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Sucrose Derivatives Preparation using *Thermomyces lanuginosus* Lipase and Their Application

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We immobilized *Thermomyces lanuginosus* lipase to catalyze transesterification reaction in DMF. This lipase was selected after screening among other commercial lipases. We found that prepared immobilized lipase is particularly useful for preparation of 6-*O*-acylsucrose with higher conversion rate even in 10 g scale. Several solvents were evaluated for selective transesterification reaction. We noticed that the immobilized lipase retained more than 80 % activity after 5 cycles of 96 h reaction. A general method was also developed to purify the products using simple crystallization and precipitation process. Furthermore, 6-*O*-vinyladipoylsucrose was subjected to synthesis of the corresponding polymer by radical initiator. The sucrose branched polymer can be used further for evaluation of its biodegradability and other biological applications.

Key Words : Sucrose, *Thermomyces lanuginosus* lipase, Immobilization, Esterification, Polymer

Introduction

Diverse applications of sucrose fatty acid esters in the field of pharmaceuticals, foods, cosmetics, oral-care, detergent and biomedical are remarkable.[1-3] The number of hydroxyl groups in sucrose should be taken into consideration prior to synthesis of the related esters for its consistency in nomenclature. Sucrose esters can be synthesized using either chemical or biological catalysts. Nowadays, the use of chemical catalyst reduced due to the formation of colored side-products and tedious purification processes. On the other hand, selective enzymatic process using appropriate enzymes for synthesis of sucrose esters gained much attention.[4] The chemical reactivity of the hydroxyl groups in sucrose follows the order as 6-OH ≥ 6'-OH > 1'-OH > secondary-OHs[5] under most experimental conditions. The regioselective esterification of sucrose using chemical catalyst is quite difficult but the enzymes are incredibly regioselective during the enzymatic synthesis of sugar esters and their derivatives.[6,7] Various studies concerning enzymatic synthesis of sugar esters catalyzed by lipases[8-12] have been reported. Recently many researchers successfully used immobilized *Thermomyces lanuginosus* lipase (TLL) for selective acylation of sucrose by vinyl esters[13-16] but there are few reports which stated the polymerizable sugar esters using divinyl esters.[17,18] In every case immobilized TLL enzyme was used to perform esterification reaction in presence of polar solvents but it has also been reported that several lipases (*e.g.*, from *Candida rugosa* and *Geotrichum candidum*) lost most of their activity when exposed to acetaldehyde[19] which is a by-product of esterification reaction using vinyl esters. As we studied, mono vinyl ester was used to obtain sucrose esters using commercial TLL enzyme only in few cases but use of divinyl ester was not reported yet. Thus, in our current research we have designed enzymatic synthesis strategy to prepare sucrose-based vinyl esters using divinyl esters by immobilized TLL enzyme to facilitate the regioselctive esterification. To overcome the solubility problem of the sucrose and to select appropriate reaction solvent, various solvents were screened. The reaction exhibited an overwhelming preference toward acylation of the primary hydroxyl group in the sucrose. We focus our interest on the substrate recognition of TLL in the acylation of sucrose by means of coherent substrate engineering to obtain regioselective sucrose esters. In addition, the prepared sucrose monoesters will be subjected to further reaction for the intramolecular transesterification to obtain sucrose diesters that could be versatile for many biological applications. This result will show us regioselective double protection of multiple hydroxyl groups containing compounds. Also, previous reports on enzymatic acylation of sucrose esters paid little attention to practical difficulties associated with the esterification process on a large scale which can be used in industrial scale. The reaction phenomena, conversion rate, regioselectivity, productivity, and catalyst stability in gram scale reactions were profoundly examined. Then we have polymerized the vinyl group that presents in the sucrose ester by radical initiator. All these outcomes and findings can be of enormous consequences in the field of sucrose esters to synthesize sucrose derivatives which will exhibit precious biological properties.

Experimental

All the reagents, solvents and enzymes were purchased from Sigma-Aldrich, TCI, Fluka and Amano Inc., *etc.* The organic solvents used in enzymatic reaction were dried over activated molecular sieves 4 Å. Screening of lipases was

performed using the following eight types of lipases (Table S1) from different origin.

