Electronic Supplementary Information

Mycobacterium smegmatis acyltransferase catalyzes the synthesis of esters and polyesters

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Materials and Methods

Chemicals and reagents

Polypropylene beads (Accurell MP 1000 surface area of 55,985 $m^2\ g^{-1}$, particle density of 1.993 cm^{-3}, and particle diameter of <1500 mm) were purchased from 3M Deutschland GmbH (Wuppertal, Germany). Vinyl acetate (>99%), vinyl butyrate (>99%), methyl acetate (>99%), methyl butyrate (>99%), ethyl butyrate (>99%), ethyl hexanoate (>99%), ethylene glycol (EG, >99%), 1,4-butanediol (BDO, >99%), 1,8-octanediol (ODO, >98%), 1-butanol (>99%), 1-octanol (>99%), 1-dodecanol (>99%), Na_2HPO_4 (>98%), NaH_2PO_4 (>98%), tetrahydrofuran (for HPLC, >99.8%), dimethyl sulfoxide (for HPLC, >99%) and toluene (>99.8%) were purchased from Merck (Sigma-Aldrich). Vinyl hexanoate (>98%), methyl hexanoate (>99%) and divinyl adipate (DVA, >99% stabilized with MEHQ) were purchased from TCI Chemicals

(Vienna, Austria). Chloroform (for HPLC, >99.8%) and ethyl acetate (>99%) were purchased from VWR Chemicals (Wien, Austria). All chemicals and solvents were used as received if not otherwise specified.

MsAcT expression and purification

Mycobacterium smegmatis acyltransferase (MsAcT) was produced and purified as previously described in Contente et al[1].

Enzymes immobilization on polypropylene beads

1.0 g of polypropylene beads were meticulously weighed in a 50-mL falcon tube and rinsed with 20 mL of acetone. A pressure of 600/700 mbar was applied for 5 min to eliminate the air and enable the enzyme binding also in the bead's cavities. This procedure was repeated three times. Afterwards, the solid support was washed with 20 mL of ddH_2O (once) and with 20 mL of the immobilization buffer, 0.1 M Na_2HPO_4/NaH_2PO_4 buffer at pH 8.0 (twice). The washing and immobilization steps were carried out using a blood rotator set at 20 rpm. After the washings, 35 mL of 0.286 mg mL^{-1} (1% *w* w^{-1} enzyme/beads), were added to the beads at 21 °C and left to react for 24 h on the rotator. Samples were withdrawn over time to monitor the reaction progression by analyzing the residual enzyme activity and residual protein concentration in the supernatant (section 2.5 and 2.6). After 24 h, the beads were filtrated using a paper filter, rinsed 3 times with 10 mL of the immobilization buffer and air-dried for 5 days at 21 °C before further use.

Determination of the extinction coefficient of *p*-nitrophenol in Na_2HPO_4/NaH_2PO_4 buffer

A solution containing 10 mmol of *p*-nitrophenol in 10 mL of 0.1 M Na_2HPO_4/NaH_2PO_4 buffer at pH 8.0 was prepared. Further dilutions with the same buffer were prepared. The absorbance of 220 µL of different solutions was measured at 405 nm (in triplicates) at 30 °C with a Tecan Reader (Tecan, Grödig, Austria) using a 96-well microtiter plate (Greiner 96 Flat Bottom Transparent Polystyrene). A blank was included using the buffer. The extinction coefficient of *p*-nitrophenol is equal to the slope of the obtained calibration curve.

Acyltransferase activity assay

The assay was adapted from a previously used protocol (Gamerith et al., 2017). Acyltransferase activity was determined by measuring the amount of *para*-nitrophenyl acetate (*P*-NPA) enzymatically hydrolyzed at 30 °C; 200 µL of the substrate solution (consisting of 82 mg of *P*-NPA and 1000 µL of dimethyl sulfoxide) was added to 20 µL of the enzyme diluted in buffer. The catalytic activity, which correlates to the increment of the absorbance at 405 nm due to the increased of released *p*-nitrophenol (ε 405 nm), was detected every 18 s for 5 min with a Tecan Reader (Tecan, Grödig, Austria) using 96-well microtiter plates (Greiner 96 Flat Bottom Transparent Polystyrene). The analysis was performed in triplicates and a blank was included using buffer. The activity was calculated in units (U), where 1 unit is defined as the quantity of enzyme required to hydrolyze 1 µmol of substrate per minute.

