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Synthesis of lactose monolaurate as influenced by various lipases and solvents

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ABSTRACT

Fatty acid sugar esters are non-ionic detergents with multiple uses in the cosmetic, food, and pharmaceutical industries. Of the many different sugar esters synthesized, lactose, a by-product of cheese manufacture, has not been investigated. The objective of this research was to investigate the synthesis of novel lactose monolaurate (LML) and sucrose monolaurate (as a comparison) (SML) using four different immobilized lipases in three different solvents at constant sugar, vinyl laurate, temperature, and enzyme concentrations. Overall, the solvent 2-methyl-2-butanol gave the highest yields and reactions rates for the synthesis of both LML and SML. Of the immobilized lipases, those from *Pseudomonas cepacia*, *Mucor miehei* and *Thermomyces lanuginosus* were effective depending on the sugar/solvent combination. Higher overall yields were obtained for the synthesis of LML with the differences in yields presumably due to the decreased solubility of sucrose as compared to lactose in 3 of the solvents used. Response surface methodology was used to determine the optimal temperature, enzyme concentration and ratio of reactants for LML synthesis using the immobilized lipase from *M. miehei* in 2-methyl-2-butanol. Based on the analysis of ridge max, the optimal synthesis conditions were predicted to occur at 61 °C, with an enzyme amount of 32 mg/mL, and a molar ratio of lactose to vinyl laurate of 1:3.8; and the optimal actual yield was 99.3%.

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1. Introduction

The enzymatic synthesis of sugar esters has been investigated for over 20 years and is typically preferred to the chemical synthesis since it is more specific and conducted under milder conditions. Uses of sugar esters, which are characterized as non-ionic biodegradable surfactants, vary and depend on the characteristics of the substrates (sugar and lipid). Typical applications are as emulsifiers for personal care products, medical supplies, and in foods and as antimicrobial agents [1–4].

The different conditions used for the synthesis of sugar esters are multitude and include the type of solvent, ratio of sugar to lipid, the specific sugar and lipid, temperature, and type of immobilized lipase. To optimize yield during synthesis, various solvents (2-methyl-2-butanol (2M2B), acetone, hexane, and methyl ethyl ketone (MEK)) have been investigated, typically with the addition of molecular sieves for water removal, which is generated during the esterification reaction of a sugar and fatty acid. Water plays an important role in the equilibrium of the reaction, with limited water favoring the esterification reaction, while resulting in limited solubility of the sugar and eventual inactivation of the enzyme. Solvents that can dissolve both sugars and lipids include dimethyl sulfoxide (DMSO), pyridine, and dimethylformamide, but these solvents often inactivate the enzyme and are incompatible with food applications [5]. To overcome this solubility issue, reaction conditions in supercritical acetone [6], supercritical carbon dioxide [1], DMSO in 2M2B [7], and ionic liquids [5,8] have been investigated.

The ratio of sugar to lipid used in past studies varies from equal [1,4] to ratios where the sugar is in excess [9,10] or the lipid is in excess [5,8,11]. The typical range of sugar to lipid ratio in the literature is from 3:1 to 1:3. The types of lipids that have been used include the fatty acids from four to sixteen carbons and virtually most known mono- and di-saccharides. The use of vinyl or methyl lipids as the substrate is also common [8,9] with the use of vinyl lipids resulting in greater yields [5,8,12]. The esterification of fatty acids to sugars results in the production of water while the transesterification with vinyl lipids results in acetaldehyde. Since water is non-toxic, the use of the fatty acids may be preferred [8] depending on the application.

Temperatures used for esterification reactions ranges from 50 to 80 °C with the immobilized form of the enzyme generally being more temperature stable than the free form. The types of immobilized lipases used include the lipase from *Thermomyces lanuginosus* (TL), *Pseudomonas cepacia* (PC), *Mucor miehei* (MM), and *Candida antarctica* (CA). CA and PC lipases are non-specific and TL and MM lipases are sn-1,3 specific with respect to triacylglycerol hydrolysis. The concentrations of immobilized enzymes for esterification

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in batch reactions generally ranges from 0.1% to 10% with some researchers using immobilized enzyme reactors [1,6,11,13] for continuous ester production. The concentration of enzyme influences the initial rate, but may not affect the equilibrium state of the reaction, which is generally measured in days.

