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Production of Pentaerythritol Monoricinoleate (PEMR) by immobilized *Candida antarctica* lipase B

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ABSTRACT

In the present study, green synthesis of pentaerythritol monoricinoleate (PEMR) was carried out using *Candida antarctica* lipase B immobilized on hydrophobic adsorbent via interfacial activation. Various reaction parameters such as reaction time, organic solvent, molar ratio, the enzyme load, and presence of molecular sieves on pentaerythritol (PE) ester synthesis were systematically studied to yield selective monoester of PE. The strategies (smart use of substrate molar ratio and polar organic solvent) were employed to suppress dimerization of ricinoleic acid (RA) to avoid by-product formation and hence to obtain superior mono-ester yield. Under optimized conditions *viz.* substrate molar ratio of 4 (PE):1 (RA) with 2% enzyme load and 200 g/L molecular sieves in the presence of *tert*-butanol, 93% substrate molar conversion in 24 h reaction time was obtained. The synthesized PEMR was also characterized using FT-IR and Mass spectroscopy. To the best of our knowledge, this is the first report describing the enzymatic synthesis of PEMR.

1. Introduction

Industrial surfactants are used as processing utilities and are recalcitrant due to their toxic and non-biodegradable nature which led to an increased interest for alternative nontoxic and biodegradable surfactants having low eco-toxicity with a range of specific properties (Hydrophile-Lipophile Balance (HLB) values, appearance, and stability) [1]. Polyol fatty acid esters, usually called sugar alcohol esters, are biodegradable, non-toxic, and non-ionic surfactants are having excellent stabilizing, conditioning, and emulsifying properties. These sugar alcohol (polyol) fatty acid esters are widely used as a surfactant in pharmaceutical, food, cosmetic, and detergent industries. Traditionally, the synthesis of these fatty acid esters has been carried out by chemical methods via esterification of a polyol with fatty acids [2–5]. These chemical processes are non-selective, resulting in multipoint esterification to form several by-products. Further, these processes make use of hazardous catalyst, organic solvents, and high temperatures, which impart color to the synthesized product. To counteract these issues, biocatalysis using lipases can be employed. The lipase usually acts under mild reaction conditions, possesses high selectivity and low carbon footprinting. However, their applications are limited due to the high cost of production and little operational stability, but that can be addressed using pre or post-expression engineering. The lipase-mediated esterification process can be tuned for high selectivity of to yield the single product (e.g., monoester, di-ester, tri-ester, etc.) using reaction engineering. Further, this green method avoids degradation of the substrate and products due to low operating reaction temperature and pressure [6,7]. Due to incremental awareness of hazards associated with chemical processes, there is a growing thrust towards green chemistry that utilizes milder reaction conditions, which makes enzyme-based methods industrially more attractive.

The polyol fatty acid esterification reaction generates water as a by-product, which has broad implications in reaction rate as well as the stability of biocatalyst. Enzymes need a small quantity of water to maintain their active conformation and on the other hand, with increasing water amount imparts adverse effects on activity. In organic media, an enzyme activity shoots high with the addition of trace amounts of water. This degree of organic solvent hydration affects the intrinsic activity of enzyme as well as initiates a nucleophile competition for an active site of an enzyme [8–10]. The ideal water content in organic media, thus, depends on the

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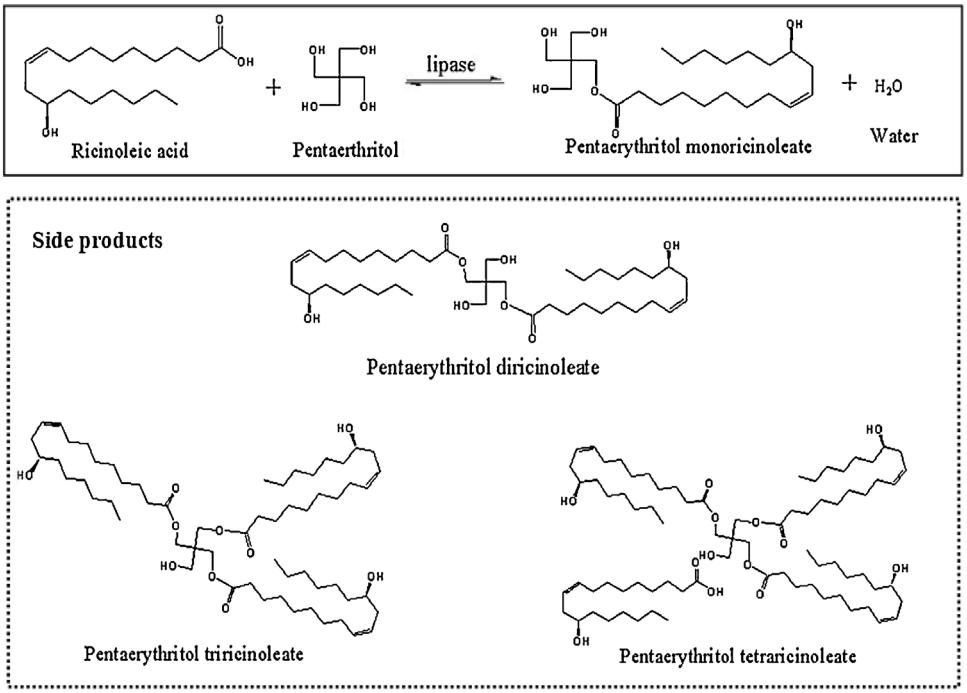
biological source of the catalyst, the nature of the organic solvent, and the type of support used [11]. The water production problems during esterification reaction could be solved using a smart selection of reaction medium polarity and the effective removal of water [12]. Another major challenge is low solubility of polyol in an organic solvent which needs to be improved for better conversion and yield. Different strategies have been reported to overcome poor solubility of polyol. Therisod et al. [13] reported the use of activated fatty acids as a substitute for fatty acid in polar solvents. Oguntimein et al. [14] reported the use of activated polyol as a substitute for polyol in non-polar solvents. In spite of the advantages associated with these strategies, the derivatization of starting material increases process costs, making it not feasible at an industrial scale. Another approach of partial solubilization of both starting materials; polyol and fatty acid in moderately polar solvents was developed and found to be useful for polyol fatty acid ester synthesis [15,16].