Protein concentration determination

Protein concentration (Bradford assay) was measured using Bio-Rad solution (Coomassie brilliant blue G-250 dye, Bio-Rad, Vienna, Austria) diluted 1:5 with ddH_2O. Bovine serum albumin was used as a standard (Bovine Serum Albumin protein standard, 2 mg/mL, Merck-Sigma-Aldrich-). Supernatant dilutions were prepared in the aforementioned immobilization buffer (0.1 M Na_2HPO_4/NaH_2PO_4 buffer at pH 8.0). Then, 10 µL of the sample were put in the plate and 200 µL of 1:5 BioRad solution were added (in triplicates). The mixture was shaken at 21 °C for 5 min at 400 rpm. The absorbance at 595 nm was measured at 30 °C with a Tecan Reader (Tecan, Grödig, Austria) using a 96-well microtiter plate (Greiner 96 Flat Bottom Transparent Polystyrene). A blank was included using only the buffer. The protein concentration was calculated using the calibration curve obtained using the BSA standard dilutions.

Planning of the design of experiments (DoE)

The full factorial design was planned with the software MODDE® Pro 13 (Sartorius). The considered independent variables, also called factors, were ester functional groups (vinyl, ethyl, methyl), alcohol chain length (C_4, C_8, and C_{12}), ester chain length (C_2, C_4 and C_6), and reaction time (4, 8 and 24 hours). The reactions were carried out in a randomized order. A further central point for the DoE was added,

which corresponded for each ester functional group at the intermediate level, meaning the reaction C_4 ester with C_8 alcohol (repeated twice). The response obtained for each experiment was the conversion rate of the acid determined by GC-FID analysis (section 2.9).

Enzymatic ester synthesis

The esterification investigations were conducted using equimolar amounts of ester and alcohol (6.0 mmol) and 2.5% of the immobilized enzyme (1% *w* w^{-1} MsAcT/beads) calculated on the total amount of the reagents (2.5% *w* w^{-1} MsAcT/total reagents weight). The experiments were performed at 25 °C in 50 mL reaction tubes continuously mixed with a magnetic stirrer (400 rpm) using the multipoint reactor Carousel 12 plus (Radleys, Shire Hill, UK). 10 µL of sample were withdrawn at specific intervals (4, 8, and 24 h) and diluted in 10 mL of $CHCl_3$; 10 µL of toluene were then added as an internal standard. A blank of each reaction was run in parallel without adding the immobilized MsAcT. The samples were then analyzed *via* gas chromatography (GC-FID) as detailed in Table S2 in ESI. Calibration curves for C_6 esters are shown in Figure S1 in ESI.

Gas chromatography (GC-FID)

Samples from the esterification investigations were diluted 1:4 with $CHCl_3$ in 1.5 mL glass vials. Gas chromatography was carried out for 45 min up to 250 °C, using $CHCl_3$ as the washing solvent. An Agilent Technologies GC system (Agilent Technologies 6890N Network GC System) connected to a J&W 122–3232 250 °C max DB-FFAP column with a capillary of 30.0 m × 250 um × 0.25 um nominal was used for the analysis. The Injector (HO 6890 series) provided an H_2 flow rate of 40 mL/min, airflow at 450 mL/min, and N_2 makeup flow equal to 20.

Liquid chromatography-mass spectrometry (LC-MS)

The LC-MS Spectra were recorded on a Microsaic 4000MiD mass spectrometer connected with Agilent 1100 HPLC. Injected samples concentration was between 300-700 µg ml^{-1}. Mass spectra were recorded in full scan with a 100-800m/z mass range, positive mode, and a Tic Voltage of 750 V. No column was used. A flow rate of 0.25 mL/min, temperature of 25 °C, volume injection 5 µL and an ultrapure water/acetonitrile with 0.1% formic acid 2:8 isocratic acid mobile phase was used.

Gas chromatography-mass spectrometry (GC-MS)

GC-MS analyses were performed on column HI-5 ms, 0.25 µm, 0.25 mm i.d., and 30 m. Analysis conditions are as follows: solvent delay 2.5 min, mass range 35−400, injector temperature 250 °C, detector temperature 250 °C, MS temperature around 200 °C, starting temperature 100 °C, starting time 3 min, temperature gradient 25 °C min^{-1}, final temperature 300 °C, flux through column 1.0 mL min^{-1}, split ratio 10.0, sample concentration 0.1 mg mL^{-1}, and amount injected 1 µL.

