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Biosynthesis of ascorbyl benzoate in organic solvents and study of its antioxygenic and antimicrobial properties

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Abstract

Ascorbyl benzoate was synthesized through lipase-catalyzed esterification in organic media, and its properties studied. A series of organic solvents with a log *P* from −1.30 to 2.50 were investigated, in which cylcohexanone (log *P* = 0.96) was found to be the most suitable. The optimum reaction conditions in cylcohexanone were pH 6.0, a_w 0.33, a substrate concentration form 0.06 M to 0.1 M, 65 °C, and above 150 rpm speeds of shaking. Experimental results also demonstrated that benzoic acid was not an ideal substrate of lipase, which led to low conversion rates, but its limitation could be overcome by excess L-ascorbic acid. Schaal oven test illustrated that the antioxidant activity of ascorbyl benzoate was comparable to that of ascorbyl palmitate, and minimal inhibitory concentration (MIC) data showed that its antimicrobial activity was weaker than that of benzoic acid.
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Keywords: Lipase; Ascorbyl benzoate; Biosynthesis; Organic solvent; Antioxidant; Antimicrobial

1. Introduction

Enzymatic reactions in organic media offer numerous possibilities for biotechnological production of useful chemicals (Klibanov, 2001), and lipase-catalyzed esterification plays a very important role in this process (Hari Krishna, 2002). Since natural substrates of lipase are long-chain fatty acid triacylglycerols, few data are available on lipase-catalyzed esterification of aliphatic alcohols with aromatic acids (Kobayashi & Adachi, 2004), although their esters find wide applications in areas such as perfumery, cosmetics and food.

Ascorbyl benzoate to be studied here is an aromatic acid ester of L-ascorbic acid. More interestingly, it is a potential bifunctional compound, because L-ascorbic acid usually works as a natural antioxidant, while benzoic acid is a traditional antimicrobial agent. Chemical synthesis of this kind of esters usually has many disadvantages such as heat sensitivity, and poor reaction selectivity leading to undesirable side reactions. In contrast, the enzymatic synthesis offers the advantages of milder reaction conditions, a selective specificity, fewer intermediary and purification steps, and a more environmentally friendly process (Hills, 2003; Villeneuve, Muderhwa, Graille, & Haas, 2000). Moreover, Compared with those produced by chemical catalysis, esters such as flavors produced through biocatalysis can be considered close to 'natural' and potentially satisfy the recent consumer demand.

The aim of this work was to study the lipase-catalyzed esterification of ascorbyl benzoate in organic media, and to explore its antioxidant and antimicrobial activity.

2. Materials and methods

2.1. Materials

2.1.1. Enzyme

A commercially immobilized lipase (triacylglycerol hydrolase, EC. 1.3.1.3, Novozym435) from Novo Nordisk Industri (Bagsvaerd, Denmark) was used in this work,

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which is a preparation of lipase B from *Candida antarctica* immobilized on macroporous acrylic resin (bead size 0.3–0.9 mm, bulk density 430 kg m^{-3}, activity 10,000 propyl laurate units) and displays a non-specific activity towards triaglycerols.

2.1.2. Chemicals

The purity of substrate is over 99.7% for L-ascorbic acid (Guangdong Guanghua Chemical Factory Co., Ltd., Shantou, Guangdong, China) and over 99.5% for benzoic acid (Shanghai Laser Fine Chemicals Factory, Shanhai, China). Refined, bleached, and deodorized peanut oil without antioxidants was bought from a local market. Ascorbyl palmitate (AP, purity >99%, Fluka AG, Buchs, Switzerland), *tert*-butylhydroquinone (TBHQ, purity >98%, Fluka AG), 2, 6-di-*tert*-butyl-4-methylphenol (BHT, purity >99%, Sigma-Aldrich, St. Louis, MO, USA) and propyl gallate (PG, purity >97%, Sigma-Aldrich) were purchased from Beijing Superior Chemical and Instrument Co., Ltd. (Beijing, China). All solvents and other reagents except which was used in HPLC analysis were of analytical grade.