This research investigated the yield and synthesis rates of novel lactose monolaurate (LML) and sucrose monolaurate (SML) (as a comparison) using four different immobilized lipases (TL, MM, PC, and CA) in three different organic solvents (MEK, 2M2B, and acetone) at constant temperature, substrate, and enzyme concentrations. One enzyme/solvent combination that showed high yields for LML, which is unique in the literature, was optimized using response surface methodology (RSM). RSM is a very useful statistical technique for complex processes and has been applied previously to optimize the synthesis of lipase-catalyzed reactions [10,14,15]. In this study, we conducted preliminary experiments to determine which enzyme/substrate combination to apply RSM. RSM was then conducted using three factors (temperature, enzyme concentration, and the ratio of lactose to vinyl laurate) to determine the optimal conditions.

2. Materials and methods

2.1. Materials

Vinyl laurate (226.4 g/mol), sucrose (324.3 g/mol), molecular sieves (3 Å), lipase acrylic resin from *C. antarctica* (Lot# 047K1672), Amano Lipase PS-C I (from *P. cepacia* (Lot# 07703EE)), Lipozyme, immobilized from *M. miehei* (Lot# 1285317), deuterated DMSO, and lauric acid were from Sigma–Aldrich (St. Louis, MO, USA). Novozyme lipase from *T. lanuginosus* (Lot# 35001701) was from Codexis (Redwood City, CA, USA), and lactose (324.3 g/mol) was from Proliant (Ames, IA, USA). Nylon syringe filters (0.2 μm) and solvents (acetonitrile, acetone, MEK, and 2M2B) were from Fisher Scientific (Pittsburgh, PA, USA).

2.2. Experimental design

Four different immobilized lipases (CA, PC, MM, and TL) were used to synthesize lactose and sucrose lauryl esters using the same temperature, enzyme, vinyl laurate, and sugar concentrations with three different solvents (2M2B, acetone, and MEK). The amount of the monoester synthesized over time (days) was determined via high-performance liquid chromatography (HPLC) with a standard curve. The rate and yield of the monoester produced was determined for each enzyme/solvent/sugar combination. One specific combination of enzyme/solvent was optimized for LML synthesis, since this ester is novel, using RSM. The solubility of lactose and sucrose in each solvent was also determined. All reactions were conducted in triplicate and data expressed as means with standard error values unless noted.

2.3. Determination of sugar solubilities

To determine the solubility of lactose and sucrose in various solvents, 0.05 g of each sugar was dissolved in 1.0 mL of water, MEK, acetone, acetonitrile, or 2M2B. These solutions were incubated at 55 °C for 3.5 h, and 900 μL of each were subsequently passed through a 0.2 μ filter. Aliquots, 600 μL, of each filtered sample were dried by a Savant SpeedVac system. The dry sugar in each tube was re-suspended in 600 μL de-ionized water. Aliquots, 20 μL, of each sample were analyzed by HPLC with water as the mobile phase and detected with an evaporative light scattering detector (ELSD) (Alltech ELSD 800) at 40 °C with a nitrogen gas pressure of 3.65 bar. The amount of sugar in each sample was determined by comparing peak areas to the lactose-in-water control.

2.4. Enzymatic reactions

Before assembling reactions, solvents (acetone, MEK, 2M2B) were dried overnight in a room temperature shaker with molecular sieves (0.1 g/mL). Reactions were assembled in 4 mL glass vials with Teflon caps. Solvent (3 mL) was added to sugar (44.16 mg or 42 mM), immobilized enzyme (0.068 g) and molecular sieves (10%). Vials were inverted several times, and vinyl laurate (0.128 mg or 0.13 M) was added which resulted in a 1:3 molar ratio of sugar:vinyl laurate. Vials were placed at 55 °C in an orbital shaker. Aliquots were removed from each vial daily for HPLC analysis.

2.5. Analytical methods

2.5.1. High-performance liquid chromatography

Analysis of the reactions was performed at room temperature by HPLC (Beckman System Gold 125 Solvent Module) equipped with a Luna 5u C18 (2) 100 Å column (250 mm × 4.6 mm, Phenomenex, Torrance, CA, USA). The mobile phase consisted of a gradient from 10% acetonitrile:water (40:60) to 100% acetonitrile:water (95:5), with a flow rate of 1.0 mL/min over 24 min. Products and standards were detected with an ELSD at 60 °C with a nitrogen gas pressure of 3.65 bar. Standards consisted of lactose, sucrose, lauric acid and vinyl laurate.

