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Optimization of aqueous enzymatic method for *Camellia sinensis* oil extraction and reuse of enzymes in the process

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Aqueous enzymatic extraction of *Camellia sinensis* oil was studied. The results suggested that saponin removal pretreatment assisted by ultrasound was effective in decreasing emulsification and in enhancing the free oil recovery. After 70% isopropanol extraction for 30 min under ultrasound, the residue of *C. sinensis* seeds was further hydrolyzed with free cellulase and Alcalase for 5 h, and calcium ions were concurrently added during enzymatic hydrolysis ($n_{Ca^{2+}}$: $n_{saponin}$ = 1:2), and free oil recovery up to 94.14% was obtained. Separate immobilization and co-immobilization of Alcalase and cellulase were performed by alginate entrapment combined with glutaraldehyde crosslinking. Specific activity and recovery of activity for Alcalase and cellulase were acceptable. After immobilization, Alcalase and cellulase exhibited higher activity at a wider pH and temperature range. Reuse experiments of immobilized enzymes were conducted. The deactivation kinetics immobilized enzymes were simulated and half-life of immobilized enzyme was estimated. The results indicated that a magnetic supporter facilitated the recovery of immobilized enzymes from tea seed slurry, and that immobilized Alcalase and cellulase had good reusability.

[**Key words:** *Camellia sinensis* seed; Aqueous enzymatic extraction; Ultrasonic pretreatment; Immobilized enzyme; Reusability]

Camellia sinensis (tea tree), which is native to East Asia, the Indian Subcontinent, and Southeast Asia, is cultivated across the world in tropical and subtropical regions. The seeds of the tea tree can be used for preparation of tea oil (29%–34%). Other valuable components consist of 17%–20% starch, 11%–15% saponin, 10%–16% protein, and 10%–14% fiber, which surround the seed bodies (1). Tea seed oil has a high content of unsaturated oleic acid (70%–85%) and natural antioxidants (2). In addition, tea seed oil has been shown to provide many health benefits, including hepatoprotection, antigastric activity against ulcers, lowering blood pressure and blood lipids, anti-obesity, care of the skin, and minimizing the signs of aging. The tea seed oil is therefore increasingly favored by consumers (3–6).

Aqueous enzymatic extraction (AEE), an attractive and emerging technology, was used for tea seed oil extraction because it provides a safer product; effectively extracts the bioactive compounds; requires a lower facility investment; and the process is environmentally friendly. AEE has been used for many oilseeds and oil-rich agricultural materials such as soybeans (7), sesame seeds (8), and pumpkin seeds (9). Nevertheless, application of AEE on a large scale is still limited by lower free oil recovery (FOR) resulting from emulsification and the high cost of the required enzymes (10). Chabrand et al. (11) reported that AEE could be used for soy flour oil recovery, and the cream was collected and subjected to various single and combined treatments, including thermal and enzymatic treatments. When enzymatic treatment was followed by a freeze-thaw step, the oil recovery increased from 3% to 46%. Later, destabilization of the emulsion was further characterized by Chabrand and Glatz (12), who showed that acidification (pH 4.5) increased the soybean oil recovery from 2% to 83%. A two-stage enzymatic demulsification process with alkaline endopeptidase or lysophospholipase A_1, led to a 95% to 100% oil recovery from the cream. These treatments all targeted the protein, peptide, and phospholipids involved in cream formation during AEE for soy oil recovery. However, each oil seed has its own compositions, so the components responsible for stabilization of the cream are different. Fang et al. (13) explored the AEE process for *Camellia* oil extraction with protease and cellulase, and found that the free oil yield was increased from 82.36% to 91.38% through de-emulsification with 20% ethanol after the AEE process. Most of the present methods focus on end-of-pipe treatments to separate the oil from the cream produced, resulting in time-consuming, operationally complicated, and unsatisfactory oil recovery. Avoiding or reducing emulsification during AEE is a desired strategy to increase the free oil recovery. Zhang et al. (14) developed a novel process for oil extraction from *Camellia oleifera* seed kernels combined with microwave puffing and AEE. To minimize emulsion formation, saponin removal by microwave extraction prior to AEE was used, and a high oil extraction yield of ~95% was obtained, regardless of industrial feasibility. Nevertheless, heating resulting from microwave treatment is undesirable for production of highly unsaturated oil. Recently, a simple and practical AEE process for tea oil extraction with the concurrent addition of calcium chloride was developed by our group. To eliminate the involvement of saponin in cream, calcium chloride solution was added in batches during the

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enzymolysis stage. Lower emusification degree and satisfactory FOR were achieved (15). Even so, the removal of saponin was still limited due to its relative lower specificity for binding with calcium ions. Ultrasound treatment is a type of cold processing, which can simplify extraction manipulation, reduce the consumption of solvent, and enhance efficiency of extraction, and is therefore a desirable, clean method with high reproducibility for extraction (16). To facilitate oil extraction, saponin removal by isopropanol extraction assisted by ultrasound, followed by AEE, was explored in this study.

