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Full Length Research Paper

Biocatalytic synthesis and antioxidant capacities of ascorbyl esters by Novozym 435 in *tert*-butanol system using different acyl donors

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Novozym 435 was used to catalyze the synthesis of fatty acid (FA) ascorbyl esters in *tert*-butanol using methyl palmitate, oleic and linoleic acids, and soybean oil as acyl donors. Response surface methodology (RSM) and three-level-four-factor central composite rotatable design (CCRD) were employed to optimize the synthesis parameters of ascorbyl palmitate, such as Novozym 435 dosage, reaction temperature, reaction duration and L-ascorbyl acid/methyl palmitate molar ratio. Under the optimized conditions, the yield of ascorbyl palmitate was up to 78.2%. The proposed model on ascorbyl palmitate yield showed a satisfactory coefficient of R^2 (87.89%), and was experimentally verified. Some other ascorbyl esters were also biosynthesized using oleic and linoleic acids and soybean oil as acyl donors under the optimal conditions. The antioxidant capacities of the derivative ascorbyl esters were evaluated by different assays, including hydroxyl and superoxide anion radical scavenging activities, reduction assay and lipid oxidative degradation. Compared to the common antioxidants of TBHQ, BHT, BHA, V_C and V_E, it is important to note that a mixture of soybean oil ascorbyl esters exhibits the highest hydroxyl radical scavenging and reduction capacities; ascorbyl palmitate showed the highest superoxide anion radical scavenging and lipid oxidative degradation activities. It can be conclude that the derivative ascorbyl esters may be used as potential antioxidants in improving food quality and stability.

Key words: Novozym 435, esterification, ascorbyl palmitate, unsaturated fatty acid ascorbyl ester, mixture of fatty acid ascorbyl esters, antioxidant activity.

INTRODUCTION

It is well known that fatty bodies have a tendency to be oxidized, even at ambient temperature, and this oxidation (or rancidness) often makes them exhibit new properties, principally in taste or smell, which are generally considered as undesirable when these fatty bodies are incorporated, for example, in food or cosmetics compositions. To protect these fatty bodies from oxidation, L-ascorbyl acid (vitamin C), a natural hydrophilic antioxidant, has been commonly used but limited in its application in hydrophobic foods and cosmetics (Wawire et al., 2011). To overcome the insolubility in any oil-based formula, it is well established that using fatty acid derivative of L-ascorbyl acid with the similar antioxidant function replaces vitamin C itself (Reyes-Duarte et al., 2011). Therefore, the derivative ascorbyl laurate, palmitate and stearate have been emphasized in several works, all which were synthesized by chemical process or enzymatic catalysis using lauric acid, palmitate and stearate as acyl donors (Chang et al., 2009; Micciche et al., 2007; Frungillo et al., 2010). Exhibiting improved miscibility with oils or fatty products, ascorbyl esters of unsaturated fatty acids (example, oleate, linoleate, DHA

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or EPA) have also been recently reported and employed as food antioxidant additives (Song et al., 2004; Viklund et al., 2003; Kidwai et al., 2009).

In comparison with chemical process, the advantages of enzymatic esterification for ascorbyl esters synthesis are mild reaction conditions, low energy requirements and a minimization of the isomerization and rearrangement side reactions (Kidwai et al., 2009). Lipases, especially that from *Candida antarctica*, have been successfully used to catalyze the synthesis of ascorbyl esters in tertiary alcohols, acetone and even in ionic liquids, employing saturated and unsaturated free fatty acids, alkyl and vinyl esters as acyl donors (Adamczak and Bomscheuer, 2009). The procedure of the biocatalytic synthesis to produce L-ascorbyl esters via esterification in water-miscible organic solvents has been comprehensively reviewed, including the parameters affecting the lipase activities on esterification reactions such as reaction time, temperature, substrate molar ratio, and acyl donors (Karmee, 2009). It has been demonstrated that response surface methodology (RSM) and central composite rotatable design (CCRD) are useful statistical techniques for complex processes investigation and have been successfully applied to optimizing ester production by lipase (Liu et al., 2010). However, so far, there are few reports dealing with the interactions between important reaction factors when ascorbyl esters are biosynthesized by enzymes in *tert*-butanol system. In addition, even few reports can be available on the comparison of antioxidant activities of ascorbyl esters biosynthesized using alkyl ester, fatty acid and triglyceride as acyl donors.

Therefore, this study focused on the enzymatic synthesis of L-ascorbyl acid fatty acid esters catalyzed by immobilized lipase from *C. antarctica* (Novozym 435) in *tert*-butanol system using alkyl ester (example palmitate), free fatty acid (example, oleic and linoleic acids), and triglyceride (example, soybean oil) as acyl donors. Our objectives were to better understand the relationships between various reaction variables (example, reaction time, temperature, enzyme amount, and substrate molar ratio) and the response (ascorbyl esters yield), and to evaluate the antioxidant activities of different ascorbyl esters specifically, (1) to optimize the significantly affecting parameters for ascorbyl palmitate synthesis by using response surface methodology (RSM) and three-level-four-factor central composite rotatable design (CCRD) and to investigate the interactions among such variables; (2) to reduce the cost of the process, using soybean oils as an alternative to fatty acids or alkyl esters as acyl donors; (3) to evaluate the antioxidant capacities of the synthesized ascorbyl esters by different assays, including hydroxyl and superoxide anion radical scavenging activity, reduction assay and lipid oxidative degradation.

