Novel biocatalytic esterification reactions on fatty acids: synthesis of sorbitol 1(6) - monostearate

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Dedicated to Professor Sukh Dev on his 80th birthday
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Abstract

Aspergillus terreus lipase has exhibited novel capability of catalyzing esterification reaction between fatty acids (C_4-C_{18}) and primary, secondary and tertiary monohydric alcohols. Although, the lipase efficiently catalyzed the esterification of saturated stearic acid ($C_{18:0}$), it failed to accept the monounsaturated oleic acid ($C_{18:1}$) as the substrate which is also a C_{18} acid, but has a double bond. Thus, this enzyme has a great potential to be used as a selective catalyst for the separation of almost identical saturated and unsaturated fatty acids in their mixtures. Extending this work, highly efficient and regioselective conversion of sorbitol to its 1(6) - monostearate has been achieved by *Aspergillus terreus* lipase - mediated esterification in *n*-hexane. Ninety four per cent conversion to the ester was achieved in 12 h on optimizing four physico-chemical factors, *i.e.* molar ratios of substrates, temperature, solvent and water activity. *Aspergillus terreus* lipase immobilized on Accurel was efficient in the synthesis of sorbitol stearate and was reused three times without any significant decline in its activity, yields were further enhanced to 96 % on using the immobilized enzyme.

Keywords: Lipase; regioselective; organic medium; esterification; sorbitol 1(6) - monostearate

Introduction

Fatty acid esters of sugars and sugar alcohols find widespread applications as surfactants/emulsifiers in food, detergents, cosmetics and pharmaceutical industries owing to their biodegradability and low toxicity.[1-3] Similarly, alcoholic esters of short chain fatty acids are important flavor and aroma compounds, whereas esters of long chain fatty acids are being explored for their use as fuel (biodiesel)[3] and as waxes[3] in the oleo-chemical industries. Among these, the fatty acid esters of sorbitol are the second largest class of carboxylic acid esters employed as surfactants.[4] Their preparation by purely chemical means requires high-energy consumption (acid catalysis and reaction temperatures of 225-250 °C) and results in the formation of toxic and colored by-products. Even preferential acylation of primary over secondary hydroxyl groups can rarely be carried out efficiently with free sugars or sugar alcohols.[3-5] New enzymatic methods involving lipases and proteases are being employed in micro-aqueous/organic media for synthesis of such esters.[2,6-9]

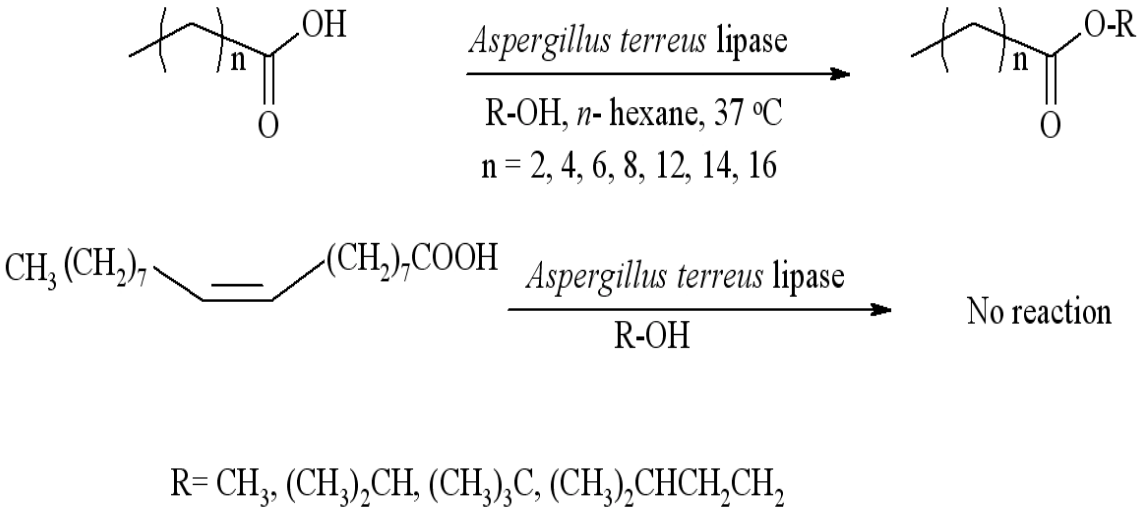
Recently we have reported the use of a lipase from *Aspergillus terreus* in the synthesis of sorbitan monooleate *via* trans-esterification on sorbitol.[3,10] In the present study, the lipase from *Aspergillus terreus* has been found to catalyze the esterification of fatty acids (C_4-C_{18}) with primary, secondary and tertiary alcohols. We also report an efficient and regioselective synthesis of sorbitol 1(6) - monostearate in quantitative yields *via* direct esterification between sorbitol and stearic acid using *A. terreus* lipase under optimized physico-chemical conditions. The esterification reactions carried out under identical conditions, but in the absence of the enzyme or in the presence of the denatured enzyme instead of the active enzyme do not yield any product.