There are many previous research reports on the lipase-mediated synthesis of polyol fatty ester from various polyols and fatty acids. Sang Yoo et al. [17] reported the enzymatic synthesis of oleic acid monoester of sorbitol and xylitol while Rufino et al. [18] reported the synthesis of xylitol fatty acid esters of oleic acid, lauric acid, and butyric acid. Patil et al., [19] reported the synthesis of sorbitol fatty acid esters of palmitic, oleic, and lauric acid using a binary solvent system of DMSO and *tert*-butanol. Continuous synthesis of erythritol-lauric acid ester was reported using a packed bed reactor (PBR) [20]. Castillo et al. [21], showed the comparative effect of various solvents such as hexane, *tert*-butyl alcohol, DMSO on selectivity for monooleoyl xylitol synthesis. Piao et al., [22] reported the impact of the excess molar ratio of oleic acid in the presence of the molecular sieve on dioleoylerythritol formation. Piao et al. [23,24] also reported continuous process in a packed bed reactor for synthesis of erythritol, arabitol, ribitol, xylitol, and sorbitol monoesters of lauric acid and oleic acid. Gulati et al. [25] reported highly efficient and region selective synthesis of sorbitol-1(6)-monostearate using *Aspergillus terreus* lipase.

Immobilization of enzyme yields superior thermal stability and activity, low inhibition by reaction products, improved selectivity towards non-natural substrates, and ensures industrial use of an enzyme [26–28]. Lipases possess interfacial activation where the active center is covered by a polypeptide chain called lid [29]. This lid needs to move for which immobilization plays a significant role to catalyze any reaction. Among practiced methods for lipase immobilization, adsorption on hydrophobic matrices is considered one of the most critical and useful techniques [30]. In the presence of a hydrophobic surface, the lipase enzyme fixes as open form with the active center being fully exposed for catalysis [31]. The binding forces, although are weak, cumulatively hold lipase firmly and preserves the native structure as well as the activity. *Candida antarctica* lipase B (CALB), a molecular weight of 33 kDa, is most industrially explored lipase used in a wide range of reactions. Simple physical adsorption for CALB by interfacial activation has been studied earlier [30,32–35]. Novozym 435 (N435) is an immobilized preparation of CALB on a Lewatit VP OC 1600 where CALB is adsorbed via interfacial activation and has been reviewed for some critical problems of support with its solutions [36].

The present study demonstrates an enzymatic synthesis of pentaerythritol monoricinoleate (PEMR), as shown in Scheme 1. PEMR, similar to pentaerythritol monooleate, has the potential to be used as raw material for the production of lubricants, plastic lubricant/dispersant, bio-surfactant, crystal retardant, solvent for emulsifiable concentrate, etc. PEMR is a ricinoleic acid monoester of pentaerythritol and hence a type of polyol fatty acid esters of polyol. However, in the case of PEMR ricinoleic acid is a hydroxy monounsaturated fatty acid which makes the synthesis of PEMR more challenging. A significant challenge is a problem of self-condensation of ricinoleic acid molecules (estolides of fatty acid) leading to reduced conversions. This is because the ricinoleic acid is bi-functional molecule and undergoes auto-catalytically polymerization to yield estolides. Earlier, there is report on synthesis PE ester of castor oil fatty acids where self-condensation occurs with ricinoleic acid before esterification with a polyols [37]. Recently, the lipase based estolide synthesis with control size has been conducted to understand the estolide enzyme polymerization [38]. Another constraint for polyol ester synthesis is the low solubility of pentaerythritol in organic solvents, which become a governing



Scheme 1. Reaction scheme showing the synthesis of PEMR by enzymatic esterification of ricinoleic acid with pentaerythritol.

factor in obtaining high conversion. Thus, the present study addresses the challenges and derives a solution for low solubility of pentaerythritol and estolides formation of ricinoleic acid to yield high content of PEMR.

2. Materials

2.1. Chemicals and lipases

All chemicals and solvents used were of analytical or chromatographic grade and purchased from SDFCL (India). *Candida antarctica* B soluble lipase was purchased from DK Enzymes & Chemicals (India). Indion®PA 500 resin was purchased from Ion Exchange, (India). *Candida antarctica* B soluble lipase was immobilized via interfacial activation (physical adsorption) on Indion® PA 500 resin and named as PyCal lipase [39,40]. Molecular sieves 3 Å were purchased from Hi-Media Laboratories (India). Ricinoleic acid and Pentaerythritol were purchased from Acme Synthetic Chemicals (India). Reference standards for fatty acids were purchased from Sigma (St. Louis, MO). All solvents and chemicals were used without any prior purification/modification.

3. Methods

3.1. Esterification reaction at batch scale

The batch reactions were carried out by mixing of pentaerythritol (4.4 mmol) and ricinoleic acid (1.1 mmol) with the selected solvent (40 ml) in 100 mL glass stopper flasks kept at 60 °C at 200 rpm. Dried molecular sieves (3 Å) were added into the reaction mixture, and esterification was initiated by adding PyCal lipase. A typical reaction mixture consisted of 4.4 mmol of PE, 1.1 mmol of RA, 40 mL *tert*-butyl alcohol, and 2% of PyCal lipase based on total reaction mixture volume. The aliquots were taken from the reaction mixture at particular time intervals for analysis. Samples were analyzed on reversed-phase high-performance liquid chromatography (RP-HPLC) as described in the section below. Degree of conversion of RA into PEMR was expressed as percent conversion and calculated using the molar concentration of RA as well as PEMR in the reaction mixture. PEMR concentration was calculated using the calibration curve of purified pentaerythritol monoricinoleate.