Bradford Assay to Determine Protein Content. Protein content of the commercial aqueous lipase was measured by Bradford assay using the standard curve as Figure 1. Absorbance values were checked using different concentration of BSA at OD_{595} and protein contents of commercial aqueous TLL was determined as 9.35 mg/mL by standard equation.

Sol-Gel Method for Immobilization of Thermomyces Lanuginosus Lipase. Granulated lipases were prepared according to a method previously described[20] with modification. In the immobilization process, tetramethylorthosilicate (3 g) and methyltrimethoxysilane (1 g) were charged at 3:1 ratio in a jacket reactor which was connected previously with circulator to maintain constant reaction temperature at 10 °C for 24 h. Then 50 mL of HCl solution (0.06 m mol/L), 1 mL of aqueous TLL and 0.5 mL of 1 M phosphate buffer were added consecutively. Buffer was used to adjust pH 6.5. After 24 h reaction gel was formed and then isolated by filtration and washed several times with distilled water to remove excess water and phosphate buffer and checked to determine the absorption of enzymes on the silane supports. The gel was then dried under laboratory atmosphere for 24 h and freeze dried for 72-96 h under high vacuum. Fine granules of immobilized lipase were obtained. Several sets of same reaction with different quantity of lipase were performed to check lipase activity in different concentration after immobilization. Prepared immobilized lipase was stored in refrigerator for future use.

Enzyme Activity Test by Potentiometric Titration using *p*-NPA (*p*-Nitrophenyl Acetate) Assay. Lipase enzymes activity was determined by the protocol of Kawauchi *et al.*[21] with significant modification. 25 mg of *p*-NPA was dissolved in 1 mL of acetonitrile containing 25 % v/v H_2O, which was used as a substrate. 0.01 N NaOH solution was used to neutralize the released acetic acid during titration at pH 7.0 at room temperature with a 718 STAT Titrino pH-stat (Metrohm, Switzerland). In the titration method the enzyme hydrolyzes the acetate ester with the help of water to produce acetic acid and *p*-nitrophenol (*p*NP), an absorption of *p*-nitrophenol was detected maximum at about 405 nm. Enzymes activities were expressed on a graph (Figure 2) by

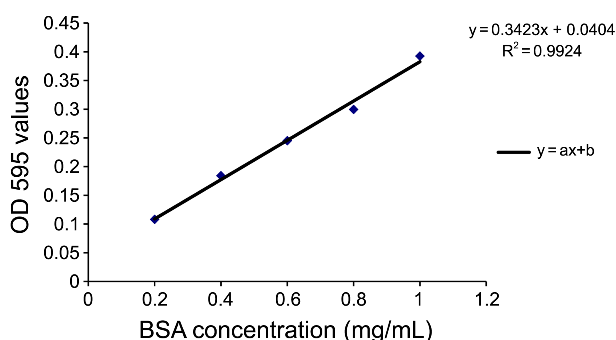
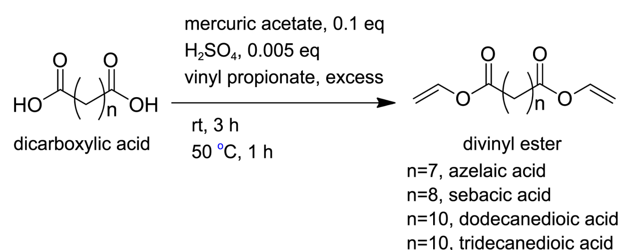


Figure 1. Determination of protein content of lipase enzyme from *Thermomyces lanuginosus* by Bradford protein assay. Protein content was calculated from the equation y = ax + b.



Scheme 1. Synthesis of divinyl esters.

plotting amounts of used NaOH solution against time values. Thus, we demonstrated that the Sol-Gel method using silanes to immobilize the TLL is an effective process where silanes absorbed the aqueous lipase efficiently during immobilization.

Synthesis of Divinyl Esters. Divinyl esters of adipic, sebacic, dodecanedioic, tridecanedioic acid were synthesized by the transvinylation reaction of acid with an excess of vinyl propionate by using mercuric acetate and sulfuric acid as catalysts.[22] In all cases product was purified by column chromatography with hexane containing 2 % ethyl acetate. Product yield was more than 90 % in all cases.

