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A TWO STEP CHEMO-ENZYMATIC METHOD FOR THE SYNTHESIS OF FATTY ACID ASCORBYL ESTERS

SANJIB KUMAR KARMEE*

ABSTRACT

Fatty acid vinyl esters were synthesised from the corresponding free fatty acids in 79%-84% yield using Hg $(OAc)_2$/H_2SO_4 under microwave irradiation. Subsequently, the obtained fatty acid vinyl esters were utilised for the lipase catalysed acylation of vitamin C under microwave heating. Various lipases viz. Pseudomonas cepacia, Porcine pancreatic, Candida rugosa, Mucor miehei, Rhizopus oryzae *and* Novozyme-435 *were screened for the acylation of vitamin C under microwave irradiation. Among these lipases,* Novozyme-435 *was found to be the best catalyst for this reaction. Five different ascorbyl esters were synthesised in 58%-85% yield in 3 hr using* Novozyme-435.

Keywords: vitamin C fatty acid esters, fatty acid vinyl esters, lipase, transesterification, microwave irradiation.

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INTRODUCTION

Vitamin C is an important and naturally occurring antioxidant. However, its biotechnological application in industry is limited because of its hydrophilicity. The fatty acids and their esters obtained from oils and fats are used as potential acylating agents for the synthesis of the hydrophobic vitamin C fatty acid ester (Karmee, 2011; 2009; 2008; Ganske and Bornscheuer, 2005; Ferrer *et al.,* 2005; Polat *et al.,* 1997; Yan *et al.,* 1999; Watanabe *et al.,* 1999; Yang *et al.,* 2003; Adamczak *et al.,* 2005; Reyes-Duarte *et al.,* 2011; Burham *et al.,* 2009). Ascorbyl fatty acid esters are also used as highly desired antioxidants and surfactants in the food and cosmetic industries (Karmee, 2011; 2009; 2008). Chemical methods are known for the synthesis of vitamin C fatty acid esters. However, there are many inherent problems associated with the conventional chemical methods *viz*.: i) low yield of ascorbyl esters due to the formation of by-products and ii) vitamin C and its esters are prone to oxidation, degradation and rearrangements under the chemo-catalysed reaction conditions (Karmee, 2011; 2009; 2008).

Alternatively, lipases can be used for the synthesis of ascorbyl fatty acid esters to overcome the problems associated with the chemo-catalysed reactions (Karmee, 2011; 2009; 2008, Ganske and Bornscheuer, 2005; Ferrer *et al.,* 2005; Polat *et al.,* 1997; Yan *et al.,* 1999; Watanabe *et al.,* 1999; Yang *et al.,* 2003; Adamczak *et al.,* 2005; Reyes-Duarte *et al.,* 2011; Burham *et al.,* 2009). However, it is observed that the enzyme catalysed reactions require longer reaction time.

To circumvent the problem of long reaction time, in this article a microwave was used to fasten the chemo-catalysed synthesis of fatty acid vinyl esters and the lipase catalysed synthesis of fatty acid ascorbyl esters. In the first step, fatty acid vinyl esters were synthesised by the vinyl exchange reaction between free fatty acids and vinyl acetate using $Hg(OAc)_2/H_2SO_4$ under microwave. By using this method, capric, caprylic, lauric, palmitic and stearic acid vinyl esters were synthesised in 79%-84% yield. Subsequently, in the second step, these fatty acid vinyl esters were subjected to the lipase catalysed synthesis of ascorbyl fatty acid esters under microwave irradiation. The *Novozyme-435* was found to be active and stable catalyst under

* Biocatalysis and Organic Chemistry Group,
Department of Biotechnology,
Julianalaan 136, 2628 BL Delft, The Netherlands.
E-mail: sanjibkarmee@gmail.com

microwave condition. Using *Novozyme-435*, ascorbyl capricate, ascorbyl caprylate, ascorbyl laurate, ascorbyl palmitate and ascorbyl stearate were synthesised with 58%-85% yield in 3 hr under microwave.

