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ARTICLE

Lipase immobilised silica monoliths as continuous-flow microreactors for triglyceride transesterification

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Lipase immobilised, silica monoliths have been prepared and applied as biocatalytic continuous-flow microreactors for the transesterification of tributyrin as a model bio-oil component. *Candida Antarctica* lipase was trapped within the pores of silica monoliths, and its successful immobilisation demonstrated by the hydrolysis of 4-nitrophenylbutyrate to 4-nitrophenol. Lipase immobilised silica monoliths were active for transesterification of tributyrin at ambient temperature, with reactivity a function of methanol:tributyrin ratio, flow rate, temperature, and textural properties. Monoliths with a high surface area, and large meso- and macropore channels, enhanced transesterification activity through improved molecule diffusion. The optimum immobilised lipase microreactor exhibited almost quantiative ester production for >100 h at 30 °C without deactivation.

1. Introduction

Global energy consumption is predicted to increase by 61 % by 2050 compared to the energy consumption in 2010 due to population growth and urbanisation.[1-3] Fossil fuels currently provide 80 % of worldwide energy demand, but also accounts for the overwhelming majority of anthropogenic greenhouse gas (GHG) emissions.[4] Despite significant recent growth in known fossil fuel reserves, concern over climate change arising from GHG emissions is driving the urgent quest for alternative energy sources.[5, 6] Renewable energy sources, geothermal, hydroelectric, ocean, solar, wind and bioenergy, are essential to deliver sustainable socio-economic development,[7] with biodiesel as the only immediate low cost solution for a drop-in liquid transportation fuel.[3, 7, 8] Second generation biodiesel comprises fatty acid methyl esters (FAME) obtained via the esterification and transesterification of free fatty acids (FFAs) and triacylglycerides (TAGs) respectively from non-edible plant, algae or waste oil sources.[9-11,12, 13]

Biodiesel is typically produced via TAG transesterification with short chain alcohols, notably methanol,[14] catalysed by enzymes (lipases),[15, 16] ionic liquids,[17, 18] and acid[19, 20] or base[21-24] catalysts, with glycerol as the by-product.[11, 20, 25] Although significant research has focused on the development of solid acid (e.g. sulphonic acids, heteropolyacids and sulphated metal oxides) and base (e.g. metal oxides and layered double hydroxides) catalysts for biodiesel production, immobilised biocatalysts such as lipase has received far less attention.[26, 27]

Lipases are carboxylesterase enzymes that hydrolyse fats[28, 29] and hence are amenable to the transesterification of TAGs to produce biodiesel.[14, 15, 27] *Candida Antarctica* is a commonly used lipase catalyst for the production of biodiesel,[30, 31] but is prone to deactivation when the transesterification of plant oils is conducted with short chain alcohols due to poor miscibility and substrate inhibition.[32-34] This has necessitated lipase catalysed transesterification employing low methanol:oil molar ratios.[8, 33] Other disadvantages of enzymes (lipase) include their comparatively high costs versus inorganic catalysts, and low reaction rate. Enzyme immobilisation on a solid support facilitates continuous operation and catalyst recovery and re-use.[8, 30, 35] Lipase can be immobilised employing different approaches including covalent bonding, entrapping, physisorption and cross-linking.[35] Commercial approaches predominantly focus on lipase adsorption onto hydrophobic polymers such as alkyl-agarose, polypropylene, and polystyrene.[36, 37]

Silica monoliths are widely employed in separation science and as 'inert' supports in heterogeneous catalysis.[38-40] Monoliths are rigid materials with interconnected pores which allow fluid transport.[40] These pores may be of uniform size and of micropore to macropore diameters, or feature multiple, interconnected pore networks to offer the optimal combination of high surface area and rapid mass transport.[30, 38, 39, 41] Monolithic microreactors have shown promise in flow chemistry as a practical, economic and environmentally friendly means to formulate catalytic, offering improved control over molecule flow rate, contact time and temperature, in addition to in-situ product separation facilitating catalyst re-use.[8, 42, 43] Porous silica monoliths are typically prepared through sol-gel syntheses in conjunction with hard/soft templating by surfactants,[44] ionic liquids,[45] or even ice.[46] The cost and toxicity of such templates are important consideration for large-scale applications, wherein e.g. pluronic