Enzymes are expensive and are difficult to separate from the *Camellia* slurry after being utilized, making it difficult to recycle and limiting the application of AEE of tea oil on an industrial scale. Using immobilized enzymes for the AEE process may therefore be a better choice. Immobilized enzymes refer to enzymes physically confined or localized in a certain defined region of space with retention of their catalytic activities (17). Compared to their free forms, immobilized enzymes are generally more stable. The heterogeneity of the immobilized enzyme systems allows an easy recovery of both enzymes and products, multiple reuse of enzymes, continuous preservation of enzymatic activity, and rapid termination of reactions. In addition, the reaction products are not contaminated with the enzyme (18,19). Zanphorlin et al. (20) found that the optimum temperature and thermal stability of alkaline protease was improved significantly after being immobilized on beads of calcium alginate, and the immobilized protease showed considerable stability for up to seven cycles of reuse at 50°C. Cherian et al. (21) found that cellulase immobilized on MnO_2 nanoparticles showed hydrolytic activity for cellulosic substances over a broader range of temperatures (60°C–80°C) and pH (pH 4–7). Magnetic materials have also shown great potential for application in enzyme immobilization due to the property of easy recovery (22). Fang et al. (23) reported that pectinase could be immobilized on amino-functionalized magnetic nanoparticles (Fe_3O_4@mSiO_2–NH_2) through the formation of Schiff bases using glutaraldehyde as a cross-linking agent, which efficiently prevented enzyme leakage and improved enzyme activity and reusability.

For a multi-enzyme reaction system, co-immobilization is desirable. By combining individual catalytic properties, multiple enzyme reactions can be carried out at the same stage, which expands the effective application scope of a single enzyme. The co–catalytic reaction effectively reduces the input of reaction steps and production equipment, and reduces the loss of intermediate products caused by separation (24). Co-immobilization of alpha-amylase, protease, and pectinase with alginate and glutaraldehyde-activated chitosan beads were carried out and evaluated by Gür et al. (25). Yu et al. (26) found that cellulase and laccase showed greater stability at higher pH and temperature and retained more than 35% of the original enzyme activities after five cycles of repeated uses with co-immoblization on reversibly soluble polymers.

Although immobilization of enzymes has the advantages of enhancing enzyme stability, widening the pH tolerance range, declining costs, and improving enzyme selectivity (27), there have been few reports describing oil seed degradation for oil recovery using immobilized enzymes. During the AEE process, the oil seed substrate exists in the form of a slurry, and some AEE processes are sequentially performed using multi-enzyme hydrolysis, which makes it difficult to recycle the immobilized enzyme. Hence, studies on oil seed hydrolysis using immobilized enzymes and co-immobilized enzymes need to be developed.

In this study, pretreatment for saponin removal by ultrasound treatment assisted isopropanol extraction, followed by AEE combined with the concurrent addition of calcium chloride during the enzymolysis stage, which was used to decrease the emulsification. To reduce the cost of the AEE process, Alcalase and cellulase were immobilized separately and co-immobilized using supporter-containing Fe_3O_4 nanoparticles, followed by evaluation of the immobilization efficiency. In the following report, the reuse of individually immobilized enzymes and co-immobilized enzymes were compared in terms of FOR and the stability of the immobilized enzymes.

MATERIALS AND METHODS

Materials *C. sinensis* seeds (with oil content of 27.68%, protein content of 8.75%) were purchased from Longwu Town, Hangzhou, China. Cellulase (EC 3.2.1.4 from *Trichoderma reesei*, enzyme activity, 100,000 U/g) was obtained from Yihao Tian Biotechnology, Jiangsu, China. Alcalase (alkaline serine endopeptidase from *Bacillus licheniformis*, enzyme activity, 280,800 U/g) was purchased from Novozyme, TianJin, China. Glutaraldehyde (50% in H_2O) and nano-sized Fe_3O_4 (99.5%, 20 nm) were purchased from Macklin Biochemical, Shanghai, China. Sodium alginate was obtained from Aladdin Bio-Chem Technology, Shanghai, China. All the other chemicals and solvents were of analytical grade from Sinopharm Chemical Reagent (Shanghai, China).