2.5.2. Nuclear magnetic resonance (NMR) and mass spectrometry analysis

LML for NMR and mass spectrometry analyses was synthesized as described above and purified using C18 solid phase extraction columns (Alltech, Englewood, CO, USA) for reactions catalyzed by TL in acetone and MM and PC in 2M2B. Columns were activated with 100% acetonitrile and rinsed with water. Reactions were added to the columns, the column washed with water and the esters were eluted with 32% acetonitrile in water. Samples were analyzed by HPLC to confirm purity. ^{1}H and ^{13}C NMR spectra of LML dissolved in d_6-DMSO were collected at 295 K on a Bruker ARX-400 at 400 and 100 MHz, respectively. For comparison, the ^{1}H and ^{13}C NMR spectra of α-lactose, vinyl laurate, and lauric acid were collected under identical conditions. Chemical shifts (δ) are referenced to the residual ^{1}H (2.50 ppm) and ^{13}C (39.50 ppm) resonances of d_6-DMSO (99.9%). Mass spectrometry data was obtained at the Mass Spectrometry Facilities in the Departments of Chemistry at the University of California, Riverside, and the University of Utah. Samples were analyzed using either APCI or ESI ionization.

2.6. Reaction rates and yields

Monoester fractions were collected from the HPLC runs using a fraction collector and were dried with a Speed-Vac and the mass measured. This dry mass was resuspended in 40:60 acetonitrile:water and serial dilutions were analyzed via HPLC to form a standard curve (mg/peak area). The standard curve was used to calculate the mg/mL of ester produced each day by each reaction and was plotted against days. A line of best fit was plotted until the maximum day of monoester production and the slope of this graph gave the rate of the reaction reported as mmol/h/g enzyme.

Each reaction vial contained 42 mM (or 0.13 mmol in 3 mL) of either lactose or sucrose which acted as the limiting substrate. The molecular weight of monoester product was determined to be 524 g/mol, which gives a maximum theoretical yield of 22 mg/mL. Measured monoester amounts were compared to this number to give actual yield.

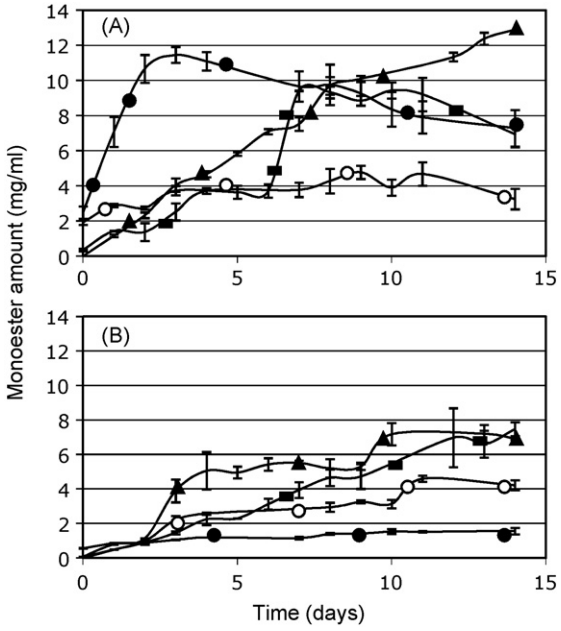

Fig. 1. Synthesis rates of lactose monolaurate (A) and sucrose monolaurate (B) with the immobilized lipase from *Thermomyces lanuginosus* in 2M2B (■); *Candida antarctica* in 2M2B (○); *Mucor miehei* in 2M2B (●); *Pseudomonas cepacia* in 2M2B (▲).

2.7. Response surface analysis

A response surface design (Roquemore R311A hybrid, Statistical Analysis System) with three factors (temperature, enzyme concentration, and lactose:vinyl laurate ratio) was conducted with MM in 2M2B to determine the optimal conditions for LML synthesis. The factor levels were 25–55 °C for temperature, 10–50 mg/mL enzyme, and 1–5 for the ratio of lactose to vinyl laurate. This resulted in 11 design points, including one center point. The average monoester yield for each design point in duplicate was analyzed by regression to fit a second-order polynomial equation as described by Chang et al. [14] and Jeong and Park [10]. The ridge max option was used to compute the estimated ridge of maximum response for increasing radii from the center of the original design. This resulted in the optimal synthesis conditions.