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surfactants and ionic liquids remain problematic. Several monolithic microreactors are reported for transesterification/biodiesel production utilising immobilised lipase.[30, 47, 48] These enzymatic microreactors displayed superior performance relative to the free enzyme. However, it should be noted that the only monolithic study employing *Candida Antarctica* lipase pre-functionalised the silica surface with a primary amine to covalently bind the glutaraldehyde cross-linker to the enzyme:[30] This approach is fundamentally flawed as it is impossible to determine whether the observed transesterification activity arose from biocatalysis by the lipase, or conventional base catalysis by the organic amine since the latter are excellent catalysts themselves for biodiesel production[49, 50] (and essential control experiments were omitted). The commercial silica capillary employed also only resulted in a modest lipase loading of 6.34 mg.

Here we present the first unequivocal demonstration of transesterification catalysed by silica immobilised *Candida Antarctica* lipase, using an amine-free silica monolith as a microreactor for the transesterification of tributyrin as a model TAG. The efficacy of lipase as a stable biocatalyst, and role of monolith textural properties, is demonstrated.

2. Experimental

2.1 Materials and instrumentation

Candida Antarctica lipase B was purchased from Sigma-Aldrich (UK) and used without further purification. Decane (99%), 4-nitrophenol (4-NP, 98%) and 4-nitrophenyl butyrate (4-NPB, 98%) were also obtained from Sigma-Aldrich. Polyethylene oxide (PEO, MW 100,000 and MW 200,000), HCl (37%), tetramethoxysilane (TMOS, 99%) and tetraethyl orthosilicate (TEOS, 99%) were purchased from Fluka. Glass capillary (0.6 /0.05 mm ID and 25 mm length) was obtained from Brand GMBH. The instruments used in fabrication and detection procedure includes a Baby bee syringe pump from Bioanalytical System Inc. (West Lafayette, USA); Model 7971 column heater (Jones Chromatography Ltd); Chemyx Fusion 100 Syringe Pump from KR Analytical Ltd (Sandbach, Cheshire); BIO Wide Pore C18 column 5 µm, 15 cm × 2.1 mm from Phenomenex (United Kingdom, Queens Avenue, Hurdsfield Ind. Est).

2.2 Catalyst synthesis

2.2.1 Preparation of silica-monoliths and characterisation

Silica-monoliths were prepared using two silica precursors, TEOS and TMOS following the protocol of Fletcher et al.[51] Typically, PEO (0.282 g, MW 100,000) was dissolved in 4 ml of 0.02 M acetic acid solution and stirred for 1 h (200 rpm) in an ice bath for complete dissolution. To this solution, 2 ml of TMOS was added and kept under stirring for an hour. The solution was subsequently poured into a plastic mould (length 6 cm, internal diameter 0.45 cm) closed at both ends using PTFE thread seal tape and aged at 40 °C for 3 days. The monolithic gel was subsequently removed from the mould and thoroughly washed with deionised water. The monolithic gel was then immersed in an incubator containing 1 M aqueous NH_4OH, and heated to 82 °C for 24 h to etch the silica surface and generate mesoporosity. The etched monolith was washed with deionised water until the washing reached pH 7, and dried at 40 °C for 1 day. Next, the dried monolith was calcined at 600 °C for 3 h under flowing air to remove the PEO surfactant, cooled and then cut to 4 cm length. The resulting silica monolith was placed between two pieces of borosilicate tube, and PTFE heat shrinkable tubing (Smith Scientific) used to encapsulate all three components. Upon heating to 350 °C for 1 h, the PTFE contracted to make a leaktight seal between the ends of the borosilicate glass capping tubes, and the monolith, as shown in **Fig. S1**. The resulting monolith was denoted M1. A similar protocol was adopted to prepare two additional silica monoliths using a heavier (0.305g, MW 200,000) PEO in conjunction with either TMOS (denoted M2), or TEOS (denoted M3). In the case of the M3 monolith, 2.537 ml of 1 M nitric acid were used to hydrolyse TEOS instead of acetic acid. Bulk and surface properties of the three parent silica monoliths were characterised by N_2 porosimetry using a Micrometrics TriStar porosimeter. Samples were degassed at 30 °C for 2 h prior to analysis by nitrogen adsorption at -196 °C. BET surface areas were calculated over the relative pressure range 0.01-0.2, while pore size distributions were determined by applying the Barrett-Joyner-Halenda (BJH) method to the desorption isotherm for $P/P_0>0.35$. Scanning electron microscopy (SEM) images were acquired on a Zeiss EVO 60 instrument and Oxford Instruments Inca System 350 at 20 kV. Samples for SEM analysis were coated with Au-Pt (~2 nm) using a SEMPREP 2 Sputter Coater (Nanotech Ltd., Sandy, UK).

