Single-step lipase-catalyzed functionalization of medium-chain-length polyhydroxyalkanoates

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INTRODUCTION
Polyhydroxyalkanoates (PHA) are known to have important industrial and biomedical applications as a result of their biodegradability, biocompatibility, compostability and versatile structural composition. In fact, the current concerns over environmental pollution and degradation, favor the application of these biodegradable polymers over their petrochemical counterparts. Despite their applications in the biomedical field spanning from surgical sutures, drug delivery devices to tissue engineering scaffolds, the biopolymers may exhibit slow degradability and resorbability especially within the extracellular matrixes. For instance, the in vivo degradability of poly-2-oxepanone was reported to take about 3–4 years in tissue in addition to lack of total elimination of the degraded monomers out of the body. Among the reported strategies employed to address this issue is the functionalization of the polymer. One such approach is functionalization by transesterification via sugar-acylation, which not only improve the hydrophilicity of these carbohydrate esters but also impart novel properties for specialty applications such as antimicrobial activity, lectin interaction, galactosyl transferase inhibition, specific ligands to the ASGPR receptor that is overexpressed in hepatocellular carcinoma, water repelling and oil absorption.

The use of conventional chemical catalysis in polymer functionalization and modification processes is viewed as unfavorable due to byproduct(s) formation that are complicated to control particularly when the main products are intended for use in biomedical and environmental applications. Alternatively, enzymatic catalysis offers excellent enantiomeric selectivity, specificity and catalytic activity under mild reaction conditions, making the enzyme catalyzed functionalization process highly desirable. Lipases (EC 3.1.1.3) were among the most commonly used triacylglycerol hydrolases that catalyze the synthesis of ester bonds in micro-aqueous media. A wide range of biodegradable functionalized polymers has been produced using enzymatic catalysis. Most reported polymer functionalization was based on polyvinyl, polyacrylate and polycaprolactone esters. In addition, most of the synthetic process is not solely enzymatic but a hybrid system (chemo-enzymatic) where enzymatic transesterification of the vinyl or acrylic ester with the sugar moiety is followed by chemical radical polymerization. In most cases, the functionalized polymer obtained through this approach has toxic chemical impurities, which make it less attractive for biomedical applications. Secondly, the pendant carbohydrate moiety is attached to the main polymeric chain via sugar-acylation, which not only improve the hydrophilicity of these carbohydrate esters but also impart novel properties for specialty applications such as antimicrobial activity, lectin interaction, galactosyl transferase inhibition, specific ligands to the ASGPR receptor that is overexpressed in hepatocellular carcinoma, water repelling and oil absorption.

Keywords: biopolymer; Candida antarctica; lipase; polyhydroxyalkanoates; sugar-ester; sucrose

Abstract
BACKGROUND: Functionalization of aliphatic biopolymers such as bacterial polyhydroxyalkanoates (PHA) using biologically active hydrophilic moieties like sugars helps to improve the hydrophilicity and biodegradability of the biomaterial.

RESULTS: The effects of reaction variables reaction time, temperature, enzyme concentration and substrate ratio on reaction rate and yield in the synthesis of poly(1'-O-3-hydroxyacyl-sucrose) using Candida antarctica lipase B (EC 3.1.1.3) were studied. Using H2O2 as micro-initiator, enzyme-mediated synthesis yielded reaction rate, vapp of 0.076 x 10−5 mol L−1 s−1. The biodegradability of the functionalized polymer was observed to increase by 1.5 fold compared with the non-functionalized material apart from showing better compostability. Increasing the reaction temperature (> 50°C), enzyme concentration (> 15 g L−1) and reactant ratio (w/w) of sucrose:PHA (> 2) did not increase further the rate or yield. The sucrose-functionalized mcl-PHA was characterized with respect to the non-functionalized material.

CONCLUSIONS: Novozym® 435 can be used effectively to synthesize poly(1'-O-3-hydroxyacyl sucrose) in micro-aqueous medium bypassing the need for chemo-synthetic steps. The synthesized biomaterials have potential applications in biomedical and industrial niches.