3. Results and discussion

3.1. Monoester yields and reaction rates

Fig. 1 shows the amount of LML (Fig. 1A) and SML (Fig. 1B) synthesized overtime (14 days) for 4 of the 12 enzyme/solvent combinations investigated at constant enzyme, temperature, and substrate concentrations. The temperature of 55 °C was chosen since previous researchers have shown that immobilized lipases are generally more active at temperatures of 50–70 °C [1]. We also wanted to stay below the evaporation temperatures of the solvents, the lowest of which was acetone with a boiling point of 56.5 °C at ambient pressure.

Several points can be made from the graphs in Fig. 1. The highest monoester yields were obtained with lactose and it was possible to determine the synthesis rate based on the time of maximum ester synthesis. An example is the synthesis of LML in Fig. 1A with MM in 2M2B, which shows a maximum at day 3, compared to the continued production of LML and SML by PC in 2M2B over the 14 day time period. In contrast to monoester yield with PC in 2M2B, most enzyme/solvent combinations reached a maximum amount of monoester in about 10–14 days with some showing a decrease in monoester content (e.g. Fig. 1A, MM and TL in 2M2B) over the time course that will be discussed below.

Table 1 shows the % monoester yields and rates for each of the enzyme/solvent combinations used. The maximum theoretical yield was 22 mg/mL based on the amount of the limiting reactant (sugar). Overall, the solvent 2M2B showed the highest yields and reaction rates for both LML and SML synthesis except for TL in acetone in which LML synthesis was slightly higher. The solvent MEK was the least effective for each of the enzyme/solvent combinations. Cauglia and Canepa [16] showed that synthesis of glucosylmyristate with CA was dependent on the solvent with the highest yields in 2M2B, followed by acetone, hexane and finally diethylether. Other studies have also shown that high ester yields are obtained in 2M2B [1,4,9].

With respect to the enzymes, PC and TL showed the highest yields with sucrose and PC followed by MM and TL showed the highest yields with lactose. CA showed similar yields and rates with sucrose and lactose. PC was also similar with both sugars in MEK and acetone, as was TL in 2M2B. The lowest yields were obtained with MM with sucrose, and CA with lactose, depending on the solvent used. Lipase from CA is very popular in literature for the synthesis of sugar esters ([1,6,8,9,11,17] are some of the most recent), yet we found this enzyme to be the least effective for LML synthesis depending on the solvent. SML yields for this enzyme were not as low as from MM. The source of our enzyme was different than the studies cited, which may account for the differences we observed.

Specifically for LML synthesis, MM in 2M2B had the highest reaction rate due to the shortest reaction time of 3 days. PC in 2M2B actually showed a slightly higher yield (56.6%) than MM in 2M2B (52.4%), but the rate is much slower due to the length of time (14 days) to reach maximum yield. Specifically for SML synthesis, TL and PC in 2M2B showed the highest synthesis rates and yields, but the yields were lower than those obtained with lactose. This dif-

Table 1
Reaction rates and yields of sucrose and lactose ester synthesis.

Enzyme	Solvent	% Lactose ester yield	% Sucrose ester yield	Lactose ester rate (mmol/h/g enz)	Sucrose ester rate (mmol/h/g enz)
Thermomyces lanuginosus	2M2B	35.5 ± 3.10	32.9 ± 2.71	4.4	4.9
	Acetone	43.1 ± 0.08	13.2 ± 0.66	5.9	2.4
	MEK	12.0 ± 0.01	1.6 ± 0.17	1.8	0.29
Mucor miehei	2M2B	52.4 ± 1.88	7.2 ± 0.78	26.7	0.81
	Acetone	32.5 ± 2.06	0.8 ± 0.35	13.8	0.31
	MEK	12.2 ± 1.72	0	1.3	0
Pseudomonas cepacia	2M2B	56.6 ± 1.45	34.2 ± 1.66	10.4	4.8
	Acetone	16.8 ± 0.21	10.0 ± 3.45	3.6	1.9
	MEK	1.3 ± 0.31	1.3 ± 0.31	0.89	0.13
Candida antarctica	2M2B	21.8 ± 1.57	20.9 ± 0.72	3.1	2.9
	Acetone	1.3 ± 0.14	3.6 ± 0.51	0.17	1.3
	MEK	0.6 ± 0.15	0.5 ± 0.12	0.10	0.10

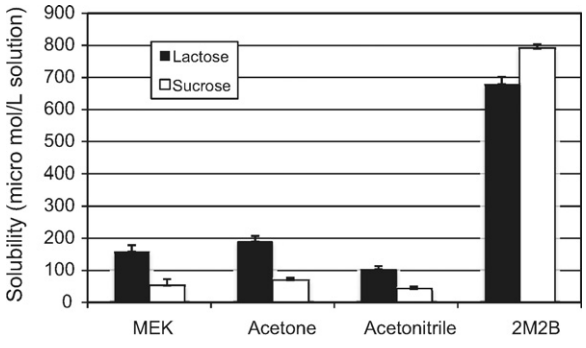

Fig. 2. Lactose and sucrose solubilities in various solvents.

ference may be due to the differing solubility of each sugar in the specific solvents, which is discussed below.