Enzymatic polycondensation reactions

Polycondensation reactions were performed with equimolar amounts of diester and diol (2 mmol) and 30% of the immobilized MsAcT (1% *w* w^{-1} enzyme/beads) calculated on the total amount of the monomers (30% *w* w^{-1} enzyme/total monomers). The syntheses were performed at 30 °C at 1000 mbar for different times in 25 mL round-bottom-flasks using a multipoint reactor Starfish multi-experiment workstation (Radleys, UK). In the second phase of the reaction, reduced pressure (20 mbar) was applied for 24 or 48 h by connecting the single reaction flasks to a Schlenk line connected to a vacuum pump V-300 (BÜCHI) equipped with a pressure controller I-300 interface (BÜCHI). Afterwards, the reaction mixture was recovered dissolving the reaction products in 2Me-THF or $CHCl_3$ and removing the biocatalyst through a filtration step using a cotton filter. The solvent was then evaporated and the polymers were analyzed without further purification steps.

Proton nuclear magnetic resonance (^{1}H-NMR)

All ^{1}H-NMR spectra were recorded using a JEOL ECZ400R/S3 at a frequency of 400 MHz using $CDCl_3$ or THF-d_8 as the solvents if not otherwise specified.

Gel Permeation Chromatography (GPC)

The samples were dissolved in $CHCl_3$ at a concentration of 2 mg mL^{-1} and filtered through cotton filters. Gel permeation chromatography was carried out at 30 °C on an Agilent Technologies HPLC System (Agilent Technologies 1260 Infinity) connected to a 17,369 6.0 mm ID × 40 mm L HHR-H, 5 µm Guard column and a 18,055 7.8 mm ID × 300 mm L GMHHR-N, 5 µm TSK gel liquid chromatography column (Tosoh Bioscience, Tessenderlo, Belgium) using $CHCl_3$ as an eluent (at a flow rate of 1.0 mL min^{-1}). An Agilent Technologies G1362A refractive index detector

was employed for detection. The molecular weights of the polymers were calculated using linear polystyrene calibration standards (250-70000 Da) purchased from Merck (Sigma-Aldrich).

Divinyl succinate synthesis

Divinyl succinate has been prepared from succinic acid following a previously described procedure.[2] However, we found the reaction rather erratic, due to the poor stability of divinyl succinate in the reaction mixture. This compound decomposes when the reaction reaches high conversions. It is important to follow strictly the procedure, performing the reaction, the work up and the column chromatography purification as fast as possible in one go.

Succinic acid (1 eq, 22.44 mmol, 2.65 g) and $Hg(OAc)_2$ (0.025 eq, 0.651 mmol, 179 mg) were dissolved in vinyl acetate (10 eq, 224.1 mmol, 21 mL) under nitrogen atmosphere and the mixture was stirred at rt for 15 minutes. After this time, a drop of concentrate sulfuric acid was added and the mixture was stirrer for other 10 minutes. Then, the reaction was refluxed for 4 hours. The reaction was quenched by adding NaOAc $3H_2O$ (0.024 eq, 0.55 eq 75 mg) and a pinch of hydroquinone to inhibit the polymerization. Then, the crude was concentrated, and quickly purified by flash chromatography (PE/Et_2O 80:20), obtaining divinyl succinate (1.041 g, 27%) as colourless oil. Rf = 0.35 (PE /AcOEt 95:5); ^{1}H-NMR (Chloroform-d) δ 7.27 (dd, J = 14.0, 6.3 Hz, 2H, 2 =CH), 4.91 (dd, J = 14.0, 1.6 Hz, 2H, 2 H of H_2C=), 4.60 (dd, J = 6.3, 1.6 Hz, 2H, 2 H of H_2C=), 2.75 (s, 4H, 2 CH_2); ^{13}C NMR (Chloroform-d) δ 169.4 (2 Cq), 141.2 (2 =CH), 98.3 (2 H_2C=), 28.7 (2 CH_2); GC-MS: Rt 4.68 min: m/z 42 (13), 43 (26), 44 (7,3), 45 (7,8), 55 (100), 56 (11), 57 (5,8), 71 (37), 73 (6,3), 99 (47), 101 (5,1), 127 ((M+ − 43, 99), 128 (6,3).

Supplementary data

Table S1. The reactions of the full factorial design. Coefficient shown are ester functional group (vinyl, ethyl and methyl), alcohol length (C_4, C_8 and C_{12}) and ester length (C_2, C_4 and C6). The last three reactions represent the central points of the DoE.