Results and Discussion

Esterification of monohydric alcohols

We had earlier reported that A*spergillus terreus* lipase is able to carry out a variety of industrially important esterification reactions for synthesis of sugars, sugar alcohols and ascorbic acid esters with both long and short chain fatty acids in organic medium.[11] In preliminary investigation, we have observed that the lipase from *Aspergillus terreus* also catalyses the esterification of fatty acids, *e.g*. butyric, caproic, caprylic, capric, myristic, palmitic and stearic acids with primary, secondary and tertiary monohydric alcohols. Thus, incubation of an equimolar mixture of a fatty acid and a primary alcohol (methanol / *iso*-amylalcohol), secondary alcohol (*iso*-propanol) or tertiary alcohol (*tert*-butanol) with *Aspergillus terreus* lipase in *n*-hexane leads to the formation of the corresponding ester in 10-79 % yields (Scheme 1, Table I). In general, the turn over of the esterification of fatty acids with primary alcohols is more than that of the one with the secondary alcohol, which in turn is more than the turn over in case of the tertiary alcohol. Although the lipase from *Aspergillus terreus* catalyses the esterification of saturated stearic acid, it failed in the case of esterification of the monounsaturated oleic acid, which is also a monocarboxylic acid with the same number of carbon atoms (Scheme 1); thus this reaction could be useful industrially for efficient separation of such fatty acids which are otherwise quite difficult to separate. Further work to establish this as a more general method by

studying a large number of saturated and unsaturated fatty acids is under progress in our Laboratories.



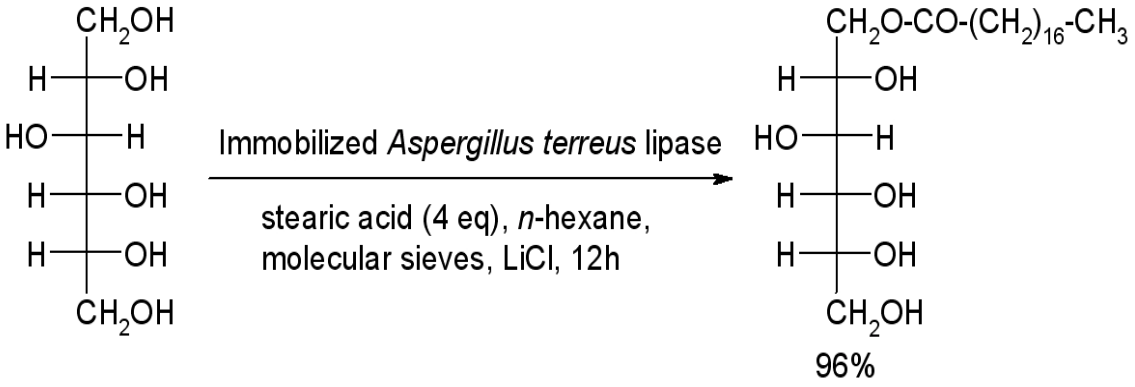
Scheme 1

Table 1. *A. terreus* lipase mediated esterification of fatty acids with primary, secondary and tertiary alcohols in *n*-hexane at 37 °C

Fatty acid	% Conversion after 48 h			
	Methanol	*iso*-Propanol	*iso*-Amyl Alcohol	*tert*-Butanol
Butyric ($C_{4.0}$)	62	33	70	24
Caproic ($C_{6.0}$)	41	16	13	10
Caprylic ($C_{8.0}$)	70	14	69	14
Capric ($C_{10.0}$)	71	32	50	29
Myristic ($C_{14.0}$)	79	51	62	35
Palmitic ($C_{16.0}$)	64	50	62	20
Stearic ($C_{18.0}$)	66	62	65	21
Oleic ($C_{18.0:1}$)	0	0	0	0