MATERIALS AND METHODS

Novozym 435 was bought from Novozymes (Bagsvaerd, Denmark). L-ascorbyl acid (V_C, 99% pure) was purchased from Sinopharm Group Company Limited (Shanghai, China). A standard sample of L-ascorbyl palmitate (99% pure) was from Alfa Company Inc. (Ohio, USA). Acetonitrile and methanol with chromatograph grade (99.9% pure) was from TEDIA Co. (New York, USA). Palmitate (95% pure), *tert*-butylhydroquinone (TBHQ, 97% pure), butylated hydroxyanisole (BHA, 96% pure), 2,6- di-tert-butyl -4- methyl phenol (BHT, 99% pure), tocopherols (V_E, 96% pure), oleic acid (85% pure), linoleic acid (90% pure) were all got from Acros Organics Co. (Morris Plains, NJ, USA). All other chemicals were of analytical reagent grade and bought from Sinopharm Group Company Limited (Shanghai, China).

Experimental design and optimization

All reactions were performed in 50 ml amber glass sealed vessels with orbital shaking (250 rpm). Ascorbic acid (0.5 mmol) and the corresponding acyl donor (alkyl ester, fatty acid, and soybean oil 1.5 mmol) were dissolved in 10 ml of dried *tert*-butanol. The mixture was equilibrated for 10 min, and the biocatalyst (125 mg) was added. Aliquots were removed at intervals, filtered using a 0.45 μm Durapore membrane coupled to an Eppendorf tube and analyzed by high performance liquid chromatography (HPLC). The preliminary single factor experiments indicated that the Novozym 435 dosage, reaction temperature, duration and L-ascorbyl acid/methyl palmitate molar ratio were the four key variables for the enzymatic synthesis of ascorbyl esters. Therefore, RSM and a three-level-four-factor CCRD requiring 27 experiments were employed to optimize the significant parameters in this study. The fractional factorial design consisted of 16 factorial points, 8 axial points (two axial points on the axis of each design variable at a distance of 2 from the design center), and 3 center points. The variables and their levels selected for the study of L-ascorbyl palmitate synthesis were: Novozym 435 dosage (4.5 to 5.5%), reaction temperature (50 to 60°C), duration (18 to 30 h) and L-ascorbyl acid/methyl palmitate molar ratio (3.5:1 to 4.5:1). Table 1 shows the independent factors (X_i, i = 1, 2, 3, 4), levels and experimental design in terms of coded and uncoded variables.

To avoid bias, 27 runs were performed in a totally random order. The general form of the second-order polynomial equation is presented in Equation (1):

$$Y = \beta_0 + \sum \beta_i X_i + \sum \beta_{ii} X_i^2 + \sum \sum \beta_{ij} X_i X_j \qquad (1)$$

Where, Y is the ascorbyl palmitate yield (dependent variables); β_0 is the constant coefficient; β_i, β_{ii} and β_{ij} are the coefficients for the linear, quadratic and interaction term, respectively; X_i and X_j stand for the variables (independent variables); subscript i and j are the number from 1 to 4.

The fitting degree of the model was evaluated by the coefficient of determination (R^2) and the analysis of variance (ANOVA). Quadratic polynomial equations were attained by holding one of the independent variances at a constant value and changing the level of the other variables. Experiments were performed in duplicate. Standard deviations were lower than 5%.

HPLC analysis and purification of product

Reaction aliquots were measured by an SSI model 2300-525 HPLC with (Scientific Systems Inc., Washington, USA) equipped with a binary pump (Series III), a UV-vis detector (Model 525), a four-channel degasser (SN-DG-2410), a column oven (Model 605), a model 7725 manual injector (Rheodyne Co. USA) and SSI ChemStation data software (CSChrom Plus). The determination of ascorbyl ester yield was performed isocratically at 30°C with Alltech

Table 1. Central composite rotatable second-order design and experimental data for 3-level-4-factor response surface analysis.

Trial	Temperature (℃)/X_1	Palmitate : L-ascorbyl acidmolar ratio/X_2	Time (h)/X_3	Lipase dosage (% w/w of L-ascorbyl acid) /X_4	Yield (%)/Y
1	-1(50)	-1(3.5:1)	0(24)	0(5.0)	68.6
2	-1(50)	+1(4.5:1)	0(24)	0(5.0)	65.5
3	-1(50)	0(4.0:1)	0(24)	-1(4.5)	63.6
4	-1(50)	0(4.0:1)	0(24)	+1(5.5)	65.5
5	+1(60)	-1(3.5:1)	0(24)	0(5.0)	53.8
6	+1(60)	+1(4.5:1)	0(24)	0(5.0)	75.1
7	+1(60)	0(4.0:1)	0(24)	-1(4.5)	68.6
8	+1(60)	0(4.0:1)	0(24)	+1(5.5)	69.4
9	0(55)	0(4.0:1)	0(24)	0(5.0)	75.9
10	-1(50)	0(4.0:1)	-1(18)	0(5.0)	67.4
11	-1(50)	0(4.0:1)	+1(30)	0(5.0)	70.1
12	+1(60)	0(4.0:1)	-1(18)	0(5.0)	68.6
13	+1(60)	0(4.0:1)	+1(30)	0(5.0)	70.5
14	0(55)	0(4.0:1)	0(24)	0(5.0)	80.1
15	0(55)	+1(4.5:1)	-1(18)	0(5.0)	75.9
16	0(55)	+1(4.5:1)	+1(30)	0(5.0)	78.5
17	0(55)	0(4.0:1)	-1(18)	+1(5.5)	76.2
18	0(55)	0(4.0:1)	+1(30)	-1(4.5)	73.2
19	0(55)	0(4.0:1)	+1(30)	+1(5.5)	68.2
20	0(55)	0(4.0:1)	-1(18)	-1(4.5)	71.7
21	0(55)	-1(3.5:1)	0(24)	-1(4.5)	70.5
22	0(55)	-1(3.5:1)	0(24)	+1(5.5)	73.2
23	0(55)	+1(4.5:1)	0(24)	-1(4.5)	77.0
24	0(55)	+1(4.5:1)	0(24)	+1(5.5)	74.3
25	0(55)	0(4.0:1)	-1(18)	+1(5.5)	76.2
26	0(55)	-1(3.5:1)	-1(18)	0(5.0)	78.5
27	0(55)	0(4.0:1)	0(24)	0(5.0)	78.5

