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Immobilization of cyclodextrin glucanotransferase on aminopropyl-functionalized silica-coated superparamagnetic nanoparticles

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Abstract

Background: Cyclodextrin glycosyltransferase (CGTase) from *Amphibacillus* sp. NPST-10 was successfully covalently immobilized on aminopropyl-functionalized silica coated superparamagnetic nanoparticles; and the properties of immobilized enzyme were investigated. The synthesis process included preparing of core magnetic magnetite (Fe_3O_4) nanoparticles using solvothermal synthesis; followed by coating of Fe_3O_4 nanoparticles with dense amino-functionalized silica (NH_2-SiO_2) layer using in *situ* functionalization method. The structure of synthesized Fe_3O_4@NH_2-SiO_2 nanoparticles was characterized using TEM, XRD, and FT-IR analysis. Fe_3O_4@NH_2-SiO_2 nanoparticles were further activated by gluteraaldehyde as bifunctional cross linker, and the activated nanoparticles were used for CGTase immobilization by covalent attachment.
Results: Magnetite nanoparticles was successfully synthesized and coated with and amino functionalized silica layer (Fe_3O_4/NH_2-SiO_2), with particle size of 50-70 nm. The silica coated magnetite nanoparticles showed with saturation magnetization of 65 $emug^{-1}$, and can be quickly recovered from the bulk solution using an external magnet within 10 sec. The activated support was effective for CGTase immobilization, which was confirmed by comparison of FT-IR spectra of free and immobilized enzyme. The applied approach for support preparation, activation, and optimization of immobilization conditions, led to high yields of CGTase immobilization (92.3%), activity recovery (73%), and loading efficiency (95.2%); which is one of the highest so far reported for CGTase. Immobilized enzyme showed shift in the optimal temperature from 50 to 55ºC, and significant enhancement in the thermal stability compared with free enzyme. The optimum pH for enzyme activity was pH 8 and pH 7.5 for free and immobilized CGTase, respectively, with slight improvement of pH stability of immobilized enzyme. Furthermore, kinetic studies revealed that immobilized CGTase had higher affinity toward substrate; with k_m values of 1.18 ± 0.05 mg/ml and 1.75 ± 0.07 mg/ml for immobilized and free CGTase, respectively. Immobilized CGTase retained 87% and 67 of its initial activity after 5 and 10 repeated batches reaction, indicating that immobilized CGTase on Fe_3O_4/NH_2-SiO_2 had good durability and magnetic recovery.
Conclusion: The improvement in kinetic and stability parameters of immobilized CGTase makes the proposed method a suitable candidate for industrial applications of CGTase. To best of our knowledge, this is the first report about CGTase immobilization on silica coated magnetite nanoparticles.

Keywords: *Amphibacillus* sp. NPST-10; cyclodextrin glucanotransferase; immobilization; magnetic nanoparticle; silica.

INTRODUCTION

Cyclodextrin glucanotransferase (CGTase, EC 2.4.1.19) is one of most industrially important enzyme that able to convert starch and related sugars, via a cyclization reaction, into non-reducing cyclic oligosaccharides called cyclodextrins (Biwer et al. 2001). Moreover, it is an important hydrolytic enzyme that carries out reversible intermolecular coupling and disproportionation of maltooligosaccharides (Savergave et al. 2008; Atanasova et al. 2009). Cyclodextrins (CDs) are of three main types: α-, β-, and γ-cyclodextrin, that composed of six, seven and eight α-(1,4) linked glucose units, respectively (Moriwaki et al. 2009). The steric arrangement of glucose units in the CD molecule results in the shape of a hollow truncated cone with a hydrophilic external surface and a hydrophobic internal cavity, which enables CDs to form inclusion complexes with various guest molecules (Moriwaki et al. 2009; Atanasova et al. 2011). Due to the fact that guest molecule is individually surrounded by a cyclodextrin (or its derivative), the molecule is micro-encapsulated from the external environment and this can led to favourable changes in the chemical and physical properties of the guest molecules, including: fixation of volatile substances, improvement of substances solubility, protection of oxygen-sensitive substances, stabilization against light, protection against microbial degradation and others (Martín del Valle, 2004; Astray et al. 2009; Otero-Espinar et al. 2010).