Reaction no°	Esters chain	Ester length [C no°]	Alcohol length [C no°]

1	Vinyl	2	4
2	Ethyl	2	4
3	Methyl	2	4
4	Vinyl	4	4
5	Ethyl	4	4
6	Methyl	4	4
7	Vinyl	6	4
8	Ethyl	6	4
9	Methyl	6	4
10	Vinyl	2	8
11	Ethyl	2	8
12	Methyl	2	8
13	Vinyl	4	8
14	Ethyl	4	8
15	Methyl	4	8
16	Vinyl	6	8
17	Ethyl	6	8
18	Methyl	6	8
19	Vinyl	2	12
20	Ethyl	2	12
21	Methyl	2	12
22	Vinyl	4	12
23	Ethyl	4	12
24	Methyl	4	12
25	Vinyl	6	12
26	Ethyl	6	12
27	Methyl	6	12
28	Vinyl	4	8
29	Ethyl	4	8
30	Methyl	4	8

Table S2. Gas chromatography (GC-FID) parameters.

GC	°C/min	Next °C°	Hold min	Run time
Initial		50	5.00	5.00
Ramp 1	5.00	230	2.00	43.00
Ramp 2	25.00	250	2.00	45.80
Post-run		50	0.00	45.80

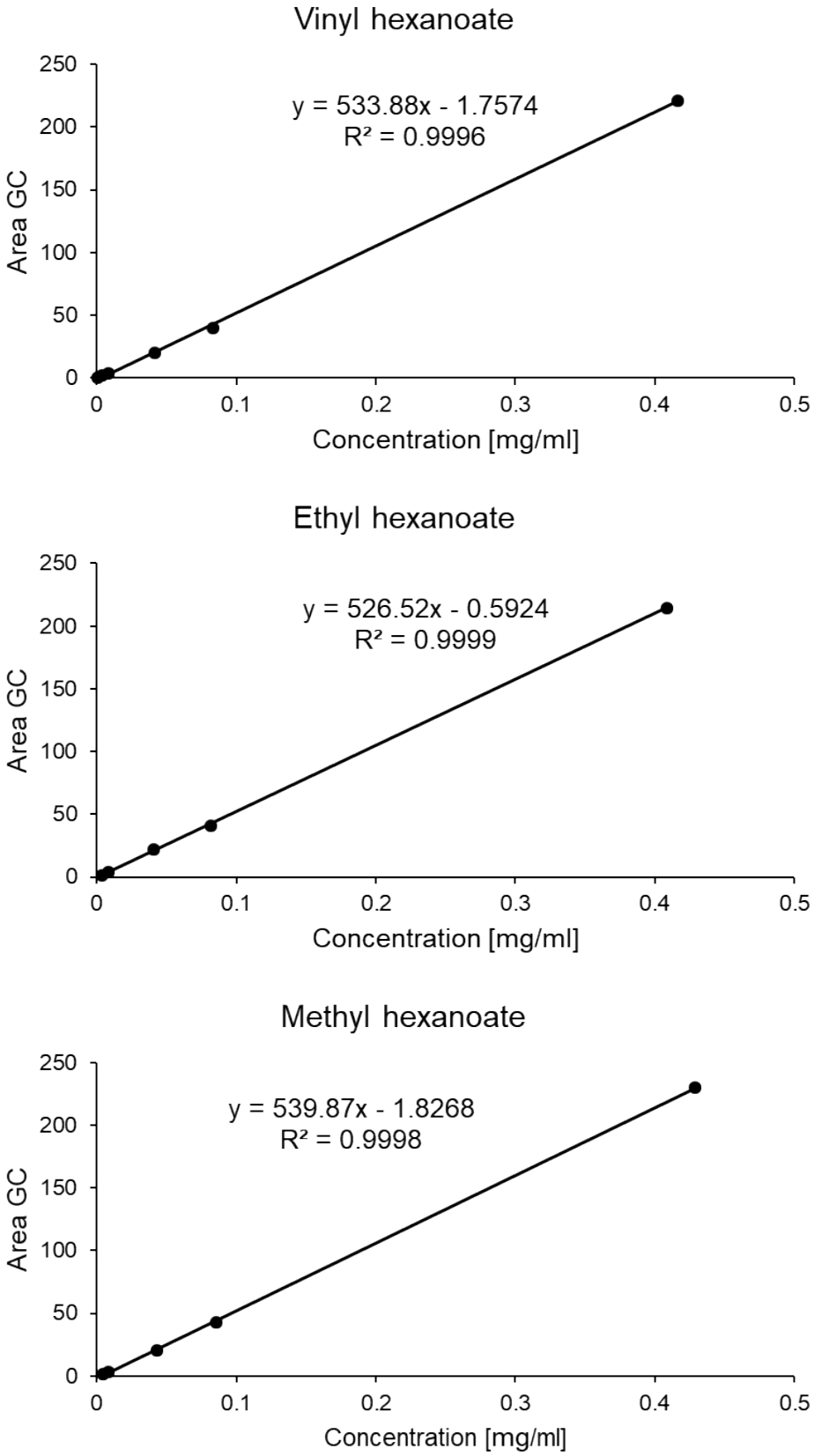

Figure S1. Gas chromatography (GC-FID) calibration curves for vinyl hexanoate, ethyl hexanoate and methyl hexanoate.