2.1.3. Microbial strains and media

For antimicrobial test, four typical microbes were selected, which were gram-negative bacterium, gram-positive bacterium, yeast and mould, respectively. Among them, *Escherichia coli* TG1 and *Aspergillus niger* AS 3.315 were preserved by our lab; *Staphylococcus aureus* ATCC 6538 and *Saccharomyces cerevisiae* ATCC 9763 were kindly provided by Institute of Bioengineering, Zhejiang University of Technology, PR China. Mueller–Hinton (MH) broth medium was purchased from Hangzhou Tianhe Microorganism Reagent Co., Ltd (Zhejiang, PRC) and RPMI 1640 broth medium (Gibco BRL) from Hangzhou SeRa Biotech Co., Ltd (Zhejiang, PRC).

2.2. Methods

2.2.1. General procedure for the enzymatic synthesis

All organic solvents were dried with molecular sieves before use in order to eliminate the influence of different water contents. Reactions (Fig. 1) were conducted in 50 ml screw-topped glass vials with 2 mmol L-ascorbic acid, 2 mmol benzoic acid, 500 mg lipase, and 20 ml organic solvent. These vials were tightly screwed, wrapped with waterproof materials, and put into a water bath shaker at 200 rpm and 70 °C, the best temperature for the lipase activity in aqueous media. These conditions were used except when otherwise stated in the text.

2.2.2. Analytical procedures

Quantitative analyses were determined by a Waters 2690 HPLC instrument (Waters Corp., Milford, Massachusetts, USA) with a LiChrospherC18 column (125 × 4 mm I.D.) (MerckKGaA, Darmsdatt, Germany) and a UV detector set at 254 nm. Interior label method was used in the analyses, where ethyl *p*-hydroxybenzoate was used as the internal standard substance. A sample (100 μl) taken out from the reaction system was vacuum evaporated, diluted to an appropriate concentration for test with 0.025 g l^{-1} ethyl *p*-hydroxybenzoate in methanol solution and injected at a volume of 20 μl. The mobile phase was methanol/water/H3PO4 (45/55/0.1, v/v/v) at a flow rate of 0.8 ml min^{-1}. The retention time of ascorbyl benzoate was around 4 min under these conditions. The total time needed for a HPLC test was about 9 min. The conversion rate was a percentage of the mole ratio of ascorbyl benzoate to the fewer of the two substrates. The yield using mmol l^{-1} as a unit was the output of ascorbyl benzoate in a certain experiment.

All the assays were done in duplicate and significantly different from tests at $P < 0.05$.

2.2.3. Initial reaction rate measurement

The initial reaction rate was measured through plotting the product concentration as a function of reaction time and determining the slope of this line at time close to zero. It was expressed as μmol of product formed per minute per gram of lipase.

2.2.4. Purification and structural analysis of the biosynthesis ascorbyl benzoate

After filtration, the reaction mixture was evaporated under reduced pressure, lyophilized and subjected to a preparative Waters 600 HPLC instrument (Waters Corp., Milford, Massachusetts, USA) equipped with a Shim-pack PREP-ODS column (250 × 20 mm I.D.) (Shimadzu, Kyoto, Japan) and a Waters 2487 UV detector (Waters Corp., Milford, Massachusetts, USA) set at 254 nm. A solution of methanol/water/H_3PO_4(40/60/0.1, v/v/v) flowing at 10 ml min^{-1} was served as the mobile phase. The sample was dissolved in methanol (0.2–0.3 g ml^{-1}) and injected 0.5 ml once. At the end, the pure ester can be acquired by collecting the efflux at desired peak, rotary evaporation and lyophilization.

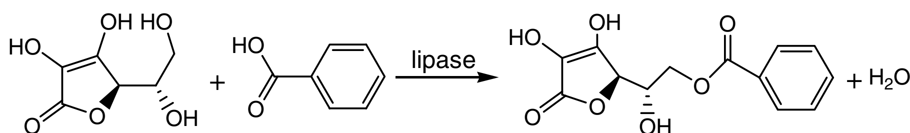

Fig. 1. Synthesis of ascorbyl benzoate catalyzed by lipase in organic media.

Subsequently, its ^{1}H NMR spectra were recorded on a Bruker Am 500 spectrometer (Karlsruhe, Germany) at 500 MHz.