Aqueous enzymatic extraction The AEE process was carried out according to our previous method with minor modifications (15). Tea seed kernels were pulverized using a grinder (AR1044; Moulinex, Grenoble, France) and then passed through a 40-mesh sieve prior to extraction. The initial amount of oil present in the seeds was measured by Soxhlet extraction. Five grams of tea seed powder was mixed with distilled water at a mass ratio of 1:8 (w/v), the slurry was heated to 90°C, held for 10 min, and then allowed to cool to the enzymatic digestion temperature. The slurry was first adjusted to pH 9.0, then cellulase (8000 U per g of tea seed powder) was added and the mixture was agitated for 1 h at 50°C. The temperature of the suspension was increased to 60°C, followed by the addition of Alcalase (22,464 U per gram of tea seed powder). The enzymatic hydrolysis was allowed to proceed for a further 4 h at this fixed temperature under constant horizontal shaking in a rotary shaker. During the enzymatic hydrolysis, 0.05 M calcium chloride ($n_{Ca^{2+}}$: $n_{saponin}$ = 1:2) was added at a flow rate of 1.35 mL/h. The suspension was then centrifuged at 3000 ×*g* for 30 min to allow phase separation. Free oil was carefully collected and weighed. FOR was calculated as the mass of free oil extracted from a given mass of kernel (g) divided by the mass of oil contained in the kernels initially processed (g) and then multiplied by 100.

Pretreatment for saponin removal by ultrasound assisted solvent extraction Before hydrolysis, 5 g tea seed powder was dispersed into the specific solvent at a mass volume ratio of 1:7 (w/v), and pretreatment by ultrasound was performed for 15 min in an ultrasonic bath at 100 W. The residue with saponin removal was obtained and subjected to the AEE process as described above. The type (methanol, ethanol, isopropanol, or distilled water) and concentration of extraction solvent for ultrasonic pretreatment were optimized in terms of removal rate of saponin and FOR obtained. The content of saponin was determined by the vanillin-sulfuric acid method (28).

Immobilization of Alcalase and cellulase Sodium alginate was dissolved in distilled water to obtain 2.0% (w/v) sodium alginate solution, and 1.6% (w/v) of Fe_3O_4 nanoparticles was added into the solution and stirred for 30 min. The mixture was heated to 80°C and stirred under a nitrogen atmosphere for another 1 h. Subsequently, the ferrofluid was further ultrasonically dispersed for 20 min and purified by removing a small amount of large agglomerates by centrifugation (1000 ×*g*, 10 min). A total of 10 mL of Alcalase (30,000 U) or cellulase (30,000 U) solution was added to 100 mL of magnetic fluid, and after mixing evenly, a syringe was used to inject ferrofluid to obtain a 10 wt% $CaCl_2$ aqueous solution to produce magnetic microspheres. After soaking for 1 h, the magnetic microspheres were washed with distilled water to remove free Ca^{2+} adhered to the surface, then crosslinked in 0.5% glutaraldehyde for 1.5 h, and washed with distilled water (29). Finally, the beads were incubated over night at 4°C to harden. After drying for 12 h at 35°C, the immobilized enzyme was obtained.

Hydrolysis activity of free and immobilized enzymes under various pHs and temperatures The effect of pH on the activity of free and immobilized Alcalase was assayed by using 0.045 M borax solution with a pH range from 6.0 to 10.0 at 60°C, and the activity of cellulase in 0.1 M acetic acid-sodium acetate buffer at pH 6.0–10.0 was then assayed at 50°C. The temperature stability of each enzyme was investigated by measuring enzyme activity at various temperatures (30°C, 40°C, 50°C, 60°C, and 70°C) at pH 9.0 in a water bath.

Enzyme assays The assay of Alcalase activity was performed according to Jin et al. (30). The activity of the Alcalase was assayed by incubating 1 mL of diluted enzyme solution with 1 mL of casein (1.0% w/v) for 10 min at 60°C in a constant temperature water bath. The reaction was terminated by adding 2 mL of 0.4 M trichloroacetic acid. The mixture was then incubated for 10 min to precipitate the total nonhydrolyzed casein. Samples and blanks were filtered using Whatman filter paper after the incubation period. The catalytic activity of the supernatant was determined by the Folin-phenol method, using casein as a substrate. One