Synthesis of Sucrose Esters. In all cases, 1 g of sucrose was added to 20 mL of anhydrous DMF in 250 mL flask and heated using oil bath at 90 °C for 20 min. When substrate was completely dissolved, oil bath was removed to maintain the reaction temperature at 30 °C. Then 0.1 mass equivalent of immobilized TLL was added and stirred for 30 min to mix properly. Following, 1.1 equivalents of vinyl ester was added and stirred at 30 °C for 30 min. Then 1.0 mass equivalent of molecular sieves 4 Å was added and reaction allowed to stirrer at 45-50 °C for 72 to 96 h. The progress of the reaction was monitored by TLC. After completion, reaction mixtures were filtered to remove molecular sieves and immobilized lipase and the solvent was evaporated. The sucrose esters were then isolated by simple crystallization and precipitation process using methanol and ethyl acetate. Similar reactions were also applied for the synthesis of sucrose esters in 10 g scale.

Synthesis of Polymer. The polymer of sucrose ester was synthesized according to Mauricio *et al*. with adaptation. 0.5 g of **2a** was dissolved in 2 mL of water containing 0.02 wt %

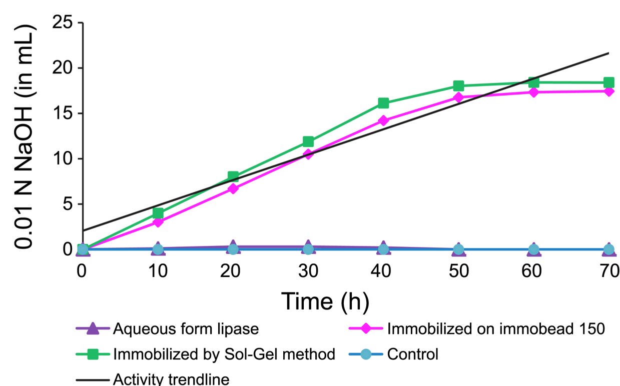
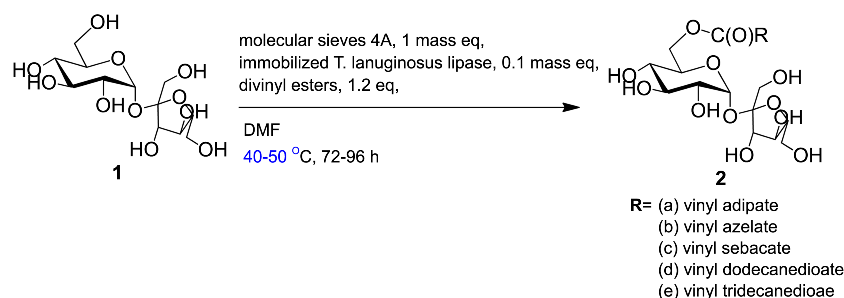
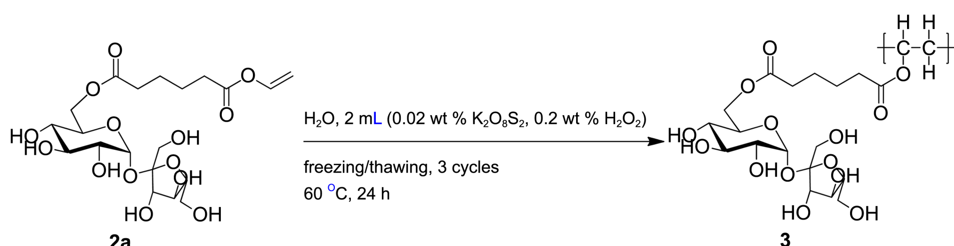


Figure 2. Enzyme activity test using 718 STAT Titrino pH-stat (Metrohm, Switzerland) at pH 7.0.



Scheme 2. Synthesis of sucrose esters.



Scheme 3. Synthesis of sucrose based polymer (**3**).

$K_2O_8S_2$ and 0.2 wt % H_2O_2 in 8 mL glass vial and sealed. Inert atmosphere was made by freezing and thawing the samples up to 3 cycles. Then free radical polymerization was carried out by maintaining constant reaction temperature at 60 °C for 24 h. The resulting polymer was precipitated in acetone and dried under high vacuum. Then the synthesized polymer was characterized.