2.2.2 Lipase immobilization

An aqueous solution of *Candida antarctica* lipase was prepared by dissolving 10 mg of the enzyme in 10 ml of Tris-HCl buffer (pH 7, 0.05 M) prepared by dissolving 0.6057 g of Tris (tris(hydroxymethyl)aminomethane) in 53.4 ml H_2O and 46.6 ml of 0.1M HCl. The resulting lipase solution was pumped through each silica monolith at a flow rate of 10 µl/min for 16 h, and the monoliths then refrigerated at 4 ºC for 1 h, prior to vacuum drying for 30 min to produce lipase immobilised analogues. The monoliths were finally washed with 0.05 M Tris-HCl buffer (flow rate 10 µl/min) for 16 h to remove physisorbed lipase, and the liquid washing collected for analysis as described below.

2.3 Determination of lipase loading

The immobilised lipase loading was quantified from the amount of unsupported lipase collected in the washing as determined from the hydrolysis of 4-nitrophenylbutyrate to 4-nitrophenol. The amount of immobilised lipase is obtained from the difference between the amount of lipase introduced into the monolith (10 mg) as described above, and that observed in the residual solution collected after the immobilisation process.

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2.3.1 4-Nitrophenylbutyrate hydrolysis

Lipase catalysed 4-nitrophenylbutyrate (4-NPB) hydrolysis was performed at 25 ºC under 200 rpm stirring in a glass round-bottomed flask. The total reaction volume was 1 ml, and comprised 0.4 ml of 10 mM 4-NPB in decane (organic phase) added to 0.6 ml of lipase in 0.05 M Tris-HCl buffer solution (the aqueous phase from the immobilisation washings). Aliquots of 100 µl were withdrawn periodically (1, 5, 10 and 15 min) from the aqueous phase and mixed with a 0.1 M Tri-HCl buffer solution (pH 7) to quench the reaction in a disposable plastic UV-vis cuvette. Samples were then analysed on a Shimadzu UVmini-1240 UV-vis spectrophotometer, with the concentration of 4-nitrophenol product determined from the 400 nm absorption intensity.

2.3.2 Assay of immobilised lipase activity

A schematic of the reactor used to evaluate the activity of immobilised lipase for 4-NPB hydrolysis is shown in **Fig. 1**, and comprised two syringe pumps to independently feed aqueous and organic solutions through the monolith, a capillary mixer, and the lipase immobilised monolithic microreactor. An aqueous solution of 0.05 M Tris–HCl buffer (without lipase) and an organic solution of 10 mM 4-NPB in decane were independently pumped through the capillary mixer and monolith and the resulting product collected from the outlet of the microreactor at steady state. The aqueous phase was then removed from the collection vial and analysed by the same methodology described above for the assay of free lipase activity. Control experiments used bare silica monoliths showed negligible hydrolysis in the absence of lipase in accordance with the literature.[52]