There is increasing interest in developing efficient industrial process for production of CDs and oligosaccharides for addressing applications in different industries. The use of CDs has increased annually around 20-30%, of which 80-90% was in food products (Astray et al. 2009). However, possibly, the main bottleneck in the industrial application of enzymes is the price and the stability of the biocatalyst (Ansari and Husain, 2011). Enzyme immobilization is one of the most useful approaches to overcome such difficulties (Mateo et al. 2007; Kim et al. 2008). Industrial production of CDs using immobilized CGTases would offer several advantages over free enzyme including: allowing reuse of the enzyme, simplifying product purification process and providing opportunities for scaling up (Leemhuis et al. 2010; Matte et al. 2012). Recent breakthroughs in nanotechnology have made various nanostructured materials more affordable for a broader range of applications including enzyme immobilization (Wang, 2006; Ansari and Husain, 2011). In this regard, nanoscale materials provide the upper limits in balancing the key factors that determine the efficiency of biocatalysts, including surface area/volume ratio, mass transfer resistance, and effective enzyme loading (Kim et al. 2006a; Mateo et al. 2007; Lei et al. 2008; Lee et al. 2009). In addition, the Brownian motion of nanoparticles, confining effect of nanopores, and self-assembling behaviours of discrete nanostructures are providing exciting opportunities in this field (Wang, 2006; Lee et al. 2009). Furthermore, to overcome the difficulty of nanoparticles separation from the bulk solution, modified magnetic nanoparticles can be applied for enzyme immobilization (Meunier et al. 2010; Sulek et al. 2010a). Among various magnetic nanoparticles, magnetite (Fe_3O_4) is a good candidate for enzyme immobilization due to its low toxicity, small size, superparamagnetism, possibility of surface modification, high field irreversibility, and high saturation field (Jiang et al. 2009; Shi et al. 2010; Ranjbakhsh et al. 2012). In order to increase the loading amount of the biomolecules on the magnetic particles and improve the stability of immobilized biomolecules, the preparation of surface functionalized magnetic particles with water insoluble, biocompatible, and reactive groups is much desired (Song et al. 2012). CGTase has been previously immobilized on traditional macro carriers, however to best of our knowledge there is very rare reports about CGTase immobilization on nanostructured materials (Ferrarotti et al. 2006; Mahmoud et al. 2009; Svensson and Adlercreutz, 2011; Matte et al. 2012).

In the present study, CGTase from recently isolated alkaliphilic *Amphibacillus* sp. NPST-10 (Ibrahim et al. 2012) was immobilized by covalent attachment on supermagnetic amino functionalized silica magnetic magnetite nanoparticles. The size and structure of nanoparticles were characterized using transmission electron microscopy (TEM), X-ray powder diffraction (XRD), and Fourier transform infrared spectroscopy (FT-IR) analysis. The enzyme immobilization was confirmed by FT-IR analysis of free and immobilized CGTase. In addition the optimum conditions for CGTase immobilization on the nanoparticles were determined. Subsequently, thermal and pH stability, kinetic parameters, and operational stability of immobilized enzyme were investigated.

MATERIALS AND METHODS

CGTase producing alkaliphilic *Amphibacillus* sp. NPST-10 used in this study was recently isolated from hypersaline Soda Lakes, located in Wadi Natrun valley in northern Egypt (Ibrahim et al. 2012). The bacterium was propagated in rich alkaline agar medium containing 0.02% (w/v) phenolphthalein, as an indicator of CGTase production (Park et al. 1989). The alkaline agar medium (pH 10.5) contained soluble starch (10 g/l), yeast extract (5 g/l), casamino acids (5 g/l), peptone (5 g/l), NaCl (50 g/l), Na_2CO_3 (15 g/L), agar (15 g/l) and 300 µl trace elements solution. The trace elements solution contained: $CaCl_2$ x $2H_2O$ (1.7 g/l), $FeSO_4$ x $7H_2O$ (1.3 g/l), $MnCl_2$ x $4H_2O$ (15.4 g/l), $ZnSO_4$ x $7H_2O$ (0.25 g/l), H_3BO_3 (2.5 g/l), $CuSO_4$ x $5H_2O$ (0.125 g/l), Na_2MoO_4 (0.125 g/l), $Co(NO_3)_2$ x $6H_2O$ (0.23 g/l) and 2.5 mL 95-97% H_2SO_4. Na_2CO_3 and trace elements solutions were autoclaved separately before addition to the medium.

CGTase production and purification

A loopful of *Amphibacillus* sp. NPST-10 culture was transferred from agar plate to 250 ml Erlenmeyer flasks containing 50 ml of alkaline production liquid medium, and incubated for overnight at 50ºC under orbital shaking (150 rpm). This culture was used to inoculate (2.5%, v/v) one liter Erlenmeyer shaking flask containing 250 ml of the same medium and incubated at 50ºC under shaking (150 rpm) for about 36 hrs. The CGTase production medium contained soluble starch (15 g/l), yeast extract (6 g/l), peptone (6 g/l), NaCl (30 g/l), Na_2CO_3 (15 g/l), $CaCl_2$ (5 mM), and 300 µl of trace elements solution. At the end of the incubation period, cells and insoluble material were removed by centrifugation at 6000 g for 15 min at 4ºC, and cell-free supernatant was used as a source of the crude enzyme.