3.2. Analysis of reaction mixture

The reaction was stopped by filtering the enzyme, and the specific amount of sample was withdrawn. Sample (0.5 ml) was diluted in methanol (0.5 ml) and subjected to HPLC analysis. The analysis was performed using reversed-phase high-performance liquid chromatography (RP-HPLC) in an Agilent 1200 Chromatography System (CA, USA) equipped with a quaternary solvent delivery module, and an Agilent evaporative light scattering detector and 10 μL sample were automatically injected on Agilent Zorbax XDB ODS 5 μM, 4.6 × 150 mm column. The separation was obtained by isocratic elution using methanol/water (90:10) as a solvent system with a flow of 1 mL/min. PE, RA, and PEMR were analyzed and quantified using the ELS detector. Degree of conversion was expressed as the percentage reduction of fatty acid in the final reaction mixture as determined by HPLC analysis. Fig. 1 shows representative overlay HPLC chromatograms of initial and final reaction mixture showing formed PEMR, consumed ricinoleic acid (RA), and pentaerythritol (PE).

3.3. Product characterization

Structure of enzymatically synthesized PEMR was analyzed using FT-IR and mass spectral analysis.

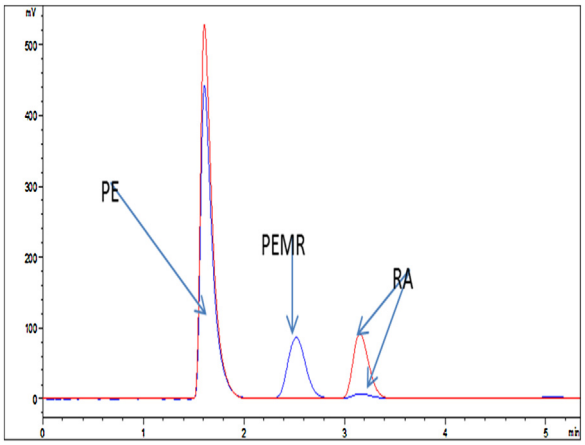


Fig. 1. Overlay HPLC chromatogram of red line represents initial and blue line represents final reaction mixture showing formed PEMR, consumed ricinoleic acid (RA), and pentaerythritol (PE).

3.3.1. FT-IR analysis

After completion of esterification, the reaction mixture was filtered and concentrated to dryness. The dried mass obtained was subjected for removal of PE and RA. Post removal of these impurities, a purified sample of PEMR was subjected to FT-IR analysis. The ester functional group in PEMR was analyzed using Fourier Transform Infrared Spectrophotometer, IR Prestige-21 (Shimadzu, Japan) equipped with DlaTGS detector. The spectrum was recorded from co-addition of 30 scans at a resolution of 4 cm^{-1}.

3.3.2. Mass spectral analysis

Mass spectral analysis was carried out using Agilent 1200 series MS system, equipped with electrospray ion (ESI) source. PEMR fraction from HPLC was collected and directly injected via autosampler into the mass spectroscopy (MS) system. Gas temperature: 300 °C, drying gas: N_2, and Flow rate: 8 L/ min and fragmentor voltage: 1.35 V.

4. Results and discussion

4.1. Immobilization of CAL B enzyme and its characterization

Candida antarctica B soluble lipase was immobilized via interfacial activation on the hydrophobic adsorbent and prepared immobilized enzyme, PyCal, was compared with commercial preparation Novozym 435 (Tables 1 & 2). Both the preparations have shown similar activity as well as reactivity to synthesize PEMR. From the data obtained, it can be concluded that PyCal lipase is competitive with merchandized Novozym 435 lipase and thus used for further optimization of PEMR synthesis.

Table 1
Comparative data of commercial Novozym 435 lipase and PyCal lipase.

Properties	Novozym 435	PyCal
Tributyrin hydrolytic activity (U)	543	528
Butyl oleate esterification activity (U)	225	210
Lipase content (mg/mL of resin)	NA	30
Water content (%)	1-2	0.5-1
PEMR Conversion* (%)	93.18	93.62

NA – Not Available.
* Under optimum reaction parameters.

Table 2
Characteristics of immobilized lipase preparation, PyCal.

Parameter	Value
Optimum Temperature (°C)	60
Optimum pH	NA
Substrate Specificity	Non-regio specific
K_m (M) for PEMR synthesis	4.205
V_{max} (mM min^{-1}) for PEMR synthesis	15.50
Space-Time Yield for PEMR synthesis (g L^{-1} h^{-1})	2.85

NA – Not Applicable.
Note – Biocatalyst loading used for calculating K_m and V_{max} is 4% w/v of the total reaction.

4.2. Effect of organic solvent on PEMR synthesis

Organic solvents play an important role by bringing conformational changes in the enzyme molecular structure and thereby affecting activity, kinetics, and selectivity of an enzyme. The degree of the effect differs depending on the organic solvents and enzyme combination used. Solvents with log P value >3, such as heptane and hexane are preferred for enzymatic esterification reactions [41]. However, pentaerythritol is not soluble in these solvents. So, a solvent with moderate or lower log P values was selected that will solubilize both substrates and provide good contact between pentaerythritol and ricinoleic acid. In this work, different solvents with varying log P values were selected to study their effect on PEMR synthesis (Fig. 2). Acetone (log P: −0.26), *tert*-butyl alcohol (log P; 0.83), diacetone alcohol (log P: −0.14) and methyl isobutyl ketone (MIBK) (log P: 1.19) were used for this purpose. As can be observed from Fig. 2, *tert*-butyl alcohol showed maximum conversion for the synthesis of PEMR as compared to other solvents. This is because being a relatively polar solvent with a log P value of 0.35; it led to greater solubility of pentaerythritol and ricinoleic acid compared to other solvents used in the study. Additionally, it makes the whole reaction system monophasic, which is beneficial for lipase based reaction [42,43]. Hence *tert*-butyl alcohol was selected as an organic solvent for PEMR synthesis.

