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High Yields of Ascorbyl Palmitate by Thermostable Lipase-Mediated Esterification

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ABSTRACT: High yields of ascorbyl palmitate (6-*O*-palmitoyl L-ascorbic acid) were obtained by lipase-mediated esterification using *Bacillus stearothermophilus* SB 1 lipase. The final yield was greatly influenced by the initial water content of the system, quantity of enzyme, and molar ratio of palmitic acid to L-ascorbic acid. Reaction rates increased directly with temperature from 40 to 100°C. Maximum conversion (97%) was achieved after 30 min at 100°C (solvent-free), 1 h at 80°C (solvent-free), and 2 h at 60°C (solvent/hexane). The synthesis was scaled up to 1-L volume with 95% conversion using 50 mmoles of ascorbic acid and 250 mmoles of palmitic acid in hexane. Similar yields of ester were obtained in five repetitive cycles using 5 g enzyme immobilized on Accurel. The present *B. stearothermophilus* SB 1 lipase was a more efficient catalyst for the synthesis of ascorbyl palmitate than other commercial lipases.

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KEY WORDS: Ascorbyl palmitate, *Bacillus* sp., biocatalysis, enzyme, esterification, immobilization, lipase, 6-*O*-palmitoyl L-ascorbic acid, thermostable enzyme.

Present-day demand for ecofriendly technologies has changed the scenario in favor of natural additives in place of synthetic compounds, especially in food and personal-care products. In this regard, fatty acid esters of ascorbic acid occupy an important place as potential antioxidants and as surfactants in high-fat-content food and personal-care products. They also have an application as browning inhibitors in fruits (1). Generally, these esters are chemically synthesized by catalyzing esterification of ascorbic acid with concentrated sulfuric acid (2,3). However, the chemical synthesis is an energy-intensive process and results in the formation of a mixture of products with a preponderance of *O*-6 substitution. It also involves cumbersome downstream processing of the final product. Enzymatic synthesis has been suggested as an alternative method as it is regioselective and also involves mild reaction conditions (1,4). Further, use of immobilized enzyme simplifies the downstream processing and makes the process economical.

There are very few reports on the lipase-catalyzed synthesis of ascorbyl palmitate. Humeau *et al.* (5) studied the synthesis of ascorbyl palmitate by *Candida antarctica* lipase and obtained 68% yield in 8 h using a high substrate molar ratio. Sakashita *et al.* (1) used immobilized lipase to catalyze the synthesis of ascorbyl stearate at 40°C in 4 h, whereas Uragaki (6) obtained ascorbyl stearate synthesis at 20–70°C with a different lipase.

In the present work, attempts were made to obtain high yields of ascorbyl palmitate in a short time using *Bacillus stearothermophilus* SB1 lipase. This paper describes the effect of various parameters on the synthesis of ascorbyl palmitate. Repeated use of immobilized enzyme in batch esterification and in a solvent-free system is also discussed.

EXPERIMENTAL PROCEDURES

Chemicals. L-Ascorbic acid and palmitic acid were purchased from SRL (Mumbai, India), molecular sieves from SD'S (Mumbai, India), and all solvents were procured from Qualigens (Mumbai, India). All the chemicals used were of analytical grade. However, acetonitrile and water were of high-performance liquid chromatography (HPLC) grade.

Enzymes. Powdered lipases from porcine pancreas, *Rhizomucor miehei, Pseudomonas cepacia,* and *C. rugosa* were obtained from Sigma (St. Louis, MO). Lipase from *B. stearothermophilus* SB-1 was produced in our laboratory.

Bacillus stearothermophilus SB-1 produced 13,000 U/L lipase with specific activity of 25 U/mg protein in minimal medium containing 2% corn oil (7). The production was carried out at 50°C and pH 3.0 in shake flasks at 250 rpm (New Brunswick Scientific shaker, Edison, NJ). After 36 h, the culture was centrifuged at 18,600 × *g* for 20 min, and the supernatant was subjected to 85% ammonium sulfate precipitation. The pellet was dissolved in 0.01 M sodium phosphate buffer (pH 7.0) and subjected to dialysis against distilled water at 4°C for 24 h. After partial purification, 80% enzyme yield with fourfold increase in specific activity was obtained. The dialyzed enzyme was subjected to immobilization/lyophilization and used for esterification.