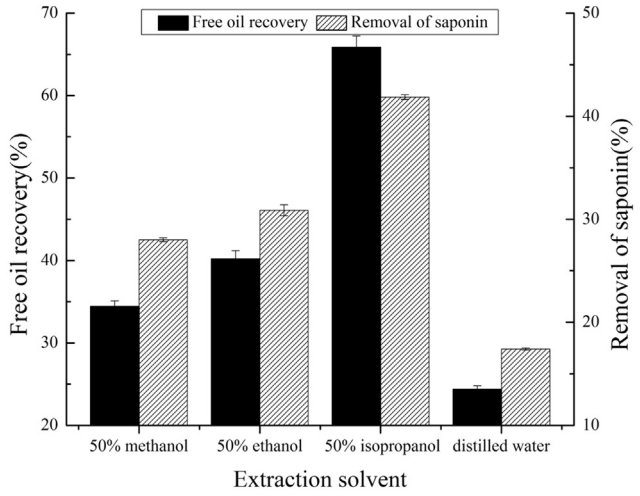

FIG. 1. The removal of saponin and free oil recovery of tea seeds with different extraction solvents.

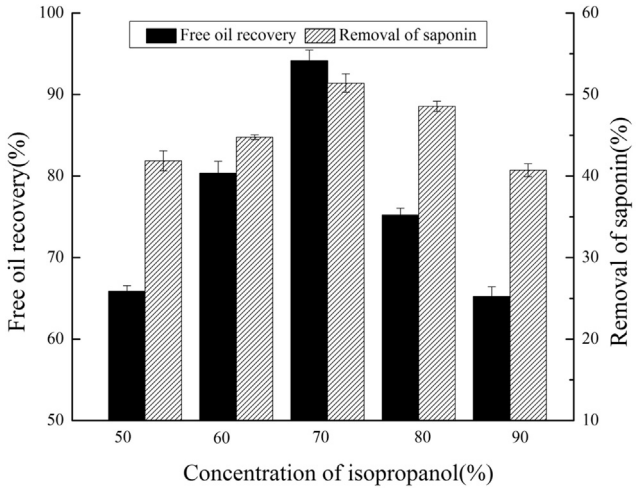

FIG. 2. The removal of saponin and free oil recovery of tea seeds with different concentrations of isopropanol.

milliliter of supernatant was mixed with 5 mL of Na_2CO_3 solution (0.4 M) and 1 mL of Folin-phenol reagent (the original Folin-phenol solution was diluted three fold). The mixture was kept at 40°C for 20 min and the absorbance was measured at 680 nm. Validation of the results was established by treating a standard enzyme solution under identical experimental conditions where the activity was known. One activity unit (U) of Alcalase was defined as the amount of enzyme preparation required to liberate 1 μmol of tyrosine from casein per min at a specified temperature and pH (31).

The cellulase activity assay was conducted by estimating the amount of reducing sugars liberated from sodium carboxymethylcellulose solubilized in sodium acetate buffer (pH 9). The mixture of cellulase and carboxymethylcellulose was incubated for 30 min at 50°C, and the reaction was stopped by the addition of 3,5 dinitrosalicylic acid solution. The treated samples were boiled for 5 min, and cooled in water until color stabilization, then the absorption at 540 nm was measured. The cellulase activity was determined by using a calibration curve for glucose. One unit of activity was defined as the amount of cellulase that released 1 μmol of glucose per minute (32).

The specific activity (activity of enzyme per gram immobilized enzyme, SA) and recovery of enzymatic activity (REA) were calculated as follows:

$$SA(\%) = \frac{\text{Total activity of immobilized enzyme}}{\text{Mass of immobilized enzyme}} \times 100 \quad (1)$$

$$REA(\%) = \frac{\text{Total activity of immobilized enzyme}}{\text{Total activity of added free enzyme}} \times 100 \quad (2)$$

Reuse of immobilized Alcalase and immobilized cellulase Immobilized enzyme equivalent to free enzyme activity was used as a substitute in the AEE process for tea oil extraction. The specific extraction steps are described above. The AEE processes were carried out using four methods: (i) free cellulase was combined with Alcalase immobilized on magnetic alginate; (ii) cellulase immobilized onto magnetic alginate was combined with free Alcalase; (iii) immobilized cellulase was combined with immobilized Alcalase; and (iv) cellulase and Alcalase were co-immobilized. After the hydrolysis was completed, the immobilized Alcalase was collected with a magnet, rinsed with distilled water, and then used for the next round of hydrolysis. The immobilized enzymes was repeated used thirteen times, the ratio of enzyme to substrate for each hydrolysis cycle remained constant. FOR and the residual activity of enzyme of each cycle were measured.

Statistical analysis All statistical analyses were conducted using Origin 8.5 software (Northampton, MA, USA). Three replicates for each set of experimental conditions were conducted, and their averages were determined. The results are expressed as the mean ± standard error.