4.3. Solubility study of pentaerythritol in tert-butyl alcohol

The major challenge during the synthesis of PEMR is the solubility of pentaerythritol in organic solvents. The solubility of pentaerythritol in the reaction mixture has a profound effect on final conversion. The various strategies, such as particle size reduction of pentaerythritol and dissolution at high temperature, were studied to address the solubility problem of pentaerythritol in an organic solvent. Commercially available pentaerythritol is of large particle size (>100μM) and does not dissolve in an organic solvent. To make it soluble, PE crystals were grounded to make them very fine. First PE crystals were grounded in a grinder followed by separation of small size (<100 μM) size particles with the help of sieves (mesh) of <100 μM size specification. After separation of small size particles of PE, they are subjected to vacuum drying. These dried small size particles were used for all the further study. Fig. 3 shows images of commercial and ground pentaerythritol. These smaller size particles showed better solubility in an organic solvent. PE powder was then dissolved in *tert*-butyl alcohol at 60 °C to make it more catalytically available as it shows very low solubility at room temperature. Earlier, metastable suspension of 10–200 μM saccharide particles in solvent-free reaction medium has been employed for lipase catalyzed the synthesis of sugar esters. The particles make suspension with product esters and increase the solubility of saccharide for esterification reaction [44].

The solubility study of pentaerythritol in *tert*-butyl alcohol at 60 °C was carried out, as shown in Fig. 4. Different amount of PE powder ranging between 40–280 mg was added to 10 mL *tert*-butyl alcohol and kept at 60 °C for 6 h. The sample was withdrawn and subjected to HPLC analysis as described in the previous section. From solubility data, the excellent solubility of PE in *tert*-butyl alcohol at 60 °C was found to be 15 mg/mL, which was further used for PEMR synthesis.

4.4. Effect of molar ratio

The molar ratio of acyl donor concerning acyl acceptor in enzymatic esterification reaction plays a critical role as an optimum molar ratio is desired for higher conversion. The molar ratio of pentaerythritol and ricinoleic acid can directly influence kinetics and yield of PEMR synthesis. The molar ratio of ricinoleic acid to pentaerythritol is important for both the product composition and the course of esterification reaction. During esterification reaction, one of the substrates is generally used in excess to favor esterification and to limit reverse hydrolysis. In the case of ricinoleic acid, two reactive functional groups, namely the hydroxyl group and the carboxyl group, are available for esterification. Pentaerythritol was taken in excess compared to ricinoleic acid to prevent esterification at hydroxyl group of one ricinoleic acid with the carboxyl group of another ricinoleic acid (dimer formation). Earlier studies also report high pentaeryhtritol to oleic acid ratio resulted in 98% conversion [45], but their aim was non-selective esterification with all –OH groups of PE. Also the

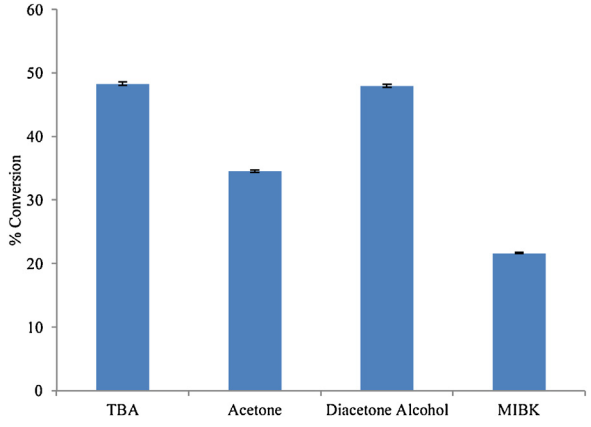


Fig. 2. Effect of organic solvent on the synthesis of PEMR. Vertical bars represent standard deviation.

Fig. 3. A: Commercial Pentaerythritol B: Ground Pentaerythritol powder of < 100-micron size.

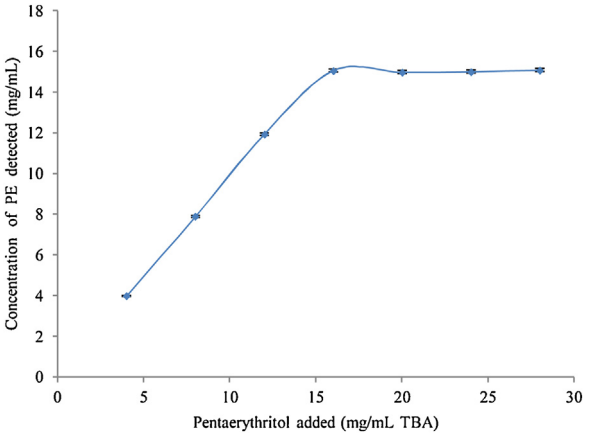


Fig. 4. Solubility profile of PE in *tert*-butyl alcohol at 60 ^{0}C. Vertical bars represent standard deviation.