Lipase assay. Lipase was assayed by the standard procedure of titrimetry (8) using 1.5% (vol/vol) olive oil as substrate at 50°C. One unit of activity is defined as the amount that liberates 1 µmol fatty acid from olive oil per min under the standard assay conditions.

Enzyme immobilization. Bacillus stearothermophilus SB-1 lipase was immobilized on Accurel EP 100 porous polypropy-

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lene supports (Enka AG, Obernberg, Germany). For immobilization, beads (2.0 g) were wetted with 12 mL ethanol, 38 mL water, 25 mL 0.1 M sodium phosphate buffer (pH 7.0), mixed with 25 mL of lipase (17 U/mL), and the volume built up to 200 mL with distilled water. The suspension was swirled on a New Brunswick shaker at 100 rpm for 24 h. The immobilized catalyst was collected by filtration, washed with 0.01 M sodium phosphate buffer (pH 7.0), rinsed with water, and air-dried to constant weight. The residual lipase activity (8) and total protein (9) in the supernatant were determined. The amount adsorbed and specific activity of the immobilized enzyme were calculated. It was observed that there was a 50-fold activation in enzyme activity (10 U/mg support) after immobilization on Accurel pretreated with ethanol.

Synthesis. The reactants, L-ascorbic acid and fatty acid in hexane, and 50 mg immobilized (10 U/mg solid)/lyophilized lipase (12.7 U/mg solid) were preequilibrated using saturated salts of varying water activity—magnesium chloride (a_w = 0.33), magnesium nitrate (a_w = 0.53), sodium chloride (a_w = 0.75), or potassium sulfate (a_w = 0.97)—to start the reaction. The preequilibrated reactants were added to 50 mg of immobilized/lyophilized lipase. The reaction was carried out at 50°C and 100 rpm. The progress of the reaction was monitored by thin-layer chromatography using Kieselgel G 60 plates (Merck, Darmstadt, Germany) using petroleum ether/diethyl ether/acetic acid (80:30:1, vol/vol/vol) as mobile phase. Spots were developed by α-naphthol/sulfuric acid (1:1, vol/vol) reagent, followed by heating at 105°C for 10 min. Ester synthesis was expressed as the percentage molar conversion of the acid to the ester after titrating residual fatty acid against 0.05 N potassium hydroxide.

Purification. The reaction medium was filtered, and the solvent was evaporated. Remaining ascorbic acid was eliminated by water-washing, and ascorbyl palmitate was isolated by recrystallization in hexane at 4°C. The product was also purified by using a silica gel column (mesh size 60–120) and eluted by petroleum ether.

Product identification. The isolated product was finally identified by spectral studies [infrared (IR), ^{1}H nuclear magnetic resonance (NMR), and electron-impact mass spectra (EIMS)].

IR spectra was recorded on a Perkin Elmer 1720 infrared Fourier transform spectrophotometer (Norwalk, CT) using a film on a sodium chloride plate. The ^{1}HNMR spectra were recorded on a Bruker-AC-300 spectrometer (Karlsruhe, Germany) at 300 MHz. The chemical shifts are expressed in δ units with tetramethylsilane as an internal standard, and the coupling constants (*J* values) are in Hz. The mass spectra was recorded on a JEOL JMA-DA 5000 mass spectrometer (Tokyo, Japan) at 70 eV in electron impact mode.

