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Enzymatic synthesis of lard-based ascorbyl esters in a packed-bed reactor: Optimization by response surface methodology and evaluation of antioxidant properties

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ABSTRACT

An optimal continuous production of lard-based ascorbyl esters (LBAEs) by transesterification of lard with L-ascorbic acid in a packed bed reactor (PBR) was developed using immobilized lipase (Novozym 435) as a catalyst in a *tert*-amyl alcohol solvent system. Response surface methodology (RSM) and central composite design (CCD) were employed to evaluate the effects of substrate flow rate, reaction temperature and substrate molar concentration ratio on the molar conversion of LBAEs. The optimum conditions were as follows: substrate flow rate 1.07 ml/min, reaction temperature 56.44 °C, and substrate molar concentration ratio 2.24:1. The optimum predicted LBAEs yield was 50.83% and the actual value was 50.50%. The above results shows that the RSM study based on CCD is adaptable for LBAEs yield studied for the current transesterification system. The antioxidant activities of LBAEs has also been studied. LBAEs represented positive antioxidant potential on superoxide anion radical and hydroxyl radicals and satisfactory antioxidant activity in lard and soybean oil. The results suggest that LBAEs has the potential to serve as natural antioxidant in food system.

1. Introduction

L-ascorbic acid (vitamin C) is the major water-soluble natural antioxidant (Parka, Viklund, Hult, & Kazlauskas, 2003). However, its highly hydrophilic behavior limits its application in cosmetics or fats and oils (Han, Yi, & Shin, 1990). Recently, L-ascorbyl fatty acid esters, derivatives of L-ascorbic acid have attracted considerable attention for preventing diseases related to oxidative stress. Being fat-soluble, they can concentrate into the lipid domains of biological systems and protect cell membranes and low-density lipoprotein (LDL) against oxidation (Liu, Ma, & Liu, 1999). They also act as antimutagens, antitumorpromoters (Rao, Rivenson, Kelloff, & Reddy, 1995) and carriers of ascorbate into neural tissue (Pokorski, Marczak, Dymeck, & Suchocki, 2003). Therefore, the modification of L-ascorbic acid via esterification or transesterification with aliphatic molecules (such as fatty acids) can be used to alter solubility in oil-based formulas and emulsion. The esterification or transesterification process can be enzymatic, which has some advantages such as high catalytic efficiency, high regioselectivity, low by-product production, low energy requirement and mild reaction conditions, comparing with chemical process (Liu & Gao, 2003). In addition, ascorbyl fatty acid esters synthesized by enzymatic reaction are considered as natural additives.

There are many reports on lipase catalyzed synthesis of ascorbyl ester in solvent system employing saturated and unsaturated free fatty acids, alkyl and vinyl esters as acyl donors using a batch reaction (Hsieh, Nair, & Wu, 2006; Parka et al., 2003; Song, Zhao, Xu, Zhou, & Wei, 2006). However, a continuous reaction would be preferred for large-scale production. Continuous production of acyl L-ascorbates in a packed-bed reactor (PBR) for use in industrial scale application had been reported (Kuwabara, Watanabe, Adachi, Nakanishi, & Matsuno, 2003). The immobilized lipase is packed into a column and the L-ascorbic acid powder is packed into another column that was connected in series and fatty acid in solvent is continuously pumped through these columns. The advantages of

Abbreviations: LBAEs, lard-based ascorbyl esters; PBR, packed-bed reactor; RSM, response surface methodology; CCD, central composite design; LDL, low-density lipoprotein; CV, coefficient of variation; POV, peroxide values; AP, L-ascorbyl palmitate.

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using PBR: it allows reuse of the enzyme without a prior separation (Chen et al., 2011); (2) it protects enzyme particles from breaking because of the mechanical shear stress (Robles-Medina, González-Moreno, Esteban-Cerdán, & Molina-Grima, 2009); (3) it permits to handle substrates of low solubility using large volumes containing low concentrations of substrate (Rahman, Kamaruddin, & Uzir, 2011). However, long-term operation of a PBR is still a challenge.

Although there are few reports on lipase-catalyzed continuous production of ascorbyl esters in a PBR, there are reports of using saturated and unsaturated fatty acid as acyl donors. In order to reduce the cost, we applied lard as acyl donors as an alternative to fatty acids or activated esters. In our previous work, lard was alcoholysis with methanol to form fatty acid methyl esters, and the formed methyl esters was used as acyl donors to form ascorbyl esters with ascorbic acid catalyzed by lipase (Zhao et al., 2011). Lard is abundantly available in China. It contains a mixture of mono-unsaturated, polyunsaturated, and saturated fatty acid. There is no report about the synthesis of lard-based ascorbyl esters (LBAE) in a continuous PBR.

In this study, a reactor system, in which a column packed with molecular sieve and another column packed with immobilized lipase that were connected in series, was proposed to continuously synthesize ascorbyl fatty acid esters. Mixtures of LBAEs were synthesized through the immobilized lipase-catalyzed transesterification of L-ascorbic acid with lard in *tert*-amyl alcohol using this system. In order to better understand the relationships between the reaction variables (substrate flow rate, reaction temperature, and molar concentration ratio of lard to ascorbic acid) and the response, conversion of LBAEs (%) and to achieve the optimal continuous transesterification condition in a PBR system, a response surface methodology (RSM) and three-level-three-factor central composite design (CCD) was used. The antioxidant activities of LBAEs were also evaluated for use in food industries.