Aollop C_{18} column (250 × 4.6 mm, 5 µm particle size (Alltech Inc., USA). The mobile phase was acetonitrile: water (90:10, v/v) at a flow rate of 1.0 ml/min. The detection wavelength was 250 nm. The injection volume was 20 µl. The relative percentage yield (molar conversion) was defined as: L-ascorbyl ester/(L-ascorbic acid + L-ascorbyl ester)×100% and was estimated using peak area integrated by SSI ChemStation data software (CSChrom Plus). The purification procedures for the reaction product of the derivative ascorbyl ester were according to the method of Watanabe et al. (1999).

Antioxidant activities

Superoxide anion radical scavenging activity

Superoxide anion scavenging activity was measured by pyrogallol antioxidation (Marklund and Marklund, 1974). The reaction mixture contained 25 µl of pyrogallol (3 mmol.L^{-1}) and 4500 µl of Tris-HCl (0.05 mol.L^{-1}, pH=10.2). A sample cell loaded with the mixture was first placed at 27℃ for 20 min in the thermostat water bath. When the cell reached the monitor, a certain weight of the derivative ascorbyl esters and V_E, V_C, BHA, BHT and TBHQ samples (2.5 mg) were injected into the cell *in situ*. The final volume was always the same (1 ml) for all spectrophotometer assays. The absorbance was simultaneously recorded by the UV-1800 PC spectrophotometer (Shanghai, China) and was recorded every 30 s (Tris-HCl was used as the control). The extent of $.O_2^-$ scavenging was determined according to Equation (2):

$$.O_2^- \text{ scavenging extent (\%)} = \frac{CL_{control} - CL_{sample}}{CL_{control}} \times 100 \qquad (2)$$

Hydroxyl radical scavenging activity

Hydroxyl radical scavenging activity was determined by a Fenton-type reaction (Zhao et al., 1989). The reaction mixture contained 1000 µl of $FeSO_4$ (7.5 mmol.L^{-1}, dissolved in deionized water), 1000 µl of 1, 10-phenanthronline solution (7.5 mmol.L^{-1}, dissolved in absolute ethanol), and 1000 µl of H_2O_2 (0.1%, v/v). The final volume of the reaction mixture was 10 ml in phosphate buffer (0.05 mol.L^{-1}, pH=7.5) and incubated at 37℃ for 1 h. The test procedure and OH scavenging extent formula were similar to those for the superoxide anion assay.

Reducing power

The reducing power of the different ascorbyl esters was modified as per the method reported by Guddadarangavvanahally et al. (2008).

Certain concentrations of ascorbyl esters (example, ascorbyl palmitate, ascorbyl oleate, ascorbyl linoleate, and ascorbyl mixture; soybean oil fatty acid methyl esters) or common antioxidants (example, V_C, V_E, BHT and TBHQ) in 0.1 ml MeOH were mixed with 2.5 ml of phosphate buffer (0.05 M, pH 7.0) and 2.5 ml of 1% (w/v) potassium ferricyanide in 15 ml test tubes. The mixtures were incubated for 20 min at 50°C. At the end of the incubation, 2.5 ml of 10% (w/v) trichloroacetic acid was added to the mixtures and centrifuged at 5000 rpm for 10 min. The upper layer (2.5 ml) was mixed with 2.5 ml of distilled water and 1.0 ml of 0.1% (w/v) ferric chloride in water, and the absorbance was measured at 700 nm. All the reducing power tests were run in triplicate. Bigger increase in absorbance of the reaction indicated the higher reducing power of the test samples.

Lipid oxidative degradation activity

Lipid oxidative degradation activity was generated from the soybean oil accelerated rancidity by high temperature and measured by titrating acid value (Yu et al., 2001). Amount of edible soybean oil was put into flask and exposed at the temperature of 225°C for 3 h. Then, it was equally divided into ten parts. The ascorbyl derivatives (200 $mg.kg^{-1}$-oil) (example, ascorbyl palmitate, ascorbyl oleate, ascorbyl linoleate, and a mixture of soybean oil ascorbyl esters) and the common antioxidants (example, V_C, V_E, BHT and TBHQ) were successively added into nine parts of soybean oil, and the rest one part of soybean oil without adding antioxidant was used as the control. All ten samples were set at 50°C in thermostat stirring bed with a speed of 250 rpm. The acid value of each sample was measured at an interval of 24 h.