RESULTS AND DISCUSSION

AEE of oil from tea seeds AEE is a common method for the extraction of oil from oil seeds (33). Nevertheless, there are two problems in the large-scale application for AEE, emulsification and the high cost of enzyme. Different methods, such as phase inversion, freezing-thawing, enzymatic treatment, and changing the pH, have been used to recover oil from emulsions (11,12). To minimize emulsion formation, Zhang et al. (14) removed the saponin from *C. oleifera* seed kernels by microwave-assisted ethanol extraction prior to AEE. In the present study, a method of ultrasonic pretreatment for saponin removal followed by AEE was used to extract tea seed oil, and calcium ions were added during enzymatic hydrolysis to precipitate the residual saponin. The $(saponin)_2Ca$ complex that formed was insoluble in ethanol, ether, and water (34). Solvent types (e.g., methanol, ethanol, isopropanol, or distilled water), concentration and ultrasonic pretreatment conditions were optimized for the removal of saponin. Fig. 1 shows that isopropanol was the optimal solvent in terms of the saponin removal rate and the FOR. The solubility of saponin in solvents was governed by the basic rule of "like dissolves attract", which indicated that polar compounds dissolve in polar solvents, and nonpolar compounds dissolve in nonpolar solvents (35). Because the polarity of aqueous isopropanol is closer to that of tea saponin, tea saponin was more soluble in aqueous isopropanol. After isopropanol extraction, the content of saponin in the seed residue was greatly reduced, which accounted for the decrease in emulsion formed in the subsequent AEE, resulting in an improved FOR. Fig. 2 suggests that pre-extraction with 70% (v/v) isopropanol solution showed the maximum saponin-removal rate and FOR. Further increases in the isopropanol concentration did not enhance the removal rate of saponin. The FOR was improved as the saponin removal ratio increased. Therefore, 70% isopropanol was selected in subsequent processes. Compared to the study we reported previously without pre-removal of saponin, FOR increased to 94.14% from 86.6% (15). The pre-removal of saponin effectively reduced the emulsification in the process of AEE. Zhang et al. (14) reported that an oil yield up to 83% was obtained after pretreatment by microwave-assisted saponin extraction. When the *Camellia* seed kernel residue was further subjected to microwave puffing and aqueous enzymatic oil extraction, the yield was increased to 95%. Nevertheless, microwave treatment is an expensive process and its heating effects are undesirable for unsaturated oil preparations, while ultrasonic pretreatment for saponin removal before AEE was effective in decreasing the emulsification.

Immobilization of Alcalase and cellulase The high cost of enzyme is another disadvantage for application of AEE on an industrial scale. In the present study, we attempted to reuse the

TABLE 1. Recovery of activity and specific activity of protease and cellulase immobilized on the magnetic alginate-based microspheres.

Form of Immobilization	Type of enzyme	SA (U/g)	REA (%)
Immobilization of single enzyme	Alcalase	13932 ± 537	92.88 ± 3.58
	Cellulase	9714 ± 129	64.76 ± 0.86
Co-immobilization of two enzymes	Alcalase	14513 ± 293	96.75 ± 1.95
	Cellulase	7874 ± 139	52.49 ± 0.93

SA, specific activity for Alcalase was determined at 60°C, pH 9.0 and for cellulase at 50°C, pH 9.0. REA, recovery of enzymatic activity.

Alcalase and cellulase via immobilization. Many techniques have been previously used for enzyme immobilization, such as adsorption, covalent binding, entrapment, encapsulation, and crosslinking. Each method has its advantages and disadvantages (36). Entrapment is one of the easiest techniques of immobilization. The major advantage of this technique is the simplicity by which spherical beads can be obtained by submersing a polymer cell suspension into a medium containing positively charged ions (37). The enzyme immobilized by the entrapment technique is restricted in movement by the structure of a gel lattice, but it is free in solution (38). We used the method of entrapment combined with crosslinking to immobilize Alcalase and cellulase. Nanoparticles of Fe_3O_4 were used to make the enzyme magnetic in order to facilate the separation of enzymes from the slurry system containing tea seed powder. Among the many matrices available, porous matrices such as alginate are the most frequently used in the formation of beads (39). Alginate is a natural anionic polysaccharide derived from marine algae, which can form thermally stable and biocompatible hydrogel in the presence of calcium cations (32). In a study by Gür et al. (25), alginate and glutaraldehyde-activated chitosan beads were used for the co-immobilization of α-amylase, protease, and pectinase. For Alcalase and cellulase, we tried to immobilize them separately and simultaneously. The recovery of enzyme activity and specific activity of Alcalase and cellulase immobilized were measured and are shown in Table 1. Alcalase immobilized on magnetic alginate-based microspheres had a high recovery. The initial activity for free Alcalase or cellulase added was 30,000 U for 2 g of magnetic microspheres. The specific activities of immobilized Alcalase and cellulase were 13,932 U/g and 9714 U/g, respectively, and the recoveries of activities were 92.88% and 64.76%, respectively. In order to simplify the hydrolysis procedure, we tried co-immobilization of cellulase and Alcalase on magnetic alginate-based microspheres. The specific activity of co-immobilized Alcalase and cellulase was 14,513 U/g and 7874 U/g, respectively. The recovery of activities for Alcalase and cellulase in the co-immobilized enzyme were 96.75% and 52.49%, respectively.