Selective esterification of sorbitol. The ability of *Aspergillus terreus* lipase for catalyzing the esterification reaction between primary, secondary and tertiary alcoholic groups and fatty acids at different rates prompted us to study the regioselective capabilities of the enzyme for the discrimination of primary and secondary hydroxyl groups present in the same molecule. In the present investigation, we have observed that the sorbitol 1(6) - monoester of stearic acid is formed in good yields by *A. terreus* lipase catalyzed esterification of sorbitol involving esterification of one of the two primary hydroxyl groups without any reaction at any of the four

secondary hydroxyl groups (Scheme 2). In order to achieve maximum conversion of sorbitol into its monostearate, various physico-chemical conditions have been optimized.



Scheme 2

Effect of molar ratio of sorbitol and stearic acid

The sorbitol-stearic acid molar ratio was varied in the range 1:1 to 1:5 in the reaction mixture. It is evident from Table II that a lower sorbitol - stearic acid ratio favored greater yields of sorbitol monostearate. Conversions of nearly 87 % were obtained in 24 h by mixing sorbitol and stearic acid in the molar ratio of 1:4. No further increase in conversion was obtained on further decreasing the molar ratio. Similar results have been obtained with *Candida viscosum* lipase catalyzed esterification between sorbitol and oleic acid and with *C. antarctica* lipase mediated synthesis of fructose oleate wherein it was reported that high concentrations of fatty acid in comparison to sugar were necessary for high yields of the ester.[5]

Table 2. Synthesis of sorbitol 1(6) - monostearate at different molar ratios of sorbitol and stearic acid by *Aspergillus terreus* lipase at 37 °C

Molar ratio	% conversion			
(sorbitol : stearic acid)	12 h	24 h	36 h	48 h
1:1	45.54	74.36	69.97	68.85
1:2	69.97	81.29	81.00	80.25
1:3	76.25	85.45	83.21	80.24
1:4	79.95	86.67	81.54	80.67
1:5	78.85	86.20	84.26	79.84

On the other hand, some workers found that an equimolar ratio of substrates favored synthesis of sugar/sugar alcohol esters using lipases from *Candida antarctica* and *Pseudomonas* sp., respectively.[4,12] It is also noteworthy that the percentage of conversion decreased for all the sorbitol to stearic acid molar ratios after 24 h (Table II). This could have been caused by water absorption by the system due to long reaction times.

Effect of temperature

The above reaction accelerated upon increase of temperature from 37 °C to 45 °C with conversion of about 90 % of sorbitol into its 1(6) - monostearate after which there was a gradual decline (Fig. 1). It is noteworthy to mention that the reaction time for maximum conversion of the acid to its ester was reduced to just 12 h at 45 °C, further the initial rate of the reaction at 37 °C was low when compared to the rate of the reaction at 45 °C, 50 °C and 60 °C. However after an incubation period of 24 h at 37°C, the % conversion was comparable to that at 45 °C. A significant decline in the conversion of sorbitol to ester was observed after 14 h of reaction run at 50 °C and 60 °C (Fig. 1). This could have been due to the inactivation of the enzyme at higher temperatures in the presence of an organic solvent.