CGTase purification was carried out, as described previously, by adsorption of CGTase to corn starch followed by enzyme elution using β-CD solution (Ibrahim et al. 2012). Briefly, corn starch and ammonium sulphate were added to one liter of cell-free supernatant to final concentrations of 5% (w/v) and 1 M, respectively. The mixture was kept at 4ºC with continuous gentle agitation for 1 hr to allow CGTase binding to starch. Then, the mixture was centrifuged at 5000g for 10 min and the starch pellet was washed twice with cold 1 M ammonium sulphate solution to remove any unbound proteins. Adsorbed CGTase was eluted from corn starch by incubating the pellet in 200 ml of Tris-HCl buffer (50 mM, pH 8.0), containing 1 mM β-CD, for 30 min at 37ºC with shaking followed by centrifugation to give eluate "A". The elution step was repeated with 80 ml of the same β-CD solution to give eluate "B". The eluates (A and B) were pooled (280 ml), dialyzed against the same buffer at 4ºC. The eluate was concentrated using an Amicon ultrafiltration membrane kit (10 kDa cut-off membrane) and stored at -20ºC till use.

Synthesis of magnetite nanoparticles

Magnetic magnetite (Fe_3O_4) nanoparticles (MNPs) were prepared by solvothermal synthesis reaction according to Deng et al. (2005) with some modifications. Briefly, $FeCl_3$ x $6H_2O$ (1.35 g, 5 mmol) was dissolved in ethylene glycol (40 ml) to form a clear solution, followed by addition of polyethylene glycol (1.0 g), and sodium acetate (3.6 g), as a stabilizing agent. The mixture was stirred vigorously for 30 min, and then sealed in Teflon lined- stainless- steel autoclave (50 ml capacity). The autoclave was heated to 190ºC for 18 hrs, and allowed to cool to room temperature. The black products were washed several times with ethanol and dried at 60ºC for 6 hrs.

Synthesis of Fe_3O_4@NH_2-SiO_2 magnetic nanoparticles

Amino functionalized silica coated magnetic (Fe_3O_4@NH_2-SiO_2) nanospheres were prepared using *in situ* functionalization protocol according to previously reported methods with some modifications (Yiu et al. 2001; Xu et al. 2006; Deng et al. 2008; Cui et al. 2010). Briefly, 0.1 g of the synthesized Fe_3O_4 nanoparticles were dispersed in a mixture of ethanol (30 ml), deionize water (2.6 ml), and concentrated ammonia aqueous solution (1.2 ml), followed by the addition of tetraethyl orthosilicate (TEOS, 0.5 ml) and 3-Aminopropyltriethoxysilane (APMS, 0.1 ml), 0.05 ml at two last TEOS addition. After stirring at room temperature for 75 min (0.1 ml/15 min), the Fe_3O_4/NH_2-SiO_2 nanoparticles were separated and washed several times with ethanol and water until the pH value of the supernatant was around neutral, and then dried at 60ºC for 24 hrs.

Characterization of the synthesized magnetic nanoparticles

Transmission electron microscopy (TEM) images of MNPs were obtained using a JEOL JSM-2100F electron microscope (Japan) operated at 200 kV. Powder X-ray diffraction (XRD) patterns of MNPs were recorded on a PANalytical X'Pert PRO MPD (Netherlands) with Ni-filtered Cu Kα radiation (45 kV, 40 mA). Nitrogen sorption isotherms were measured at 77 K with a Quantachrome NOVA 4200 analyzer (USA). Fourier transform infrared (FT-IR) spectra were recorded using a Bruker Vertex-80 spectrometer. Magnetic characterization was carried out on a superconducting quantum interference device (SQUID) magnetometer.

Support activation and CGTase immobilization

The purified CGTase was covalently immobilized onto the surface of amino functionalized silica coated magnetic nanoparticles (Fe_3O_4@NH_2-SiO_2). First, the support was activated by glutaraldehyde as bifunctional (two aldehyde groups) cross linker agent, followed by coupling of purified CGTase to the activated nanoparticles (Kim et al. 2007; Cui et al. 2010; Song et al. 2012). Briefly, 50 mg of magnetic support was suspended in 10 ml of 2% (v/v) of glutaraldehyde, prepared in distilled water, and the mixture was incubated for 2 hrs at room temperature with stirring. Then, the activated MNPs were collected from the solution using an external magnetic field, and rinsed several times with distilled water to remove any excess glutaraldehyde. Thereafter, the activated MNPs were re-suspended in one ml of 50 mM glycine buffer (pH 8) containing purified CGTase, and kept for overnight at 4ºC with gentle shaking. CGTase bound Fe_3O_4@NH_2SiO_2 nanoparticles were recovered by magnetic separation, and washed twice with glycine buffer (50 mM, pH 8) to remove any unbound CGTase, and the washed solution was collected. The amounts of protein in free enzyme and in the washed solution were determined by the Bradford method (Bradford, 1976), using bovine serum albumin as the standard protein. The amount of immobilized CGTase was calculated by subtracting protein recovered in the supernatant (washed solution) from the protein subjected to immobilization, and loading efficiency was calculated from the following equation: Loading efficiency= $[(P_i-P_{unb})/P_0]$ x 100, where P_i and P_{unb} are the initial protein subjected to immobilization, and unbound protein, respectively. CGTase activity of free and, immobilized enzyme; and unbound enzyme (washed solution) was measured as described below. Immobilization yield and activity yield were calculated according to the following equations: Immobilization yield (%) = [(A- B)/A] x 100, where A is total activity of enzyme added in the initial immobilization solution; B, activity of unbound CGTase. Activity yield= (C/A) x 100, where A is total activity of enzyme added in the initial immobilization solution, and C is activity of the immobilized enzyme.