Characterization of free and immobilized enzymes The catalytic potential of enzymes is affected by several environmental factors, including pH and temperature. The optimal temperatures (T_{opt}) for the free and immobilized Alcalase and cellulase were examined and are listed in Table 2. For Alcalase, whether or not it was immobilized, the T_{opt} was found to be 60°C. Immobilization and co-immobilization with cellulase on alginate did not change its characteristic T_{opt}; however, it improved its temperature stability. For free cellulase, the T_{opt} was 50°C, with the highest activity at 99,599 U/g, and above or below this T_{opt}, the enzymatic activity decreased abruptly. After immobilization, the T_{opt} dropped to 40°C. Andriani et al. (32) and Dinçer and Telefoncu (40) found that the T_{opt} for cellulase remained unchanged or slightly increased after immobilization. Nevertheless, this small difference of enzymatic activity between 40°C and 50°C had little effect on the subsequent hydrolysis reaction. In general, the rate of enzymatic reaction increases with the increase of reaction temperature to a certain level, and thereafter, the temperature increase causes protein denaturation and thus decreases the reaction rate (41). However, the immobilized cellulase exhibited high activity in a broader temperature range (30°C–70°C) compared to its free form. Hence, 40°C was taken as the T_{opt} for immobilized cellulase, in our experiments.

At varied pH, the catalytic activity of Alcalase and cellulase at each T_{opt} was reported as shown in Table 3. The optimum pH (pH_{opt}) for both free and immobilized Alcalase activities was pH 9.0. The free cellulase had a pH_{opt} at approximately pH 6.0, and the activity decreased sharply at higher pH values. The pH_{opt} for immobilized cellulase was shifted to pH 8.0. This might be due to the polyanionic microenvironment of alginate caused by mannuronic and glucuronic sugar residues, which surrounded and protected the enzyme molecule (40,42). This pH_{opt} for the immobilized cellulase was close to that of immobilized Alcalase, which was beneficial for the one-step hydrolysis of the co-immobilized enzyme. Immobilized Alcalase and cellulase showed acceptable pH stabilities over a broad pH range. Khoshnevisan et al. (43) also found that cellulase could be used at broader temperature and pH ranges after immobilization on superparamagnetic nanoparticles. It can be seen from Tables 1–3 that under the same conditions, the activity of the co-immobilized Alcalase was slightly higher than that of the separately immobilized enzymes, while the result of cellulase was reversed. Nevertheless, the activity difference between co-immobilization and individual immobilization was no higher than 5%–10%, as was within the normal error range. The decreased activity of cellulase in co-immobilized enzymes also could be due to partial proteolysis of cellulase by Alcalase. This agreed with our previous finding that simultaneous hydrolysis by Alcalase and cellulase resulted in significantly lower FOR compared to that with a two-step hydrolysis (15). Whelan and Pembroke (44) also found that cellulase activity was unstable in the presence of protease, and ascribed the activity loss of cellulase to its association with proteases. While the peptides from partial hydrolysis of cellulase favored the activity recovery of Alcalase during immobilization, this could account for the increased proteolytic activity of co-immobilized Alcalase. Chae et al. (45) investigated the effects of peptides on the immobilization of protease using glutaraldehyde-activated Lewatit R258-K resin and found that when soy peptide was added at 0.1 g/L, the activity recovery increased by 20%.

TABLE 2. Specific activity of enzymes at different temperature.

Temperature (°C)	Alcalase (U/g)			Cellulase (U/g)		
	Free enzyme	Immobilization	Co-immobilization	Free enzyme	Immobilization	Co-immobilization
30	50493 ± 83	11585 ± 654	12150 ± 640	70945 ± 4616	9118 ± 255	9104 ± 151
40	115094 ± 534	12176 ± 853	12717 ± 587	83639 ± 5476	9881 ± 126	9796 ± 149
50	214088 ± 1899	12906 ± 548	13862 ± 107	99599 ± 1256	9745 ± 221	9688 ± 103
60	250188 ± 3201	13698 ± 152	14943 ± 107	74209 ± 6528	9267 ± 179	8875 ± 96
70	209434 ± 2953	12792 ± 103	11585 ± 407	60426 ± 6648	8693 ± 333	8580 ± 128

Both Alcalase and cellulase were determined at pH 9.