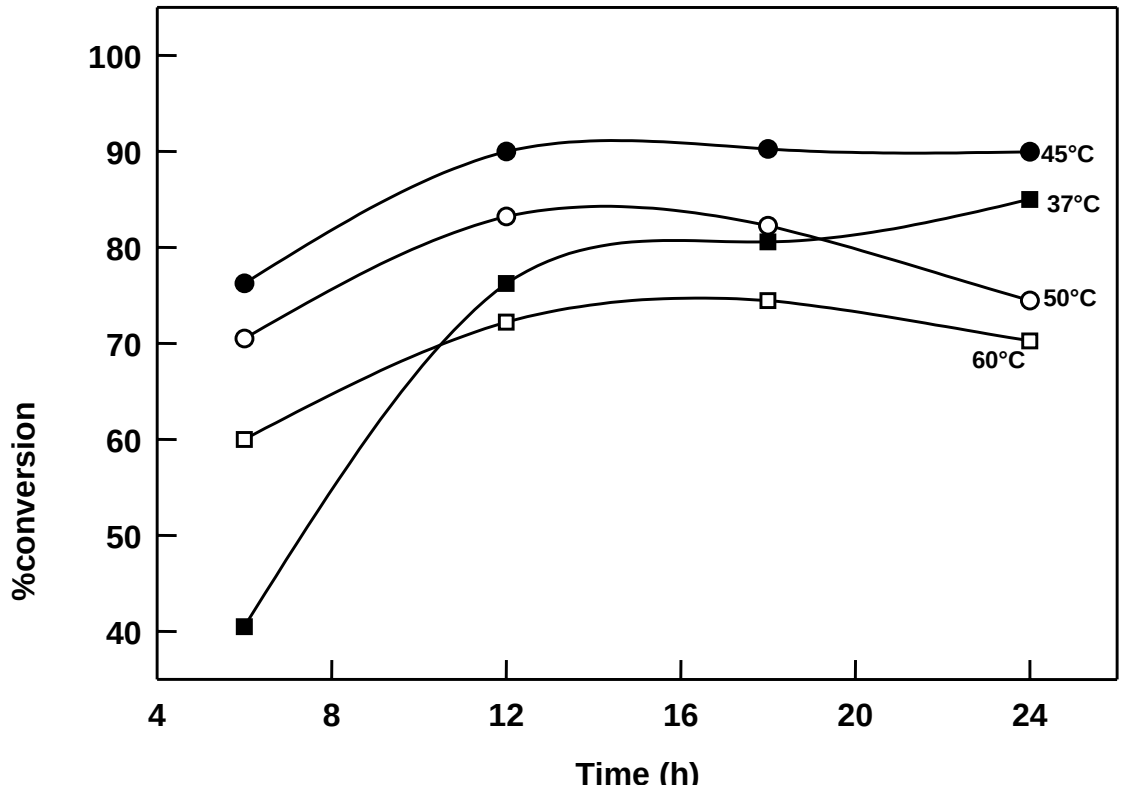


Figure 1. Effect of temperature on the synthesis of sorbitol 1(6) - monostearate in *n*-hexane by *Aspergillus terreus* lipase at initial a_w of 0.33.

Effect of organic solvents

Esterification reactions are, generally affected by the type of the solvent. Organic solvents with a higher log P value are known to favor greater enzyme stability.[8,13,14] In the present study, among the different solvents tried (Fig. 2), *n*-hexane (log P 3.5) and iso-amyl alcohol (log P 1.3) affected maximal conversion of 89 % of sorbitol into its 1(6) - monostearate. Pyridine also gave high conversions of 89 % despite of its lower log P value (0.71), which may have been due to higher solubility of the substrate sugar alcohol in this solvent. Although the log P values of pyridine and *n*-butanol are quite comparable, there are appreciable differences in the % conversion of sorbitol to ester, this may be because of the differences in solubility of the reactants in these solvents. Lowest yields were obtained in methanol, which has a log P value of -0.76.