RESULTS AND DISCUSSION

RSM optimization and mutual interaction of parameters

To optimize the enzymatic synthesis parameters of ascorbyl esters, we used ascorbyl palmitate as an example. As stated afore, the crucial parameters (Novozym 435 dosage, reaction temperature, duration and L-ascorbyl acid/methyl palmitate molar ratio) affecting ascorbyl palmitate yield were optimized through RSM. The statistical combination of the independent variables in coded and actual values along with the response (ascorbyl palmitate yield, %) is presented in Table 1. The RSREG procedure for SAS (statistics analysis system) was employed to fit the second-order polynomial Equation (I) to the experimental data (Table 1). Among the various treatments, the highest yield (80.1%) was trial 14 (time 24 h, temperature 55°C, enzyme dosage 5%, substrate molar ratio 4:1), and the smallest yield (53.8%) was trial 5 (time 24 h, temperature 60°C, enzyme dosage 5%, substrate molar ratio 3.5:1). From the SAS output of RSREG, the second-order polynomial Equation (1) could be shown as Equation (3):

$$Y1 = 0.6113 + 0.0079*X1 + 0.0321*X2 - 0.0059*X3 + 0.0024*X4 - 0.1271*X1*X1 + 0.0790*X1*X2 - 0.0027*X1*X3 - 0.0034*X1*X4 - 0.0242*X2*X2 + 0.0301*X2*X3 - 0.0199*X2*X4 - 0.0144*X3*X3 - 0.0343*X3*X4 - 0.0512*X4*X4 \quad (3)$$

In order to determine whether or not the second-order polynomial model was significant, it was necessary to conduct the analysis of variance (ANOVA) (Table 2), in which the regression sum of square was subdivided into two parts that attributed to linear regression and quadratic model, respectively. The P-value was used as the tool to check the significance of each coefficient, which also indicates the interaction strength between every two parameters. The smaller the P-value is, the bigger the significance of the regression coefficient. In other words, the higher the F-value for the model (F_{model}>5), the lower probability the value for the model (P< 0.005), which also indicates the bigger significance of the fitted model. In this study, the P-value of the model is ≤ 0.0015, suggesting that the model is suitable for the experimental prediction of ascorbyl palmitate yield. The P-value of "lack of fit" is 0.4456 (P>0.01), demonstrating that "lack of fit" is insignificant relative to the pure error. The coefficient of determination (R^2=87.89%) implies that the accuracy and general availability of the polynomial model were adequate.

The regression coefficients and the corresponding P-values are also shown in Table 2. From the P-value of each model term, it is concluded that the regression coefficients of the linear term X_2 and the quadratic term X_1^2 and X_4^2 and the mutual term $X_1.X_2$ have significant effect on the ascorbyl palmitate yield. Among them, X_2 and X_4^2 are significant at 5% level, while X_1^2 and $X_1.X_2$ are significant at 1% level. The relationships between reaction factors and response can be better understood by examining the planned series of contour plots (Figure 1) generated from the predicted model (Equation 3). All six contour plots exhibited the interaction behavior between two parameters. Obviously, the reaction temperature around 55°C, enzyme dosage around 5%, reaction time around 17 h and substrate molar ratio 1:4 could result in the predicted high yield (*c.a.* 78%). Therefore, the optimal synthesis condition for L-ascorbyl palmitate was analyzed by the ridge max analysis which computes the estimated ridge of maximum response from the center of original design. The outcome of ridge max analysis showed that higher enzyme amount will result in higher yield of L-ascorbyl palmitate with different conditions of all other parameters. So, according to the ridge max analysis outcome, the optimum reaction condition with the maximum predicted yield was: lipase dosage 5.2 wt% (based on palmitate weight), temperature 54.9°C, molar ratio of L-ascorbyl acid to palmitate 1:3.9, and reaction duration 17.4 h. Under the optimized conditions, ascorbyl palmitate yield could be achieved up to 78.2%.

Verification of the model and other ascorbyl esters synthesis

The adequacy of the predicted model was examined by

Table 2. Analysis of variance (ANOVA) for regression equation.

Source	DF	Sum of square	Mean square	F-value	Pr > F
X1	1	0.0007	0.0007	0.4550	0.5127
X2	1	0.0124	0.0124	7.5693	0.0176*
X3	1	0.0004	0.0004	0.2533	0.6239
X4	1	0.0000^{7}	0.0000^{7}	0.0440	0.8374
X1*X1	1	0.0861	0.0861	52.6619	0.0001**
X1*X2	1	0.0250	0.0250	15.2829	0.0021**
X1*X3	1	0.0000^{3}	0.0000^{3}	0.0175	0.8970
X1*X4	1	0.0000^{5}	0.0000^{5}	0.0278	0.8703
X2*X2	1	0.0031	0.0031	1.9048	0.1927
X2*X3	1	0.0036	0.0036	2.2222	0.1619
X2*X4	1	0.0016	0.0016	0.9702	0.3441
X3*X3	1	0.0011	0.0011	0.6764	0.4269
X3*X4	1	0.0047	0.0047	2.8810	0.1154
X4*X4	1	0.0140	0.0140	8.5395	0.0128*
Model	14	0.1423	0.1423	6.2193	0.0015**
Linear term	4	0.0136	0.0136	2.0804	0.1466
Quadratic term	4	0.0938	0.0938	14.3368	0.0002**
Interaction term	6	0.0350	0.0350	3.5669	0.0290*
Error	12	0.0196	0.0196		
Lack of fit	10	0.0174	0.0174	1.5975	0.4456
Pure error	2	0.0022	0.0022		
Coefficient (R^2)	87.89%				