2.2.5. Antimicrobial activity determination

A broth microdilution method, which was slightly adjusted based on the method recommended by the National Committee for Clinical Laboratory Standards (NCCLS, 2002), was applied to determine minimal inhibitory concentrations (MICs) of antimicrobial agent. It was performed on 96-well microplates in a sterile environment. To the first well of a row was added 200 μl broth without antimicrobial agent; while to others were added 100 μl antimicrobial broth solutions with the prepared concentration. By transferring 100 μl broth from one well to the next, discarding the 100 μl broth from the eleventh well, a serial dilution of the original antimicrobial solution could be obtained (from 1:2, 3:4 to 1023:1024 ending with the prepared concentration). By this method, relatively high MICs can be estimated more accurately.

Mueller–Hinton broth and RPMI 1640 broth were separately used as the medium for microdilution in antibacterial and antifungal test. RPMI 1640 (supplemented with 2% glucose for yeasts) broth was buffered to pH 7.0 with 0.165 M 4-morpholinepropanesulphonic acid (MOPS) (Sigma, Madrid, Spain) before use. The standard inoculum was added to give a final concentration of 5×10^5 CFU ml^{-1} for bacteria and 2×10^3 CFU ml^{-1} for fungi. After incubation of the 96-well microplates with bacteria at 35 °C and microplates with fungi at 28 °C for 48 h in ambient air, MICs were recorded at 12, 24, and 48 h as the lowest concentration of antimicrobial inhibiting visible growth. Controls without antimicrobial agent were included to verify the effect of diluents on growth of microbes. Three trials were concluded for each experiment.

2.2.6. Antioxygenic property determination

Schaal oven test (Fennema, 1976) was carried out to study the antioxidant activity during the accelerated storage of oils. Refined, bleached, deodorized peanut oil without any added synthetic antioxidant was used for the storage study. Triplicate samples of peanut oil (50 g) with added ascorbyl benzoate, benzoic acid, BHT, TBHQ and PG at 50, 100, 200 ppm level and three control sets without antioxidant were stored in 100 ml open uniform glass beakers at 60 ± 1 °C in a dark drying oven for 30 days. The rate of oil oxidation was monitored by the increase of peroxide values (PV). Samples were analyzed after 5, 10, 15, 20, 25, and 30 days for PV by iodometric determination (AOCS, 2004) to follow the oxidative changes.

3. Results and discussion

3.1. Optimization of organic solvents

Hydrophobicity of an organic solvent is important for the enzyme activity. $\log P$, where P is the partition coefficient of a given solvent between *n*-octanol and water (Lyman, 1990), is a widely used parameter to describe solvent hydrophobicity and their possible effects on enzyme activity. Generally, solvents with a $\log P > 4.0$ are recommended in enzyme-catalyzed reactions.

Nine organic solvents listed in Table 1 were selected in a $\log P$ range from −1.30 to 2.50. Only in four of them with a $\log P < 2.0$, acetone ($\log P = -0.23$), tetrahydrofuran ($\log P = 0.49$), cyclohexanone ($\log P = 0.96$) and *t*-amyl alcohol (2-methyl-2-butanol, $\log P = 1.31$) produced ascorbyl benzoate. This is somewhat inconsistent with general reports that solvents with $\log P < 2$ are less suitable for biocatalysis (Jyh-Ping, 1996). However, considering the big polarity difference between L-ascorbic acid and benzoic acid/ascorbyl benzoate, the above result is also reasonable. L-ascorbic acid is hydrophilic and easy to dissolve in polar solvents, but the other two are hydrophobic and preferred by non-polar solvents. Therefore, organic solvents with an appropriate polarity are significant for the substrate and product solubility and mass transfer in the reaction. Meanwhile, if the enzyme's microenvironment favored high substrate but low product solubility, the conversion rate would be high. In the second place, enzymes in nonaqueous systems could be active provided that the essential water layer around them is well retained. High polarity solvents might strip off the essential water and thus affect the active conformation of the enzyme. This might be a reason why no ascorbyl benzoate was detected in organic solvents with a very low $\log P$. Nevertheless, there are various exceptions such as porcine pancreatic lipase that could retain its bound water and be active even in water-miscible solvents (Zaks & Klibanov, 1985).

Higher conversion rates could be distinctly observed in acetone (2.38%) and cyclohexanone (6.28%) than those in tetrahydrofuran (0.53%) and *t*-amyl alcohol (0.94%), which indicated that the type of organic solvent besides $\log P$ value is also important for enzyme. In the following study, cyclohexanone was chosen as the solvent.