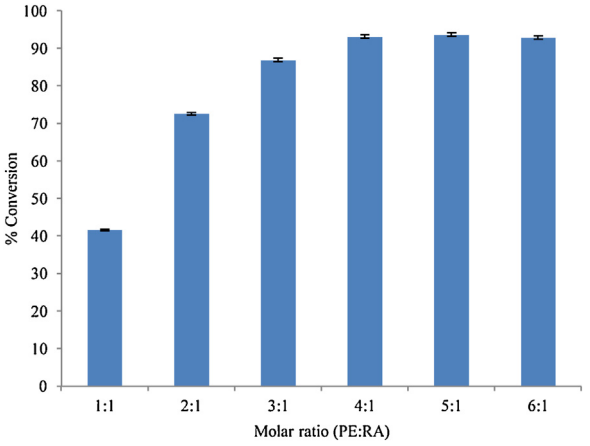


Fig. 5. Effect of the molar ratio of pentaerythritol and ricinoleic acid on the synthesis of PEMR. Reaction mixture composed of 4.4 mmol of Pentaerythritol, 40 mL *tert*-butyl alcohol, 2% (w/v) PyCal lipase, 200 g/L 3 Å molecular sieve. The reaction temperature was 60 ^{0}C, and reaction time: 24 h. Vertical bars represent standard deviation.

start polymer consisting of polymeric ricinoleic acid & pentaeryhtritol has been synthesized, and they observed that ricinoleic acid polymerized before it esterifies with pentaerythritol [46].

The different molar ratio of pentaerythritol and ricinoleic acid were studied to understand its effect on the molar conversion of

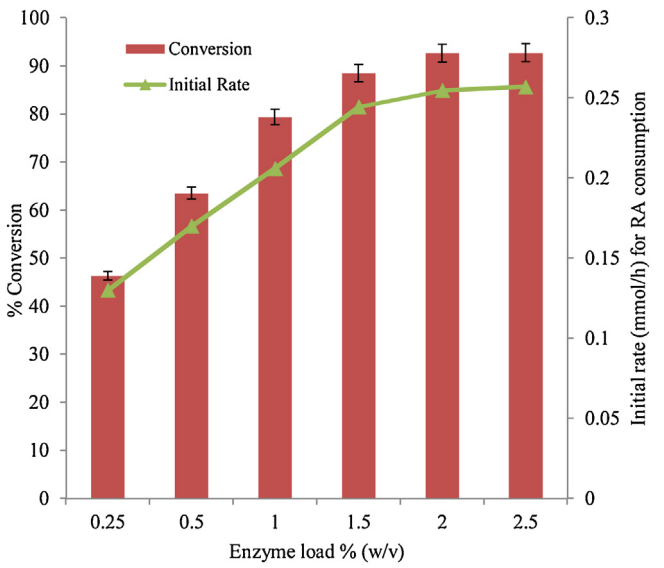


Fig. 7. Effect of enzyme load on the synthesis of pentaerythritol monoricinoleate. Reaction mixture composed of 4.4 mmol of pentaerythritol, 1.1 mmol of ricinoleic acid, 40 mL *tert*-butyl alcohol, 0.25%–2.5% (w/v) PyCal lipase, 200 g/L 3 Å molecular sieve. Reaction temperature 60^{0} C and reaction time: 24 h. Vertical bars represent standard deviation.

ricinoleic acid into pentaerythritol monoricinoleate. The time period selected for this study was fair enough to get thermodynamic yield with minimal dosage of enzyme to reach equilibrium conversion. Fig. 5 shows the effect of different molar ratios of pentaerythritol and ricinoleic acid on pentaerythritol monoricinoleate synthesis. As molar ratio increased from 1:1 to 4:1, a significant increase in conversion was observed from 41% to 93% and on further increasing molar ratio from 4:1 to 6:1, no further increase in conversion was observed. Hence the molar ratio of 4:1 (PE: RA) was selected as optimum for PEMR synthesis. Wei et al. [47] reported the synthesis of PE ester of behenic acid using the molar ratio of 15:1 (PE; behenic acid).

The reaction was also carried out using an excess of ricinoleic acid to demonstrate dimer formation, which occurs at higher concentrations of RA. Here, the linear decrease of ricinoleic acid was not found with the formation of PEMR because of polymerization of RA before esterification and multipoint attachment of RA on PE backbone (Fig. 6). The substantial decrease in RA concentration was attributed to the polycondensation reaction of RA molecules (formation of dimer and oligomer of ricinoleic acid

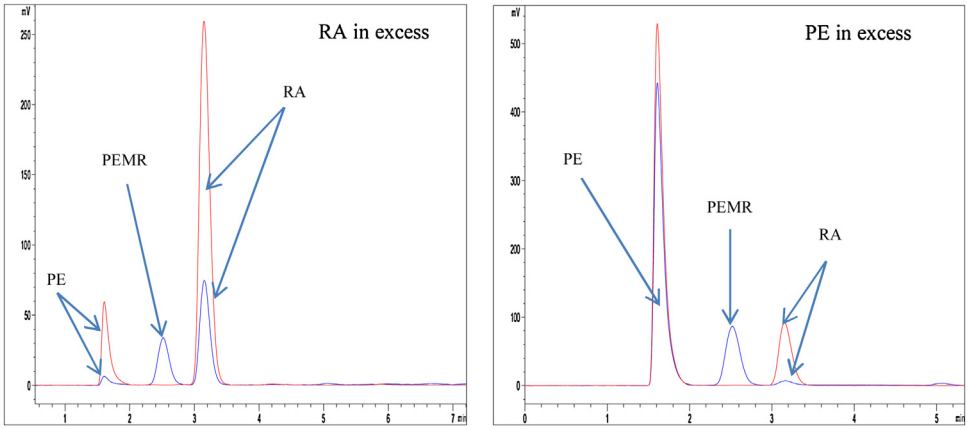


Fig. 6. HPLC chromatogram of PEMR reaction mixture in *tert*-butyl alcohol with ricinoleic acid in excess and pentaerythritol in excess. A red line represents the initial and blue line represents the final reaction mixture.