Results and Discussion

We have synthesized several sucrose vinyl esters using short to long carbon chain (4-13) divinyl esters by immobilized TLL in DMF. We found higher conversion rate when short and long carbon chain vinyl esters were used but conversion rate was reduced in the case of medium carbon chain vinyl esters. We optimized overall reaction conditions to obtain excellent yield. Removal of unreacted sucrose was made very simple by crystallization and precipitation procedure, one notable modification of the synthetic procedures for such enzymatic reaction. Recovery and recycle of the immobilized lipase up to 3 cycles with high yield are the most important advantages in our current research work.

Selection of Suitable Organic Solvent. Sucrose is scarcely soluble in ordinary organic solvents. Containing multiple hydroxyl groups, sucrose is only soluble in polar solvents like pyridine, DMSO and DMF, sometimes *tert*-amyl alcohol together with water can be used. Selection of the appropriate solvent for a reaction, with enzyme being active and stable, is always a critical point. Although the use of DMSO and *tert*-amyl alcohol has been reported by research group of Masaru *et al*. and Ferrer *et al*., but removal of these solvents is troublesome due to high boiling point. Furthermore, pyridine[23] is not suitable one as non volatile, expensive and toxic solvent although some researchers used in their works. We designed simple process for the regioselective acylation of sucrose using only DMF[24] after screening various solvent systems as described in Figure 3.

In the presence of DMF, monoester formation was very significant with yield above 90 % using sucrose as substrate. In contrast, formation of diester was low as less than 5 %. It

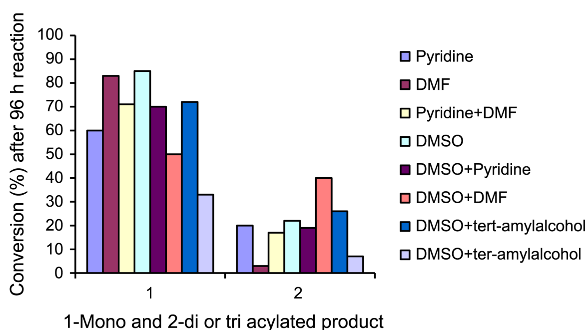


Figure 3. Screening of solvent for enzymatic reaction by *Thermomyces lanuginosus.*

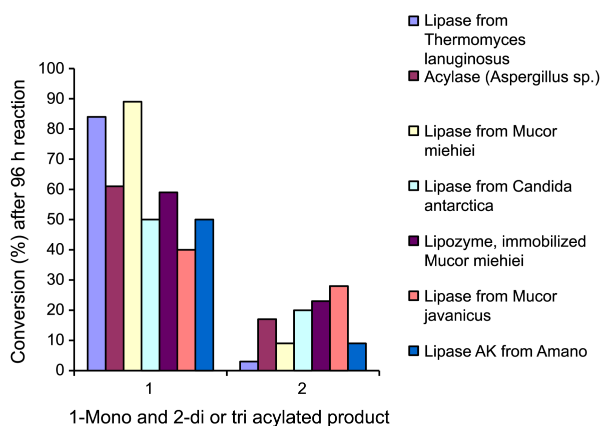


Figure 4. Screening of the enzymes for transesterification reaction.

is worth mentioning, DMF was removed using evaporator under high vacuum at 45-50 °C. Our immobilized lipase was remained active in the DMF during reaction time up to 96 h.

Enzyme Immobilization & Regioselectivity of the TLL. Ferrer and his colleagues immobilized TLL by Sipernat 22, sipernat D17, Accurel EP100, Celite and Eupergit C. for their acylation of sucrose using vinyl laurate. Effects of TLL immobilized on silica (TMOS : MTMS) for synthesis of sucrose esters using divinyl esters yet not reported as per our knowledge. In our current research, total eight enzymes were screened and their activities on acylation of sucrose were observed with great interest. As described by the Figure 4, we found that the immobilized TLL was the most efficient enzyme for the sucrose acylation and obtained products are regiospecifically the hydroxyl 6-OH on sucrose molecule in all cases.