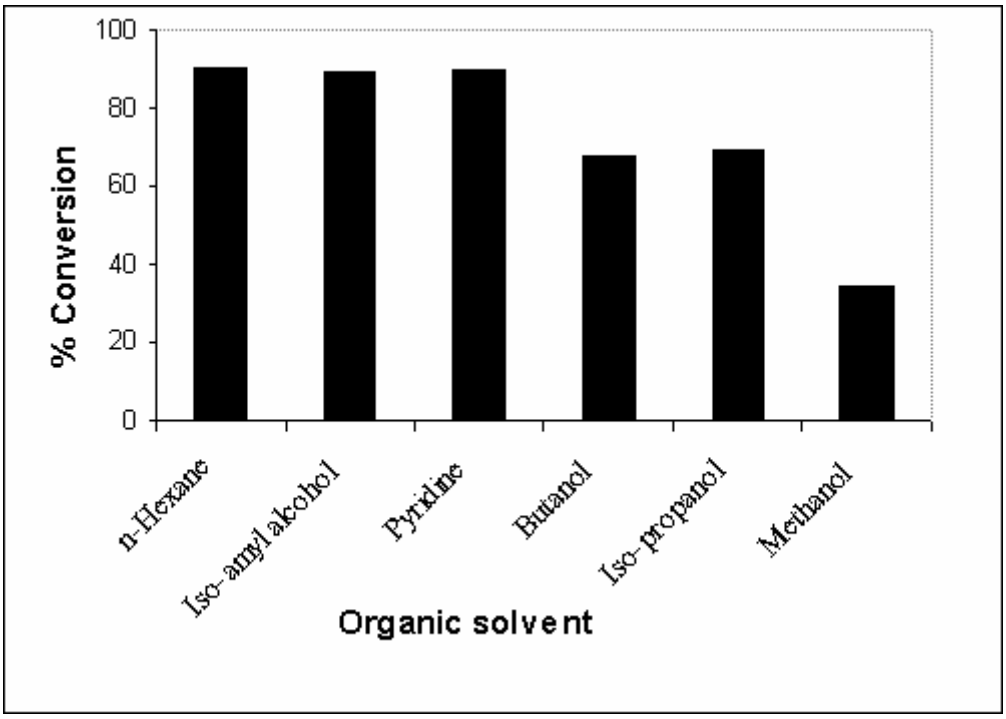


Figure 2. Effect of various organic solvents on the synthesis of sorbitol 1(6) - monostearate at 45 °C and initial a_w of 0.33. Log P values of different solvents used are: *n*-hexane : 3.5; *iso*-amyl alcohol : 1.3; *n*-butanol : 0.8; pyridine : 0.71; *iso*-propanol : 0.28; methanol : -0.76.

Further, the % conversion of sorbitol to its monoester seems unaffected by protic and aprotic nature of organic solvents as the turn over of the esterification reaction is almost the same in *n*-hexane, pyridine and isoamyl alcohol (Fig. 2). However, the turn over of the esterification reaction is directly proportional to the lipophilicity of the protic solvents, e.g. the yield of the sorbitol esterification reaction decreases from isoamyl alcohol to butanol and isopropanol, and further to methanol (Fig. 2). A major problem for synthesis of sugar esters is the low solubility of sugar/sugar alcohol in organic solvents. To overcome this problem, generally pyridine or dimethylformamide are used, but these solvents inactivate the enzyme and are non-compatible

with the industry.[6,7,15] Other solvents, like chloroform, hexane, acetone, etc. are being used now even though the precursor sugar is not soluble in them.[16] Investigations on these solvents has, however, revealed that as the reaction proceeds, reactants dissolve because of the higher solubility of the ester formed in such non-polar solvents. Similar results were obtained in the present investigation for synthesis of sorbitol 1(6) - monostearate in *n*-hexane which perhaps is industrially compatible, non-toxic, has a low boiling point and thus can easily be removed from the reaction system. Hexane has also been used for synthesis of 6-*O*-palmitoyl glucose using *Pseudomonas* sp. lipase.[12] Arcos *et al.*[4] have used acetone as solvent for the synthesis of 1,6-diacylsorbitol derivatives.

Effect of water activity

The enzyme-catalyzed reactions in organic media critically depend on the amount of water in the reaction system.[17-19] The water released during the esterification reaction may disturb the equilibrium and hydrolyze the ester formed. The use of salt hydrates and molecular sieves (type 4Å, at a concentration of 10 % w/v of the reaction mixture) is a convenient and simple method to maintain a constant a_w in the reaction medium.[14] In the present investigation, the effect of varying initial water activity (using salt hydrates) of the reaction system showed that lower water activity of the reaction system favored higher conversion rates (Table III). It was found that low initial a_w of 0.11 and 0.33 (obtained with LiCl and $MgCl_2$, respectively) gave optimal conversion of sorbitol into sorbitol monostearate, *i.e*. 87.75 % and 85.45 % (Table III). However, higher initial a_w of 0.97 (using K_2SO_4) gave conversion of only 70.91 %. Kim *et al*.[14] used salt hydrate pairs to control a_w in the reaction mixture for *Mucor miehei* lipase catalyzed regioselective monoacylation of sucrose. Further, it is interesting to note that even a slight change in the pH of the reaction causes a considerable change in the % conversion of sorbitol. Thus LiCl, $MgCl_2$ and $Mg(NO_3)_2$ that are the salts of weaker bases and strong acids lower the pH of the reaction system than the salts NaCl and K_2SO_4 (of strong acid and strong base) show higher conversion rates of sorbitol into its ester (Table III).