Assay of free and immobilized CGTase

CGTase activity of free and immobilized enzyme was measured as β-CD forming activity, based on ability of the formed cyclodextrin to form inclusion complexes with phenolphthalein, according to previously described method with some modifications (Martín et al. 2003). Seven hundred and fifty micro liter of 1% (w/v) starch solution prepared in glycine buffer (50 mM, pH 8) was pre-incubated at 50ºC for 5 min. Then, 100 µl of enzyme samples or 30 mg of immobilized CGTase was added to the reaction mixture; and after incubating for 20 min at 50ºC, the reaction was quenched by adding 375 µl of 0.15 M NaOH. Subsequently, 100 µl of 0.02% (w/v) phenolphthalein prepared in 5 mM Na_2CO_3 was added, and after standing at room temperature for 15 min, the colour intensity was measured at 550 nm. One unit of CGTase activity was defined as the amount of enzyme releasing 1 µmol of β-CD per min under the defined assay conditions. A calibration curve was made using 0.001-0.5 µmol of β-CD in 50 mM glycine buffer (pH 8).

Catalytic properties of immobilized CGTase

Effect of temperature on activity and stability of immobilized CGTase. The influence of temperature on activity of free and immobilized CGTase was investigated by measuring the enzyme activity at various temperatures in range from 35ºC to 75ºC under standard assay conditions.

The thermostability of free and immobilized CGTase was determined by incubating free and immobilized CGTase, prepared in 50 mM glycine buffer (pH 8), at different temperatures ranging from 35ºC to 70ºC for 1 hr in a shaking water bath. Thereafter, the reaction mixture was immediately cooled

in an ice bath, and the residual enzyme activity was determined under standard assay conditions. The residual activity of free and immobilized CGTase was calculated compared with untreated samples. All experiments and enzyme assays were performed in triplicate and the mean values were reported.

Effect of pH on activity stability of immobilized CGTase. The enzyme activity of free and immobilized CGTase was measured at various pH values ranging from pH 4.0 to 12.0, using suitable buffers. These buffers included 50 mM sodium acetate (pH 5.0 and 6.0), 50 mM Trise-HCl (pH 7.0 and 8.0), 50 mM glycine-NaOH buffer (9.0 and 10.0) and 50 mM carbonate-bicarbonate buffer (pH 11.0 and 12.0). In addition, pH stability of free and immobilized CGTase was investigated by incubating the free and immobilized CGTase in buffers at the designated pH for 1 hr at room temperature, and the residual activity were assayed under standard assay conditions. All experiments and enzyme assays were performed in triplicate and the mean values were reported.

Kinetic studies

Kinetic studies of free and immobilized CGTase were performed by by measuring initial rates of the reaction at different concentrations of soluble starch (1 to 20 mg/ml) as a substrate in glycine buffer solution (50 mM, pH 10) at 50ºC. The kinetic constants, K_m and V_{max}, were estimated using Michaelis-Menten equation and double reciprocal plot known as Lineweaver-Burk plot (Mathews et al. 1990).

Operational stability

The reusability of immobilized CGTase was assayed in successive batches in 2 ml of standard assay mixture. The samples were incubated in a shaking water bath with a shaking speed of 120 rpm at 34ºC for 20 min. At the end of each cycle, immobilized enzyme was magnetically separated from the reaction mixture using an external magnetic field, thoroughly washed with 50 mM glycine buffer (pH 8), to remove any substrate or products remaining in the nanoparticles, and finally resuspended in a freshly prepared substrate solution to restart a new cycle. Activity was estimated at the end of each cycle, and the residual activity was calculated and expressed relative to the initial enzyme activity.

RESULTS AND DISCUSSION

Preparation and characterization of Fe_3O_4@NH_2-SiO_2 nanoparticles

The synthesized magnetic magnetite (Fe_3O_4) nanoparticles were characterized by XRD patterns analysis (Figure 1). All of detected diffraction peaks can be indexed as spinel magnetite, which is in good agreement with standard literature data (JCPDF card number: 86-1354), (Gong et al. 2010). These sharp diffraction peaks clearly revealed that spinel magnetite product is well defined crystallites, with no impurity diffraction peaks, indicating a pure phase of synthesized magnetite nanoparticles. Figure 2a shows TEM image of synthesized magnetic nanoparticles. Particle size distribution measurements showed that Fe_3O_4 nanoparticles sample has broad distribution with mean particle size of 50 nm (Figure 3a).