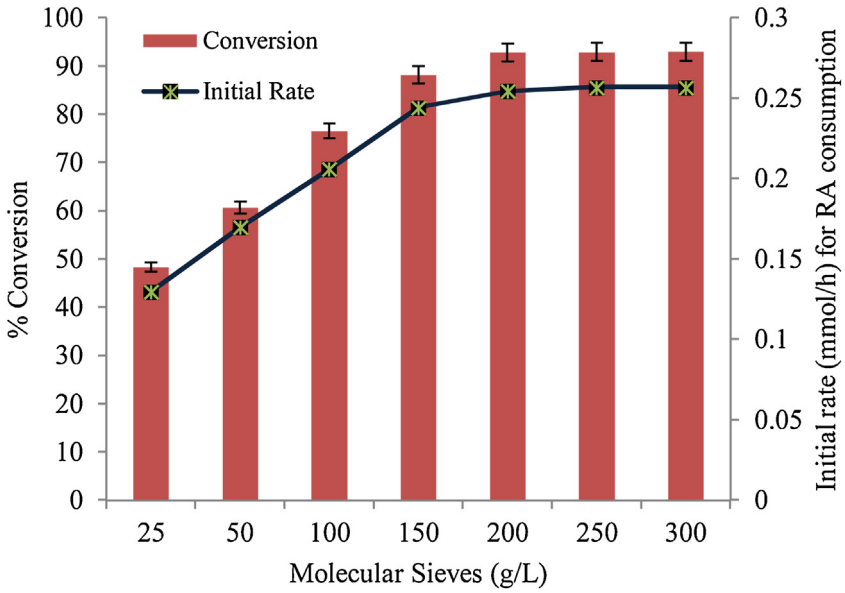


Fig. 8. Effect of molecular sieves concentration on pentaerythritol monoricinoleate synthesis. Reaction mixture composed of 4.4 mmol of pentaerythritol, 1.1 mmol of ricinoleic acid, 40 mL *tert*-butyl alcohol, 2% (w/v) PyCal lipase, 25 g/L to 300 g/L 3 Å molecular sieve. Reaction temperature 60 ^{0}C and reaction time: 24 h. Vertical bars represent standard deviation.

through esterification at hydroxyl group of one ricinoleic acid with the carboxyl group of another ricinoleic acid) [48,49]. When an excess of PE used, the product formation was found to be linear with the decrease in PE, indicating no multipoint esterification of PE with RA. Also, the solvent plays a crucial role in the prevention of estolides. The polar organic solvent (*tert*-butanol in our case) solvates the hydroxyl group of ricinoleic acid and stabilizes the molecules and hence making it less reactive than the hydroxyl group of PE. Therefore, combining the crucial factors such as the use of polar organic solvent, and ricinoleic acid as limiting substrate, monoester yield was much improved.

4.5. Effect of enzyme load

In an enzymatic process, enzyme load has a significant effect on process efficiency and economics. To enhance industrial profitability, it is desired to have high conversion at low enzyme concentration. To study the effect of enzyme concentration on the synthesis of PEMR, PyCal lipase load of 0.25% to 2.5% (based on total reaction mixture volume) was used. Effect of enzyme load on the conversion of ricinoleic acid is shown in Fig. 7. As an amount of PyCal lipase increased from 0.25% to 2%, increase in conversion was observed. Enzyme load higher than 2% did not lead to any significant increase in conversion as the rate of reaction remains nearly constant. The enzyme has occupied all the active sites and its work at a maximum of rate for PEMR synthesis. Hence the optimum enzyme concentration of 2% was used for further evaluation of PEMR synthesis.

4.6. Effect of molecular sieves

For lipase based processes, water plays a very decisive role in reaction equilibrium. Lipase catalyzes both esterifications of fatty acid into esters and hydrolysis of esters into fatty acid depending

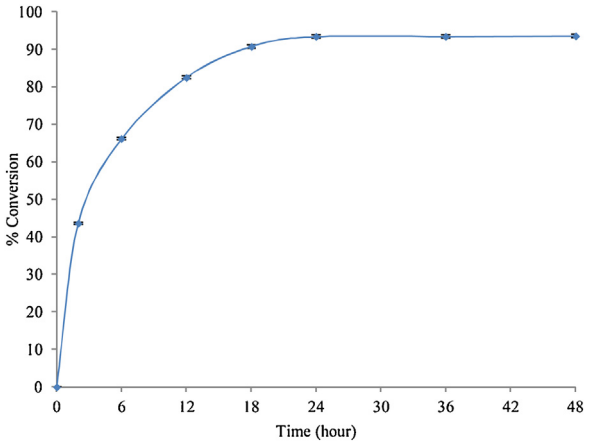


Fig. 9. Effect of reaction time on the synthesis of L-ascorbyl palmitate.Reaction mixture consisted of 4.4 mmol of pentaerythritol, 1.1 mmol of ricinoleic acid, 40 mL *tert*-butyl alcohol, 2.0% (w/v) PyCal lipase, 200 g/L 3 Å molecular sieve. Reaction temperature 60^{0} C and reaction time: 0–48 h. Vertical bars represent standard deviation.

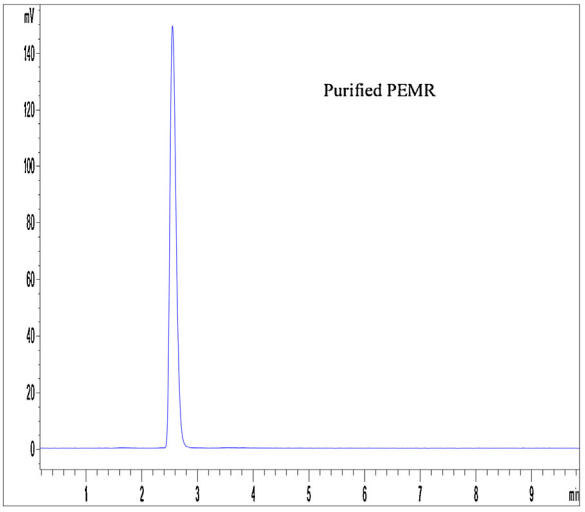


Fig. 10. RP-HPLC chromatogram of purified PEMR.