*Spectral data for 6-*O*-Palmitoyl L-ascorbic acid.* IR: 3443 (–OH), 2958 (C–H), 1731 (–C–) and 1286 (C–O) cm^{-1}; ^{1}H NMR (300 MHz, acetone-d_6) δ 4.78 (*d*, *J* = 1.71Hz, H-4, 1H), 4.15–4.29 (*m*, H-5 and H-6, 3H), 2.93 (*brs*, 3 × OH, 3H), 2.35 (*t*, H-2′, 2H), 2.03–2.06 (*m*, H-3′, 2H), 1.59–1.64 (*m*, H-4′, 2H), 1.28 [*brs*, 22H, $(CH_2)_{11}$], and 0.88 (*t*, H-16′, 3H); mass spectrum *m/z* (relative abundance), 396 $[M - H_2O]^+$ (5), 256 (43), 239 (10), 213 (15), 157 (5), 129 (25), 73 (45), 60 (50), and 44 (100).

Analysis. Quantification of the product was done by HPLC using a Shimadzu pump system with a Shimadzu refractive index monitor on Shimadzu C 18 CLC-ODS (4.6 × 25 cm) column (Kyoto, Japan). Elution was conducted at 40°C with acetonitrile/water (1:1, vol/vol) and a flow rate of 1.5 mL/min. Quantitative data were obtained with an integrator after calibration with standards. Retention time for the ester was obtained at 7.83.

RESULTS AND DISCUSSION

Bacillus stearothermophilus SB 1 lipase (50 mg lyophilized/immobilized solid) could catalyze the synthesis of various fatty acid esters of ascorbic acid at a_w 0.33 and 50°C after 24 h using equimolar concentrations (50 mmoles) of palmitic acid and ascorbic acid prepared in hexane. However, conditions for maximal synthesis of ascorbyl palmitate, an important antioxidant, were standardized.

Time course of ascorbyl palmitate synthesis. Maximal conversion (50%) to ascorbyl palmitate at 50°C under the above reaction conditions occurred after 18 h of incubation whereafter there was a decline in the percentage of conversion (Fig. 1). This decline could be due to the water released during ester synthesis which promotes lipase-mediated hydrolysis. Macrae (10) reported that in excess of water, hydrolysis dominates esterification in lipase-catalyzed reactions. Therefore, addition of molecular sieves during reaction is recommended to control water activity of the system (11,12).

Effect of molecular sieves addition. Molecular sieves (5%) were added at different time intervals in the above reaction conditions. Maximal conversion (92%) was obtained immediately after the addition of molecular sieves irrespective of the time of addition (6, 12, 18, 24 h) except when they were added at 0 h (Fig. 1). Addition of sieves at the beginning of

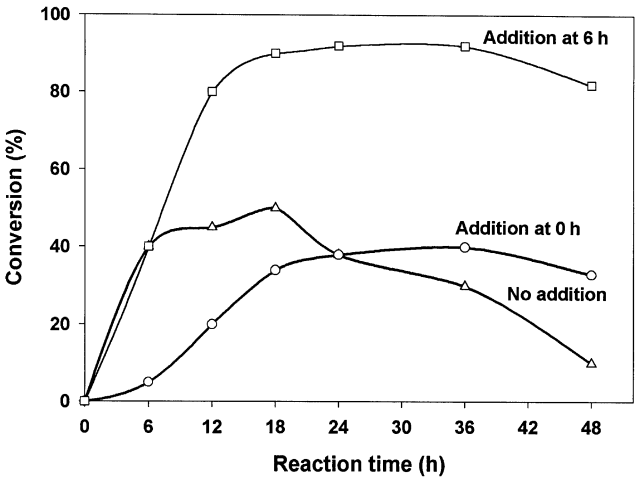

FIG. 1. Effect of addition of molecular sieves after varying hours of reaction on ascorbyl palmitate synthesis by *Bacillus stearothermophilus* SB1 lipase at a_w 0.33 and 50°C.