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by a di-carboxylic acid spacer arm,19,22,24 which could probably increase the hydrophobicity of the polymer rather than the hydrophilicity. Literature reporting bacterial PHA functionalization is very scarce,25 particularly reports on the single-step enzymatic functionalization of medium chain length PHA (mcl-PHA).

This study reported Candida antarctica lipase B catalyzed functionalization of bacterial mcl-PHA with sucrose in the absence of the spacer arm to yield poly(1'-O-3-hydroxyacyl-sucrose). Effects of reaction variables such as temperature, enzyme loading, reaction time, and enzymatic reaction rate, \( v_{\text{ENPP}} \) and reactant conversion were studied. In addition to characterization of the functionalized mcl-PHA, biodegradability and compostability of the biomaterial were also studied.

MATERIALS AND METHODS

Materials

Candida antarctica lipase B (Novozym 435) immobilized on acrylic resin beads (Sigma-Aldrich; L4777), p-nitrophenyl palmitate (Sigma-Aldrich; N2752), dimethyl sulfoxide (Sigma-Aldrich; D8418), molecular sieves 4 Å (Sigma-Aldrich; 334308), NaCl crystal window (Sigma-Aldrich; Z267724). Sucrose (Merck; 107651), hydrogen peroxide (Merck; 822287), methanol (Merck; 106012), chloroform (Merck; 102395), dichloromethane (Merck; 106051), acetone (Merck; 100299), N-(trimethylsilyl) imidazole (Merck; 818204). All chemicals were of analytical grade.

Biodegradable mcl-PHA with co-monomeric compositions of \( C_{60} \) to \( C_{140} \) was obtained based on reported fermentation culture conditions,26 using Pseudomonas putida Bet001 as producer microorganism and fatty acids as sole carbon and energy source.

Methods

Enzyme activity

The enzymatic activity was measured as reported by Teng and Xu.27 Novozym 435 (150 mg) was added to a vial containing 10 mL of a 10 mmol L\(^{-1}\) 4-nitrophenyl palmitate solution in n-hexane. To this mixture 60 \( \mu \)L of 1 mol L\(^{-1}\) absolute ethanol was added. The resulting slurry was incubated at 40 °C, 200 rpm for a period of 20 min. Aliquots (30 \( \mu \)L each) of the reaction mixture were withdrawn at intervals and quenched by mixing with 1 mL of 0.1 mol L\(^{-1}\) NaOH in a quartz cuvette. The 4-nitrophenol liberated by the reaction was measured at 412 nm (UV–Vis spectrophotometer V-630; Jasco, Japan) against distilled water as blank. The enzyme activity was calculated as the slope of a plot of converted sucrose concentration versus time.

Enzymatic transesterification

All reactions were performed in triplicate. A solution of sucrose (0.16 g) dissolved in 2 mL DMSO under mild warming (40 °C, 3 min) was mixed into 20 mL capped reaction vials containing 0.08 g mlc-PHA dissolved in 8 mL chloroform. To this mixture, 150 mg lipase, 30 mg molecular sieve 4Å and 10 \( \mu \)L H\(_2\)O\(_2\) (micro-initiator) were added. The initial water activity (\( a_w \)) of the mixture was then recorded using Rotronic hygropalm water activity meter (HP23-AW; www.rotronic.co.uk). The reaction was then allowed to progress for a total period of 24 h at 50 °C, 200 rpm. All reactions were carried out according to the aforementioned procedure except if stated otherwise.