3.2. Effect of pH value

It was discovered (Zaks & Klibanov, 1985) that the enzyme catalytic behavior in the organic solvent reflects

Table 1
Effect of solvent on the reaction in 25 ml screw-caped glass vials with 1 mmol L-ascorbic acid, 1 mmol benzoic acid, 250 mg lipase and 10 ml organic solvent at 55 °C and 200 rpm for 48 h

Solvent	$\log P$	Conversion rate (%)
Dimethylsulfoxide	−1.30	0
N,N-Dimethylformamide	−1.00	0
Acetone	−0.23	2.38
Tetrahydrofuran	0.49	0.53
Cyclohexanone	0.96	6.28
t-amyl alcohol	1.31	0.94
n-Butyl acetate	1.70	0
Benzene	2.00	0
Toluene	2.50	0

the pH of the last aqueous solution to which they were exposed (e.g. from which they were lyophilized). In order to control the reaction pH, 250 mg of lipase was added to a 5 ml tube with 1 ml buffer (0.2 M citric acid and dibasic sodium phosphate), freeze-dried, and applied to the ascorbyl benzoate synthesis system.

Fig. 2 shows that when the pH of the buffer changed from 5.0 to 7.0, the conversion rate increased remarkably compared with that of cyclohexanone in Table 1, because the pH of lipase used in Table 1 was not adjusted and kept at that favorable for storage, but not reaction. Both maximum conversion and maximum initial rate were achieved at pH 6.0. Increasing pH from 6.0 to 7.0, the initial rate decreased more drastically than that during reducing pH from 6.0 to 5.0, but the conversion rates almost decreased to the same level. This result demonstrates that the optimal pH for the esterification reaction was 6.0, and the lipase was more stable during reaction in the range from 5.0 to 6.0. From these results, it is easy to conclude that unless enzyme powders are obtained from an aqueous solution of the pH affording maximal activity, enzymatic performance in organic solvents is doomed to be suboptimal.

3.3. Influence of the thermodynamic water activity (a_w)

Thermodynamic water activity, a_w, instead of water content is used to study water effects on enzyme reactions in organic media (Goderis et al., 1987). In this research, both lipases (pH = 6.0) and cylcohexanone were pre-equilibrated through vapor phase in sealed containers with saturated salt solutions of known a_w at 25 °C for 7 days. The salts used were LiBr ($a_w = 0.06$), LiCl ($a_w = 0.11$), $MgCl_2$ ($a_w = 0.33$), $Mg(NO_3)_2$ ($a_w = 0.55$), NaCl ($a_w = 0.75$) and K_2SO_4 ($a_w = 0.97$).

Fig. 3 revealed that the initial rate increased steadily from 1.57 to 12.85 μmol min^{-1} g^{-1} of lipase with the rising of a_w, but the conversion rate reached a maximum value at a_w 0.33. These results confirmed the conclusion that the lipase is an excellent catalyst at low a_w (Wehtje & Adlercreutz, 1997). Since a certain amount of water on surface is essential for keeping active conformation of enzyme (Faber, 2000), increase of a_w might satisfy the essential

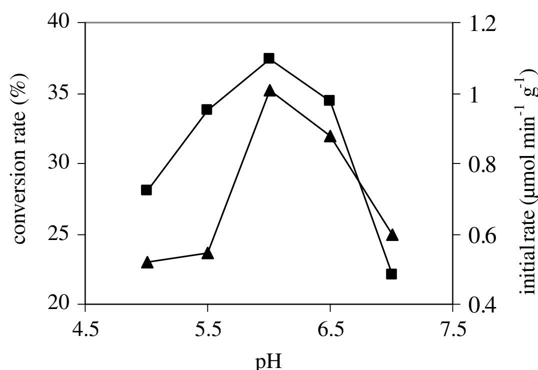

Fig. 2. Effect of pH on the initial rate (■) and conversion rate (▲).