TABLE 3. Specific activity of enzymes at different pH.

pH	Alcalase (U/g)			Cellulase (U/g)		
	Free enzyme	Immobilization	Co-immobilization	Free enzyme	Immobilization	Co-immobilization
6	171321 ± 11206	11915 ± 493	13283 ± 53	137320 ± 2176	7006 ± 26	6131 ± 77
7	214339 ± 5337	12478 ± 509	13887 ± 480	116283 ± 4530	9579 ± 73	8563 ± 205
8	227673 ± 1067	13072 ± 320	14679 ± 160	101412 ± 1539	9881 ± 26	9796 ± 149
9	250188 ± 3201	13698 ± 152	14943 ± 107	99599 ± 1256	9745 ± 221	9688 ± 103
10	203522 ± 4099	11962 ± 287	13886 ± 640	82914 ± 2176	9615 ± 103	9198 ± 128

The activities of Alcalase and cellulase were determined at 60°C and 50°C, respectively.

Entrapment immobilization by alginate/Fe_3O_4 nanoparticles broadened the pH and temperature ranges at which Alcalase and cellulase functioned. The T_{opt} and pH_{opt} for immobilized Alcalase remained the same values as that of the free enzymes. However, for cellulase, the pH_{opt} after immobilization shifted from pH 6 to 8, which was advantageous to facilitate the hydrolysis process.

Reuse of immobilized Alcalase and cellulase The reuse of the immobilized enzyme is important for reducing the cost of the enzyme production, which is an important factor when considering its suitability for commercial application (46). In order to determine the reusability of immobilized Alcalase and immobilized cellulase, the two immobilized enzymes were used for the hydrolysis reaction and recycled several times, separately. Three different combinations of Alcalase and cellulase were used in the AEE process, including free cellulase combined with immobilized Alcalase, immobilized cellulase combined with free Alcalase, and immobilized Alcalase combined with immobilized cellulase. The co-immobilized Alcalase and cellulase were also used to replace free cellulase and free Alcalase. After the reactions, the immobilized enzymes were collected with magnets, washed with phosphate buffer (pH 8.0), and reused in the next batch reaction. This recycling was repeated thirteen times.

Free cellulase combined with immobilized Alcalase Fig. 3 shows the repeated performance of the Alcalase immobilized on magnetic alginate-based microspheres. The reaction time of Alcalase is 4 h each round. The FOR for the first run hydrolysis was 92.59%, which was close to the results for hydrolysis by free enzyme (94.14%), suggesting good performance of immobilized Alcalase. The FOR decreased gradually during the first four cycles of reuse (83.56%), and thereafter decreased sharply. The residual enzymatic activity showed a similar trend with FOR as the number of cycles increases. After 13 cycles, 52.64% of the original enzymatic activity was maintained. While the corresponding FOR was a bit low with value of 45.48%, this should be related with the breakage of immobilized Alcalase after multi-use. In the study of Anwar et al. (47), protease from a newly isolated strain of *Bacillus subtilis* KIBGE-HAS was immobilized on calcium alginate beads, and then the immobilized enzyme was reused. The immobilized enzyme showed 80% activity during the second reuse and 35% activity on its third reuse, while complete loss of activity of the entrapped enzyme was observed during the fourth cycle. This decrease in activity could be due to conformational changes, instability of the enzyme, and/or leakage of enzyme from the carriers occurring during the reaction and/or the washing of beads at the end of each cycle (48). Differences in enzyme type may also be an important cause. In our study, the immobilized Alcalase was more stable. This should be ascribed to crosslinking by glutaraldehyde. And it was easier to recover the immobilized Alcalase from the hydrolyzed system containing tea seed slurry, with the assistance of magnets.

Immobilized cellulase combined with free Alcalase The results for reuse of immobilized cellulase (1 h each round) are depicted in Fig. 4. The FOR of the first run hydrolysis was 88.12%, which was lower than the results obtained by hydrolysis with free enzyme (94.14%), suggesting that immobilization performance of cellulase was not as good as expected. However, immobilized cellulase showed good stability after reuse. As the number of reuse increased, the activity loss of immobilized cellulase was not significant. After 13 batches of use, 63.24% of the original enzyme activity was maintained. Andriani et al. (32) reported that cellulase immobilized on an alginate support showed rather weak stability. After four cycles of reuse, the remaining activity of the immobilized cellulase was less than 10%.