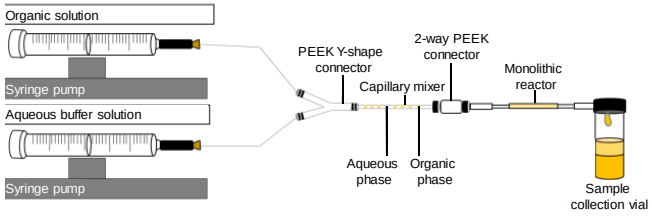

Fig 1. Schematic of the reactor system to assay the activity of the immobilised lipase [53]

2.4 Transesterification

Transesterification of glyceryl tributyrin with methanol (**Fig. 2**) was performed in an isothermal flow reactor supplied by a syringe pump. A selected molar ratio of tributyrin and methanol were charged to the syringe pump and delivered into the reactor at a given temperature (**Fig. 2**). Samples were collected at different time intervals, and analysed off-line on a Perkin Elmer Clarus HPLC with UV detector and BIO Wide Pore C18 column (5 µm x 15 cm x 2.1 mm), using a methanol/ultra-pure water (80:20 v/v) mobile phase, operated under isocratic conditions at 23 °C to quantify tributyrin conversion and methyl butyrate formation. Multi-point calibrations were obtained for methyl butyrate to calculate the response factors. Errors in FAME yield are the standard deviation of three experiments.

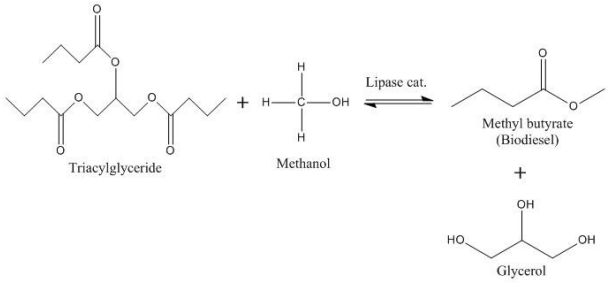

Fig 2. Transesterification reaction of tributyrin with methanol.

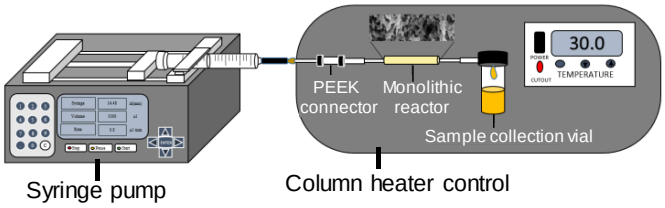

Fig 3. Schematic of continuous flow reactor for testing.

3. Results and discussion

3.1 Characterisation

The silica monolith morphology is expected to be strongly influenced by the synthesis protocol, notably the choice and concentration of silane precursor, the molecular weight of polymer surfactant, and the water:silane precursor ratio. Here, 'coral-like' bicontinuous architectures with interpenetrating macropores form as a result of spinodal decomposition of silica-rich and solvent-rich phases during gelation,[51, 53] in the presence of PEO as a surfactant-template to impart meso-/macroporosity.[54] Such hierarchically porous frameworks maximise the accessible internal surface area and pore volume, and minimise the pressure drop across a monolith.[51, 55, 56] Successful monolith formation was confirmed by SEM, with micrographs in **Fig. 4** evidencing coral-like porous architectures for all three samples[55, 57] after lipase immobilization. Macropore diameters calculated from SEM (**Table 1**) were similar in all cases, indicating a common liquid volume fraction co-existed with silica sol-gel.[56, 58, 59] However, textural differences in the architectures are apparent, with the M1 and M3 frameworks exhibiting smooth surfaces, while of M2 is constructed from approximately 1 µ spherical beads. **Table 1** summarises textural properties of the three parent monoliths. Mesopore generation on the silica skeleton (wall) was confirmed by N_2 porosimetry, revealing an increase in both mesopore and macropore diameter, and total pore volume, with the surfactant molecular weight (M2 versus M1), and use of TEOS (M3) versus TMOS (M1) as the silica source. These changes are accompanied by an inverse trend in BET surface area (representative isotherm shown for M1 in **Fig. S2**), which decreased with surfactant molecular weight and the use of TEOS, reflecting the enhanced porosity. The observation that TMOS created a higher surface area monolith than TEOS is consistent with previous literature.[60] The increase in mesopore diameter for TEOS is attributed to the faster rate of hydrolysis for TMOS, which results in the formation of highly

