acyl migration.
 270 When compared to our work, they observed much higher degrees of branching, which the
 271 authors hypothesized could be due to the use of solvent or carboxylic acids as reagents.^[29]
 272 Solvent affects acyl migration in different ways. Polarity has been shown to have an effect,
 273 with low polarity solvents dispersing the charge of the transition state, lowering its energy
 274 state and decreasing the rate of the migration reaction.^[57] However when compared to
 275 solventless systems, Wang *et al.* found that all solvents tested had an inhibitory effect on
 276 acyl migration (albeit to different degrees).^[59] The use of solvent may also have a beneficial
 277 effect on molecular weight, as solventless polycondensations often lead to a highly viscous
 278 reaction matrix. This was most obvious in reactions N28 – N30, where polymers were
 279 viscous and gel-like, and had visibly hindered stirring. Use of diesters as an alternative to
 280 dicarboxylic acids would also reduce the rate of acyl migration, due to the different leaving
 281 groups. Although both water and methanol have been found to increase the rate of acyl
 282 migration,^[57,60] methanol is easier to remove, and is done so via reduced pressure in this
 283 work.

284 Aside from this, there are quite a number of factors affecting acyl migration that were not
285 investigated here. The CaLB used was immobilized on an acrylic resin, which is a polar
286 carrier, and more polar materials have been known to increase acyl migration.^[57]
287 Furthermore, the structure of Triol-citro may also have an influence. Unlike glycerol or other
288 sugars such as sorbitol, only two hydroxy groups are adjacent in this monomer; instead of an
289 α - β - γ there is an α - β - ϵ triol structure. A previous work attempted to induce acyl migration
290 over the same number of C atoms in mannopyranoside structures, but were unsuccessful,
291 suggesting that two hydroxy groups must be spatially close for this reaction to occur.^[61]

292 **Conclusions**

293 Overall the desired outcome must be carefully considered. For example, reaction N15 gave
294 the highest molecular weight, but also resulted in the most branched structure, at 16%. It is
295 worth performing several more experiments to obtain a valid model for both molecular weight
296 and conversion. In this way, all three responses can be predicted, and parameters can be
297 carefully selected based on the desired properties of the final polymer. With respect to
298 obtaining a high molecular weight, the design space with regard to reaction time should be
299 extended to 96 hours, and the use of solvent should be considered.

300 The utility of CaLB in selectively polymerizing bulky triol monomers has been clearly shown.
301 This work investigated the use of MODDE to determine significant factors in the branching
302 behavior of poly(Triol-citro adipate), and determined that monomer ratio, temperature and
303 reaction time are all significant factors. It was clearly demonstrated that branching was
304 minimal, with a maximum of 16% observed, and this was attributed to acyl migration rather
305 than enzyme catalyzed transesterification. Extending the reaction time to 96h resulted in a
306 polymer with a number average molecular weight of up to 13 kDa and only 13% of
307 branching. CaLB has been shown to be a suitable biocatalytic alternative to traditional metal
308 catalysts in this reaction. Polyesters produced using this method can be further
309 functionalized through reactions with pendant hydroxy groups for more specialized purposes.

310 **Experimental Section**

311 **Materials**

312 Tetrahydrofuran (inhibitor-free, for HPLC, $\geq 99.9\%$), Methanol (for HPLC, gradient grade,
313 $\geq 99.9\%$), Chloroform (HiPerSolv CHROMANORM), and *Candida antarctica* lipase B
314 immobilized on acrylic resin (CaLB, code: L4777, $\geq 5,000$ U/g) were purchased from Sigma
315 Aldrich. Dimethyl adipate (99%) was purchased from Alfa Aesar. All deuterated solvents
316 were purchased from Eurisotop.

317 Trio-citro was synthesised according to a previously published procedure.^[13]

318 **Design of Experiment (DoE)**

319 The software MODDE® Pro 13 (Sartorius) was used to optimize polycondensation
320 conditions. Reactant ratio, enzyme wt%, temperature and reaction time were selected as
321 quantitative factors. Conversion, number average molecular weight (M_n) and degree of
322 branching were the responses measured, with target for conversion set to 99%, M_n target set
323 high and degree of branching set at 1%. A central composite orthogonal (CCO) design (star
324 distance: 1.54671) was chosen with a quadratic model, to generate a set of experiments for
325 optimisation.