Monoester yields that are reported in the literature vary and depend on the conditions that were investigated in this study. Examples yields include 43% glucosylmyristate with CA in 2M2B [16], 13% sucrose laurate with CA in 2M2B [1], and 30% sucrose laurate with TL in 2M2B [4].

The rates obtained for all enzyme/solvent combinations presented here are in the low mmol/h/g enzyme range. Other researchers synthesizing xylitol myristate ([6], in acetone), fructose monopalmitate ([11], in 2M2B), glucosylmyristate ([16], in 2M2B), and glucose laurate ([18], in 2M2B) obtained similar rates.

3.2. Solubilities of sugars in solvents

Fig. 2 shows the solubility of lactose and sucrose in MEK, acetone, acetonitrile and 2M2B. The solubility test was done with 5% sugar solutions to ensure complete solubility in water. Each sugar showed limited solubility in each solvent with the solubility in 2M2B being the highest at approximately 700–800 μmol/L solvent. The solubilities in the other solvents were much lower at 50–200 μmol/L solvent with sucrose about half as soluble as lactose. This difference in solubility may result in a generally higher LML synthesis than SML synthesis. The yield obtained for LML synthesis with PC in 2M2B is 56.6%; the amount of lactose solubilized over the synthesis time was greater than 50%, with is 100 times higher than the yield predicted based on the data in Fig. 2. Therefore, as the esters are synthesized, the insoluble sugars solubilize to maintain equilibrium.

The limiting factors in the synthesis and yield may be a combination of the sugar solubility and inactivation of the enzyme. Flores et al. [18] showed that the initial synthesis rate of glucose laurate in 2M2B was dependent of the dissolved sugar concentration with a 70% conversion in the presence of molecular sieves. Reactions with the highest rates of conversion allow more of the sugar to be solubilized hence, the reactions have a higher yield. The higher solubility of lactose and sucrose in 2M2B resulted in the highest yields and synthesis rates, excluding LML synthesis by TL in acetone.

If solubility were the only limiting factor, we would assume a higher, or at least equal yield for SML in 2M2B with each enzyme. But this was observed for only two of the four enzymes (TL and CA). The sugar type and enzyme specificity may also influence the rate of esters synthesized.

3.3. NMR and mass spectrometry analysis

Analysis of the ^{13}C NMR features of the purified LML esters synthesized by TL, MM and PC revealed that the LML products were all esterified at the C6′ carbon with lactose primarily in the alpha configuration. The key indications of esterification at the C6′ position are: (1) the downfield shift of the C6′ ^{13}C NMR resonance from 60.57 ppm in α-lactose to 63.30 ppm (Table 2) in LML, and (2) an upfield shift for the resonance of the adjacent C5′ carbon resonance in LML [19]. The atom numbering scheme for LML is given in Fig. 3. Other reducing sugars, maltose and leucrose, were enzymatically esterified at the C6′ and the C1 and C6′ locations respectively [4,12]. The esterification of sucrose, a non reducing sugar, with lipids has been shown to occur most frequently in the C6 position but is dependent on the enzyme type. Ferrer et al. [4] showed that C6 and C6′ SML were synthesized with CA but as minor products compared to the synthesis of C6, 6′di and only C6 SML was produced with TL with minor amounts of diesters. The difference may be due to the presence of DMSO in their reactions, which they have already shown changes the final degree of esterification and the site of esterification [4].

Table 2
$^{13}C\{^1H\}$ NMR resonances for α-lactose and α-C6′ lactose monolaurate ester in d_6-DMSO at 295 K.