* Significant at 5% level; ** Significant at 1% level; Sum of squares: the sum of squared predicted values in a standard regression model. D.F.: degree of freedom.

performing three additional independent experiments at the suggested optimum synthesis conditions. The predicted value of yield product was 78.1±5.6% and the actual experimental value was 79.5±3.8%. A chi-square test (P_{value} = 0.982, DF = 5) indicated that the observed values were essentially the same as the predicted values and that the generated model could precisely predict the ascorbyl palmitate yield. Therefore, the model is considered to be accurate and reliable for predicting the ascorbyl palmitate yield by enzymatic esterification. Under the optimized conditions, we also biosynthesized the mixture of soybean oil ascorbyl esters with a yield of 79.03%, using soybean oil as acyl donors. Simultaneously, we have also biosynthesized the ascorbyl oleate and linoleate using oleic and linoleic acids as acyl donors, respectively.

The yield of these derivatives of ascorbyl esters were respectively from 80.1 and 84%. Compared to the previous reports using Novozym 435 to synthesize ascorbyl esters, although, the target product and solvent type were different from Song et al. (2004, 2005) and Lerin et al. (2010), it was interesting to find that all investigations obtained similar results with a yield of 33.5 to 44%, which represented at least 40% lower product yield than the results obtained in this study. Chang et al. (2009) optimized synthesis of L-ascorbyl laurate catalyzed by immobilized Novozym 435 in acetonitrile system using RSM and CCRD, and obtained a product yield of 93.2%. It is obvious that RSM and ridge max statistical analysis are useful to obtain the optimum conditions.

Hydroxyl radical scavenging activity

The hydroxyl radical scavenging activity of the derivative of ascorbyl esters was examined and compared to that of the common antioxidants. The results are shown in Figure 2. It can be seen that a mixture soybean oil ascorbyl esters have the best hydroxyl radical scavenging activities, followed by ascorbyl palmitate, ascorbyl oleate, and ascorbyl linoleate. The extent of hydroxyl radical scavenging of a mixture of soybean oil ascorbyl esters is similar to that of V_E and BHA, more than 3.5-fold over that of TBHQ. The 50% hydroxyl radical scavenging concentrations (IC_{50}) of the mixture soybean oil ascorbyl esters, ascorbyl palmitate, ascorbyl oleate, and ascorbyl linoleate were calculated from their dose-activity curves; they were 0.1, 0.19, 0.24 and 0.31 $mg.ml^{-1}$, respectively.

