

APPLICATION OF SILVER NANOPARTICLE PRODUCTION FOR LIPASE MODIFICATION USING PUER TEA

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Abstract. Application of cystine capped silver nanoparticles synthesized using puer tea as the reducing agent to decorate lipase has been reported in the present work. Biodiesel production technology is competitive in terms of low cost and alternative source of energy which should be not only sustainable but also environmentally friendly. Designing of the lipase immobilization for biodiesel production has a remarkable impact and is still challenging. In this work, biodiesel production from soybean oil was enhanced and facilitated by using a novel biocatalyst consisting of commercial lipase (EC 3.1.1.3), silver nanoparticles, and polydopamine. Various parameters such as cystine concentration, pH, temperature and amount of reducing agent were standardized and their effect on the synthesis process has been initially evaluated by surface plasmon resonance peak analysis. Further the synthesized nanoparticles were also characterized using X-ray diffraction (XRD) and scanning electron microscope (SEM). The transesterification reaction conditions were as follows: oil/alcohol molar ratio 1:4; temperature 40 °C and total reaction time 6 h. Lipozyme TL-100L lipase provided the highest yield of fatty acid methyl esters as 92%. Operational stability was determined with immobilized lipase and it indicated that a small enzyme deactivation occurred after used repeatedly for 10 consecutive batches with each of 24 h. Since the process is yet effective and enzyme does not leak out from the polymer, the method can be proposed for industrial applications. Research highlights Lipozyme TL-100L and Novozym 388 were immobilized onto micro porous polymeric matrix by both physical adsorption and covalent linking. Immobilized enzymes were used for synthesis of fatty acid methyl esters by transesterification of canola oil and methanol using semi-continuous operation system. According to chromatographic analysis, Lipase Lipozyme TL-100L resulted in the highest yield of methyl ester as 92%. The bioconjugates saw 15% loss in its initial activity at the end of five reusability cycles. This decorated reusable system has the potential to be utilized for various applications pertaining to the exploitation of lipase in various industries.

Keywords: *silver nanoparticles, cystine, application, puer tea, reducing agent*

Introduction

Biodiesel production from vegetable oil has been increasing in the last decade in ASEAN countries, for example, Thailand, Malaysia, Indonesia, Vietnam, and The Philippines, since many of them have diverse and high yield crops of oleaginous plants, for example, palm, soybean, and sunflower (Mukta et al., 2010; Murphy et al., 2012). This is relevant to the world's rapid industrialization and increasing population (Preechajarn et al., 2012). Since the use of enzyme in its free form makes the process very expensive, efforts are being made worldwide to develop a stable decorated enzyme system so that the enzyme can be reused and the processing can be made cost

effective. Transesterification of vegetable oils by short chain alcohols (methanol or ethanol) catalyzed by lipase (EC 3.1.1.3) is a worldwide biochemical mechanism to produce biodiesel (Fjerbaek et al., 2009; Fan et al., 2012) and there are two modes of lipase usage: free or soluble lipase and immobilized lipase. In early 2001, using free lipases from *Candida rugosa* and *Pseudomonas cepacia* along with methanol as acyl acceptor, a method was reported to yield biodiesel from soybean oil up to 80 and 90%, respectively (Kaieda et al., 2001). Various matrixes has been successfully used for enzyme immobilization application (to name some alginate, gelatin, polyacrylamide, cellulose, polyvinyl fibers, silica and nanoparticles etc.), among them nanoparticles can be a promising choice (Tan et al., 2010; Salis et al., 2008). Immobilized lipase methods have been introduced to improve lipase stability and reusability. Herein immobilized enzymes are defined as “enzyme physically confined or localized in a certain defined region of space with retention of their catalytic activities, which can be used repeatedly and continuously” (Lee et al., 2007).