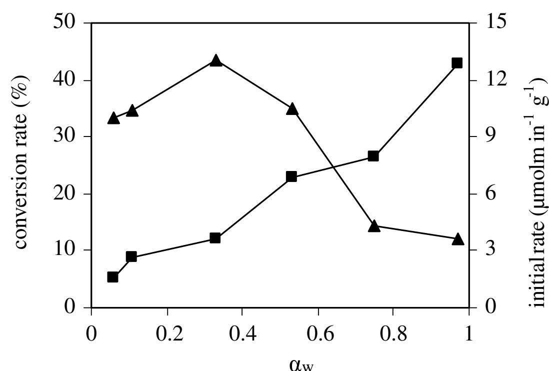

Fig. 3. Effect of water activity (a_w) on the initial rate (■) and conversion rate (▲).

water of lipase, which subsequently leads to an increase of initial rate. In addition, accumulation of water around the catalyst particles would facilitate substrates, especially for L-ascorbic acid to the enzyme molecules. However, as water is also a reaction product that can affect the equilibrium between esterification and hydrolysis of the presumed acyl-enzyme intermediate, high a_w might lead to a low conversion.

3.4. Effect of substrate

The effect of substrate concentration on the reaction is depicted in Fig. 4. When the substrate concentration increased, the conversion rate firstly increased and reached a maximum, then decreased, while the output increased to the highest and kept unchanged. This result reflected the change of lipase/substrate concentration ratio. Only at low substrate concentration, the conversion rate increases with rising of substrate concentration because all substrates available can form the lipase/substrate complex.

Fig. 5 shows the effect of the mole ratio of L-ascorbic acid to benzoic acid on the conversion rate. When the mole ratio increased from 0.1 to 1.0, the conversion rate decreased slowly, while it increased rapidly when the mole ratio

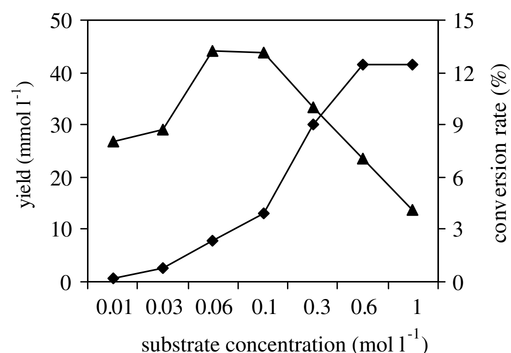

Fig. 4. Effect of substrate concentration on the conversion rate (▲) and yield (◆). Reactions were conducted in 25 ml screw-caped glass vials with 250 mg lipase, 10 ml cyclohexanone and different concentrations of equimolar L-ascorbic acid/benzoic acid at pH 6.0, 55 °C, 200 rpm for 48 h.

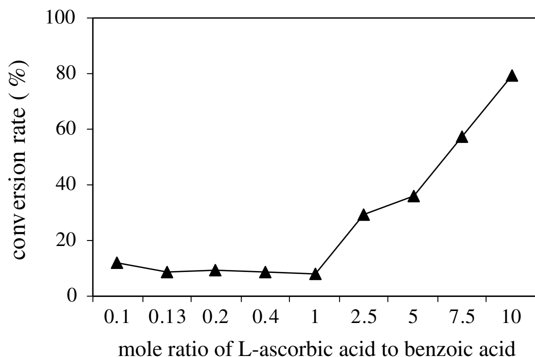

Fig. 5. Effect of mole ratio of L-ascorbic acid to benzoic acid on the conversion rate. The result was acquired by fixing L-ascorbic acid concentration at 0.01 M and varying benzoic acid concentration from 0.01 M to 0.1 M and vice versa. Reactions were conducted in 25 ml screw-caped glass vials with above substrate concentration, 250 mg lipase, 10 ml cyclohexanone, at pH 6.0, 55 °C and 200 rpm for 48 h.

increased from 1.0 to 10. The maximum conversion rate (79.48%) was achieved at a mole ratio of 10:1, and higher mole ratios were not tested for the limitation of solubility. This result indicated that excess L-ascorbic acid had a stronger promotion effect on the esterification than that of excess benzoic acid, and benzoic acid was not an ideal substrate of this lipase, which led to low conversion rates, but its limitation could be overcome by excess L-ascorbic acid.

3.5. Effect of temperature

The influence of temperature was studied at a_w 0.97, because the initial rate was the highest and therefore the reaction equilibrium could be acquired as soon as possible at this water activity. Fig. 6 displays that slopes of the time course of conversion rate increased markedly with the increase of temperature form 50 °C to 70 °C in the first thirty minutes, which confirmed a rise of the enzyme activity with temperature. Thirty minutes later, the conversion profile changed appreciably, and a lower conversion rate was obtained at 70 °C than those at 60 °C and 65 °C because of enzyme deactivations caused by a long time exposure at high temperatures.