EXPERIMENTAL PROCEDURES

Materials

Pseudomonas cepacia lipase, *Porcine pancreatic* lipase, *Candida rugosa* lipase, *Mucor miehei* lipase, *Rhizopus oryzae* lipase and *Novozyme-435* were purchased from Sigma-Aldrich Co., St Louis, Missouri, USA. Vitamin C and *t*-butanol were procured from local sources and used as such. A modified microwave oven (LG) equipped with a reflux condenser and magnetic stirrer was used for the chemical and enzymatic reactions. The modification of the microwave oven was done as reported earlier (Loupy, 2003).

Method

Procedure for the synthesis of fatty acid vinyl esters. Fatty acid (0.025 mol) and Hg $(OAc)_2$ (0.003 mol) were dissolved in vinyl acetate (0.5 mol). After stirring at room temperature for 30 min, concentrated H_2SO_4 (1 drop) was added to the reaction mixture and then it was refluxed under microwaves (360 W, 30 min). The work-up of the reaction was carried out according to the reported procedure (Yang *et al.*, 1999; Adelman, 1949). The mixture obtained was cooled to room temperature and then NaOAc (500 mg) was added to the mixture to quench the catalyst. The resulting mixture was filtered off and the filtrate was concentrated under reduced pressure. The obtained mass was purified by silica gel column chromatography (ethyl acetate/heptane, 1:99, v/v) to obtain the pure fatty acid vinyl esters. Using this method capric, caprylic, lauric, palmitic and stearic acid vinyl esters were synthesised. The results are presented in *Table 1*.

TABLE 1. MICROWAVE ASSISTED SYNTHESIS OF FATTY ACID VINYL ESTERS

Product	R	Yield/%[a,b]
Capric acid vinyl ester	CH_3-$(CH_2)_6$	79
Caprylic acid vinyl ester	CH_3-$(CH_2)_8$	83
Lauric acid vinyl ester	CH_3-$(CH_2)_{10}$	80
Palmitic acid vinyl ester	CH_3-$(CH_2)_{14}$	84
Stearic acid vinyl ester	CH_3-$(CH_2)_{16}$	80

Note: [a]Isolated yield after column chromatography.
[b]All products were characterised by ^1H and ^{13}C NMR.

Screening of lipases for the synthesis of vitamin C fatty acid esters. Vitamin C (1 mmol) and lauric acid vinyl ester (1 mmol) were added to a 25 ml round bottomed flask. To this, different lipases (50 mg) *viz.* *Pseudomonas cepacia* lipase, *Porcine pancreatic* lipase, *Candida rugosa* lipase, *Mucor miehei* lipase, *Rhizopus oryzae* lipase and *Novozyme-435* and *t*-butanol (2 ml) were added. The mixture was stirred under discontinuous microwave irradiation (160 W, 3 hr). Progress of the reaction was monitored by TLC using methanol/ethyl acetate (60:40, v/v) as solvent system and compounds were detected under UV. Work-up of the reaction was carried out according to the reported procedure (Yan *et al.*, 1999). Ascorbyl laurate was isolated as a white solid and characterised by ^{13}C NMR using DMSO (d6) as solvent (Yan *et al.*, 1999). The results obtained are presented in *Figure 1.*

Lipases catalysed synthesis of ascorbyl laurate at different molar ratio. Vitamin C and lauric acid vinyl ester were taken in a particular molar ratio (1:1, 1:2, 1:3 and 1:4) were added to a 25 ml round bottomed flask. To this *Novozyme-435* (50 mg) and and *t*-butanol (2 ml) were added. The mixture was stirred under discontinuous microwave irradiation (160 W, 3 hr). Progress of the reaction was monitored by TLC using methanol/ethyl acetate (60:40, v/v) as solvent system and compounds were detected under UV. Work-up of the reaction and isolation of the product was carried out according to the reported procedure (Yan *et al.*, 1999). The obtained results are summarised in *Figure 2*.