Employed lipases for immobilization have been derived from microorganisms to animals, for example, from pancreatic porcine, *Burkholderia cepacia*, *Pseudomonas* spp., and *Candida* spp. (Bu et al., 2012; Peng et al., 2013). Immobilization methods are also various, for example, adsorption on acrylic resin, celite, and anion resin, covalent bonding using silica-PVA and styrene-divinylbenzene, entrapment using hydrophobic sol-gel support, and cross-linking using glutaraldehyde (Ren et al., 2011). These methods can yield biodiesel from soybean oil using adsorbed lipase (Lee et al., 2009) and from mahua oil using cross-linked lipase (Veerapandian et al., 2011). However, these techniques are still sophisticated and require multiple-steps material consumption in case of *Pseudomonas cepacia* cross-linked lipase (Jierwiryapant et al., 1992), as well as time consumption for oil transesterification (49 h) in case of adsorbed *Pseudomonas fluorescens* lipase on macroporous polypropylene matrix (Sangla et al., 2006).

In current study silver nanoparticles were synthesized using puer tea extract as the reducing agent. The formation and morphology of each composition were characterized by UV-Vis spectroscopy and scanning electron microscope (SEM). Polydopamine has also been studied for enzyme immobilization, for example, trypsin (Kanmani et al., 2016), glucose oxidase, and lipase (Rivera et al., 2012). Enzymes can be covalently immobilized on a polydopamine surface via nucleophiles (Walt and Agayn, 1994). The effects of various parameters like temperature, pH and reducing agent concentration were standardized during the course of nanoparticle synthesis. Therefore, nanoparticles were characterized for their surface morphology using SEM. Initial screening of the nanoparticle synthesis was done by monitoring the surface plasmon resonance with help of UV-Vis spectroscopy for each parameter under consideration. This study brings us one step closer to the continuous use of lipase for various biotechnological processes.

Materials and methods

Chemicals

Silver nitrate, glutaraldehyde, Triton X-100 and sodium dodecyl sulfate (SDS) were obtained from Aladdin Chemistry (Shanghai, China). P-nitrophenol palmitate (pNP) was obtained from sigma; L-Cystine was obtained from Aladdin Chemistry (Shanghai,

China). Deionized water was used throughout the process from Millipore and puer tea was procured from Puer, Yunnan province, China.

p-nitrophenol palmitate substrate preparation

420 μM of substrate solution was prepared in Tris-Cl buffer (0.1 M, 8.2 pH) by weighing 0.004 g of pNP and adding it to 25 mL of buffer along with 125 μL of Triton X-100 and 0.0045 g of SDS. The mixture was heated at 65 °C for 20 min and then stored at 4 °C to be used within a week.

Puer tea extract preparation

3.0 g of puer tea leaves were boiled with 50 mL of deionized water at 65 °C for 10 min in a closed container. The extract was first filtered through whatman filter paper No.1 and then centrifuged at 10000 rpm for 10 min at room temperature. The supernatant was collected in a clean falcon tube and stored at 4 °C for further use. The extract was used within a week.

Enzyme production and enzyme activity assay

Lipase was isolated from *B. subtilis* cultured in nutrient broth supplemented with 1% olive oil for 24 h. The broth was then centrifuged at 8000 rpm for 10 min at 4 °C. The supernatant was collected after centrifugation was stored at 4 °C as enzyme source for further use. The crude enzyme was tested for activity using assay mixture comprising of p-nitrophenol palmitate as a the substrate (Gupta et al., 2003). For the activity assay 2.5 mL of substrate was added to 1.5 mL of Tris-Cl buffer and 1 mL of crude enzyme preparation. The assay mixture was incubated at 37 °C for 30 min. The absorbance was recorded at 410 nm. The enzyme activity was calculated as U/mL based on the concentration of p-nitrophenol obtained from the standard curve. The total protein content of the crude enzyme was calculated using the method of (Lowry et al.).