Magnetic Fe_3O_4 nanoparticles have been coated with dense silica layer by sol-gel technique. Sol-gel process is based on hydrolysis of silicon alkoxide (TEOS) in alkaline medium. In addition, silica functionalization with amino group has been conducted using *in situ* functionalization protocol. This process provides homogenous and smooth silica layer due to slow hydrolysis of TEOS precursor (Deng et al. 2008). The TEM images shown in Figure 2b revealed that the core-shell structured and aminopropyl-functionalized silica coated (Fe_3O_4/NH_2-SiO_2) nanospheres with the shell thicknesses of about 25 nm were successfully prepared, and the particle size distribution tended to agglomerated to larger particle size with two mean diameters of 50 and 70 nm (Figure 3b). The magnetic hysteresis loops Fe_3O_4 and Fe_3O_4/NH_2-SiO_2 nanoparticles indicated that the saturation magnetization of magnetic magnetite and Fe_3O_4/NH_2-SiO_2 are 80 and 65 $emug^{-1}$, respectively (Figure 4). This slight loss of magnetic properties of the silica coated MNPs can be attributed to shielding effect of silica layer (Zhu et al. 2009). However, it had no significant effect on magnetic separability of the MNPs.

Moreover, the magnetic silica coated magnetite nanospheres can be quickly separated to the wall of the container using an external magnet magnet within 10 sec (Figure 5). Furthermore, FT-IR

measurements have been conducted to confirm coating of magnetite nanoparticles with silica functionalized with aminopropyl groups. The strong bond at 580 cm^{-1} (Figure 6a) corresponds to Fe-O vibrations of the magnetite core (Bruce and Sen, 2005). Upon silica coating for Fe_3O_4, Si-O peak can be seen formed at 1050-1250 cm^{-1} (Figure 6b). Fe-O-Si peak that refer for chemical binding between Fe_3O_4 and silica, cannot be seen in the FT-IR spectrum because it appears at around 584 cm^{-1} and therefore overlaps with the Fe-O vibration of magnetite nanoparticles. However, the co-existence of peaks characteristics for Fe_3O_4 and silica together indicated successful coating of Fe_3O_4 with silica layer. On the other hand, functionalization of silica with aminopropyl groups can be elucidated from the existence of N-H peak at 1637 cm^{-1}. Moreover, OCH_2CH_3 stretching vibrations peaks that appeared at 2930 cm^{-1} provide further evidence for silica functionlization with aminopropyl groups (Yamaura et al. 2004). Thus, magnetite was successfully synthesized and functionalized with amino groups mediated the reaction of silanization.

CGTase immobilization

Silica-coated magnetic nanoparticles provide many surface reactive silanol (Si-OH groups) to be directly employed in subsequent surface functionalization. Furthermore, derivatization of additional reactive moieties on the particle surface can serve to covalently bind various kinds of biomolecules (Sulek et al. 2010b). Here, functionalization of silica surface with amino groups has been carried out using *in situ* functionalization process, which involves the addition of the target species (APMS) during the preparation of the gel. In contrast to *post* synthesis, *in situ* functionalisation process is characterized by enriched incorporation of the functional groups (Yiu et al. 2001).

To induce CGTase immobilization by covalent attachment to amino functionalized silica coated nanoparticles, the free amino groups (-NH_2) lying outwards the magnetic silica surface was further activated by glutaraldehyde as a bifunctional crosslinker. In which, the free amino groups of Fe_3O_4@NH_2-SiO_2 reacts with terminal aldehyde groups of glutaraldehyde to form a Schiff-base linkage and provides a free terminal aldehyde, which can be then condensed with free amino groups in CGTase molecule to form a second Schiff-base (Migneault et al. 2004; Cui et al. 2010; Sulek et al. 2010a; Sulek et al. 2010b). The results indicated that the activated Fe_3O_4/NH_2-SiO_2 surface was effective forimmobilization of the *Amphibacillus* sp. NPST-10 CGTase. Binding of CGTase to magnetic nanoparticles was confirmed by FT-IR analysis of free and immobilized enzyme. As shown in Figure 7, free CGTase showed characteristic peaks at 1400 cm^{-1} for carboxylic (COO) bond and another peak at 1640 cm^{-1} that could be either for NH or OH bond (Xiangkai and Qianwang, 2013). The binding of CGTase to magnetic nanoparticles was confirmed from the appearance of carboxylic COO bond characteristic for enzyme together with those characteristic for amino functionalized magnetite nanoparticles (Figure 7).

Optimization of CGTase immobilization procedure

To enhance and optimize CGTase immobilization by covalent attachment on Fe_3O_4/NH_2-SiO_2 nanoparticles, the effect of glutaraldehyde concentration, activation time, proteins concentration and enzyme coupling time on the immobilization efficiency was investigated, and the results are summarized in Table 1. It was found that the immobilization yield and loading efficiency increased by increasing glutaraldehyde concentration, reaching maximum value at 5% glutaraldehyde of 93.4% and 94.5%, respectively. Further increase of the glutaraldehyde concentration had no significant effect on the immobilization yield or loading efficiency. In addition, activation of Fe_3O_4/NH_2-SiO_2 nanoparticles with glutaraldehyde (5%) for 2 hrs was enough to get maximum immobilization yield, as depicted from Table 1. It was found that, by increasing the reaction time of CGTase with activated Fe_3O_4/NH_2-SiO_2 from 6 to 18 hrs, the amount of immobilized CGTase increased and remained constant after about 18 hrs. It seems that most of the free aldhydes groups on the MNPs surface were blocked by the amino-group of CGTase after this time. Therefore, optimal coupling time was 18 hrs. Furthermore, to investigate the loading efficiency of the nanoparticles, different amounts of the CGTase (60 to 1900 µg protein) were used for immobilization on 10 mg MNPs. As shown in Figure 8, CGTase activity increased with increasing the initial concentration of CGTase up to 470 mg, and decreases thereafter.