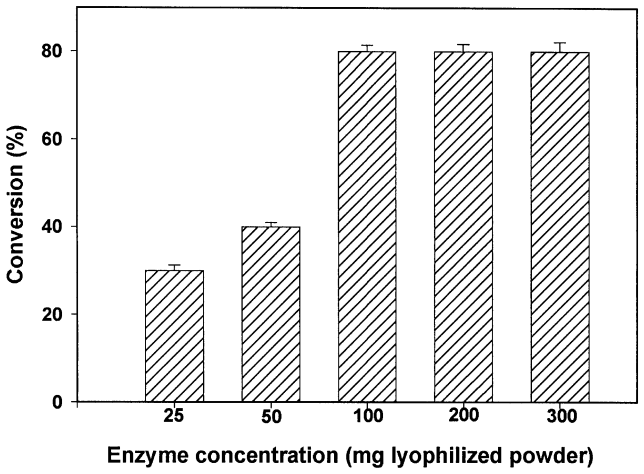

FIG. 2. Effect of lipase quantity on the yield of ascorbyl palmitate after 6 h of incubation at 50°C and a_w 0.33. Error bars represent standard error.

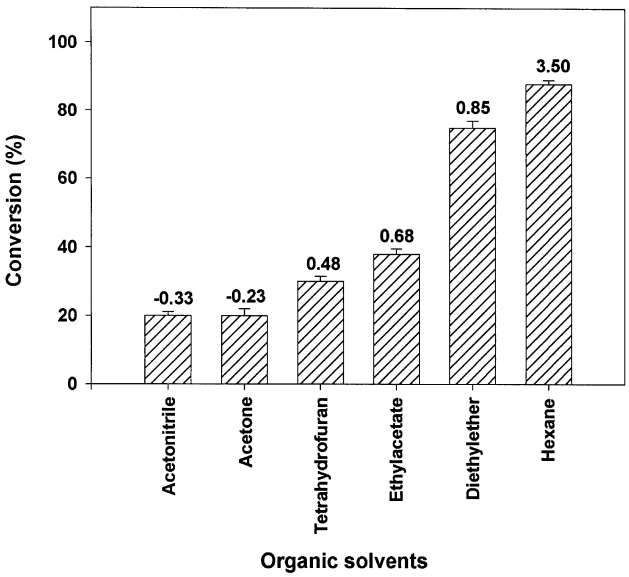

FIG. 3. Solvent influence on the synthesis of ascorbyl palmitate using 100 mg lyophilized enzyme after 6 h of incubation at 50°C and a_w 0.33. Value above the bar indicates log *P* (where *P* = partition coefficient between water and solvent) value of the particular solvent. Error bars represent standard error.

the reaction drastically lowered the product formation since enzymes need a small amount of water to maintain their active conformation (11).

Increase in molecular sieve concentration (5 to 20%) did not have an influence on the rate of esterification. Thus the important parameter is not the total amount of sieves added but the time of addition of sieves. A similar observation was reported by Ergan *et al.* (11) for triglyceride synthesis by Lipozyme; they obtained 70% triolein with no diolein or monolein at the end of the reaction when sieves were added later in contact with the enzyme.

Effect of enzyme concentration. Ascorbyl palmitate synthesis as a function of enzyme concentration was studied at 50°C, a_w 0.33, for 6 h. Esterification extent increased with increasing lipase concentrations with maximal conversion achieved with 100 mg lyophilized enzyme whereafter it became constant (Fig. 2).

Solvent effect on catalytic activity. The hydrophobicity of the organic solvent is known to greatly influence the activity of the biocatalyst acting in a microaqueous organic solvent system. Log *P* is widely used to represent the characteristics of the organic phase (13) where *P* is the partition coefficient of the solvent between water and octanol. The effect of various organic solvents on ascorbyl palmitate synthesis was studied using 100 mg lyophilized enzyme, 50 mmoles of ascorbic and palmitic acids at a_w 0.33, 50°C for 6 h. It was observed to be a function of the solvent's log *P* values (Fig. 3) with highest yield obtained in hexane (log $P = 3.5$). It is generally reported that solvents with log $P < 2$ are less suitable for biocatalytic purposes (14).

Effect of a_w. Effect of a_w was studied at 50°C using 50 mmoles of ascorbic acid and palmitic acid prepared in hexane and 100 mg lyophilized enzyme for 6 h. Lipase-catalyzed hydrolysis and synthesis largely depend upon water activity of the system (15). In an excess of water, hydrolysis dominates, whereas esterification proceeds under low water conditions. In the present case, the reaction rate was maximal at a_w 0.53 with comparatively similar activity at a_w 0.33. However, there was a decrease in reaction rate as a_w increased further (Fig. 4). Thus, water content plays an important role not only in determining the selectivity for esterification over hydrolysis but also in maintaining the enzyme activity (15).