Table S3. Polycondensation of DVA with BDO catalysed by 10% (w/w) of immobilized MsAcT at 30°C at different vacuum (20 mbar) duration times.

N°	Diol	Reaction time	Vacuum	M_n [a]	M_w [a]	Đ [a]	M_o	DP [c]	Conv. [%] [b]
1	1,4-butanediol	24h	18h	-	-	-	162.1	-	5
2		72h	/	-	-	-		-	9
3			24h						4

[a]Calculated *via* GPC calibrated with low molecular weight polystyrene standards 250-70 000 Da.
[b]Calculated *via* ^{1}H-NMR by comparing the ratio between the signal methylene groups adjacent to -OH of BDO and the methylene groups of DVA (assumed as constant).
[c]Degree of polymerization (DP) = M_n/molecular weight of the repeating unit (M_o).

Table S4. Polycondensation of DVS with EG catalysed by 30% (w/w) of immobilized MsAcT at 30°C for 72 h at different vacuum (20 mbar) duration times.

N°	Diol	Reaction time	Vacuum	M_n [a]	M_w [a]	Đ [a]	M_o	DP [c]	Conv. [%] [b]
1	Ethylene glycol	72h	/	-	-	-	162.1	-	6
2			24h	-	-	-		-	11
3		168h	/						14

[a]Calculated *via* GPC calibrated with low molecular weight polystyrene standards 250-70 000 Da.
[b]Calculated *via* ^{1}H-NMR by comparing the ratio between the signal methylene groups adjacent to -OH of EG and the methylene groups of DVS (assumed as constant).
[c]Degree of polymerization (DP) = M_n/molecular weight of the repeating unit (M_o).

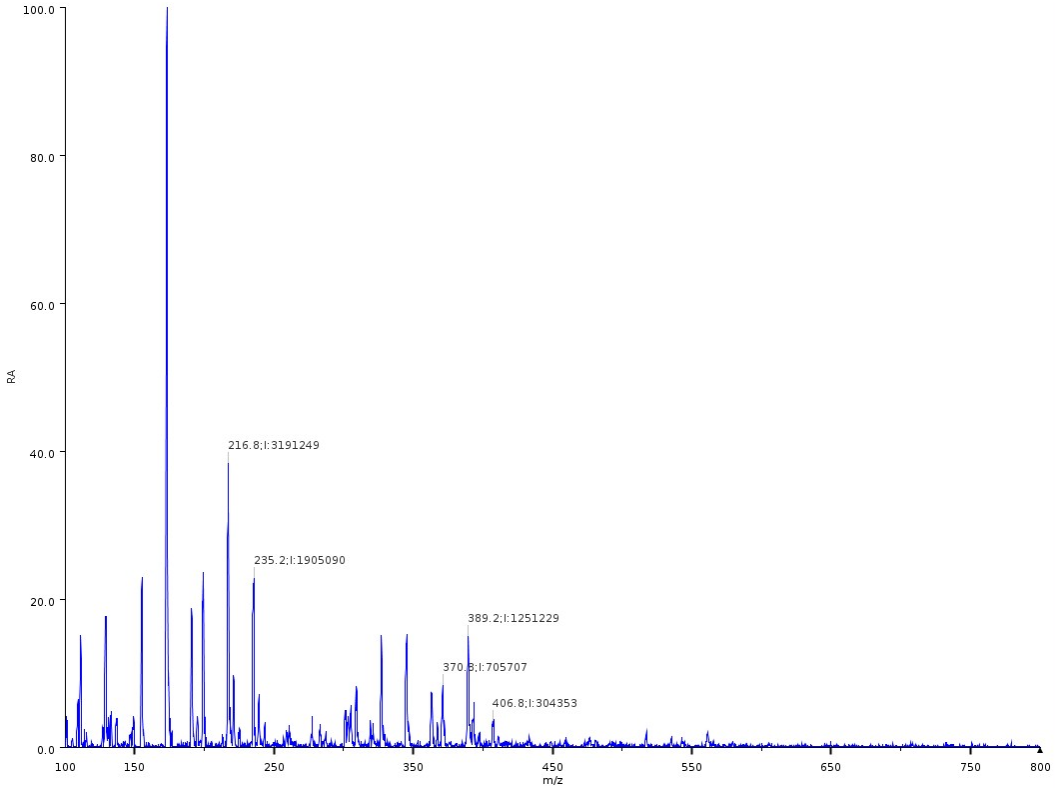

Figure S2. LC-MS spectra of poly(ethylene adipate) synthesized using a 72 h reaction time at 1000 mbar.