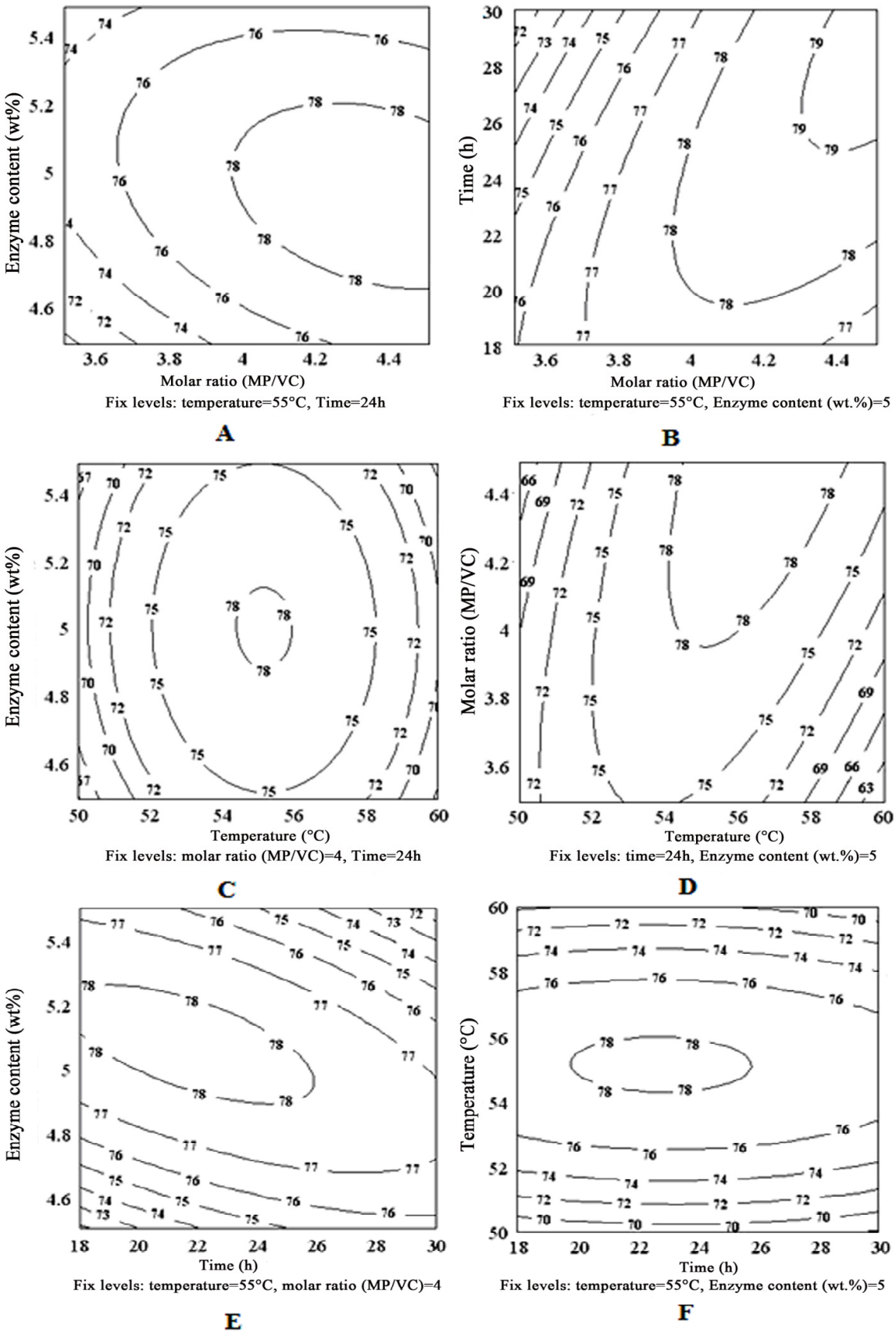

Figure 1. Contour plots of temperature, molar ratio and reaction time variables.

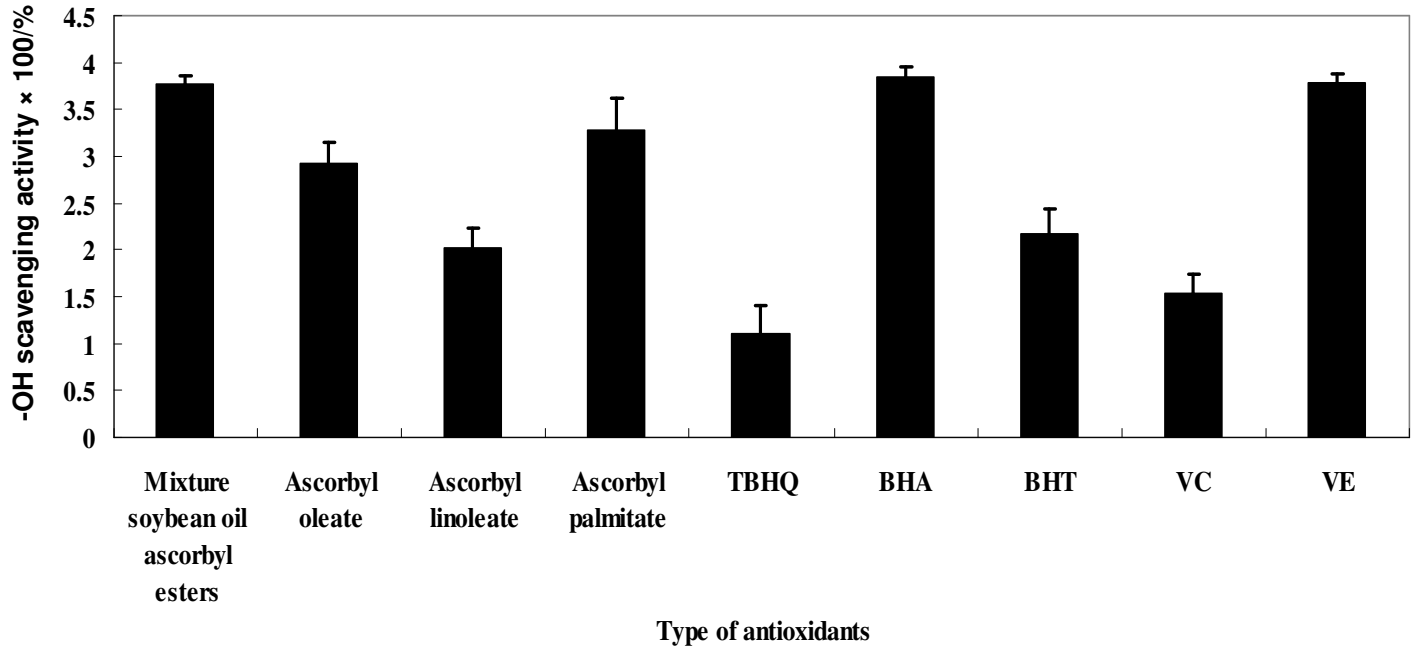

Figure 2. Hydroxyl radical scavenging activity of different antioxidants at the concentration of 4.0 mg.ml^{-1}.