Assignment[a,b]	α-Lactose (ppm)	LML[c] (ppm)
C-1′	103.86	103.58
C-1	92.85	92.02
C-4	81.34	81.21
C-5′	75.48	72.77
C-3′	73.22	72.38
C-5	72.13	72.18
C-3	71.36	71.24
C-2′	70.60	70.27
C-2	69.80	69.70
C-4′	68.14	68.25
C-6′	60.57	63.30
C-6	60.38	60.43
C-1L		172.91
C-2L		33.28
C-3L		24.32
C-4L to C-9L		29.02[d], 28.91, 28.72[d], 28.49
C-10L		31.30
C-11L		22.11
C-12L		13.98

[a] For atom numbering scheme see Fig. 3.
[b] Assignment of sugar ring carbon resonances based on comparison to literature spectra [19].
[c] LML sample produced using the immobilized lipase from *Thermomyces lanuginosus*. LML produced using the lipases from *Psuedomonas cepacia* and *Mucor miehei* produced similar ^{13}C NMR features. In selected samples an α/β mix of sugars was present.
[d] Signal intensity indicates the overlap of two resonances.

Mass spectrometry analysis of the LML produced using lipases from TL, PC, and MM gave a molecular ion peak at m/z 547, which is consistent with the formulation $[NaLML]^+$ and the monoesterification of lactose.

3.4. HPLC chromatograms of reactions

Figs. 4 (lactose reactions) and 5 (sucrose reactions) show HPLC chromatograms of the products synthesized for representative reactions. In both figures, peaks that have been identified include lactose, sucrose, SML/LML and lauric acid. With free fatty acids as

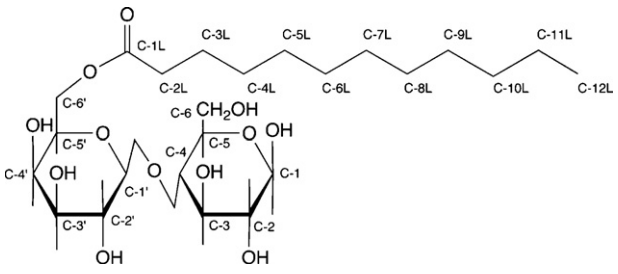

Fig. 3. Atom numbering scheme for lactose monolaurate.

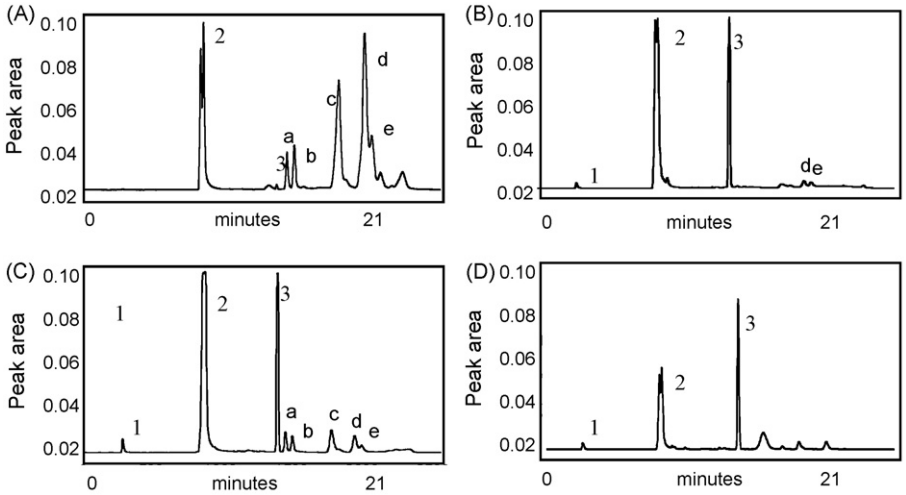

Fig. 4. HPLC chromatograms of lactose ester reactions with various enzymes and solvents. (A) Reaction in acetone with lipase from *Thermomyces lanuginosus* after 7 days. (B) Reaction in 2M2B with lipase from *Mucor miehei* after 3 days. (C) Reaction in 2M2B with lipase from *Pseudomonas cepacia* after 14 days. (D) Reaction in 2M2B with lipase from *Candida antarctica* after 9 days. Identified peaks; 1, lactose (2.2 min), 2, lactose monoester (6.8–7.9 min), 3, lauric acid (11.4 min). Peaks sharing the same letter have the same retention times.

substrates, product yields can be determined by the decrease in free fatty acid amount, while we found this not to be possible with the vinyl lipid with our HPLC conditions. The vinyl laurate did not elute from the column within the 30 min run time, presumable due to its hydrophobicity, which increased the retention time (Fig. 5).