Residual sugar quantification

At specific intervals the residual carbohydrate moiety was quantified by withdrawing aliquot samples (20 \( \mu \)L) of the reaction mixture and mixed with 5 \( \mu \)L absolute ethanol to stop the reaction. This mixture was then mixed with 1.0 mL chloroform and thereafter derivatized by adding 5 \( \mu \)L N-(trimethylsilyl) imidazole (TMSI) followed by sonication at 37 kHz, 1.9 W and 30 °C for 2 min and immediately filtered into GC vials. The residual sugar was then measured on Agilent triple quadrupole 7000B instrument (Agilent, USA) equipped with GCMSMs triple axis detector carrying Agilent HP-5ms column (30 m long × 0.25 mm internal diameter × 0.25 \( \mu \)m film thickness). A sample (2 \( \mu \)L) was automatically injected into the GCMSMS at a split ratio of 1:10. The injection temperature was 280 °C. The column oven temperature ramping was as follows: 110 °C for 1 min then increased to 200 °C at 20 °C min\(^{-1}\); held at 200 °C for 2 min then increased to 280 °C at 20 °C min\(^{-1}\); held at 280 °C for 5 min. Helium at 0.76 bar and 14 mL min\(^{-1}\) was used as the carrier gas. Mass spectra were acquired at 1250 scan speed using electron impact energy of 70 eV at 230 °C ion-source and 250 °C interface temperatures. The data obtained per each reaction time was used to quantify the residual sucrose by comparing with the calibration plot prepared by analyzing different concentrations of sucrose standard solutions in DMSO. The enzymatic reaction rate (mol L\(^{-1}\) s\(^{-1}\)) was calculated as the slope of a plot of converted sucrose concentration versus time.

Product extraction

At the end of the reaction, the functionalized polymer (poly(1'-O-3-hydroxyacyl-sucrose)) was extracted by diluting the reaction mixture with 20 mL of dichloromethane, followed by filtration using glass fritted Buchner funnel to separate the immobilized enzyme and molecular sieves. The filtrate was then concentrated under reduced pressure to about 3 mL at 40 °C. The functionalized polymer was precipitated out from the filtrate by adding the concentrate into 10 mL of cold acetone (4 °C). The solvent was then decanted and the precipitate was re-purified by subjecting it to three-cycle precipitation. At each stage, the functionalized polymer was dissolved in dichloromethane (3 mL) then added to cold acetone (10 mL, 4 °C) and the white precipitate was recovered. The final extracted product was dried to a constant weight at 30 °C under vacuum. A portion of the dried sample was subsequently subjected to further authentication analyses.

Product authentication

Perkin-Elmer FTIR RX 1 spectrometer (Perkin-Elmer Inc., Wellesley, MA, USA) was used to record FTIR-ATR spectra of the poly(1'-O-3-hydroxyacyl-sucrose) as previously reported.25 Proton NMR spectra were recorded on a JEOL JNM-GSX 270 FT-NMR (JOEL Ltd, Tokyo, Japan) machine at 250 MHz according to reported literature.26 Gel permeation chromatography of the poly(1'-O-3-hydroxyacyl-sucrose) was recorded on Waters 6000 (Waters Corp, Milford, MA, USA) equipped with a Waters refractive index detector (model 2414) and the following gel columns (7.8 mm internal diameter × 300 mm each) in series: HR1, HR2, HR5E and HR5E Waters Styrogel HR-THF. Monodisperse polystyrene of different molecular weights (3.72 × 10\(^3\), 2.63 × 10\(^3\), 9.10 × 10\(^3\), 3.79 × 10\(^3\), 3.55 × 10\(^3\), 7.06 × 10\(^3\), 3.84 × 10\(^3\) and 6.77 × 10\(^3\) Da) were used as standards to produce the calibration curve. The polymer samples (2.0 mg mL\(^{-1}\)) were dissolved in dichloromethane (DCM), filtered through a 0.22 \( \mu \)m PTFE filter and then injected into the GPC (100 \( \mu \)L) at 40 °C. DCM was used as mobile phase at a flow rate of 1.0 mL min\(^{-1}\).

Differential scanning calorimetric (DSC) analysis of the poly(1'-O-3-hydroxyacyl-sucrose) was performed on a Perkin-Elmer Diamond
The biochemical oxygen demand (BOD) and percentage BOD biodegradability were determined according to Equations (3) and (4), respectively:

$$\text{BOD} = \frac{DO_t - DO_0}{\varphi}$$  \hspace{1cm} (3)

where $DO_0$ and $DO_t$ is the dissolved oxygen (DO) initially and at time $t$, respectively; $\varphi$ is the fractional oxygen volume defined as the ratio of the experimental DO volume to that of theoretical DO volume calculated from the ideal gas equation and according to stoichiometry in Equation (5).