Effect of molar ratio of ascorbic acid to palmitic acid. The effect of varying palmitic acid concentration on the synthesis of ascorbyl palmitate was studied. Figure 5 shows that highest conversion (97%) occurred when 1 mole of ascorbic acid was reacted with 2.5 moles of palmitic acid. At molar ratios greater than 1:2.5, a decrease in conversion was observed, possibly

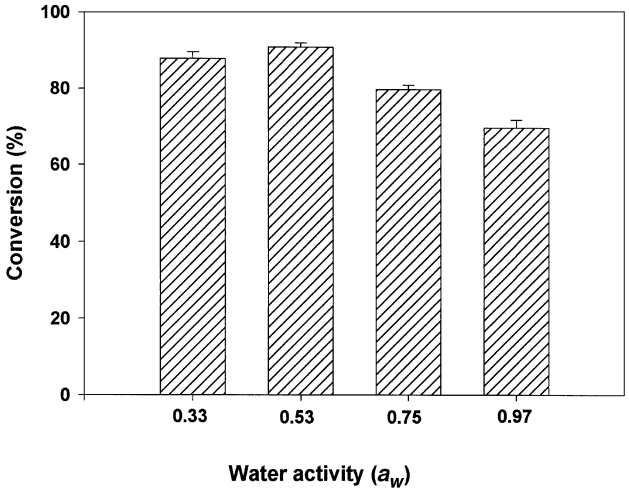

FIG. 4. Effect of water activity on the synthesis of ascorbyl palmitate in hexane using 100 mg of *Bacillus stearothermophilus* SB1 lipase after 6 h of incubation at 50°C. Error bars represent standard error.

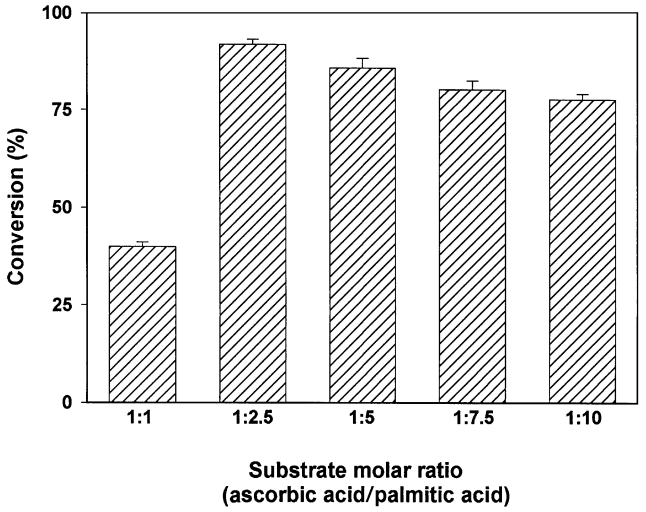

FIG. 5. Effect of varying molar ratio of ascorbic acid to palmitic acid on ascorbyl palmitate synthesis at a_w 0.53 and 50°C after 6 h of incubation. Error bars represent standard error.

owing to substrate inhibition. Thus, high yields were obtained with lower substrate ratio as compared to a previous report with *C. antarctica* (5) lipase where higher ascorbic acid to palmitic acid ratio (1:5) gave comparatively lower yields (68%).

Effect of temperature. The reaction temperature has a profound effect on the reaction rates, equilibrium kinetics, and enzyme stability (16). In the present case, the initial reaction rates were found to increase directly with the temperature with maximal rate obtained at 100°C (Fig. 6).