Procedure for the synthesis of different vitamin C fatty acid esters. Vitamin C (1 mmol) and fatty acid vinyl esters (3 mmol) were added to a 25 ml round bottomed flask. To this *Novozyme-435* (50 mg)

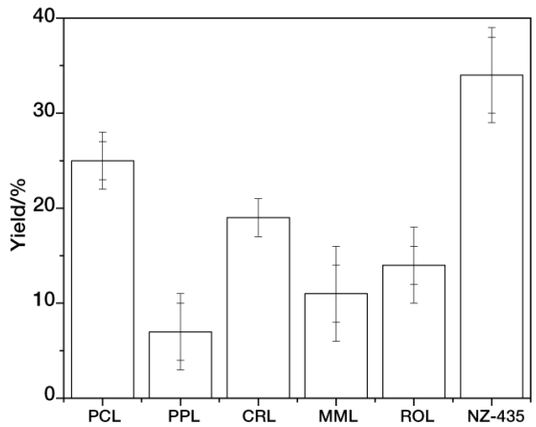

*Figure 1. Synthesis of ascorbyl laurate using different lipases: (*Pseudomonas cepacia *lipase (PCL),* Porcine pancreatic *lipase (PPL),* Candida rugosa *lipase (CRL),* Mucor miehei *lipase (MML),* Rhizopus oryzae *lipase (ROL) and* Novozyme-435 *(NZ-435) at 1:1 molar ratio of vitamin C to lauric acid vinyl ester.*

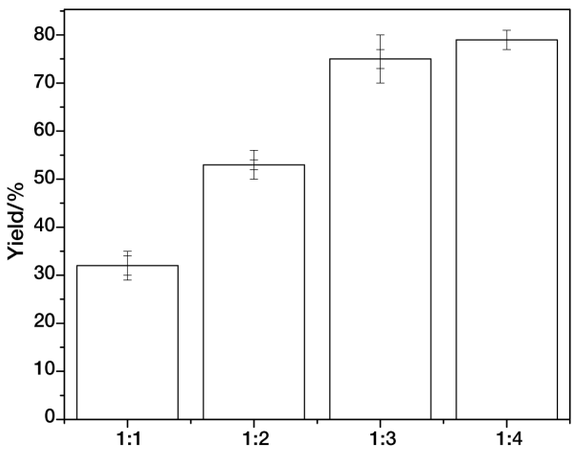

Figure 2. Novozyme-435 *catalysed synthesis of ascorbyl laurate at different molar ratio (vitamin C: lauric acid vinyl ester) under microwave irradiation.*

and t-butanol (2 ml) were added. The mixture was stirred under discontinuous microwave irradiation (160 W, 3 hr). Progress of the reaction was done as described above. Work-up of the reaction was carried out according to the reported procedure (Yan *et al.* 1999). All the vitamin C fatty acid esters were obtained as white solids and characterised by ^{13}C NMR using DMSO (d_6) as solvent (Yan *et al.*, 1999). The ^{13}C NMR data of a representative compound, *i.e.* 6-O-octadecanoyl-L-asorbic acid synthesised during this study is given below.

6-O-octadecanoyl-L-asorbic acid: ^{13}C NMR (DMSO-d_6, 100 MHz) δ (ppm): 172.3, 169.9, 151.7, 117.8, 74.6, 65.1, 64.0, 33.0, 30.9, 28.6, 28.5, 28.3, 28.0, 23.9, 21.7, 13.5.

***Reusability of* Novozym-435.** Vitamin C (1 mmol) and decanoic acid vinyl ester (3 mmol) were added to a 25 ml round bottomed flask. To this, *Novozym-435* (50 mg) and *t*-butanol (2 ml) were added and the reaction was carried out under microwave irradiation as stated earlier. The work-up of this reaction was carried our according to Yan *et al.* (1999). After the reaction, the immobilised lipase was recovered and reused again for the next run.

RESULTS AND DISCUSSIONS

Fatty acid vinyl esters are potential acylating agents for the lipase catalysed synthesis of surfactants and lipophilic antioxidants (Ganske and Bornscheuer, 2005; Ferrer *et al.*, 2005; Polat *et al.*, 1997). However, till to date the syntheses of fatty acid vinyl esters are basically carried out according to reported procedures with few modifications (Yang *et al.*, 1999; Adelman, 1949; Wang *et al.*, 2004). The long reaction time of the reported methods is a major drawback. In this work, it was circumvented by using microwaves; thereby, reducing the time of the reaction to 30 min (*Figure 3*). Five fatty acid vinyl esters *viz*. capric, caprylic, lauric, palmitic and stearic were prepared in 79%-84% yields under microwave irradiation in 30 min (*Table 1*).