Nanoparticle synthesis and characterization

The nanoparticles synthesis was initiated by mixing 100 mL of 1 mM silver nitrate solution and 1 mL of 1 mM L-cystine. This solution was slowly stirred at room temperature for 10 min. 5 mL of puer tea extract was then added slowly with continuous stirring at room temperature. pH of the reaction mixture was adjusted to 11 by dropwise addition of 1 M sodium hydroxide. The change in the colour of the reaction mixture was recorded at 65 °C for 30 min. The colour of reaction mixture changes from transparent (before addition of tea extract) to light brown (after addition of tea extract) to dark brown (after adjusting pH and heating at 65 °C for 30 min). This change in the colour of the reaction mixture corresponds to synthesis of silver nanoparticles. Thus obtained nanoparticles were then centrifuged at 20000 rpm for 40 min to pelletize the nanoparticles which were dried at 37 °C overnight. The formation of nanoparticles was also confirmed using nanodrop spectrophotometric method in terms of surface plasmon resonance (SPR) at 405 nm. The particles were analyzed by particle size analyzer to determine average size of the particles synthesized. Transmission electron microscopy was performed to observe the surface characteristics of the synthesized silver nanoparticles, while XRD analysis was done to confirm the crystalline structure of the synthesized nanoparticles.

Results and discussion

Optimization of nanoparticle synthesis parameters

Different parameters affecting the synthesis of silver nanoparticles were optimized so that mono- dispersed nanoparticles of uniform size and shape can be obtained. The parameters optimized were analyzed in terms of SPR peaks and the effect of each parameter was recorded on the basis of the SPR peaks achieved for each parameter. To check the consistency of data the experiments were carried out in triplicates.

Effect of varying concentrations of cystine

The effect of cystine concentration on nanoparticle synthesis was analyzed by changing the concentrations of cystine in the reaction mixture. Cystine concentration was varied from 0.5 mM, 1 mM, 2 mM, 5 mM and 10 mM. The effect was observed by monitoring SPR peaks in the complete UV-Vis range for each test concentration of cystine.

Effect of varying concentrations of reducing agent (puer tea)

Various concentrations of silver nitrate solutions: puer tea extract was used to carry out the synthesis of nanoparticles i.e. 100:1, 100:2, 100:3, 100:4 and 100:5. The effect of varying the concentration of reducing agent on the synthesis of nanoparticles was observed by viewing the SPR peaks. *Figure 1a* shows the effect of various concentrations of cystine on the synthesis of nanoparticles. Higher concentration of cystine resulted in slight shift of the peak towards higher wavelength with a broader peak size. This could be because of the aggregation of nanoparticles to form super clusters leading to redshift. Redshift is a phenomenon which is represented by increase in the emitted wavelength by the nanoparticles because of increase in the crystalline structure (size) of the particles. As the size of the particles increases, the distance between the valance band also increases resulting in the increase of the dielectric constant (Yon-Rui et al., 2013).

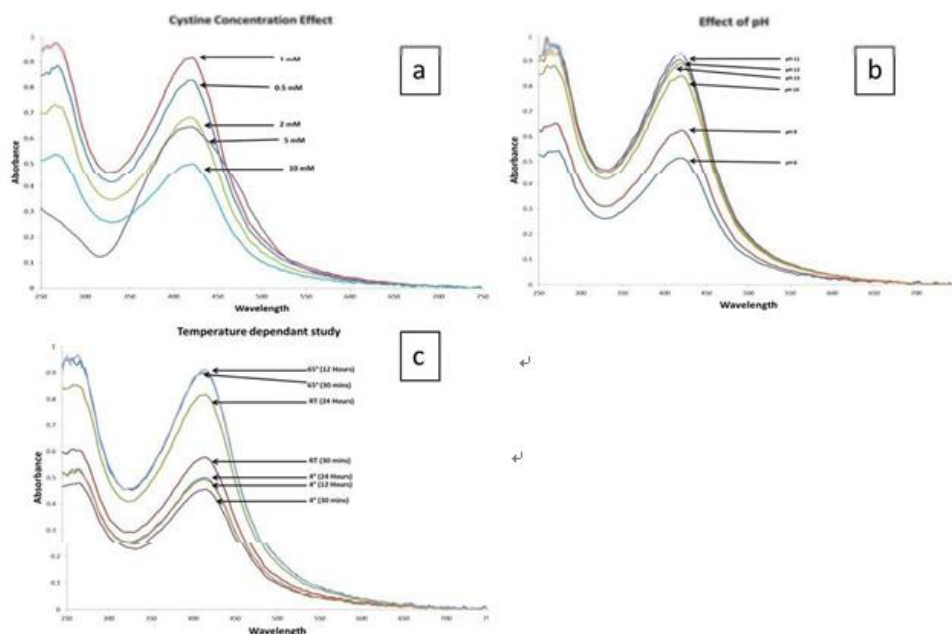


Figure 1. Process optimization for synthesis of cystine capped silver nanoparticles