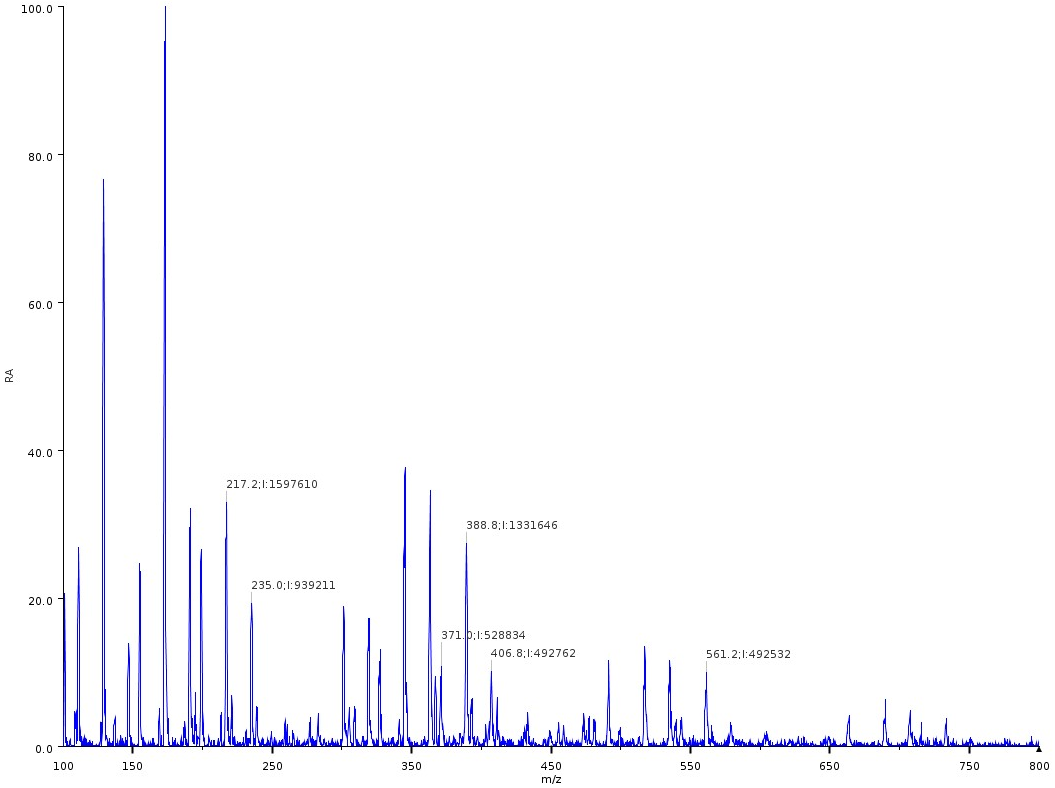

Figure S3. LC-MS spectra of poly(ethylene adipate) synthesized using a 72 h total reaction time (48 at 1000 mbar + 24 h at 20 mbar).