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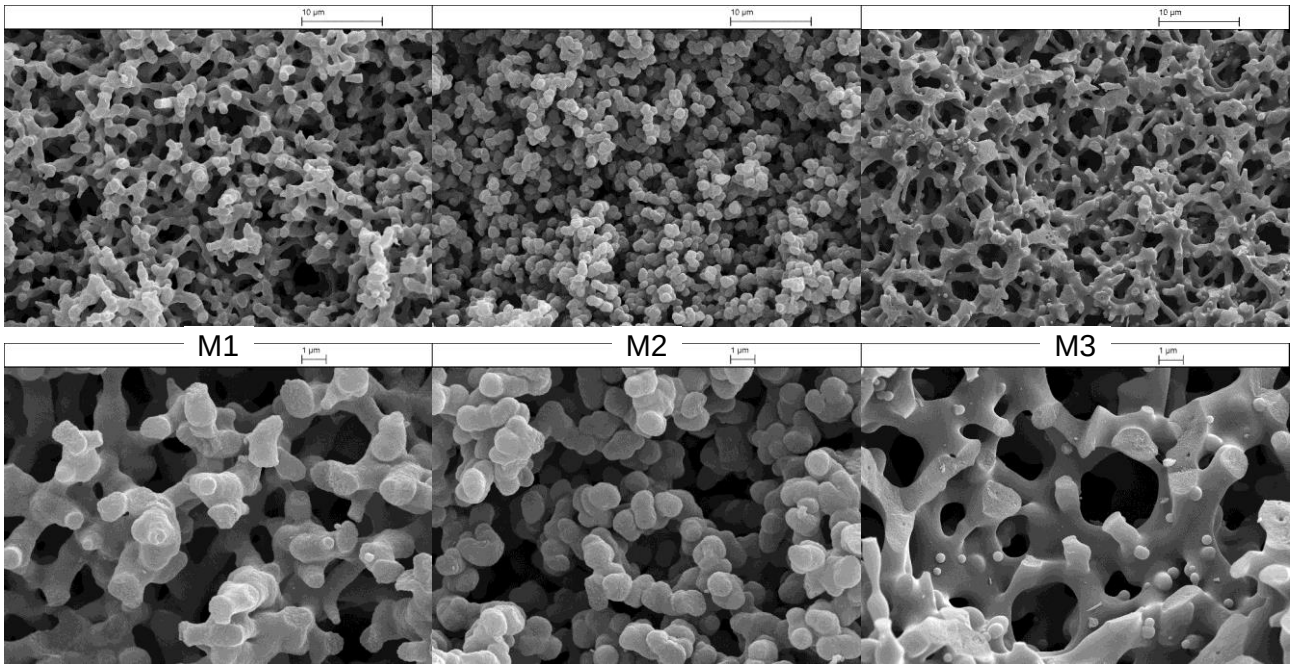

Fig 4. SEM images of silica monolith samples (a) M1, (b) M2, and (c) M3.

branched silica clusters for the latter which link to form a monolith dominated by small pore diameter. Similar observations reported by Can et al who observed an increase in mesopore size from 9 to 16 nm on switching from TMOS to TEOS.[61] Macroporosity is essential for immobilisation of Candida Antarctica lipase B. within the monolith architecture due to its high molecular weight (33,273 Da) and size (120.43 nm^2).[62]