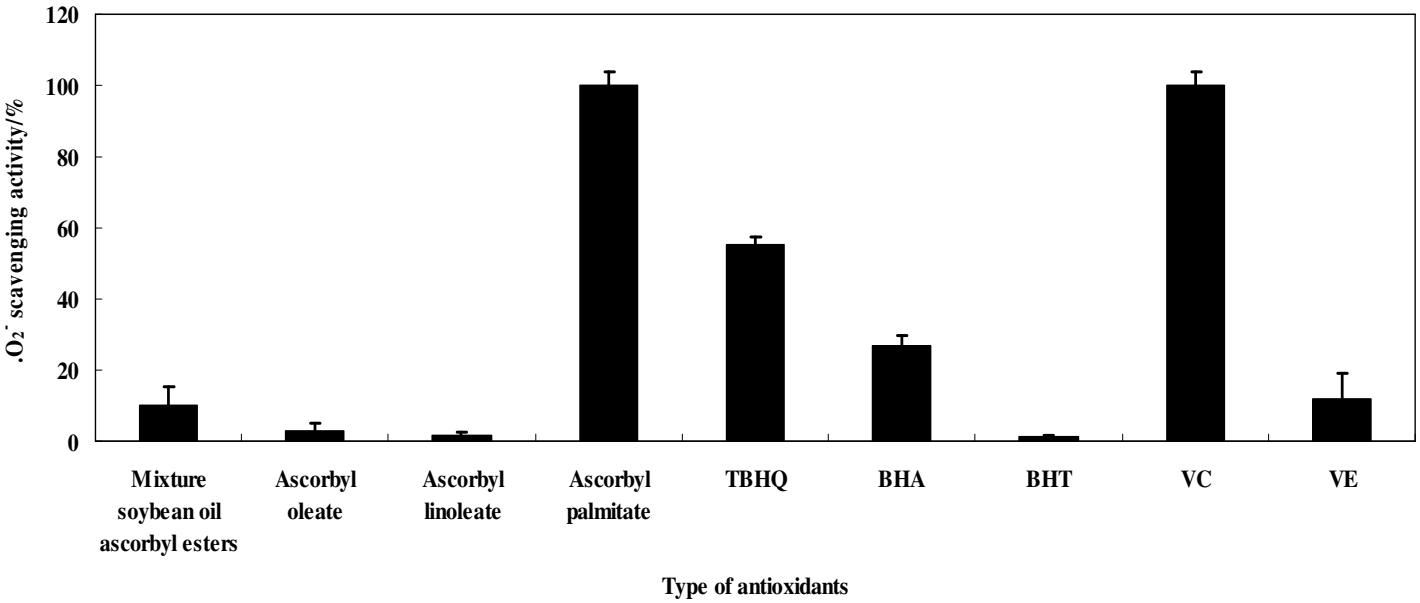

Figure 3. Scavenging effect of different antioxidants at the concentration of 0.55 mg.ml^{-1}on superoxide anion for 180 s.

As positive controls, 50% inhibitory concentrations of V_E, BHA, BHT, V_C and TBHQ were detected as 0.1, 0.1, 0.33, 0.45 and 0.50 mg.ml^{-1}, respectively.

Superoxide anion scavenging activity

It can be seen from Figure 3 that ascorbyl palmitate and V_C have the best superoxide anion scavenging activity, followed by TBHQ, BHA, BHT, V_E and a mixture of soybean oil ascorbyl esters. However, ascorbyl oleate, ascorbyl linoleate and BHT show very low superoxide anion scavenging activity. The reasonable explanation may be partially due to ascorbyl palmitate and Vc react with superoxide anion at a very fast rate, while other antioxidants in our work react with superoxide anion at a

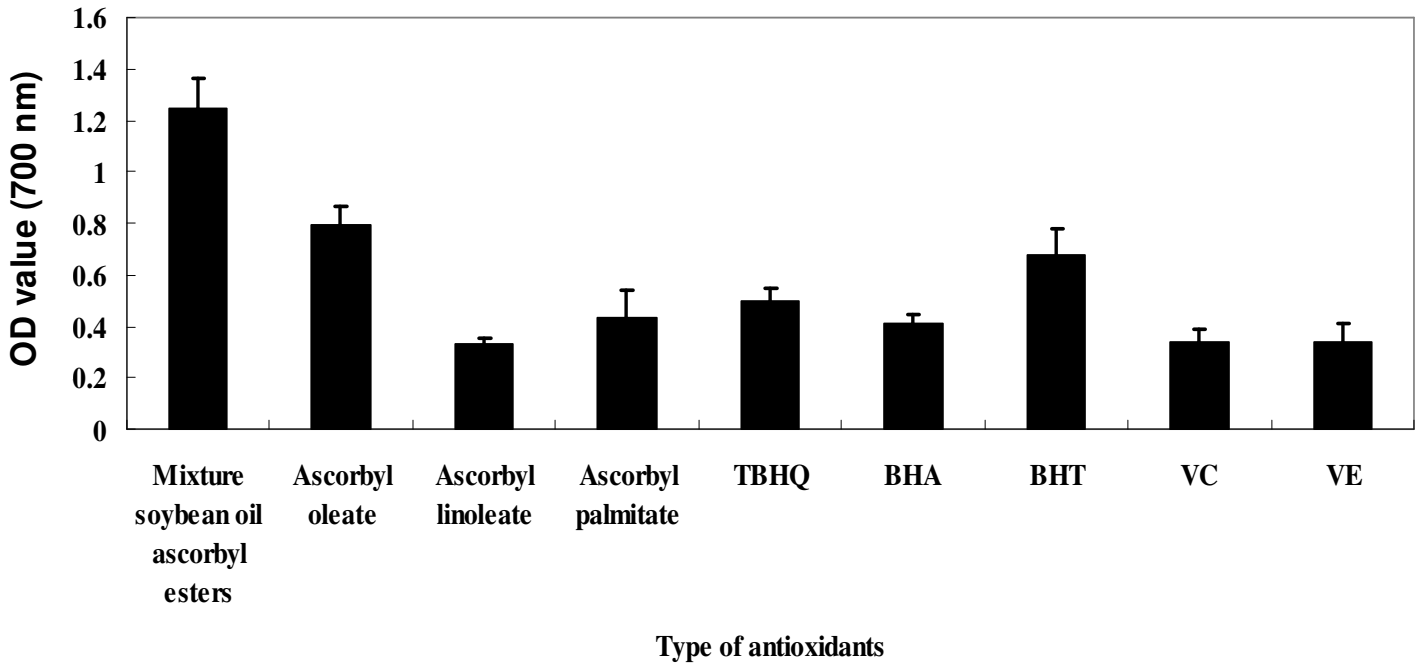

Figure 4. Reducing power of different antioxidants at the concentration of 4 mg.ml^{-1}.

relative low rate.