This could be due to the steric hindrance of CGTase molecules on the surface of the supporting particles, or that the over loading of enzyme molecules on MNPs causes some unfavorable protein-protein interactions that, subsequently, reduces the enzyme activity (Guo et al. 2003, Jiang et al. 2009; Ranjbakhsh et al. 2012). In addition, the amount of loaded CGTase increased with increasing the initial

concentration of CGTase and remains constant at about 47 µg/mg carriers. This loading efficiency of CGTase on Fe_3O_4/NH_2-SiO_2 (47 µg protein/mg carrier) nanoparticles is much higher than previously reported by Shi et al. (2010) for immobilization of porcine pancrease lipase on magnetic microspheres (11.21 µg/mg carrier); or by Ranjbakhsh et al. (2012) for lipase immobilization on magnetic nanoparticles (35.8 µg/mg carrier). Higher activity yield and loading efficiency of CGTase in our work is probably due to amino functionalization of silica surface using *in situ* functionalization method, rather than *post* synthesis process, which led to incorporation of high density of free amino groups available for activation and subsequently enzyme immobilization (Xu et al. 2006; Deng et al. 2008; Cui et al. 2010).

Table 1. Optimization of the immobilization process of CGTase on amino functionalized silica coated magnetic nanoparticles. Results represent the mean of three separate experiments, and standard deviation was in the range of 1-3.5%.

Parameters	Immobilization Yield (%)	Activity Yield (%)	Loading efficiency (%)
Glutaraldehyde concentration (%):			
1.0	61.4	42.2	58.7
2.0	71.2	56.4	70.3
3.0	83.8	66.5	79.8
4.0	87.1	71.4	88.5
5.0	**93.4**	**81.5**	**94.5**
6.0	92.4	80.2	94.6
Activation time (hrs):			
1	81.8	71.6	82.4
2	**93.6**	**82.1**	**94.1**
4	94.0	83.2	93.5
6	90.1	79.0	91.1
1	86	77.2	89.1
CGTase reaction time:			
6 hrs	45.1	31.0	43.2
12 hrs	71.0	55.3	6.9.5
18 hrs	**92.3**	**83.4**	**95.2**
24 hrs	91.5	84.8	92.0
30 hrs	92.4	85.0	93.0

Characterization of immobilized CGTase

Effect of temperature. The effect of temperature (25-75ºC) on the activity of free and immobilized is shown in Figure 9a. The results indicated that the optimal temperature for CGTase activity was increased from 50 to 55ºC for free and immobilized CGTase, respectively. In addition, the immobilized enzyme showed higher relative activities than free enzyme, particularly at high temperature. The shift of the optimal temperature for enzyme activity up on immobilization has been previously reported by others (Martín et al. 2003; Matte et al. 2012). Furthermore, as shown Figure 9b, there was significant enhancement in the thermal stability of immobilized CGTase in comparison to free enzyme. After incubation for 1 hr at 70ºC, Free and immobilized CGTase retained 76.0% and 89.5%, respectively. Increment of optimal temperature and enhanced thermostability of immobilized CGTase is probably due to covalent conjugation of the enzyme molecule onto the amino-functionalized magnetic nanoparticles, which increase the rigidity of three-dimensional structure and, hence, protect CGTase against thermal denaturation particularly at high temperatures (Kim et al. 2006b; Ranjbakhsh et al. 2012).

In addition, a “vicinal effect” induced by the enzyme immobilization might favor the configuration of the enzyme through H-bonds, which contributed to the improved thermal stability of the immobilized enzyme (Song et al. 2012).

Effect of pH on activity stability of immobilized CGTase. The influence of pH on the activity of free and immobilized CGTase was established by measurement of enzyme activity at varying pH values ranging from 5.0 to 12.0 at 50ºC, under standard assay conditions. The results shown in Figure 10a indicated that the optimum pH of immobilized CGTase was slightly higher than free enzyme that shifted from pH 8 and 7.5 for free and immobilized enzyme, respectively. Similar result has been reported by Zeng and Zheng (2002) where the pH/activity profile of immobilized chitosanase was shifted toward acidic value. However, optimal pH of superoxide dismutase was increased from 6.0 10.0 up on enzyme immobilization (Song et al. 2012). Generally, the pH optimum value of an immobilized enzyme can shift to a higher or lower pH, depending on surface charges and structure of the carrier (Reshmi et al. 2006; Shi et al. 2010). Investigation of the pH stability of *Amphibacillus* sp. NPST-10 CGTase was performed by pre-incubation of the free and immobilized enzyme in buffers at the designated pH for 1 hr at room temperature. Both free and immobilized CGTase was stable in a wide range of pH values, between pH 6.0 and 10.0, retaining more than 90% of its initial activity (Figure 10b). However, there was slight enhancement of pH stability of immobilized CGTase, particularly at low and high pH values, which may be attributed to the stabilization of immobilized CGTase via multipoint attachment on the surface of the nanoparticles (Kim et al. 2006a).