Since *B. stearothermophilus* SB 1 lipase is thermostable, ester synthesis was obtained at all temperatures from 40 to 100°C (Fig. 6). At 40°C, only 78% conversion could be achieved after 6 h, whereas maximal conversion (95%) was achieved after 2 h at 50–70°C, after 1 h at 80°C and within

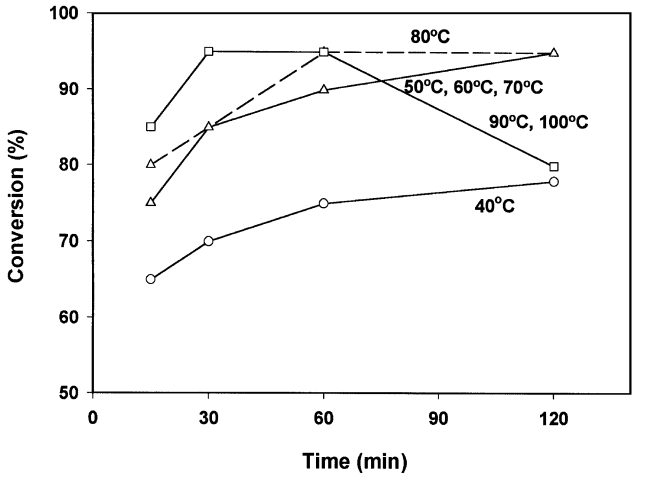

FIG. 6. Kinetics of ascorbyl palmitate synthesis as a function of temperature using 50 mmole of ascorbic acid, 225 mmole of palmitic acid, and 100 mg of lyophilized *Bacillus stearothermophilus* SB1 lipase. Reactions conducted at temperatures up to and including 70°C were conducted in hexane. Those above 70°C were solvent-free.

TABLE 1
Synthesis of Ascorbyl Palmitate Using Various Commercial Lipases[a]

Lipase source	Enzyme unit (U/mg solid)	Conversion (%)
Porcine pancreas (PPL)	110–220	25
Rhizomucor miehei (RML)	4000	70
Pseudomonas cepacia (PCL)	50	63
Candida rugosa (CRL)	100,000–400,000	58
Bacillus stearothermophilus SB1	12.7	97

[a]At a_w 0.53 in hexane (5 mL) containing 50 mmole of ascorbic acid and 225 mmole of palmitic acid for 6 h at their respective temperature optima (60°C for *Bacillus stearothermophilus* SB1 lipase and 37°C for other lipases) and 100 rpm.

30 min at 90–100°C. Higher temperatures help in maintaining lower water activity in the system by rapid evaporation, thus increasing the rate of synthesis, as has also been reported by Ergan *et al.* (11).

In this experiment series, hexane (boiling point approx. 70°C) was present for reactions conducted at up to 70°C Above that temperature no solvent was added to the reaction. Similar solvent-free syntheses by immobilized *R. miehei* lipase have also been reported for triglyceride synthesis (11) and for interesterification of triglyceride and fatty acid at 90 and 100°C (17). Solvent-free synthesis is of particular advantage in the food industry where the use of organic solvents is restricted. In this regard, thermostable lipases have immense importance in solvent-free synthesis of important compounds.

Scaleup and repeated esterification in batch. Synthesis of ascorbyl palmitate was scaled up to 1 L volume using 5 g of immobilized enzyme. It was possible to use this lipase for five cycles of batch synthesis of 20 g ascorbyl palmitate with 95% conversion using 50 mmoles of ascorbic acid and 250 mmoles of palmitic acid.

Microbial specificities. Ascorbyl palmitate synthesis was carried out using various commercial lipases at 60°C for *B. stearothermophilus* SB1 lipase and 37°C for the others with a 1:2.5 molar ratio of ascorbic acid to palmitic acid for 6 h in hexane. The reaction mixture was preequilibrated at a_w = 0.53. From the results (Table 1) it is observed that all the commercial lipases could carry out this synthesis with maximal conversion obtained with *R. miehei* lipase (70%), followed by lipases from *P. cepacia* (63%) and *C. rugosa* (58%). Lowest conversion was obtained with porcine pancreas lipase (25%). However, the conversion rate was maximal with *B. stearothermophilus* SB 1 lipase.

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