Selectivity in organic synthesis under microwave irradiation has gained considerable attention in recent years (Loupy, 2003; De La Hoz *et al.*, 2004). Especially, advantages like short reaction time and regioselectivity are the significant features of organic synthesis under microwaves (Loupy, 2003; De La Hoz *et al.*, 2004; Moreuende *et al.*, 1994; Caddick *et al.*, 2001; Ley and Mynett, 1993). Therefore, microwave assisted systems are often used for the rapid synthesis of organic compounds. In this study, six different lipases *viz*. *Pseudomonas cepacia*, *Porcine pancreatic*, *Candida rugosa*, *Mucor miehei*, *Rhizopus oryzae* and *Novozyme-435* were screened for the acylation of vitamin C using lauric acid vinyl ester at 1:1 molar ratio (vitamin C: lauric acid vinyl ester). The *Pseudomonas cepacia* and *Novozyme-435* catalysed reaction gave 25% and 33% yield; whereas, *Porcine pancreatic*, *Candida rugosa*, *Mucor miehei* and *Rhizopus oryzae* catalysed reaction gave 7%, 19%, 10% and 14% yield of ascorbyl laurate respectively (*Figure 1*). Clearly, *Novozyme-435* was found to be the best enzyme for this reaction. Further, reactions were carried out at different molar ratios of vitamin C to acylating agent. Increase in yield from 32% to 75% was observed by increasing the molar ratio from 1:1 to 1:3 (*Figure 2*). However, only a marginal increase in yield was observed when molar ratio vitamin C to acylating agent was increased from 1:3 (75%) to 1:4 (79%) (*Figure 2*).

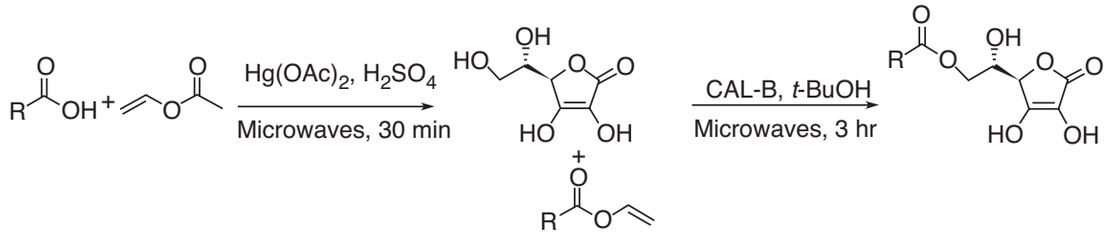

Figure 3. Chemo-enzymatic synthesis of vitamin C fatty acid esters using microwave irradiation.

Having optimised the molar ratio, vitamin C was regioselectively acylated using a varieties of fatty acid vinyl esters (capric, caprylic, lauric, palmitic and stearic) as acylating agents under microwaves (*Table* 2). *Novozyme-435* was found to be an efficient biocatalyst for the synthesis of vitamin C fatty acid esters under microwave irradiation. Earlier, Réjasse *et al.* have reported the influence of microwave heating on the stability of *Novozyme-435* at 100°C and found that the enzymatic stability was higher under microwave than the conventional thermal heating (Réjasse *et al.*, 2004; Karmee, 2006). The reason behind the increased stability of *Novozyme-435* under microwave irradiation is unknown (Réjasse *et al.*, 2004). It was reported that the microwave field alters the interactions between the lipases and their microenvironment, which causes the prevention of enzymes from thermal denaturation (Réjasse *et al.*, 2004).

Various vitamin C fatty acid esters were synthesised in 58%-85% yield (*Table* 2). The purity of the products was checked by ^{13}C NMR. No side product or decomposed product was observed during the reaction. This seems to be an efficient method to synthesise vitamin C fatty acid esters. Yan *et al*. have obtained high yield (65%-91%) of vitamin C fatty acid esters after long reaction time (48 hr) (Yan *et al.*, 1999). Along this line, earlier work reported 40% conversion after 5 hr during the transesterification of palmitic acid methyl ester with vitamin C; however, no significant increase in conversion was observed even after a long reaction time (Humeau *et al.*, 1998a, b). In ionic liquid the *Novozyme-435* catalysed vacuum driven acylation of ascorbic acid using oleic acid gave 61% of the esterified product in 30 hr (Park *et al.*, 2003).