Effect of pH

pH plays an important role in the formation of nanoparticles since acidic conditions interfere and hinder the synthesis of nanoparticles in most of the cases studied and reported. Under our standard condition, also reduction of silver nitrate was not observed even with prolonged incubation under acidic conditions. Therefore, pH of the reaction mixture was set towards alkaline side by addition of 1 M sodium hydroxide. pH 8, 9 and 10 exhibited slight change in reaction mixture colour and it gave very low intensity peaks indicating low or negligible reduction rate. At pH 11 and 12, a very rapid change in the colour was observed with an intense peak within 30 min of incubation (*Fig. 1b*). Result shown in *Figure 1c* suggests that the synthesis was achieved significantly at high temperature.

Effect of reaction temperature

The study of temperature effect was performed at 3 different temperatures, such as 4 °C, room temperature (25 °C) and 65 °C. The effect of temperature on nanoparticle synthesis was observed by viewing the SPR peaks. Effect of temperature on the synthesis of nanoparticles was studied at three different temperatures i.e. 4 °C, RT and 65 °C. The temperatures selected were from low to medium to high range. Initiation of nanoparticle synthesis took place at every temperature tested which is evident from the colour development at all three temperatures. Result shown in *Figure 1c* suggests that the synthesis was achieved significantly at high temperature. Low temperature resulted into low intensity peaks after 12 h of incubation and did not enhance further even after prolonged incubation, while room temperature lead to medium intensity peaks in about 4 h of incubation and took 24 h to match the intensity observed at high temperature incubation. At 65 °C, a high intensity peak was obtained within 30 min of incubation.

Nanoparticle synthesis and characterization

The puer tea used as reducing agent for the synthesis of cystine capped silver nanoparticles in present work exhibits reducing properties mainly due to presence of anti-oxidant compounds such as catachins in it. The mechanism for the formation of cystine capped silver nanoparticles involves formation of silver cystine complex followed with reduction in presence of anti-oxidant components of puer tea at pH 11 at 65 °C. Cystine binds to silver through thiolate bonding (Mandal et al., 2001). Initially formation of nanoparticle was confirmed by checking SPR peaks spectrophotometrically. A sharp peak at 405 nm (*Fig. 2a*) indicates the formation of silver nanoparticles. The peak arises due to the absorption of electromagnetic waves in the visible region by the nanoparticles that provides them the ability to give a peak at a specific wavelength. The reaction colour changes from light brown to dark brown within 10 min of incubation at 65 °C under alkaline conditions (*Fig. 2b*). The time-dependent study was carried out for formation of silver nanoparticle for 2 h. It can be concluded from the SPR peaks obtained that the maximum nanoparticles synthesis is achieved within 30 min of incubation as there is no significant change in the peak value after that. A higher temperature mediated synthesis was preferred over synthesis at RT to avoid super-clustering of the nanoparticles due to carboxyl or amino group present on the cystine molecules. The peaks obtained from the spectrophotometric analysis even gave a rough idea about the size of nanoparticles since the SPR peaks obtained were broad; it indicates that the particles formed might be of larger size.