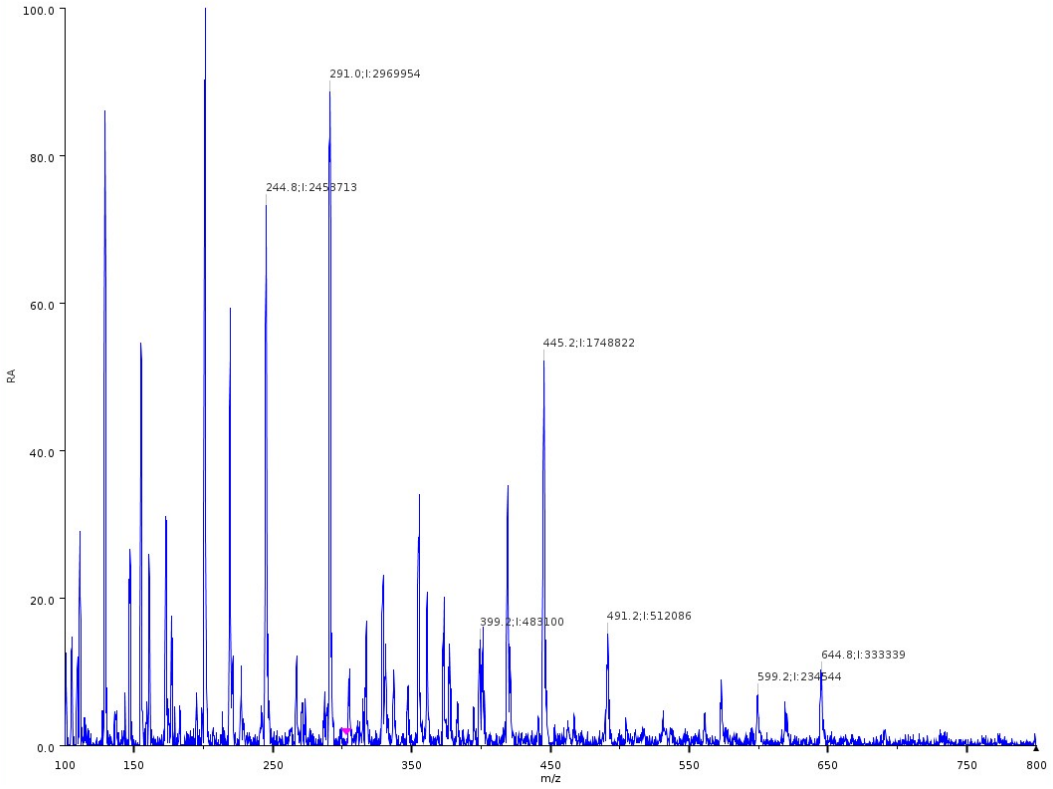

Figure S4. LC-MS spectra of poly(1,4-butylene adipate) synthesized using a 72 h reaction time at 1000 mbar.

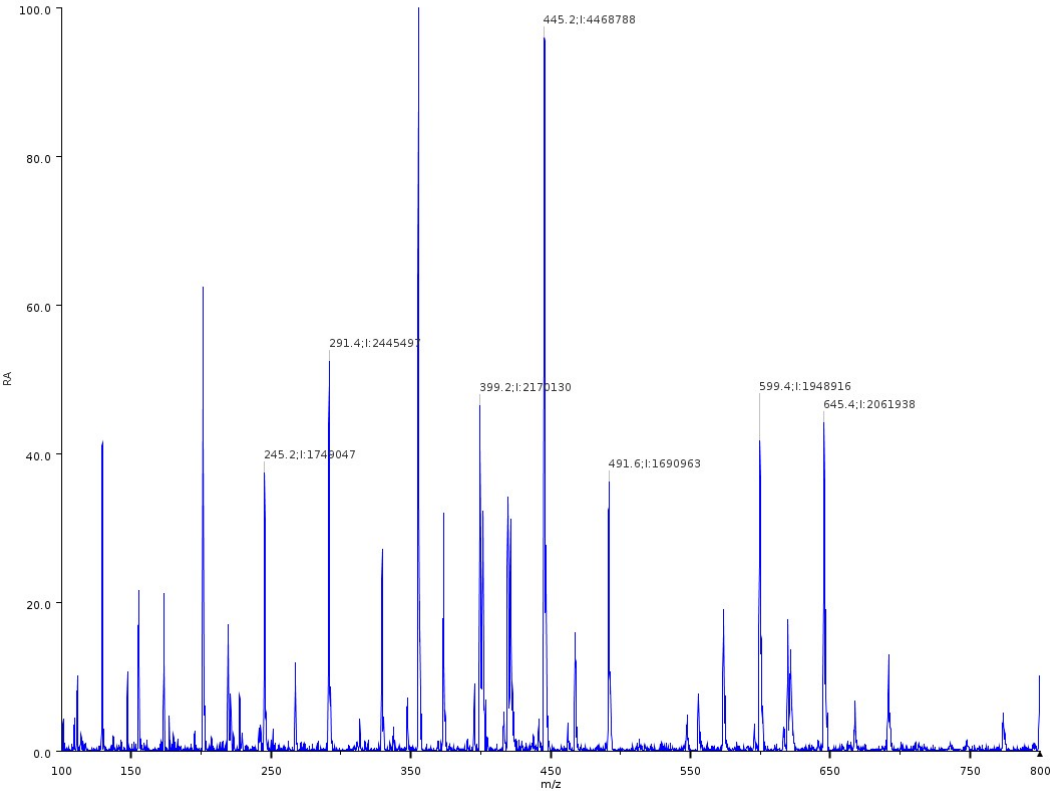

Figure S5. LC-MS spectra of poly(1,4-butylene adipate) synthesized using a 72 h total reaction time (48 at 1000 mbar + 24 h at 20 mbar).