Table 1. Textural properties of lipase immobilised silica monoliths

Sample	Surface area[a] / m^2g^{-1}	Total pore volume[b] / cm^3g^{-1}	Mesopore diameter[b] / nm	Mean macropore diameter[c] / µm	Lipase loading / mg[d]
M1	529	0.97	6.5	4 ± 2	8.6
M2	460	1.04	8.7	4 ± 2	8.2
M3	218	1.07	18.6	4 ± 2	9.2

[a]BET; [b]BJH; [c]SEM; [d]from 4-NPB hydrolysis

The amount of enzyme immobilised onto each monolith was calculated from hydrolysis of 4-NPB to 4-NP (**Table 1**), and revealed a common loading of around 8.5 mg lipase. This represents immobilisation of 80-80 % of the lipase introduced to the monolith, similar to that previously reported for an amine-functionalised monolith.[30]

3.2 Transesterification

Candida Antarctica lipase is prone to deactivation by high concentrations of short chain alcohols[34] and hence optimisation of the methanol:oil molar ratio is critical for its application in transesterification.[32-34] The impact of methanol:tributyrin molar ratio was therefore first explored over the M2 catalyst to identify the optimum reactant stoichiometry. **Fig. 5** shows a strong dependence of methyl butyrate yield on reactant stoichiometry, with a maximum of 66 % reached for 2:1 MeOH:tributyrin; this corresponds to the maximum theoretical TAG conversion to FAME for such a stoichiometry. Further increases in methanol, which usually promote transesterification over conventional solid acid/base catalysts, resulted in a significant loss in lipase activity. This difference between heterogeneous and biocatalysts is ascribed to enzyme denaturation due to methanol toxicity,[63] although other reports suggest that lipase remains active for rapeseed oil methanolysis even for MeOH:oil of 6:1.[64] Staged methanol introduction is proposed as a solution to the avoid lipase deactivation and achieve complete oil conversion either in batch or flow.[63]

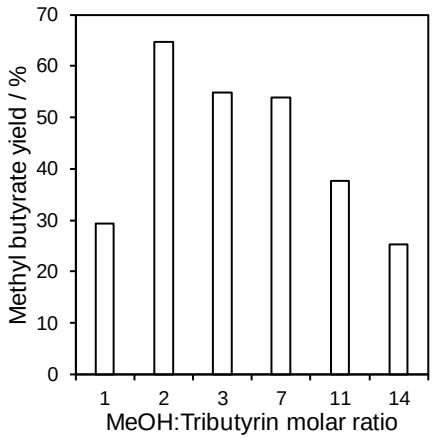

Fig. 5 Methyl butyrate yield as a function of methanol:tributyrin mole ratio over M2 catalyst. Reaction conditions: 0.8 µL.min^{-1}, 30 °C and 20 h on-stream.

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The impact of liquid flow rate was subsequently explored over M2 (**Fig. S3**), and revealed a monotonic decrease with increasing flow rate from 66 % at 0.8 µL/min to 12 % for 12.8 µL/min. These flow rates are similar to literature ranges, when relative enzyme loadings are accounted for.[47, 48, 65] This decrease simply reflects the corresponding fall in residence time (from 400 to 25 min) within the catalyst bed. Catalyst stability at the longest residence time (and hence highest conversion) was also assessed for the optimum 2:1 MeOH:tributyrin stoichiometry identified above, and at the 3:1 molar ratio necessary to achieve complete TAG conversion (**Fig. S4**). While negligible deactivation was observed for the 2:1 MeOH:tributyrin stoichiometry >100 h reaction on-stream, the higher methanol concentration resulted in 69 % activity loss over the same time period confirming the lipase intolerance to methanol.[63, 66] The steady state productivity of M2 after 100 h on-stream under these optimal conditions was 0.7 µmol.min^{-1}.mg$_{lipase}^{-1}$. This compares favourably with literature values of 0.16 µmol.min^{-1}.mg$_{enzyme}^{-1}$ for the transesterification of crude safflower oil to ethyl linoleate catalysed by Thermomyces Lanuginosus lipase immobilised on epoxy-functionalised silica monoliths,[67] 0.17 µmol.min^{-1}.mg$_{enzyme}^{-1}$ for the transesterification of cottonseed oil with methanol over resin immobilised Candida antarctica B lipase (Novozym 435),[65] and 0.007 µmol.min^{-1}.mg$_{enzyme}^{-1}$ crude Jatropha oil methanolysis over a *n*-butyl-functionalised silica monolith immobilised with Burkholderia cepacia lipase.[47]