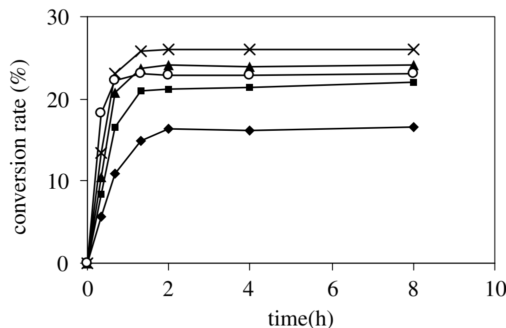

Fig. 6. Time course of ascorbyl benzoate synthesized at 50 °C (◆), 55 °C (■), 60 °C (▲), 65 °C (×) and 70 °C (○).

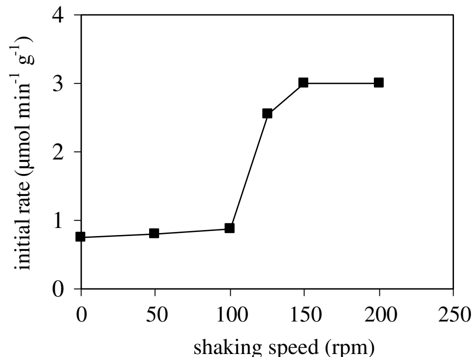

Fig. 7. Effect of shaking speed on the initial rate.

3.6. Effect of shaking speed on the initial rate

It can be seen from Fig. 7 that the initial rate increased simultaneously with shaking speed below 150 rpm, and it increased slowly below 100 rpm due to the mass transfer limitations. Higher than 150 rpm, the initial rate was independent of the shaking speed, because the mass transfer limitations had been overcome for the reaction system.

3.7. Antioxygenic property of ascorbyl benzoate

The peroxide value (PV) development showed that ascorbyl benzoate (AB) and other antioxidants remarkably retarded the oxidation in Schaal oven test (Fig. 8). Peanut oil without antioxidants (control) reached a maximum PV of 232.5 meq kg^{-1} after 30 days of storage, while those with 200 ppm of TBHQ, PG, BHT, AB and AP were only 12.1, 39.5, 102.4, 150.4 and 156.1 meq kg^{-1}, respectively. During the whole test, samples with TBHQ always maintained the lowest PV of the five, which confirmed the outstanding antioxidant activity of TBHQ (Kamil, Jeon, & Shahidi, 2002; Zhang, Wu, & Weng, 2004). PG was the second best antioxidant in the test. AB and AP showed similar antiox-

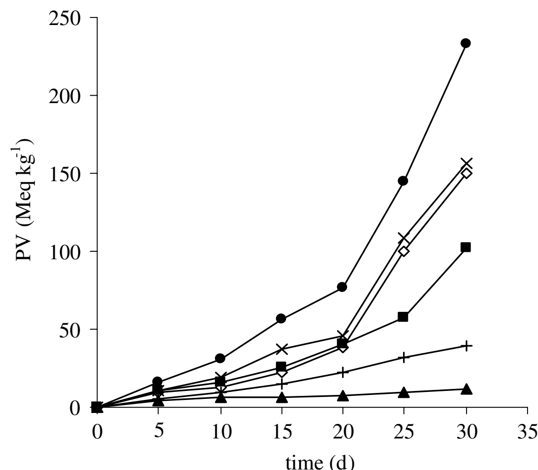

Fig. 8. Peroxide values (PV) of peanut oil without antioxidant (●) and with added one of 200 ppm AP (×), ascorbyl benzoate (◇), BHT (■), PG (+) and TBHQ (▲) during storage at 60 °C.