The effect of fatty acid chain length on the yield of the ascorbyl esters has been reported (Yan *et al.*, 1999; Cao *et al.*, 1997). Such a trend was observed during the microwave assisted enzymatic synthesis of vitamin C fatty acid esters using different chain lengths of fatty acid vinyl esters (C8-C18) (*Figure 4*). When octanoic acid vinyl ester was used for the acylation of vitamin C, only 58% of the 6-O-Octanoyl-L-ascorbic acid was obtained; whereas, use of decanoic acid vinyl ester and dodecanoic acid vinyl ester yielded 72% and 76% of the corresponding vitamin C fatty acid esters in 3 hr. Furthermore, 6-O-Palmitoyl- and 6-O-steroyl-derivatives of L-ascorbic acid were isolated in 80% and 85% yield respectively.

TABLE 2. SYNTHESIS OF VITAMIN C FATTY ACID ESTERS UNDER MICROWAVE IRRADIATION

Product	R	Yield[a,b]/%
Ascorbyl capricate	CH_3-$(CH_2)_6$	58
Ascorbyl caprylate	CH_3-$(CH_2)_8$	72
Ascorbyl laurate	CH_3-$(CH_2)_{10}$	76
Ascorbyl palmitate	CH_3-$(CH_2)_{14}$	80
Ascorbyl stearate	CH_3-$(CH_2)_{16}$	85

Note: [a]Isolated yield after purification. [b]All products were characterised by ^{13}C NMR.

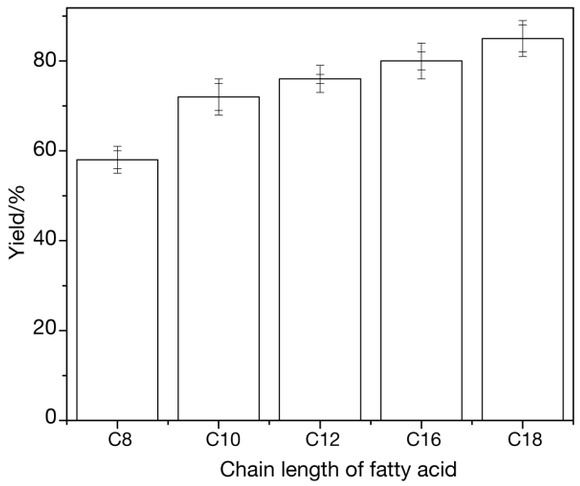

Figure 4. Influence of fatty acid chain length on product formation.

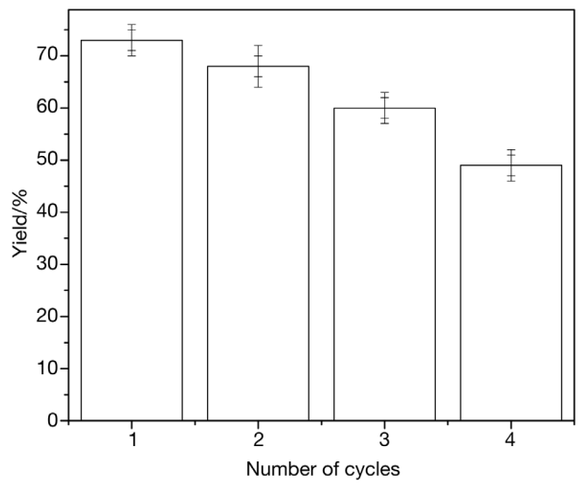

Figure 5. Reusability of Novozyme-435 *under microwave irradiation.*

The enhancement of the reaction rate of the *Novozyme-435* catalysed reaction under microwave irradiation is well documented (Loupy, 2003). The reusability of this biocatalyst was studied for the acylation of vitamin C under microwaves. In this case, *Novozyme-435* was reused for five cycles with 24% loss in activity (*Figure 5*).

CONCLUSION

A sequential chemo-enzymatic method was developed for the synthesis of vitamin C fatty acid esters. Firstly, fatty acid vinyl esters were synthesised in 30 min under microwaves. The fatty acid vinyl esters which were obtained

then subjected to the *Novozyme-435* catalysed irreversible transesterification with vitamin C, to yield ascorbyl the fatty acid esters in 58%-85% yield under microwave irradiation.

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