Finally, the temperature sensitivity of monolith immobilised *Candida Antarctica* was investigated. **Fig. S5** revealed a significant increase in conversion from ~30 to 63 % as the reaction temperature was raised from 25 °C to 30 °C, followed by a steady loss in activity between 37-55 °C due to enzyme denaturation.[68] Note that negligible reaction occurred in any experiments in the absence of lipase at temperatures <55 °C. The optimum reaction conditions were thus 2:1 methanol: tributyrin at 30 °C and a reactant flow rate of 0.8 µL.min^{-1}.

The performance of the three, lipase immobilised monolithic reactors was subsequently compared under these optimised conditions, as shown in **Fig. 6**. It is evident that M2 exhibited the highest FAME production, and that activity was strongly correlated with the textural properties (since all monoliths possessed a similar amount of lipase). M1 and M2 possess similar high surface areas, however the higher MW PEO templated M2 possess wider mesopore which may offer superior mass transport to/from the active site, as previously reported for mesoporous[69] and hierarchically porous solid acid[70] and base[22] catalysts for tributyrin transesterification. M2 was also superior to M3, despite the latter possessing the largest mesopores, presumably a consequence of the far lower surface area of the (TEOS-derived) M3 monolith. The superior activity of M2 may also reflect its unique textural properties, which may result in a different surface roughness and/or hydrophobicity and hence affinity for methanol and oil; kinetic modelling of heterogeneous base catalysed transesterification indicates that the reaction follows a Langmuir−Hinshelwood−Hougen−Watson (LHHW) mechanism, with adsorption of polar methanol rate-limiting.[71] A balance of high surface area, wide meso- and macropores, and an appropriate surface texture/functionality are required to provide a high density of accessible (enzyme) active sites.

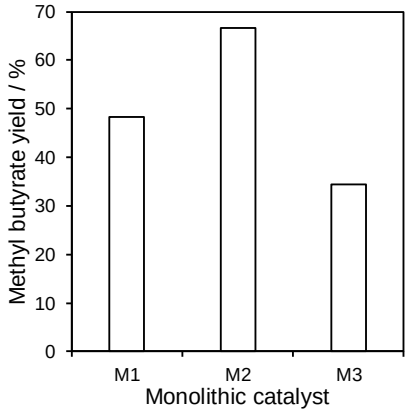

Fig. 6 Methyl butyrate yield using different lipase immobilised microreactors. Reaction conditions: 0.8 µL.min^{-1}, methanol:tributyrin molar ratio = 2:1, 30 °C and 20 h on-stream.

4 Conclusions

Lipase immobilised silica monoliths have been successfully synthesised and screened for the transesterification of tributyrin in continuous-flow at mild temperatures. Successful *Candida Antarctica* immobilisation was demonstrated by calculating the amount of trapped enzyme via 4-nitrophenylbutyrate hydrolysis. Reaction conditions were optimised for the methanol:tributyrin molar ratio, flow rate, and reaction temperature. Although enzyme deactivation occurred at high methanol concentrations and temperatures >30 °C, excellent stability and quantitative methyl tributyrate yields were obtained for >100 h reaction for a 2:1 methanol:tributyrin feed and flow rate of 0.8 µL.min^{-1} at 30 °C. Although lipase immobilised monoliths prepared using different silica precursors and molecular weight surfactants were all active for tributyrin transesterification, their different textural properties significant influenced catalytic activity. A combination of high surface area and large meso- and macropores were necessary to overcome diffusions limitations.

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