Substrate Ratio and Influence of Acyl Donor. Usually in the lipase-catalyzed reactions acylation proceed through the formation of acyl-enzyme intermediate and the nature of the acyl donor (both fatty acid and leaving group) has a distinguished effect on reactivity.[25,26] We observed that, except medium chain fatty acids (divinyl azelate and divinyl sebacate), the longer carbon chain also gives the higher conversion rate. In case of divinyl laurate and divinyl tridecanedioate, monoesters conversion were observed around 75 % while overall yield of monoester increased at almost 90 % for short chain divinyl adipate when used without changing the other parameter of transesterification reactions. In the presence of excess of acyl donor, 80 % conversion of sugar ester was achieved within 36 h and later diester formation was gradually increased in course of time. With respect to the product purification, reaction at equimolar concentration of acyl donor and sucrose found to be the best choice.

Influence of Enzyme Load: Due to the nature of highly polar solvent and immobilized enzyme mixtures, it is obvious that effective mixing of reactants and enzyme is important to provide good transport and contact of the reaction partners. The optimum enzyme concentration depends on the stirring status as the reaction medium contains molecular sieves. As expected, overall yields increased with the immobilized lipase used quantity ranging from 0.1 to 0.5 mass equivalents. It was found that the yield was increased with increasing enzyme concentration until 0.5 mass equivalents within 24 h but diester formation drastically increased. We studied, less amount of immobilized enzyme (0.1 mass equivalents) provide the maximum monoester in prolonged reaction time 96 h with less diester as below 5 %.

Use of Molecular Sieves: The initial water activities of the substrates were adjusted according to Goderis *et al.*[27] by adding the molecular sieves in the reaction mixture. It was found that the esterification reaction was stable when mass equivalent of molecular sieves was used. Acetaldehyde formation reached high in case of reaction more than 96 h which initiate the degradation of monoester in the reaction medium. Molecular sieves were removed immediately by filtration just after completion of the reaction.

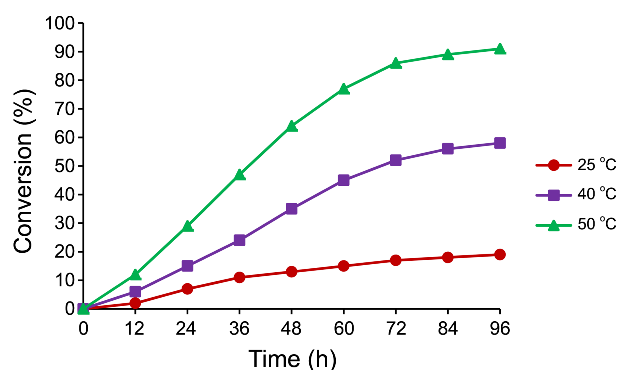


Figure 5. Effect of temperature at 25 °C, 40 °C and 50 °C on conversion rate.

Influence of Reaction Temperature. Figure 5 shows a plot of conversion rate *vs* time for three different set of temperatures. As expected, conversion was found to increase with increasing temperatures. After 96 h, an increase in product yield of 40 % was observed when the temperature was raised from 25 to 40 °C, whereas an increase from 40 to 50 °C led to further 30 % higher yields.

Recycle of Immobilized TLL and Enzyme Stability. One of the most vital factors regarding immobilized enzymes is the maintenance of enzyme activity under operational conditions. Significant improvement of the reaction rate by immobilization of the TLL has been discussed earlier. The apparent stability of immobilized lipase was estimated by filtering, washing and drying the immobilized lipase after every reaction cycle. Although recycling of immobilized TLL lipase was reported by Ferrer *et al.* but only mono vinyl ester was used and applied for very small scale reaction. On the other hand, our recycle enzyme was applied on large scale reaction where activated divinyl esters were used. Such result has not been reported yet specially using immobilized TLL. Our investigated results (Figure 6) showed that the stability of silica granulated lipase in DMF is quite satisfactory.