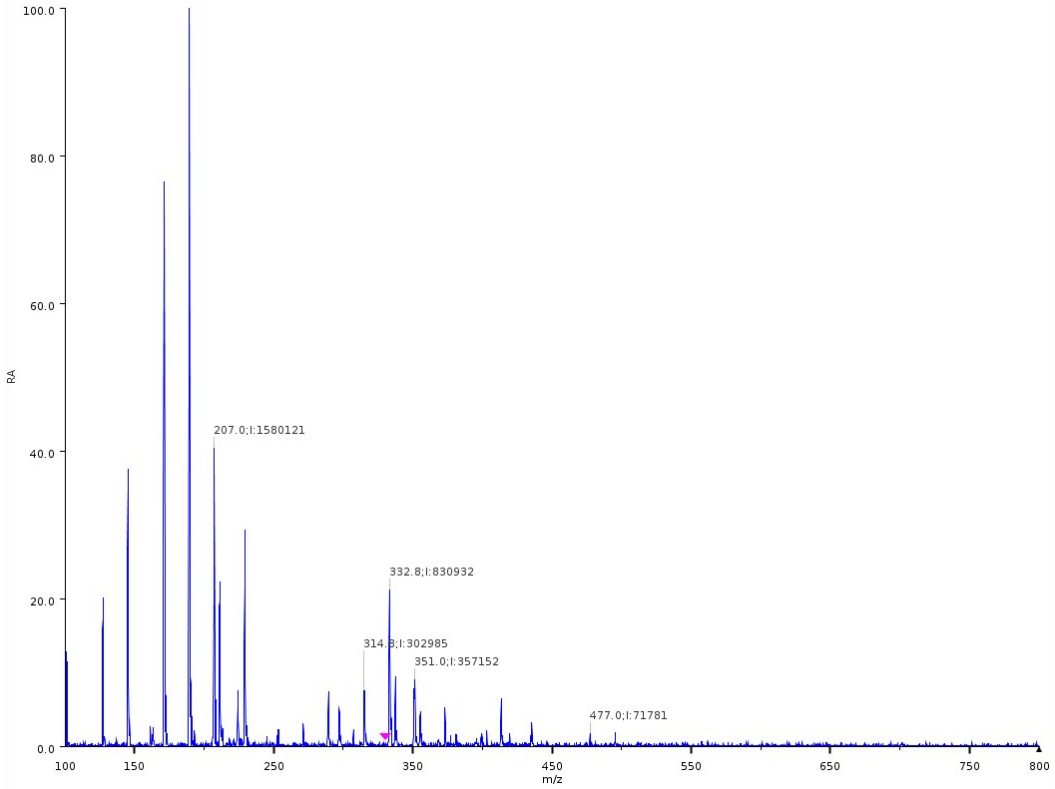

Figure S6. LC-MS spectra of poly(ethylene succinate) synthesized using a 72 h total reaction time (48 at 1000 mbar + 24 h at 20 mbar).

Supplementary information for Figure 2:

4D-contour response of *Mycobacterium smegmatis* acyltransferase (MsAcT) in ester synthesis at 25 °C. The figure is divided into 9 graphs, each displaying the results obtained for a particular ester chain group (vinyl, ethyl, methyl), ester carbon chain length (from C_2 to C_6), alcohol carbon chain length (from C_4 to C_{12}) and at a specific reaction time (4 h, 8 h, 24 h). The figure columns display a fixed ester chain (vinyl, ethyl, methyl, as shown on top of every column) while the rows show a fixed reaction time (4, 8, 24 h, as reported on the right side of each row). The X-axis of each graph represents the alcohol chain length, varying from C_4 to C_{12}. The Y-axis displays the ester chain length, varying from C_2 to C_6. The color scale indicates percentual conversion, as shown in the legend at the right end side of the figure. Each numerical label inside the graph refers to the conversion rate, according to the legend. The “Min” label stands for “Minimum” and equals a conversion rate of 0%.

References

[1] Contente M. L., Pinto A., Molinari F., Paradisi F. Biocatalytic N-acylation of amines in water using an acyltransferase from Mycobacterium smegmatis. Advanced Synthesis & Catalysis 2018, 360, 1-7.

[2] a) Paterson D. L., Flanagan J. U., Shepherd P. R., Harris P. W. R., Brimble M. A. Variable-Length Ester-Based Staples for α-Helical Peptides by Using A Double Thiol-ene Reaction. Chem. Eur. J. 2020, 26, 10826; b) Magrone P., Cavallo F., Panzeri W., Passarella D., Riva S. Exploiting enzymatic regioselectivity: a facile methodology for the synthesis of polyhydroxylated hybrid compounds. Org. Biomol. Chem., 2010,8, 5583-5590.