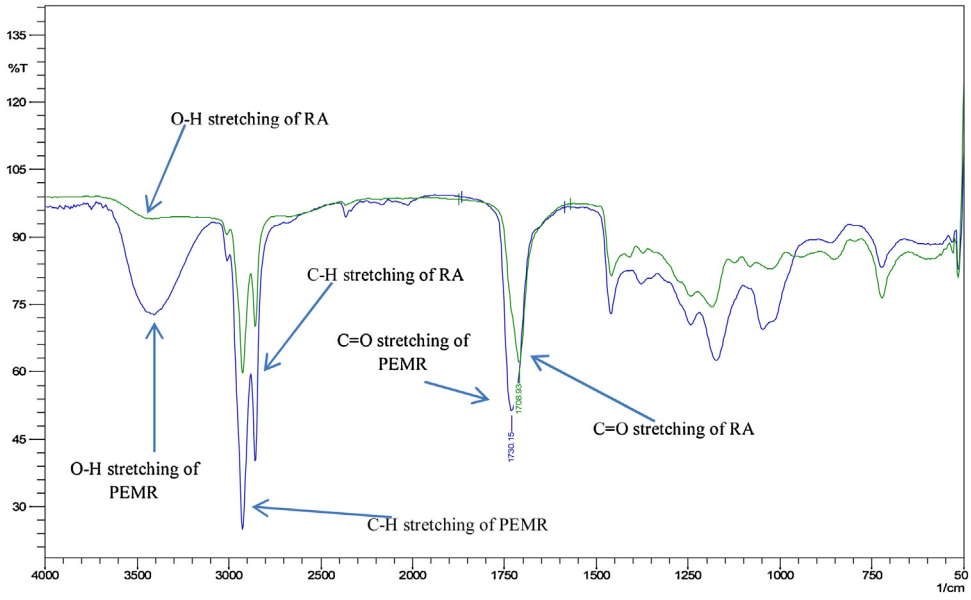


Fig. 11. ATR-FTIR overlay spectra of PEMR represented by blue lines and starting material RA represented by green lines.

on the amount of water present in the microenvironment of reaction. Hence, to increase ester product formation, water generated in the reaction needs to be removed to prevent reverse hydrolysis of the formed ester. It was necessary to remove water formed in the reaction to avoid back hydrolysis of built product to push conversion to a higher value for pentaerythritol monoricinoleate synthesis.

For this purpose, molecular sieves were used to remove water formed in the reaction mixture for the synthesis of PEMR. A 3 Å molecular sieve with varying concentration from 25 g/L to 300 g/L was used to study the effect of molecular sieve on the synthesis of PEMR. Fig. 8 shows the impact of molecular sieve concentration on the synthesis of PEMR. It was observed that when the concentration of molecular sieve increased from 25 g/L to 200 g/L, conversion of ricinoleic acid into PEMR risen from 48% to 93%. Further increase in molecular sieve concentration did not result in any significant increase in conversion as the added molecular sieves were enough to remove water generated in the reaction. This is also evident from the initial rate for RA consumption as it shows plateau when it goes beyond 200 g/L of molecular sieves. Hence molecular sieve concentration of 200 g/L was selected as optimum for the synthesis of pentaerythritol monoricinoleate. The obtained result was very similar to our previous finding for the use of molecular sieves to enhance ascorbyl palmitate production [40].

4.7. Effect of reaction time

For any enzymatic process to be efficient, it is desired to have shortened reaction time and high conversion [50]. Optimum interaction between substrate and enzyme is required to have a good conversion rate. However, after a certain time interval, no increase is generally observed in the conversion of substrate into product. Here, we studied reaction time course for PEMR synthesis throughout 48 h using a 4:1 M ratio of PE: RA at 60°C in the

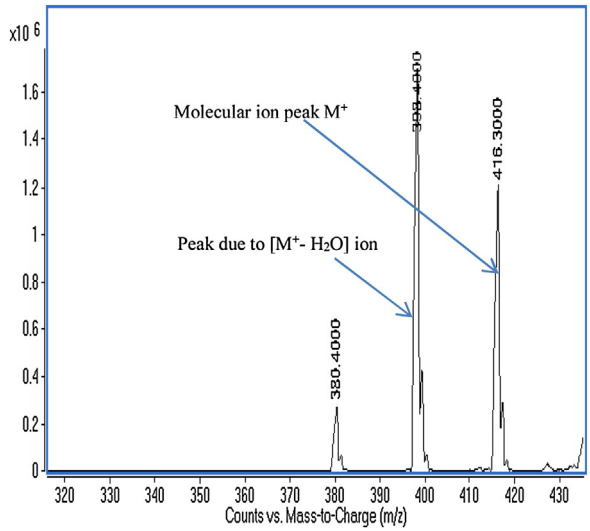


Fig. 12. Mass spectrum of synthesized PEMR, Gas temperature: 300°C, drying gas: N_2, Flow rate: 8 L/min, fragmentor voltage: 1.35 V.

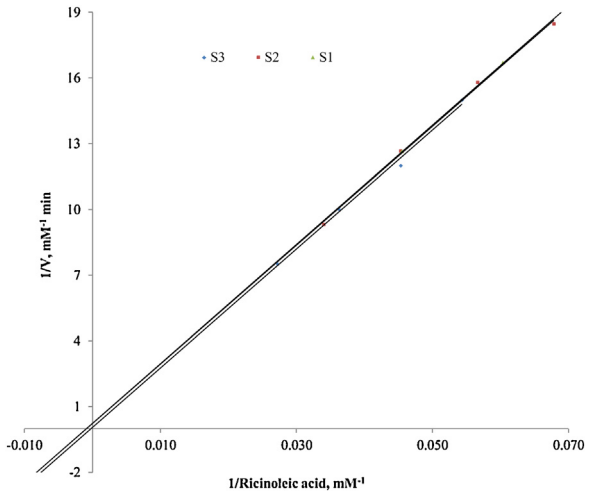


Fig. 13. Lineweaver-Burk plots of initial velocities of esterification of ricinoleic acid with pentaerythritol as a function of ricinoleic acid concentration at a different fixed concentration of pentaerythritol. (S1: 66 mM^{-1}, S2: 88 mM^{-1}, S3: 110 mM^{-1}).