$$\text{BOD degradability} = \frac{DO_0 - DO_t}{BOD_0} \times 100$$  \hspace{1cm} (4)

where $BOD_0$ is the theoretical BOD which equals $DO_0$ in a batch process.

$$C_{\text{PHA} + \text{biomass}} + O_2 \rightarrow CO_2 + H_2O + C_{\text{residues}} + C_{\text{biomass}}$$  \hspace{1cm} (5)

Polymers with average molecular weight. For the enzyme catalytic activity,31 in Fig. 1, the overall reaction of this enzyme is composed of a catalytic triad of three polar amino acids Asp187 and Ser105. These amino acids are responsible for the enzyme catalytic activity.31 In Fig. 1, the overall reaction of this enzyme is composed of a catalytic triad of three polar amino acids Asp187 and Ser105. These amino acids are responsible for the enzyme catalytic activity.31

$$\text{Polydispersity index} = \frac{M_w}{M_n}$$  \hspace{1cm} (6)

where $M_w$ is weight-averaged molecular weight and $M_n$ is number averaged molecular weight.

RESULTS AND DISCUSSION

Characterization of sucrose-based functionalized mcl-PHA

The synthesis of sucrose-based functionalized mcl-PHA (poly(1'-O-3-hydroxyacyl-sucrose)) was achieved using Novozym 435 from Candida antarctica as a catalyst in a co-solvent system (Fig. 1). In this process, a sucrose conversion yield of 34.1% (±1%) was observed within 24 h. The solvent-cast film of the extracted polymeric product (white amorphous film) was subjected to analytical authentication. FTIR-ATR (Fig. 2): wave number 3389 (\(\nu\) -O-H), 2979 (\(\nu\) -CH2-CH3), 2891 (\(\nu\) -CH2-), 1734 (\(\nu\) C=O), 1295 (\(\nu\) -COO-). 1H NMR (Fig. 3) relative to trimethylsilane (TMS): \(\delta\) 5.30 (1H, 1a-H of glucose), 4.72 (1H, J\(=\)g,p-HA\(\beta\)), 4.19 (1H, J\(=\)g,p-HA\(\alpha\)), 3.83 - 3.20 (1H, 3\'β-H of sucrose), 3.19 (1H, J\(=\)g,p-HA\(\beta\)), 2.99 (1H, 4\'β-H of sucrose), 2.91 - 2.72 (2H, 3\'α-H of sucrose), 2.66 - 2.41 (2H, 5\'β-H of sucrose), 2.30 - 1.50 (2H, 4\'β-H of sucrose). The 1H NMR spectrum indicated the presence of the sucrose backbone and the terminal acyl group.

Candida antarctica lipase B (CALB) is reported to be composed of 317 different amino acid residues and has a total molecular weight of about 33 kDa.31,32 Like other hydrolases, the catalytic site of this enzyme is composed of a catalytic triad of three polar amino acid residues i.e. serine-histidine-aspartic acid (Ser105, His224 and Asp187) as depicted in Fig. 1. These amino acids are responsible for the enzyme catalytic activity.31 In Fig. 1, the overall reaction mechanism is proposed to follow a two-step mechanism with an initial acylation step where the PHA polymer interact with the Ser105 residue resulting in a covalent acyl-enzyme intermediate.33 In this step, the primary hydroxyl group on Ser105 serves as a nucleophile, which attack the terminal acyl-carbonyl-carbon of the PHA forming a transition state which leads to the formation of covalently bonded tetrahedral intermediate. This intermediate is then stabilized by the proton-accepting His224 residue due to the formation of two important hydrogen bonds between itself (His224) and the protonated primary hydroxyl group on Ser105.30
and the other two amino acids (Ser_{105} and Asp_{187}). This is followed by deacylation as a result of interaction between the Ser_{105} primary amide nitrogen and the ester bond hydroxyl group resulting in the release of water molecule and Ser_{105} activated complex. Further nucleophilic attack by alcohol (sucrose) results in the glycosylation of the sugar moiety onto the polymer chain (Fig. 1).