2. Methods

2.1. Materials

Novozym 435 (*Candida antartica* B, a nonspecific lipase immobilized on a macroporous acrylic resin with a specific activity 10,000 PLU/g and water content 1–2% (w/w)) was purchased from Novozym A/S Bioindustrial, Inc. (Bagsvaerd, Denmark). The refined bleached and deodorized lard was kindly provided by Oil Processing Company of Lianyungang (China), with the composition of C14:0 (1.5%), C16:0 (26%), C16:1 (2.5%), C18:0 (13%), C18:1 (45%), C18:2 (12%) determined by GC with internal standard method. L-Ascorbic acid (purity > 99%), *tert*-amyl alcohol, and molecular sieve 4Å were purchased from Sinopharm Chemical Reagent Co., Ltd. All other chemicals used were of analytical grade or chromatography grade.

2.2. Enzymatic transesterification in a continuous reactor

Supplementary materials Fig. 1 shows the experimental rig setup of the packed bed reactor. The packed bed reactor had two series of stainless steel column with dimensions of 2 cm (i.d) × 10 cm length (6) and 2 cm (i.d) × 25 cm length (8), respectively. The substrates mixture of L-ascorbic acid and lard (molar concentration ratios of 1:1–1:3) in *tert*-amyl alcohol was preheated to a set temperature (55 °C) in a feed container (2) by a temperature- controlled magnetic stirrer (1), then fed upwards through the column using a peristaltic pump (4) at flow rate of 0.5–2.0 ml/min. The column 6 was packed with immobilized enzyme (particle diameters 0.3–0.9 mm) and column 8 was packed with 4Å molecular sieve. The upper and lower end of the columns were layered with glass wool. The column temperature was maintained by a water bath. At the outlet of the reaction vessel, samples were collected and analyzed.

2.3. HPLC Analysis of reaction products

HPLC analysis of LBAEs was carried out as preciously described (Zhao et al., 2011). Conversion was calculated as followed:

$$\text{Conversion}(\%) = \frac{\text{Total molar number of LBAEs}}{\text{Molar numbers of L-ascorbic acid added}} \times 100$$

2.4. Experimental design and data analysis

A 3-level-3-factor CCD with three replicates at the center point was employed in this study, requiring 17 experiments. The variables and their levels selected for LBAEs synthesis were substrate flow rate (0.5–1.5 ml/min), reaction temperature (50–60 °C), and substrate molar concentration ratio (lard to L-ascorbic acid; 1:1–3:1) according to our preliminary experiment results. All experiments were performed in a *tert*-amyl alcohol system with a packed-bed reactor in present work. Table 1 shows the independent factor (x_i), levels, and experimental design in terms of coded and uncoded values. The experimental data were analyzed by RSM using the software Design Expert version 7.1.6 (Stat-ease Inc., Minneapolis, USA). The quadratic response surface model was fitted to the Eq (1):

$$y = \beta_0 + \beta_1 x_1 + \beta_2 x_2 + \beta_3 x_3 + \beta_{11} x_1^2 + \beta_{22} x_2^2 + \beta_{33} x_3^2 + \beta_{12} x_1 x_2 + \beta_{13} x_1 x_3 + \beta_{23} x_2 x_3 + \beta_{123} x_1 x_2 x_3 \quad (1)$$

The predicted response (y) was therefore correlated to the set of regression coefficients: the intercept (β_0), linear (β_1, β_2, β_3), interaction (β_{12}, β_{13}, β_{23}, β_{123}) and quadratic (β_{11}, β_{22}, β_{33}) coefficients.

2.5. Purification of LBAEs for antioxidant activity assay

The reaction mixture was filtered via PTFE microporous membrane to remove lipase and solid L-ascorbic acid. The reaction filtrate was concentrated under vacuum and was precipitated by the addition of hexane. The precipitate was obtained after centrifugation (3000× *g*, 20 min), and mixed with ethyl acetate and distilled water (v/v, 1:4), then allowed to stand for 30 min to separate into two layers, the upper layer solution was carefully evaporated just to dryness with vacuum rotatory evaporator (LABOROTA 4001, Heidolph) at 40 °C. A white powdered solid product was obtained. The purity of LBAEs was determined by HPLC to be >95% and its composition were 1.81% ascorbyl myristate, 2.66% ascorby palmitoleate, 0.75% ascorbyl laurate, 46.45% ascorbyl palmitate, 14.29% ascorbyl oleate and 34.04% of ascorbyl stearate. The structural conformation of LBAEs had been done by HPLC-ESI-MS (data not shown).

Table 1
Level and code of variables chosen for CCD.

Variables	Uncoded symbol	Coded level		
		−1	0	1
Flow rate (ml/min)	x_1	0.5	1.0	1.5
Temperature (°C)	x_2	50	55	60
Lard:Ascorbic acid (molar concentration ratio)	x_3	1	2	3