Table 2
Antibacterial activity of ascorbyl benzoate, benzoic acid, sorbic acid and propyl-*p*-hydroxy benzoate

Species	Ascorbyl benzoate		Benzoic acid		Sorbic acid		Propyl-*p*-hydroxy benzoate	
	MIC_{24} (mg l^{-1})	MIC_{48} (mg l^{-1})	MIC_{24} (mg l^{-1})	MIC_{48} (mg l^{-1})	MIC_{24} (mg l^{-1})	MIC_{48} (mg l^{-1})	MIC_{24} (mg l^{-1})	MIC_{48} (mg l^{-1})
E. coli	1200	1400	962.5	1065.6	550	962.5	150	262.5
S. aureus	1200	1400	825	1031.3	875	1218.8	281.3	437.5

Data read after 24 h and 48 h were marked as MIC_{24} and MIC_{48} separately. MIC ranges were 800 to 1600 mg l^{-1} ascorbyl benzoate and 550 to 1100 mg l^{-1} benzoic acid for all bacteria, 550 to 1100 mg l^{-1} sorbic acid and 150 to 300 mg l^{-1} propyl-*p*-hydroxy benzoate for *E. coli*, 650 to 1300 mg l^{-1} sorbic acid and 250 to 500 mg l^{-1} propyl-*p*-hydroxy benzoate for *S. aureus*.

Table 3
Antifungal activity of ascorbyl benzoate, benzoic acid, sorbic acid and propyl-*p*-hydroxy benzoate

Species	Ascorbyl benzoate		Benzoic acid		Sorbic acid		Propyl-*p*-hydroxy benzoate	
	MIC_{12} (mg l^{-1})	MIC_{24} (mg l^{-1})	MIC_{12} (mg l^{-1})	MIC_{24} (mg l^{-1})	MIC_{12} (mg l^{-1})	MIC_{24} (mg l^{-1})	MIC_{12} (mg l^{-1})	MIC_{24} (mg l^{-1})
A. niger	3346.9	4191.8	1575	2325	1400	2625	750	998.1
S. cerevisiae	3396.7	4195.9	1050	2395.3	1400	2450	750	1095.7

MIC ranges were 2100–4200 mg l^{-1} ascorbyl benzoate, 1200–2400 mg l^{-1} benzoic acid, 1400–2800 mg l^{-1} sorbic acid, and 550–1100 mg l^{-1} propyl-*p*-hydroxy benzoate for both *A. niger* and *S. cerevisiae*.

idant curves, but the PV of samples with AB was lower than that with AP. BHT inhibited the formation of hydroperoxides less than AB but more than AP during the first twenty days of storage. After that the PV of samples with AB increased rapidly and exceeded that with BHT.

A series of experiments at 50 ppm and 100 ppm were also conducted in this work (results not shown here). Test results showed a similar trend with that at 200 ppm. Nevertheless, it was found that antioxidant effect of TBHQ and PG at 50 ppm was far better than that of BHT, AB and AP at both 100 and 200 ppm.

3.8. Antimicrobial activity of ascorbyl benzoate

The MICs of ascorbyl benzoate, benzoic acid, sorbic acid and propyl-*p*-hydroxy benzoate were tested against two bacteria and two fungi using broth microdilution method (Tables 2 and 3). Propyl-*p*-hydroxy benzoate possessed the best antibacterial and antifungal ability, and sorbic acid behaved a little better than benzoic acid in its ability against *E. coli*, but nearly the same with benzoic acid when it was applied to *S. aureus*, *A. niger* and *S. cerevisiae*. In contrast, ascorbyl benzoate performed a relatively weaker antimicrobial ability than that of others. This will not influence the antimicrobial application of this product, since esters as antimicrobial agent also have some advantages such as a wide range of active pH over the acids range.

3.9. Structural analysis

The purity of isolated ascorbyl benzoate was over 98%. ^{1}H NMR (500 MHz, CH_3OD): $\delta = 4.25$ (m, 1H, H-6), 4.47 (m, 2H, H-5, H-6), 4.85 (t, $J = 12.79$, 1H, H-4), 7.47 (t, $J = 7.67$, 2H, H-3′, H-5′), 7.60 (t, $J = 7.30$, 1H, H-4′), 8.06 (d, $J = 7.73$, 2H, H-3′, H-5′).

4. Conclusions

Organic solvents with $\log P < 2.0$, slightly acidic environments, low water activities, excess L-ascorbic acid, an appropriate substrate and lipase concentration were found favorable for production of ascorbyl benzoate. The hydrophilicity and solubility of L-ascorbic acid, the essential water and equilibrium theory of enzyme reaction, mass transfer, the activation and deactivation of temperature on the enzyme were supposed to explain the observed results. At the end of reaction, the product was purified and identified, and a subsequent experiment found that it had obvious antimicrobial and antioxidant activity. To the best of our knowledge, the lipase-catalyzed esterification condition of this product was studied for the first time.

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