Elimination of Unreacted Sugar and Product Purification. Products were concentrated using evaporation at 45 °C under high vacuum by removing DMF. Primary isolation was carried out by crystallization, dissolving crude product

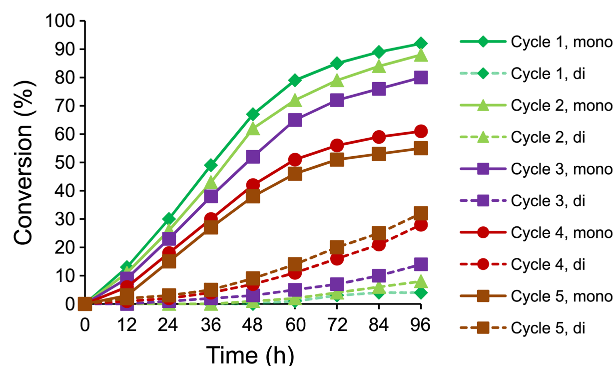


Figure 6. Retention of the enzyme activity during 5 cycles of reaction.

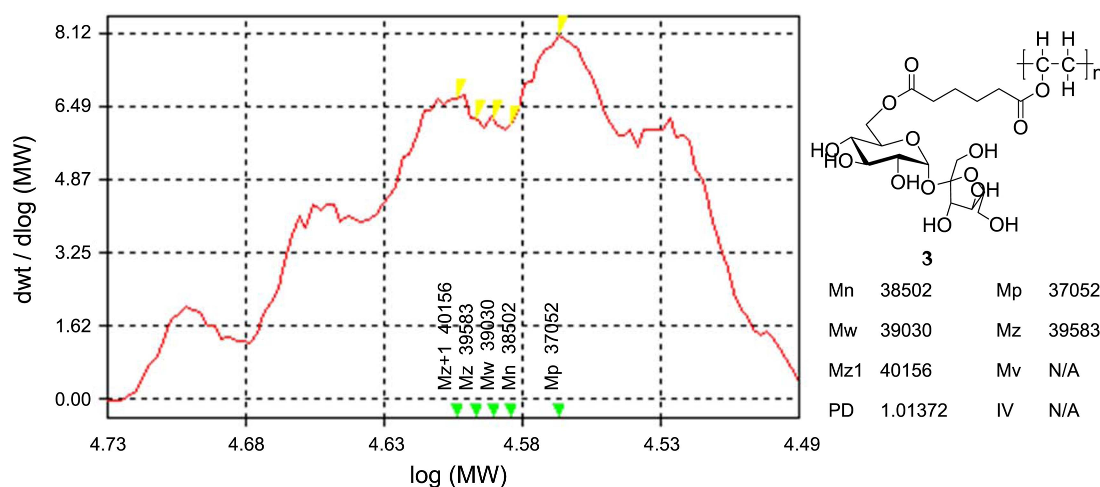


Figure 7. GPC analysis of the sucrose branched polymer (**3**).

in less amount of methanol and applying excess ethyl acetate with shaking. Crystals of sucrose with small portion of monoesters, which was removed again following the same crystallization procedure. After separation, the monoester was again dissolved in less methanol and excess ethyl acetate, was kept in the refrigerator for 24 h to get very pure crystals of monoester with high yield. We found, this simple and inexpensive process of product purification is very effective.

Use of Product 2a and 2e for Further Reaction. In this study, we examined modification sucrose moiety, that is, synthesis of polymerizable **2a** using divinyl adipate and then the vinyl group in the main chain was polymerized further in water with free radical polymerization using radical initiator. Polymer compound **3** was isolated by precipitation in acetone and dried under high vacuum. Resulted polymer was examined by Gel Permeation Chromatography (GPC). The obtained values for polymer analysis by GPC were the number-average molar mass (*M*n), weight-average molar mass (*M*w) and polydispersivity (PD) 3.85×10^4 g/mol, 3.90×10^4 g/mol and 1.013 respectively (Figure 7). The infrared spectra of monomer and the respective polymer were determined by FTIR (Figure 8). The interested frequency region to accompanying the polymerization on vinyl group is between 1640 cm^{-1} and 1660 cm^{-1} ($\nu_{C=C}$). The absorption band of vinyl group disappears during the polymerization. It can be noticed that the absorption band at 1646 cm^{-1} ($\nu_{C=C}$) for sucrose monomer, disappeared in comparison to the polymer. This spectroscopic result has confirmed the free radical polymerization in the vinyl group.