In each chromatogram in which the solvent was 2M2B (all except Fig. 4A), there is a sugar peak present, which supports the lactose solubility data in Fig. 2. Depending on the enzyme used, there are multiple products present that have greater hydrophobicity (e.g. retention times) than lauric acid. We assume these are sugar esters with multiple lauric acids esterified. The greatest number of these products is present in reactions with lactose as the substrate with TL, followed by reactions with either lactose or sucrose with PC, MM and CA. Peaks with the same letter among the chromatograms have the same retention times and may be similar esters with multiple lauric acids esterified.

Doublet LML peaks were observed for most enzyme/solvent reactions except with the lipase from PC. The doublet peaks are presumably from the lactose in the alpha and beta configurations while the doublet peaks for the SML are presumably from the presence of both the C6 and C6′ products as previously determined [4,12].

The data in Fig. 1A shows that some of the enzyme/substrate combinations exhibit a decrease in yield over time. Specifically, reactions involving MM in 2M2B and acetone, TL in acetone, and PS in acetone showed a decrease in yield. It is possible that the monoester is being converted to di- or multi-ester sugar products for reactions that are synthesized by TL and PC since the chromatographs for these enzymes show multiple hydrophobic products. This is probably not the case for reactions with MM since the chromatograms show limited multi-ester peaks. We are not sure why the yield decreases over time with this enzyme. There was no obvious decrease in any of the yields in the sucrose reactions in Fig. 1B.

3.5. RSM analysis

The RSM analysis was conducted for the synthesis of LML using MM in 2M2B because this combination resulted in a high yield,

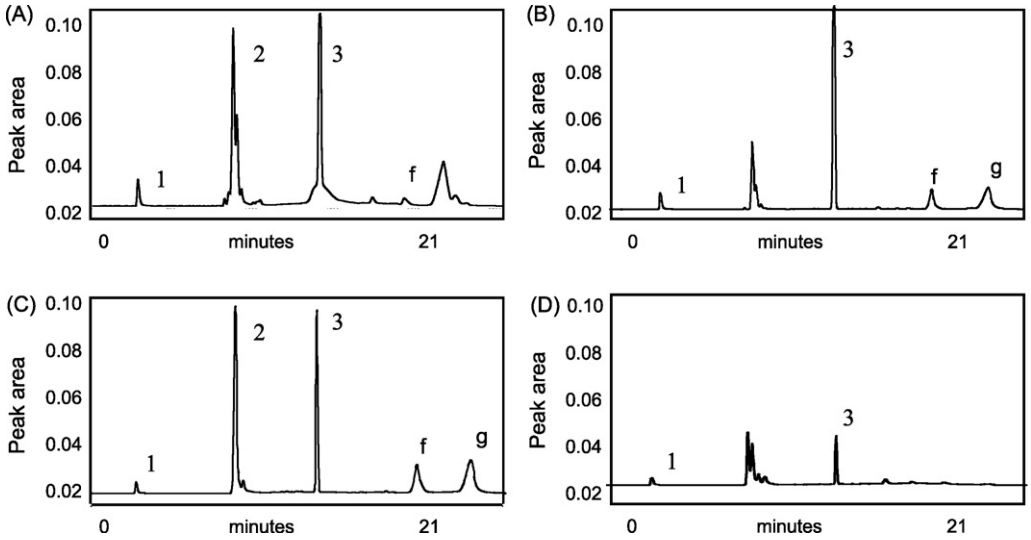

Fig. 5. HPLC chromatograms of sucrose ester synthesis with various enzymes in 2M2B. (A) Reaction with lipase from *Thermomyces lanuginosus* after 10 days. (B) Reaction with lipase from *Mucor miehei* after 14 days. (C) Reaction with lipase from *Pseudomonas cepacia* after 14 days. (D) Reaction with lipase from *Candida antarctica* after 8 days. Identified peaks; 1, sucrose (2.2 min), sucrose monoester (6.8–7.9 min), 3 lauric acid (11.4 min). Peaks sharing the same letter have the same retention times.

Table 3
Response surface design and experimental results.