326 **Enzymatic Polycondensations**

327 Triol-citro (233.2 mg, 8.5×10^{-4} mol) and the appropriate amount of dimethyl adipate ($6.4 \times$
328 10^{-4} mol – 1.9×10^{-3} mol) were added to a 25-mL round bottom flask with CaLB (1 – 25% by
329 weight of monomers). The flask was heated (25 – 100 °C) and stirred at 400 rpm for 6 hours,
330 at which point the system was placed under vacuum (20 mbar) in order to remove the

331 methanol by-product. Experiments were run from 1 – 30 hrs, and experiments that were run
332 for ≤ 6 hrs were not placed under vacuum.

333 Upon completion of the reaction, the polymers were washed with approx. 2 mL THF and
334 filtered through cotton packed into a glass Pasteur pipette to remove the insoluble enzyme
335 pellet. The flask was washed with 1 mL THF a further two times, and all fractions were
336 filtered and collected. THF was removed via a rotary evaporator, and the resulting polymer
337 was characterized by NMR and GPC analysis.

338 If a wash step was performed, the polymer was transferred into a 1.5 mL or 5 mL Eppendorf,
339 precipitated through addition of ice-cold methanol (amount as specified), and centrifuged (10
340 min at 4 °C, 3700 rpm for 5 mL Eppendorfs and 12700 rpm for 1.5 mL Eppendorfs). The
341 supernatant was removed and the washing step repeated as specified. The resulting polymer
342 was characterized by NMR analysis.

343 **Characterization**

344 *Gel permeation Chromatography (GPC)*

345 Polymers were dissolved in CHCl_3 or THF to a concentration between 2.0 and 2.5 mg/mL
346 and filtered through cotton. The analysis was performed at 30 °C on an Agilent Technologies
347 HPLC System (Agilent Technologies 1260 Infinity) and an Agilent Technologies G1362A
348 refractive index detector was employed for detection. Linear polystyrene calibration
349 standards (250 – 70,000 Da) purchased from Sigma-Aldrich were used to calculate the
350 molecular weights of the polymers.

351 For those polymers dissolved in CHCl_3 , a 17,369 6.0 mm ID \times 40 mm LHHR-H, 5 μm Guard
352 column and a 18,055 7.8 mm ID \times 300 mm L GMHHR-N, 5 μm TSK gel liquid
353 chromatography column (Tosoh Bioscience, Tessenderlo, Belgium) was used, with CHCl_3 as
354 an eluent (at a flow rate of 1 mL min^{-1} for 20 min). For those polymers dissolved in THF, a
355 guard column and chromatography column of the same specification was used with THF as
356 the eluent (at a flow rate of 1 mL min^{-1} for 20 min).

357 *Nuclear Magnetic Resonance (NMR)*

358 ^1H -NMR, ^{13}C -NMR, COSY, HMBC and HSQC spectroscopy was performed using a JEOL
359 ECZ400R/S3 at a frequency of 400 MHz, with some ^1H -NMR performed on a Bruker Avance
360 II 400 (resonance frequencies 400.13 MHz for ^1H) equipped with a 5 mm N_2 -cooled cryo
361 probe (Prodigy) with z-gradients at room temperature with standard Bruker pulse programs.
362 Samples were dissolved in either CDCl_3 or $\text{DMSO}-d_6$ as a solvent.

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370 **Data availability statement**

371 All data not present in the main text or the ESI are available from the authors upon request.

372 **Conflicts of Interest**

373 The authors declare no conflicts of interest.

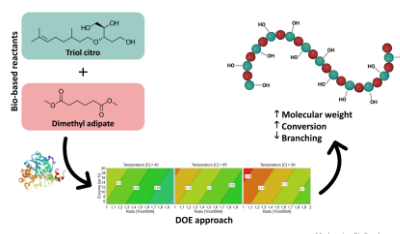
374 **Keywords:** Bio-based, Enzyme catalysis, Green Chemistry, NMR spectroscopy, Polyesters

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 473

474 Entry for the Table of Contents



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476 Polyesters were enzymatically synthesised from a bio-based triol, and a Design of
 477 Experiment approach was used to investigate factors that affect the branching during
 478 synthesis of this polymer.