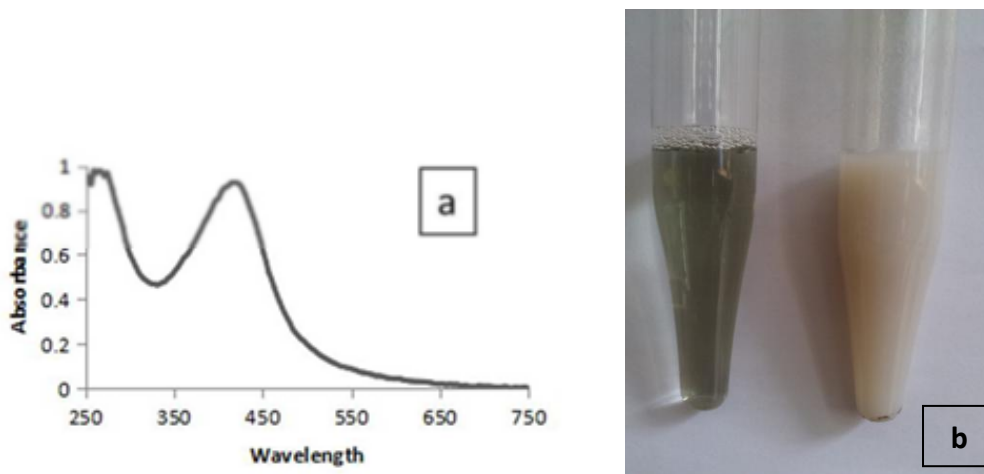


Figure 2. (a) SPR analysis of synthesized cystine capped silver nanoparticles. (b) Confirmatory colour change during the synthesis of nanoparticles

SEM analysis of nanoparticles

The particle size analysis of the nanoparticles revealed the average size of the particles to be around 20 nm (Fig. 3a). The same was also confirmed in the SEM analysis of the samples. Spherical nanoparticles which were around 20 nm in diameter can be clearly seen in the SEM analysis result represented in Figure 3b. Nanoparticles of different sizes have been reported using green synthesis like Prakasha et al. (2013) synthesized silver nanoparticles of 55-83 nm in size using leaf extract of *Mimusops elengi*. Another research by Jyoti et al. (2016) demonstrated the synthesis of silver nanoparticles of 36 nm in diameter using the leaf extract of *Urtica dioica*. Thus, it can be concluded that reducing agents and reaction conditions play very critical role in deciding the shape and size of the nanoparticles synthesized. These reflections correspond to the facets of face centered cubic crystal of silver nanoparticles (Jyoti et al., 2016).

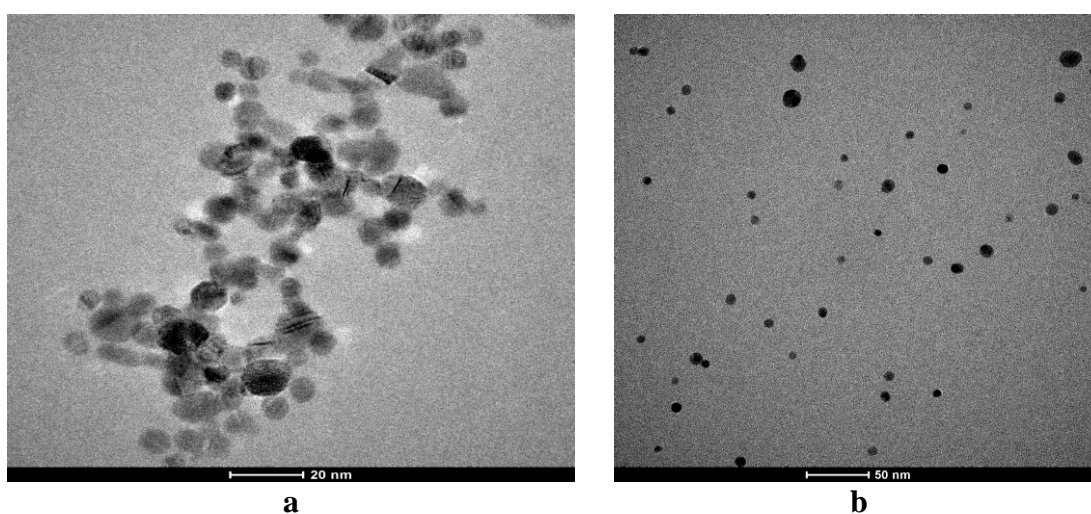


Figure 3. (a) Particle Size analysis of the synthesized silver nanoparticles. (b) SEM images of the synthesized nanoparticles