Run	Temperature (°C)	Enzyme amount (mg/mL)	Substrate molar ratios (lactose:vinyl laurate)	Lactose ester yield (mg/mL)
1	40	30	1:5.83	11.00
2	40	30	1:0.17	0.43
3	25	10	1:0.17	2.13
4	55	10	1:4.41	21.43
5	25	50	1:4.41	4.22
6	55	50	1:4.41	21.33
7	61	30	1:1.59	22.00
8	18	30	1:1.59	1.70
9	40	58.28	1:1.59	4.02
10	40	1.72	1:1.59	0.81
11	40	30	1:3	13.95

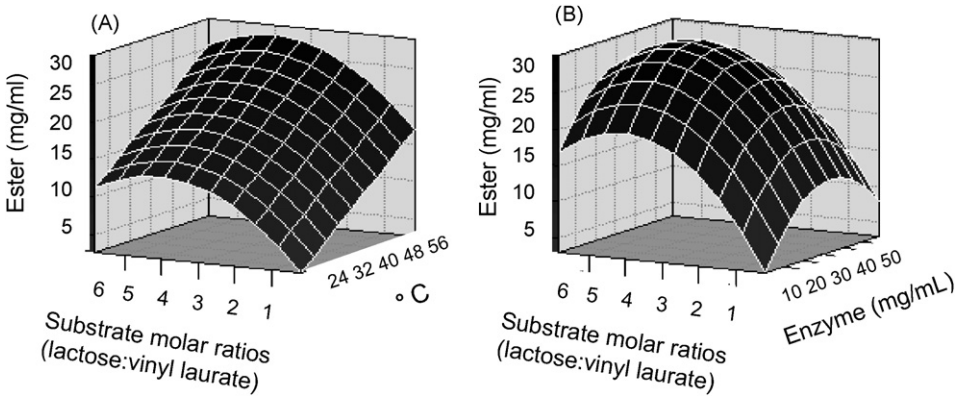

Fig. 6. Response surface plots showing the mutual effects of substrate ratios with temperature ((A) at a constant enzyme concentration of 32 mg/mL) and with enzyme concentration ((B) at a constant temperature of 61 °C) on the synthesis of lactose monolaurate in 2M2 M with *Mucor miehei* lipase.

fast rate, and the enzyme is more economical than the others. The experimental design and concentration of LML synthesized at each design point are given in Table 3. Among the various treatments, the highest yields were obtained with runs 4, 6 and 7, while runs 2 and 10 showed the lowest yields.

ANOVA results revealed that all three variables and the interactions of temperature × temperature and ratio × ratio exhibited statistically significant effects ($p < 0.05$) on the yield of LML. The estimate response model equation, without the insignificant variables, was used to estimate the enzymatic synthesis of LML with MM and is as follows:

$$Y = -353.78 + 5.81\,X_1 + 6.9\,X_2 + 101.13\,X_3 - 0.11\,X_2X_2 - 13.50\,X_3X_3 \quad (1)$$

where Y is the response factor in peak area and X_1, X_2, and X_3 are the independent factors of temperature, enzyme concentration (mg/mL) and ratio of lactose to vinyl laurate. The coefficient of determination (R^2) was 0.95 indicating that the model was suitable to represent the factors.

Canonical analysis of the three variables determined that the most critical factor was temperature, with the concentration of enzyme being the second most influential factor on the yield. Fig. 6 shows the effect of ratio, temperature and enzyme concentration on the amount of LML synthesized. The stationary point for maximum yield was determined to be a saddle point, therefore there was no unique optimum. This can be seen in Fig. 6 where there is a narrow range of ratios (3.7–3.8) at 61 °C that gives maximum LML yield. Fig. 6 also shows the influence of temperature on yield is linear, with increasing yields with an increase in temperature while the influence of substrate ratio and enzyme concentration have narrow optimum values.

The ridge maximum analysis was conducted as described by Chang et al. [14], which determines the optimal reaction conditions with the maximum, predicted yield. The conditions of 61 °C, 32 mg/mL of enzyme and a lactose:vinyl laurate ratio of 1:3.8 was predicted to yield 28 mg/mL LML. Our experimental results were in agreement with a concentration of 27.8 mg/mL obtained with conditions listed above. Therefore RSM was successful in determining the optimal conditions for LML synthesis in 2M2B with MM.

4. Conclusions

The enzymatic synthesis of LML was conducted with 4 different immobilized lipases in three different solvents and compared to the enzymatic synthesis of SML under the same conditions. The yields of the monoesters was dependent on the type of solvent and enzyme, with the solvent 2M2B generally showing the highest yields and MEK showing the lowest yields for both sugars. Of the enzymes used, CA showed the lowest yields for both sugars. RSM was successfully used to optimize the synthesis of LML using MM lipase in 2M2B. The activity of LML can now be investigated and compared to the cited activities of other sugar esters.

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