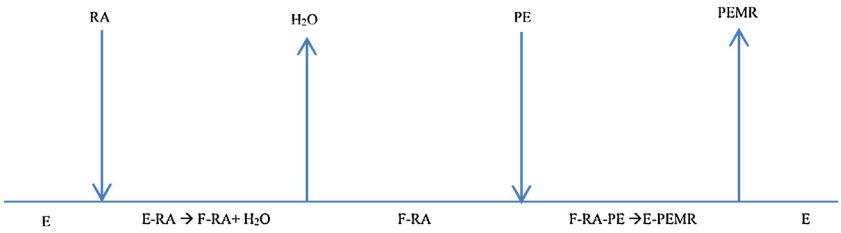


Fig. 14. Representation of probable mechanism for lipase-mediated esterification of ricinoleic acid with pentaerythritol. E and F: an initial enzyme in natural structural state and rearranged architectural state after formation of the acyl-enzyme complex.

presence of molecular sieves. Fig. 9 shows the effect of reaction time on the esterification of ricinoleic acid. Conversion of ricinoleic acid to PEMR increased from 43% to 93% from 2 h to 24 h, post 24 h no significant increase in conversion of ricinoleic acid was observed. From these results, 24 h reaction time was found to be optimum for the synthesis of PEMR using PyCal lipase. Previously, Patil et al. [19] have reported the synthesis of sorbitol esters of oleic, lauric, palmitic acid with the conversion of 92%, 85%, and 82% respectively in 48 h reaction time.

4.8. Separation of PEMR from the reaction mixture

Batch process for PEMR synthesis resulted in a 93% conversion of ricinoleic acid into PEMR. The final reaction mixture was subjected to distillation to recover *tert*-butyl alcohol. The dry residue left after distillation was treated with water to recover unreacted pentaerythritol. The residue left after PE removal was then washed with hexane to remove unreacted ricinoleic acid. The final residue left was dried under vacuum and obtained PEMR was analyzed using RP-HPLC. Fig. 10 shows the HPLC chromatogram of purified PEMR.

4.9. FT-IR analysis of reaction mixture

The FT-IR spectrum of the purified PEMR sample is presented in Fig. 11. As seen in Fig. 11, the band in region 1708.93 cm^{-1} is due to C=O stretching vibration typical of fatty acid whereas band in the region 1730.15 cm^{-1} is due to C=O stretching vibration of formed PEMR.

4.10. Mass spectral analysis

The mass spectrum of the final product PEMR (Fig. 12) shows a mass-to-charge ratio of the molecular ion peak (M^+) of 416.30 and molecular mass of PEMR is 416.61. The peak at 398.400 is due to the formation of M^+- H_2O, which further confirms the structure. These results confirm that the synthesized product is PEMR with a molecular weight of 416.61.

4.11. Study of mechanism for PEMR synthesis

Reactions at a different substrate concentration of ricinoleic acid were tested at different fixed concentrations of pentaerythritol to investigate the mechanism for esterification reaction of pentaerythritol with RA. Reactions were carried out at three fixed substrate concentrations of pentaerythritol, 66 mM^{-1}, 88 mM^{-1}, and 110 mM^{-1}. At each fixed pentaerythritol concentration, various concentrations of ricinoleic acid were taken for the esterification reaction. Initial velocities for esterification of pentaerythritol with ricinoleic acid as a function of ricinoleic acid concentration at a different fixed concentration of pentaerythritol were measured. Double reciprocal plots of different initial velocities upon varying ricinoleic acid concentration were plotted for each set, as shown in Fig. 13. As can be observed from Fig. 13, a collection of straight lines has been obtained which are parallel to each other having nearly the same slopes. When in a double reciprocal plot of initial velocity against substrate concentration results in parallel lines, then a ping pong bi-bi mechanism is inferred [51]. Hence, it can be concluded that esterification between pentaerythritol and ricinoleic acid catalyzed by lipase follows ping pong bi-bi mechanism.

Hence, based on a ping pong bi-bi reaction model, the probable mechanism for the esterification reaction of pentaerythritol with ricinoleic acid can be represented, as shown in Fig. 14.

As can be seen from Fig. 14; lipase (E) with the help of catalytic triad aspartate-histidine-serine amino acid residues forms an enzyme- acyl complex (E-RA) with ricinoleic acid (RA) via nucleophilic attack of the serine-OH group to the carbonyl carbon of ricinoleic acid. This enzyme-acyl complex (E-RA) rearranges itself to form a molecule of water and a new rearranged enzyme-acyl complex (F-RA). This rearranged activated enzyme-acyl complex (F-RA) subsequently undergoes a nucleophilic attack from Pentaerythritol (PE) to form another complex (F-RA-PE) which rearranges in such a way that enzyme comes to its initial structural state and creates ester product, pentaerythritol mono-ricinoleate (PEMR).

5. Conclusion

Enzymatic esterification process for the synthesis of PEMR was successfully developed using immobilized (via interfacial activation) PyCal lipase preparation. Critical reaction conditions were established step by step, which favored selective monoester formation and suppressed dimerization of ricinoleic acid. Water formed in the reaction medium was removed with the help of molecular sieves leading to substrate conversion of 93%. Structural characterization of synthesized PEMR was carried out using FT-IR and mass spectrometry, which confirmed its functional group and molecular weight. The developed reaction system can be used as a useful reference for enzymatic synthesis of PEMR on an industrial scale as well as for other polyol fatty acid monoesters.

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