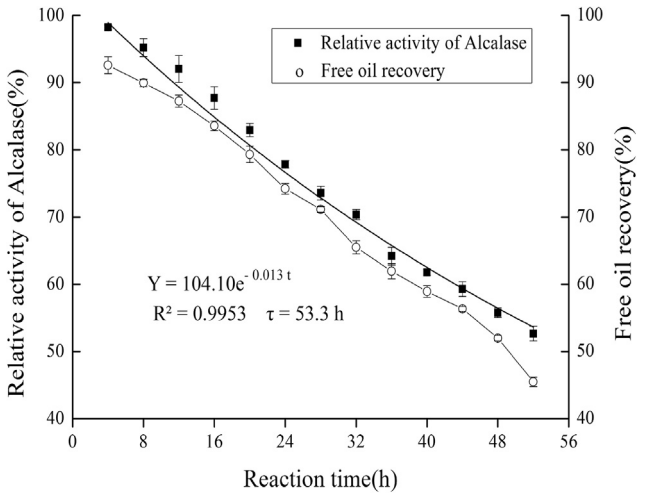

FIG. 3. Deactivation kinetics of immobilized Alcalase and its free oil recovery for repeated hydrolysis.

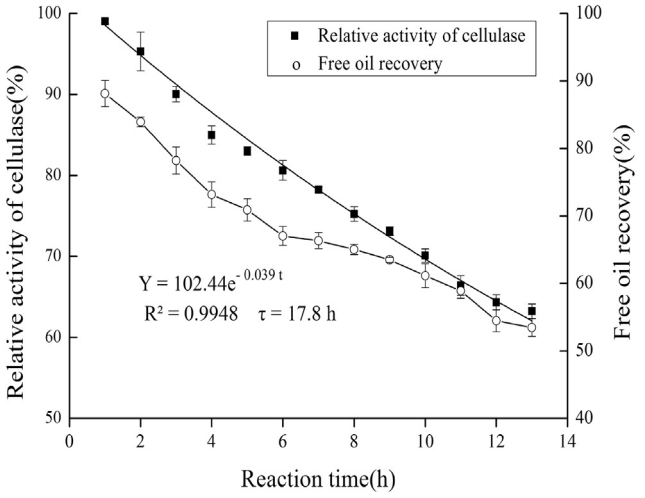

FIG. 4. Deactivation kinetics of immobilized cellulase and its free oil recovery for repeated hydrolysis.

The deactivation dynamic equations of immobilized cellulase and Alcalase was simulated with Eq. 3, and the K_0 and half-time were estimated and are shown in Figs. 3 and 4. The results indicated the R^2 of immobilized Alcalase and cellulase were 0.9953 and 0.9948, respectively. The half-life for immobilized Alcalase and cellulase were 55.3 h and 17.8 h, respectively. The immobilized enzymes showed good stability for repeated use. Predicated half time of enzyme agrees with the practical values well.

The equation used is:

$$K = K_0 \cdot e^{-\frac{(\ln 2)\ t}{\tau}} \quad (3)$$

where K represents the residual activity of the enzyme, K_0 is the constant of reaction, t is the reaction time, and τ is half time of enzymatic activity.

Use of immobilized Alcalase, cellulase, and co-immobilized Alcalase and cellulase After simultaneous replacement of two free enzymes by two immobilized enzymes in the hydrolysis for tea seed oil extraction, up to 82.58% of FOR was obtained. The FOR of the first run hydrolysis was lower than that using only one kind of immobilized enzyme. Unsatisfactory immobilization of cellulase may have contributed to the lower FOR. To facilitate the hydrolysis process further, co-immobilized cellulase and Alcalase were also tested in AEE of tea seed oil, and the FOR value was 72.02%, which was lower than that obtained using the enzymes immobilized separately. This once again verified our previous finding that two-step hydrolysis was superior to simultaneous one-step hydrolysis. The immobilization of the two enzymes on the same carrier resulted in insufficient hydrolysis. In addition, the presence of protease and cellulase together may also have resulted in proteolysis of cellulase. After the three-batch repeated reuse, the residual activities of the Alcalase and cellulase in the co-immobilized enzymes were 77.27% and 73.35%, respectively. The results of reuse of immobilized Alcalase and cellulase suggested that both immobilized enzymes were stable during recycle, and could be reused for more than ten cycles. The FOR for the two-step immobilized enzymatic hydrolysis was superior to simultaneous hydrolysis and co-immobilized enzyme hydrolysis. The magnetic supporter facilitated the recovery and reuse of immobilized enzymes. However, further studies directed to improving the hydrolysis performance of co-immobilized cellulase and Alcalase are needed.

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