In Fig. 2, the FTIR spectrum of the poly(1'-O-3-hydroxyacyl-sucrose) shows an increased absorption at wave number ($\nu$) 3389 cm$^{-1}$ corresponding to increased −OH group and the persistence of ester absorption at 1734 cm$^{-1}$ reveals a successful functionalization of the PHA with the sugar moiety compared with the spectra of non-functionalized PHA that has been used as control (Fig. 2).

This observation was found to be in accord with the proton NMR spectrum of the product (Fig. 3), which reveals a series of chemical shifts at δ 5.30 to 3.20 ppm, signifying the presence of sugar in the product. These observations were found to be in good agreement with reported literature on sucrose polyesters characterization.$^{19,22}$

In contrast to control mcl-PHA ($T_m$ 42°C; $T_d$ 264.4°C, $X_{ap}$ 0.7), the thermal analyses of the poly(1'-O-3-hydroxyacyl-sucrose) was observed to have low degree of crystallinity ($X_{ap}$ = 0.3), two peak endothermic melting temperature ($T_m$) of 123.3 and 131.8°C (±0.2°C) corresponding to the distinct melting of the sucrose moiety and PHA, respectively. The thermogravimetric analysis revealed a two-step degradation temperature $T_d$ (Fig. 4) at 186.2°C.
and 275.5 °C (±0.2 °C), signifying the decomposition of both the hydrophilic sugar moiety and the hydrophobic mcl-PHA backbone, respectively. Furthermore, the GPC analysis of the poly(1'-O-3-hydroxyacyl-sucrose) revealed a general decrease in weight average molecular weight ($M_w$ 35.4 kDa) with increasing number average molecular weight ($M_n$ 48.7 kDa) and 0.7 PDI compared with the non-functionalized PHA ($M_w$ 41.2 kDa, $M_n$ 21.3 kDa, PDI 1.9). This reduction in $M_w$ and the increase in $M_n$ of the poly(1'-O-3-hydroxyacyl-sucrose) were attributed to the possible enzymatic hydrolysis of the polymeric backbone by random chain scission, which explains the increase in $M_n$. The observed increase in $T_m$ could be attributed to the increased hydrophilicity of the polymer as a result of sugar moiety hydrogen bonding causing strong intermolecular attraction, thus at the same time increasing the amount of thermal energy required to break the bond. This observation is further supported by the differences in the $T_d$ between 264.4 °C for non-functionalized mcl-PHA and 275.5 °C for functionalized mcl-PHA.

**Biodegradability and compostability of the poly(1'-O-3-hydroxyacyl-sucrose)**

The biodegradability of polymeric materials has been documented to rely substantially on their basic physical and chemical structure properties. These are not only restricted to the polymeric molecular structures but also the length of the polymeric chain and crystallinity of the polymer since it is more difficult to degrade the crystalline part compared with the amorphous parts. Reaction conditions (vessel’s volume and shape, temperature, pH, aerobic or anaerobic, mixing and duration) and polymeric polarity were also reported to affect the rate of degradation process. Indeed, the hydrophilicity of the sugar moiety on the polymer will also influence the absorption of water favoring high microbial growth and attract more microbial cells to the sample.

As expected the biodegradability of the poly(1'-O-3-hydroxyacyl-sucrose) was observed to surpass that of non-functionalized polymer by about 1.5 fold (Fig. 5). In fact, poly(1'-O-3-hydroxyacyl-sucrose) was observed to be degraded by 56.4% after 5 days compared with 38.1% of non-functionalized mcl-PHA. In Fig. 5, a short lag period between incubation times of 0 to 12 h was observed, which could be attributed to the microbial adaptation in the culture medium and the substrate. After that time a progressive increase in degradation was observed up to day 3 indicating successful use of the polymer as a carbon and energy source by the yeast. A plateau was observed after day 3, thus, indicating a probable end of the degradation under the mentioned incubation conditions. In contrast to the control polymer this difference in percentage degradation could be due to the increase hydrophilicity and microbial growth in the sucrose-based polymer sample. These results are in agreement with the reported increase in biodegradation of sugar-based polymers. For instance, Barros et al. investigated the biodegradation of poly(6-O-methacryloyl sucrose-co-styrene) and poly(6-O-crotonyl sucrose-co-styrene) using Aspergillus niger, the authors reported a very good biodegradability after 90 days of incubation. Lu et al. reported a decrease in $M_n$ from 33 kDa to 1080 Da in their studies on the biodegradation of poly(1'-O-vinyladipoyl-sucrose) using Bacillus subtilis alkaline protease (EC 3.4.21.62) at 37 °C in a shake flask process.