The di or tri ester compound of sucrose receives the special awareness due to their use as surfactants.[28] Dordick *et al.*[29] had used the diester of sucrose for the synthesis of linear polymer. Considering importance of such compounds we continued our work towards synthesis of di and tri ester sucrose products. Product **2a** and **2e** were used further for second time esterification to check the intramolecular reaction by TLL following the same reaction condition. We have also tried for the synthesis of di or tri ester compounds by addition of activated vinyl esters during our reaction. Total 9 lipases and 5 proteases have been used for this enzymatic reaction but none was able to catalyze the reaction further to give desired products as listed in Table 2. We demonstrated that **2a** and **2e** is not the appropriate substrate for our target esterification reaction using enzymatic process as its structural conformation has changed during the synthesis of sucrose esters from sucrose using divinyl esters. Once the sucrose vinyl ester formed then it becomes unfavorable for further modification on it using enzymatic process under the same reaction conditions. Although we have just tried these

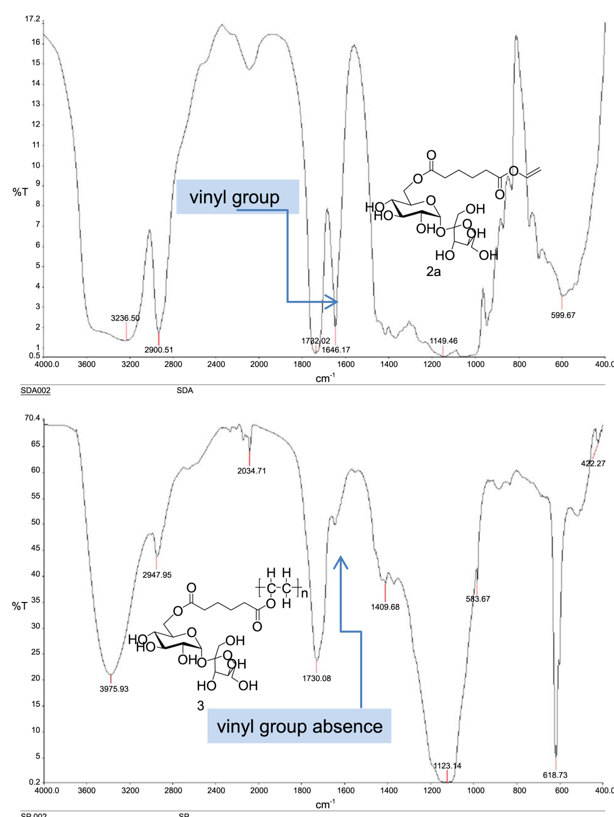


Figure 8. FTIR spectra of the sucrose monoester **2a** and polymer **3** respectively.

reactions following same conditions but we cannot conclude that these reactions are impossible, it might have some optimistic results if same esterification processes carry out after changing the other parameters of the reaction in future. Our truthful efforts toward such modification to receive noble sucrose esters will be continued in future.

Conclusion

We found that immobilized TLL is particularly useful for the preparation of 6-*O*-acylsucrose ester with higher conversion rate even in the 10 g scale reaction. Several solvents were evaluated for the selective transesterification reaction and DMF was proved as the suitable solvent. During experimental process we found that the immobilized lipase retained more than 80 % of its activity after 5 cycles of 96 h reaction. TLL was similarly effective for the regioselective synthesis of short to long (4-13) carbon chain 6-*O*-acylsucrose. For intramolecular transesterification of 6-*O*-acylsucrose **2a** and **2e**, there was no further reaction under the same enzymatic reaction condition. This result showed, once the 6-*O*-acylsucrose was obtained, it is no more suitable as substrate for TLL. For the second transesterification, other enzyme might be required. Unfortunately, under the screening of many other enzymes, no appropriate one was obtained and this work is ongoing. 6-*O*-Vinyladipoylsucrose was further subjected to synthesize respective sucrose branched polymer. The polymer will be used for evaluation of its biodegradability and other biological activities. All the sucrose vinyl esters will be valuable for future investigation as well as biological application. Finally, sugar branched polymer is expected to have interesting biological applications such as surfactants, cryopresevatives and so on, which is in progress and will be reported consequently.

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