Kinetic studies

Kinetic study of free and immobilized CGTase was investigated by measuring initial rates of enzyme reaction at different concentrations of soluble starch at 50ºC and pH 8. The kinetic parameters (K_m and V_{max}) were estimated using Michaelis-Menten equation and double reciprocal plot known as Lineweaver-Burk plot (Mathews et al. 1990). The results shown in Figure 11 revealed that there was no significant difference in the V_{max} value of the free and immobilized CGTase (101.2 ± Umg^{-1}). However, K_m (value of immobilized CGTase 1.180.05 mg/ml) was lower than free enzyme (1.75 ± 0.07 mg/ml), indicating higher affinity of immobilized enzyme toward the substrate. This could be due to more efficient conformation of immobilized CGTase, or owing to enzyme expanding on the surface of the nanoscale particles with a better orientation leading to higher affinity to substrate and more available active sites (Ranjbakhsh et al. 2012).

Operational stability

The reuse stability of immobilized enzymes is one of the most important factors affecting the utilization of an immobilized enzyme system in industrial process. As assayed, it was indicated that the immobilized CGTase could retain 87% and 67% of its initial activity after reutilizations for five and ten times, respectively (Figure 12), indicating that immobilized CGTase on Fe_3O_4/NH_2-SiO_2 had good durability and magnetic recovery. Furthermore, the Fe_3O_4/NH_2-SiO_2 nanosphere system performed well compared with other systems. For instance, the operational stability of immobilized Thermoanaerobacter sp. CGTase on Eupergit C reported by Martín et al. (2003) showed that after 8 cycles the system kept only 40% of residual activity. Thus, the immobilised CGTase on this nano-structured carrier is a promising approach for industrial bioprocess for production of cyclodextrins.

CONCLUDING REMARKS

Aminopropyl functionalized silica coated magnetite nanoparticles with were successfully synthesized and characterized and first used as carriers for *Amphibacillus* sp NPST-10 CGTase immobilization. CGTase was successfully immobilized by covalent attachment on the activated Fe_3O_4/NH_2-SiO_2. This approach for support synthesis and activation led to produced high yields of CGTase immobilization, activity recovery, and loading efficiency; which is one of the highest so far reported for CGTase. In addition, the immobilized CGTase can be easily magnetically recovered using an external magnetic field. The results demonstrated significant improvement of thermal and pH of CGTase upon immobilization. Moreover, kinetic study showed higher enzyme affinity toward substrate compared with free CGTase. The immobilized CGTase could retain 87% of its initial activity after reutilizations five cycles, indicating that immobilized CGTase on Fe_3O_4/NH_2-SiO_2 had good durability and magnetic recovery.

The improvement in kinetic and stability parameters of immobilized CGTase makes the proposed method a suitable candidate for industrial applications of CGTase. To best of our knowledge, this is the first report about CGTase immobilization on silica coated magnetite nanoparticles.

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Figures

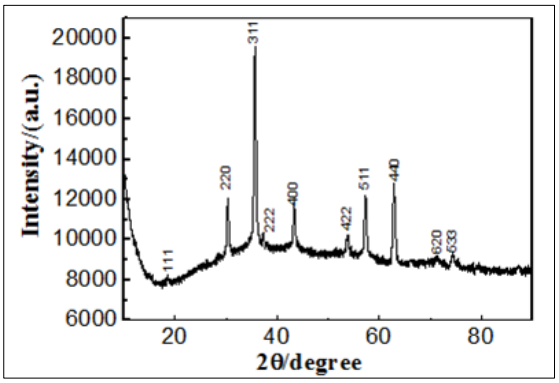

Fig. 1 X-ray diffraction patterns (XRD) of synthesized magnetic magnetite (Fe_3O_4) nanoparticles.

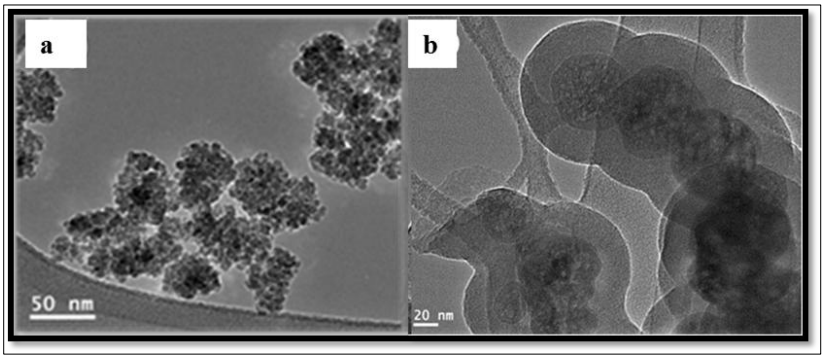

Fig. 2 TEM images of (a) magnetic magnetite (Fe_3O_4); (b) NH_2-SiO_2 coated magnetic Fe_3O_4 nanoparticles.