Table 3. Effect of initial a_w of the reaction system on synthesis of sorbitol 1(6) -monostearate after 12 h at 45 °C in the presence of different salts

Salt	a_w	% conversion
LiCl	0.11	87.75
$MgCl_2.6H_2O$	0.33	85.45
$Mg(NO_3)_2$	0.53	82.45
NaCl	0.75	71.22
K_2SO_4	0.97	70.91

The water released during the esterification reaction was controlled by addition of molecular sieves to the reaction system. It was found that the addition of molecular sieves (10% w/v)

favored higher reaction rates with 96 % conversion in just 12 h (Fig. 3). This also stabilized the product preventing a backward reaction as observed in the system without the molecular sieves wherein product yields were reduced after 24 h. The addition of molecular sieves had two advantages, firstly it shifted the equilibrium towards ester synthesis. Secondly, it stabilized the product by preventing its hydrolysis by absorbing excess water in the system in comparison to the system without the molecular sieves. Ducret *et al.*[20] and Sarney *et al.*[13] also used molecular sieves for the removal of water for the esterification of sorbitol with oleic and lauric acids, respectively; higher yields of the esters were obtained which supports our findings.

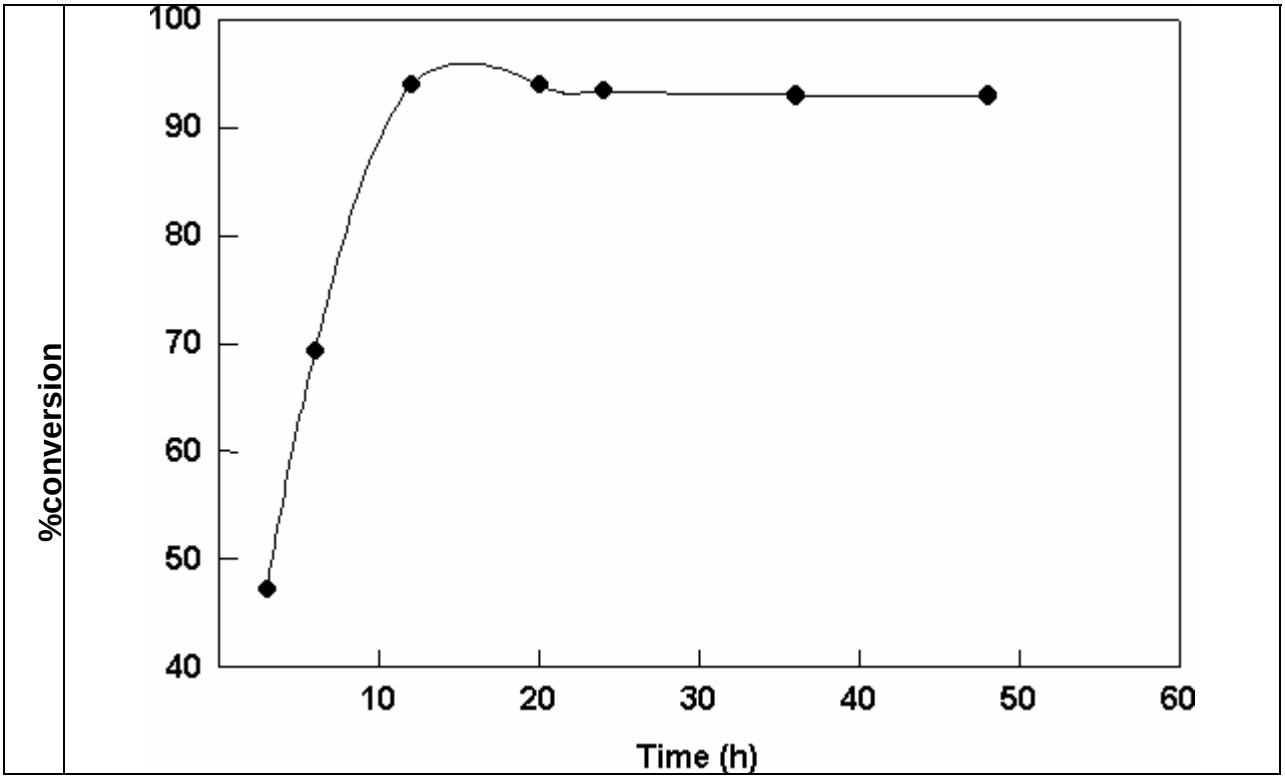


Figure 3. Effect of the addition of molecular sieves (type 4 Å, 10% w/v) on the synthesis of sorbitol 1(6) - monostearate by *Aspergillus terreus* lipase in *n*-hexane at 45 °C.