Lipase isolation and activity assay

The crude lipase was isolated from strain of *Bacillus subtilis* (Acc. No. KT591343). The crude lipase was quantified for its activity using p-nitrophenol palmitate as the substrate. The lipase acts on the substrate and hydrolyzes the palmitate group resulting in release of p-nitrophenol. The release of p-nitrophenol brings a change in colour of the assay mixture from colorless to light yellow. The change in the colour intensity is spectrophotometrically recorded at 410 nm. The standard plot for p-nitrophenol is represented in *Figure 4*. The activity of the enzyme was calculated as the amount of p-nitrophenol released from 1 mole of p-nitrophenol palmitate per minute per mL of reaction mixture. The activity of lipase from an unidentified strain isolated from dairy waste (Sahu et al., 2011) and from a *Staphylococcus sp.* isolated from oil contaminated area has been reported by (Sirisha et al., 2010) to be 38-46 and 25 U/mL respectively.

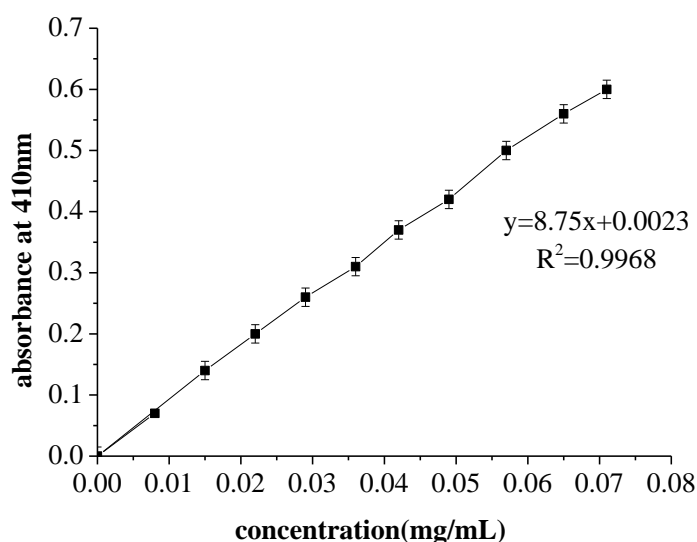


Figure 4. Standard curve for p-nitrophenol

Stability and reusability

The enzyme system so formed was stable for a period of 2 months with a loss of 15% of its original activity which is marginally better than the soluble enzyme which showed the loss of 25% from its original activity. Even though the activity lost comparison does not reflect a significant change, the reusability of the system gives it a significant advantage over the soluble enzyme. The use of enzyme system repeatedly showed the average loss of activity by 3-4% per cycle over the course of 5 cycles as shown in *Table 1*. This loss in the activity can also be attributed to the loss of nanoparticles after every assay cycle which could not be recovered during centrifugation step. The loss can also be possible because of leeching out of the enzyme from the decorated nanoparticles. Even though the covalent bond binds the nanoparticles with the enzyme the possibility of the nanoparticles releasing the enzyme into the system cannot be ruled out either during the activity assay or during the centrifugation step to pelletize the system.

Table 1. Reusability test for lipase-silver nanoparticles bioconjugates

Cycle number	Absorbance	Activity U/mL
Cycle 1	0.17	24.9
Cycle 2	0.16	23.1
Cycle 3	0.16	23.1
Cycle 4	0.15	21.2
Cycle 5	0.15	21.2

Conclusion

Original research article represents the immobilization of lipase on the surface of economically synthesized capped silver nanoparticles. The synthesis involved the production of cystine capped silver nanoparticles using puer tea as the reducing agent. The covalent coupling of the enzyme to the amino group present on the cystine capping acted as a bridge to decorate the enzyme to the nanoparticles. This improved the shelf life of expensive soluble enzyme with a reasonable specific activity in the decorated state. The development of such decorated system can be taken to the next level as to work on specific industrial applications with minor modifications as per application demand. This decorated reusable system has the potential to be utilized for various applications pertaining to the exploitation of lipase in various industries. Further experiments will include studies regarding the recovery of AgNPs from other media and the implementation of other metal nanoparticles (Au, Pt and Pd).

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