The observations in this study were supported by the results of the solid-phase surface texture analysis. The functionalized mcl-PHA seems to be well degraded under the simulated composting conditions. In Fig. 6, relative to the initial PHA film in both samples (Fig. 6 ax, bx), the non-functionalized PHA (Fig. 6 ay) was observed to have less surface degradation after 3 weeks of composting compared with poly(1'-O-3-hydroxyacyl-sucrose), which showed more degraded surface (Fig. 6 by). In both samples maximum surface degradation was observed after 8 weeks of composting; as expected poly(1'-O-3-hydroxyacyl-sucrose) (Fig. 6 bz) degraded more than the non-functionalized PHA (Fig. 6 az). This could be due to the presence of sucrose moiety improving the hydrophilicity of the functionalized mcl-PHA. In general, the degradation in both samples was localized to specific areas. This could be the amorphous phase of the film, which is more readily degraded by the composting microorganisms in relation to the more organized crystalline phase.

**Effects of reaction time on reactant (sucrose) conversion in the lipase-catalyzed functionalization of mcl-PHA**

The effect of reaction time on the enzymatic reaction rate and acyl acceptor conversion was observed at w/w ratio of 0.5 PHA to sucrose (Fig. 7). Strong increase in both the apparent reaction rate $v_{app} = 0.073 \times 10^{-3} \text{ mol L}^{-1} \text{ s}^{-1}$ and corresponding sucrose conversion (23.4%) were observed during the early hours of the reaction (~10 h). Both the reaction rate and the conversion increased gradually up to 20 h; thereafter the reaction rate and
4.9 g L\(^{-1}\) of sugar esters in organic media. They reported a reaction rate of 0.075 x 10\(^{-5}\) mol L\(^{-1}\) s\(^{-1}\) and molecular weight (0.073 x 10\(^{-5}\) mol L\(^{-1}\) s\(^{-1}\)) and conversion (34.4%) and molecular weight in the Novozym 435 catalyzed synthesis of poly(1’-O-3-hydroxyacylsucrose) in chloroform-DMSO ratio 4:1 (v/v) was observed (Table 1). It has been previously stated that in micro-aqueous sugar ester synthesis, the solubility and rate of sugar dissolution play an important role in product yield.\(^{37,39}\) In fact, in such reaction, the solubility of sugar moiety has been reported to largely rely on the reaction temperature and solvent polarity.\(^{37,39,40}\)

Effects of reaction temperature on \(v^{app}\) and molecular weight

The effect of reaction temperature on the \(v^{app}\) and molecular weight in the Novozym 435 catalyzed synthesis of poly(1’-O-3-hydroxyacylsucrose) was studied using 8 g L\(^{-1}\) PHA and 15 g L\(^{-1}\) Novozym 435 incubated at 50°C, 200 rpm for 24 h. It is observed that changing the sugar concentration from 0 to 40°C; between 40 and 60°C the conversion remained constant (95.1–95.9%). These findings were in agreement with previously reported literature,\(^{39–41}\) which cited the optimal temperature for lipase reaction to be between 40 and 60°C.