Synthesis of sorbitol stearate using immobilized *A. terreus* lipase

The efficiency of the synthesis of sorbitol monostearate was also checked with *A. terreus* lipase immobilized on Accurel. Enhanced yields of 96 % were obtained on using immobilized lipase. The process could be repeated for three cycles without any significant loss in the selective esterification ability of the enzyme (Fig. 4).

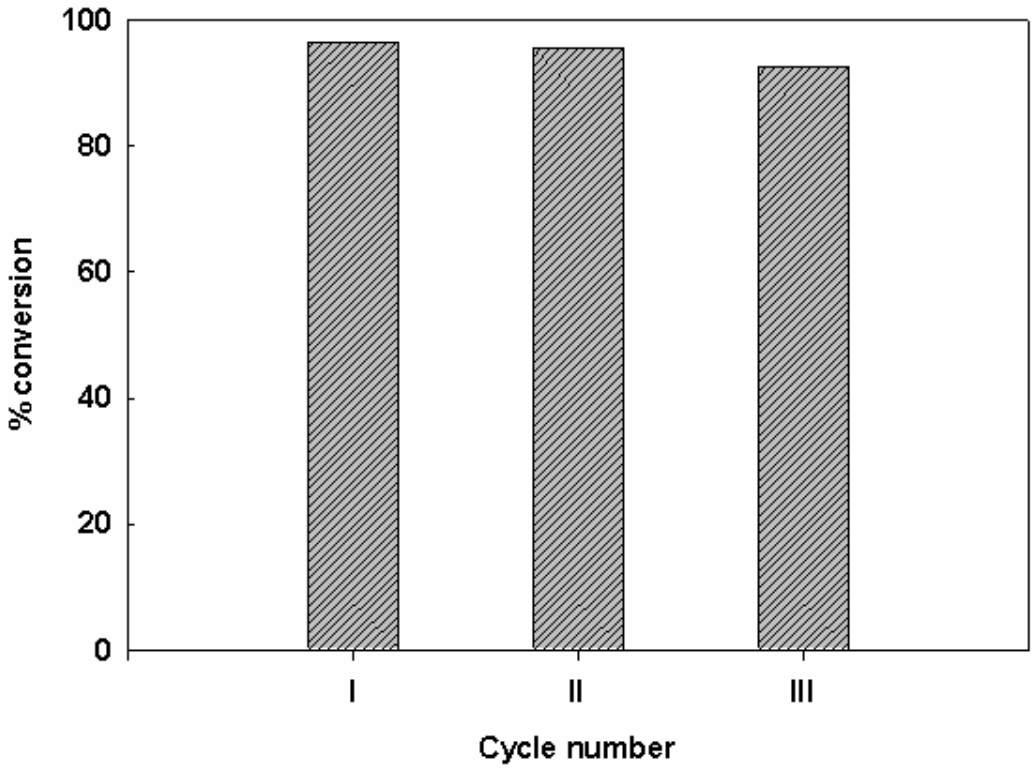


Figure 4. Effect of immobilised *Aspergillus terreus* lipase on synthesis of sorbitol 1(6) - monostearate at 45 °C and initial a_w of 0.33 in *n* – hexane.

Experimental Section

Microorganism and production medium

Aspergillus terreus (RKS 101) was grown at 37 °C and subsequently maintained at 4 °C on potato dextrose agar slants. Lipase was produced in the medium as previously described using corn oil as the lipidic substrate in a 10 L fermentor containing 5 L of the production medium (New Brunswick Sci. Co. Inc., Bio-flow IV fermentor).[10,11] The medium was autoclaved at 121 °C (15 psi) for 15 min. and inoculated with $1x10^7$ spores/50 ml of the medium using 96 h old culture of *A. terreus*. Standard operating conditions were: agitation (300 rpm), aeration (1 vvm) and dissolved oxygen concentration not less than 20 % saturation. After 60 h of the fermentation run, the culture broth containing extracellular lipase was filtered through Whatman No. I filter paper. The culture filtrate containing lipase was partially purified by ammonium sulphate precipitation (60 % saturation). The precipitate containing lipase was dialyzed against 0.01 M

phosphate buffer (pH 7.0) for 24 h. The dialyzed lipolytic preparation was lyophilized to a dry powder and used in different esterification reactions after storing over P_2O_5 in a vacuum dessicator for 24 h.