Reducing power

The relative reducing powers of the mixture of soybean oil ascorbyl esters, ascorbyl oleate, ascorbyl linoleate and ascorbyl palmitate also corroborated with those obtained with their radical scavenging activities. In this assay, the ability of all ascorbyl esters to reduce Fe^{3+} to Fe^{2+} was determined and compared to that of TBHQ, BHA, BHT, V_E and V_C, which are known to be the common reducing agents. The increase in the absorbance at 700 nm of the reaction mixture, caused by the afore-mentioned antioxidants, is indicative of their increased reducing power, and the results are presented in Figure 4. It can be seen that a mixture of soybean oil ascorbyl esters showed the highest reducing potential activity than all other antioxidants at 4 mg.ml^{-1} concentration. Fe^{3+} reducing activity of ascorbyl oleate is similar to that of BHT, which is higher than those of ascorbyl palmitate, ascorbyl linoleate, TBHQ, BHA, V_E and V_C. However, it has been reported that the correlation between Fe^{3+} reducing activity and antioxidant contents may not be always linear (Yildirin et al., 2000).

Lipid oxidative degradation activity

Figure 5 shows the lipid oxidative degradation activities of different antioxidants, such as a mixture of soybean oil ascorbyl esters, ascorbyl palmitate, ascorbyl oleate, ascorbyl linoleate, TBHQ, BHA, BHT, V_E and V_C. It can be seen that all the examined antioxidants can improve the quality of oil to some extent, and it seems that ascorbyl oleate and palmitate have the same antioxidant activity of retarding lipid oxidative degradation. In this context, Reyes-Duarte et al. (2011) reported that both ascorbyl oleate and palmitate had the same oxidative stability of soybean oil by the accelerated test of Rancimat's method. Viklund et al. (2003) compared the efficiency of ascorbyl oleate and palmitate to retard lipid oxidative degradation of rapeseed oil. They concluded that the oleate derivative was more effective than the palmitate one in a 144 h period.

Conclusions

The study demonstrates the feasibility of using immobilized *C. antarctica* lipase (Novozym 435) to synthesize fatty acid ascorbyl esters with palmitate, oleic and linoleic acids and soybean oil as acyl donors in *tert*-butanol system. The crucial parameters significantly affecting ascorbyl palmitate yield was optimized by RSM and CCRD. The derivative ascorbyl esters manifested strong antioxidant and radical scavenging activities in four model systems, such as hydroxyl radical and superoxide anion scavenging, reducing power and lipid oxidative degradation. It is concluded that the derivative ascorbyl ester, facilitates its use in foods, and also as a micellar vehicle, it can solubilize and stabilize hydrophobic drugs and other

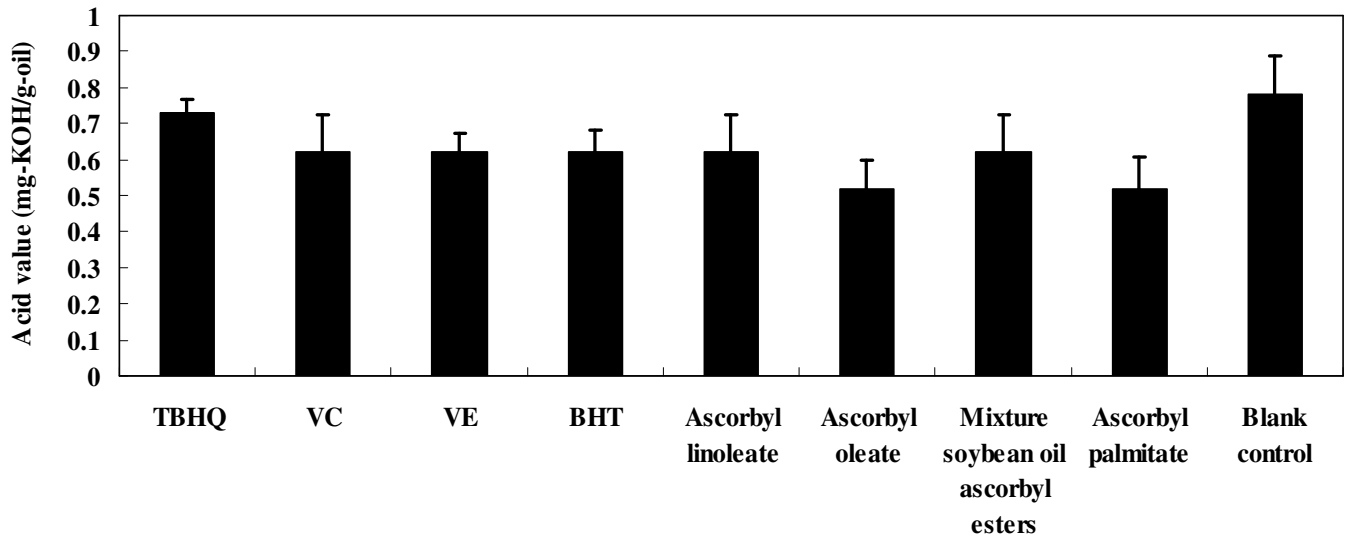

Figure 5. Lipid oxidative degradation activity of different antioxidants at a concentration of 200 mg.kg^{-1}-oil.

substances.

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