Effects of reactant ratio on \(v^{app}\) and reactant conversion

The relative amount and proportion of reactants in a reaction system is said to influence the kinetic behavior of the process.\(^{6,37,40}\) This is because in this kind of heterogeneous catalysis, the solubility of the carboxylic group is affected by the concentration of dissolvable alcohol, which increases the polarity of the medium. The effect of reactant ratio (w/w sucrose to PHA) in the lipase catalyzed synthesis of poly(1’-O-3-hydroxyacylsucrose) was studied using 8 g L\(^{-1}\) PHA and 15 g L\(^{-1}\) Novozym 435 incubated at 50°C, 200 rpm for 24 h. It is observed that changing the sugar concentration from 1 to 2.0 (Fig. 8), progressively influenced both the \(v^{app}\) and the corresponding conversion. At a sucrose weight ratio of 2, highest \(v^{app}\) of 0.075 x 10\(^{-5}\) mol L\(^{-1}\) s\(^{-1}\) and conversion (34.1%) was achieved. In our experimental condition (10 mL reaction solvent), increasing the ratio beyond 2, resulted in no further improvement in the \(v^{app}\) (0.073 x 10\(^{-5}\) mol L\(^{-1}\) s\(^{-1}\)) and the conversion (33.4%). A probable explanation for high \(v^{app}\) at ratio of 2 could be increased availability of soluble sugar for the transesterification. Since the solubility of sucrose in micro-aqueous media is known to be poor, increasing the concentration will increase the availability of the soluble sugar. However, the
low solubility of the sugar moiety in this kind of micro-aqueous media restricts the possible mass increment to a certain limit per reaction volume. Therefore, continuous loading of the low solubility sugar moiety resulted in altered media viscosity, causing complication in mass transfer of the reactants to the active site of the enzyme resulting in the decrease in $v_{app}$. Moreover, the increased solvation of the sugar moiety within the constant media volume increases the polarity of the media, which in turn drives the reaction equilibrium towards ester-bond hydrolysis instead of synthesis culminating in decreased conversion. Mat Radzi et al. reported a similar observation in attempting to achieve highest conversion (97.1%) of oleyl alcohol to oleyl oleate ester at alcohol:carboxylic acid ratio of 2. Contrary to this observation, Ha et al. reported achieving highest conversion of glucose to glucosyl laurate in ionic liquid at molar ratio of 1. However, these authors reported low solubility of the lauric acid in their ionic liquids, which could probably explain the high conversion at equimolar ratio.

**Effects of enzyme loading on $v_{app}$**

The influence of enzyme concentration on the $v_{app}$ is shown in Fig. 9. Increase in enzyme loading from 1 to 15 g L$^{-1}$ resulted in increased $v_{app}$ ($0.014–0.069 \times 10^{-5}$ mol L$^{-1}$ s$^{-1}$). The increase in excess enzyme concentration beyond 15 g L$^{-1}$ was not positively contributing to further increase in the $v_{app}$. For example, increasing enzyme loading from 20–25 g L$^{-1}$ resulted in almost constant $v_{app}$ of $0.070–0.071 \times 10^{-4}$ mol L$^{-1}$ s$^{-1}$, most likely due to the altered and unconductive mixing dynamics in the batch reaction system used. This observation was found to agree well with earlier reports. Preceding, Kitagawa and Tokiwa reported an increase in reaction rate with increasing *Alcaligenes* sp. lipase loading achieving a maximum reaction rate of $3.05 \times 10^{-3}$ mol L$^{-1}$ s$^{-1}$ at enzyme loading of 10 g L$^{-1}$ during sugar based polyvinylsebacate ester synthesis. The researchers observed that increasing the concentration beyond this value resulted in an almost constant reaction rate.

**CONCLUSIONS**

The industrial applications of bacterial mcl-PHA could be further broadened by functionalizing the biopolymers for specific characteristics like increased hydrophilicity and degradability. While most of the previously reported functionalization processes relied on hybrid chemo-enzymatic syntheses, this study demonstrated that *C. antarctica* lipase B can be used to synthesize poly(1′-O-3-hydroxyacyl sucrose) in a single-step within a micro-aqueous medium. The biodegradability of the functionalized polymer was observed to increase 1.5-fold compared with the non-functionalized material. The effects of different variables such as reaction time, temperature, enzyme concentrations and reactant ratio were studied: time 20 h, temperature 50°C, enzyme loading 15 g L$^{-1}$ and reactant ratio 2 improved the reaction rate and yield. Indeed, these values could serve as initial points for further optimization.

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