***Aspergillus terreus* lipase catalyzed esterification of sorbitol with stearic acid**

Sorbitol (50 mM) and stearic acid (50 mM) were mixed together in *n*-hexane (4 ml) in screw-capped vials (15 ml). The reactants and *A. terreus* lipases (50 mg, equivalent to 25 U/mg protein) were equilibrated separately to attain a water activity (a_w) of 0.33 with a saturated aqueous solution of $MgCl_2.6H_2O$ for 24 h in an evacuated dessicator. The reactants and the enzyme were then mixed and incubated at 37 °C and 150 rpm for 48 h in a shaker incubator. Periodically, sample aliquots were withdrawn and product formation was qualitatively assessed by TLC analysis. Ester synthesis was expressed as the percentage molar conversion of the acid to the ester after titrating the residual fatty acid against 0.01 M NaOH. The maximum conversion of fatty acid to ester achieved under the conditions was 68.85 % (Table II).

Different physico-chemical factors were studied in order to achieve maximum formation of sorbitol monostearate. These were: molar ratio of substrates, temperature, organic solvent and water activity (a_w) control. To study the effect of water activity on the esterification reaction, molecular sieves (type 4 Å) were added at a concentration of 10 % w/v of the reaction mixture. Any deviations from the standard reaction conditions are mentioned at appropriate places.

Lipase immobilization

For immobilization, Accurel EP 100 beads (200 mg, Enka AG, Obernberg, Germany) were wetted with ethanol (2 ml). To this, distilled water (4 ml) and sodium phosphate buffer (0.01 M, 2.5 ml) were added. Lipase powder (200 mg, 20 U/mg) in distilled water (13.5 ml) was added to the beads and the suspension was shaken at 100 rpm for 12 h at room temperature for immobilization to occur. The beads were filtered off and washed twice with phosphate buffer to remove any unbound protein and subsequently dried at room temperature under vacuum. The immobilized lipase (50 mg) was added to the optimized reaction mixture of sorbitol and stearic acid. Esterification was carried out at 45 °C, 150 rpm and for 12 h. All other reaction conditions were the same as standardized earlier. The immobilized enzyme was used for three cycles to check the efficiency of the enzyme in terms of reusability.

The esterification reactions carried out under identical conditions, but in the absence of the enzyme or in the presence of the denatured enzyme instead of the active enzyme did not yield any product.

Spectral analysis of the reaction products

After the desired reaction time, the enzyme was removed by filtration. The filtrate was concentrated by evaporation of the solvent *in vacuo* and the products subsequently purified by column chromatography over silica gel. TLC analysis of the products were carried out on E. Merck pre-coated silica gel plates using the solvent system: petroleum ether - ethyl acetate (19:1). The purified products were analyzed by IR and ^1H NMR spectroscopy; the IR spectra

were recorded on a Perkin –Elmer RX/FT- IR spectrophotometer and the ^{1}H NMR spectra were recorded on a Bruker Avance 300 MHz instrument in $CDCl_3$, the spectra of different esters obtained in this study were fully compatible with their structures and matched well with those reported in the literature for the corresponding compounds.

Conclusions

A. terreus lipase discriminates between saturated and unsaturated fatty acids towards their esterification reactions with alcohols. Further, very high conversions (96 %) of sorbitol into its 1(6) - monostearate were obtained in only 12 h by immobilized *A. terreus* lipase-catalyzed esterification of sorbitol with stearic acid in *n*-hexane on optimizing various physico-chemical conditions. Temperature, water activity control and organic solvent type were the major controlling factors in the conversion of sorbitol to its 1(6) - monostearate. The efficiency of the immobilized lipase, which could be used repeatedly, established the cost-effectiveness of the whole process. Thus, the biocatalytic route described here is economical, efficient and environmentally benign for carrying out esterification reactions of commercial significance.

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