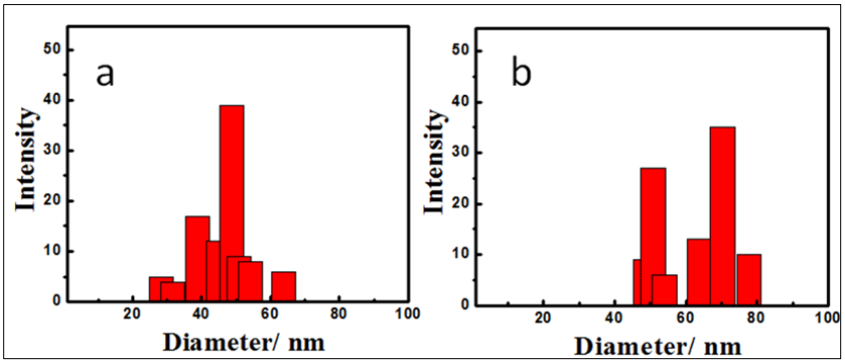

Fig. 3 Particle size distribution of (a) magnetic magnetite (Fe_3O_4); and (b) NH_2-SiO_2 coated Fe_3O_4 nanoparticles.

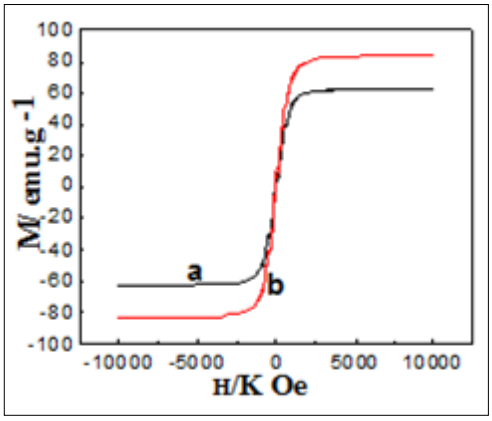

Fig. 4 Room-temperature magnetization curve of (a) magnetic magnetite Fe_3O_4 and (b) NH_2-SiO_2 coated magnetic Fe_3O_4 nanoparticles.

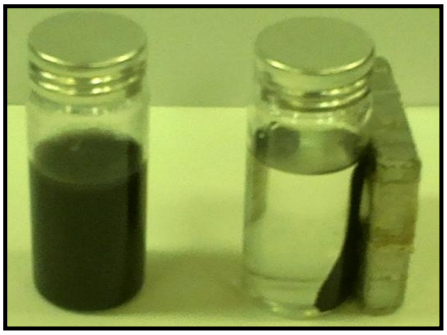

Fig. 5 Separability of CGTase immobilized on NH_2-SiO_2 coated magnetic Fe_3O_4 nanoparticles, using an external magnetic field.

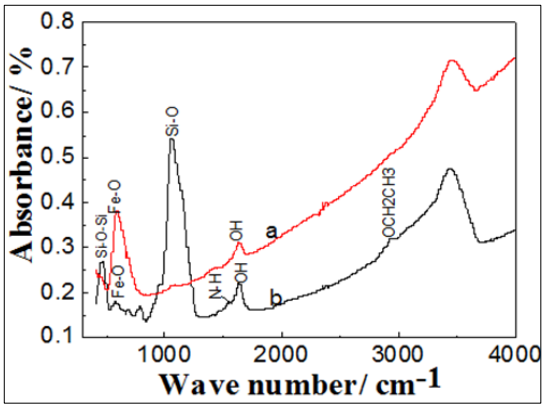

Fig. 6 FT-IR spectra of (a) magnetic magnetite Fe_3O_4; and (b) NH_2-SiO_2 coated magnetic Fe_3O_4 nanoparticles.

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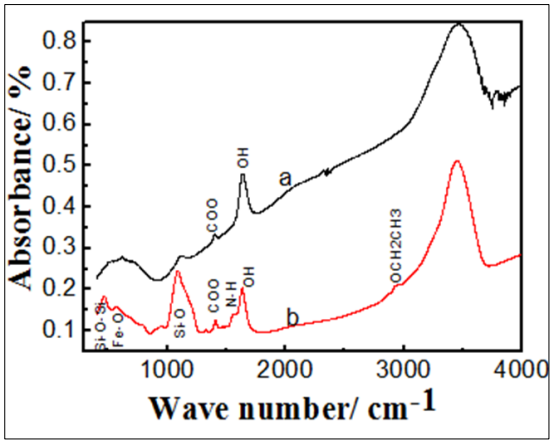

Fig. 7 FT-IR spectra of (a) free CGTase; and (b) CGTase immobilized on Fe_3O_4@NH_2-SiO_2 nanoparticles.

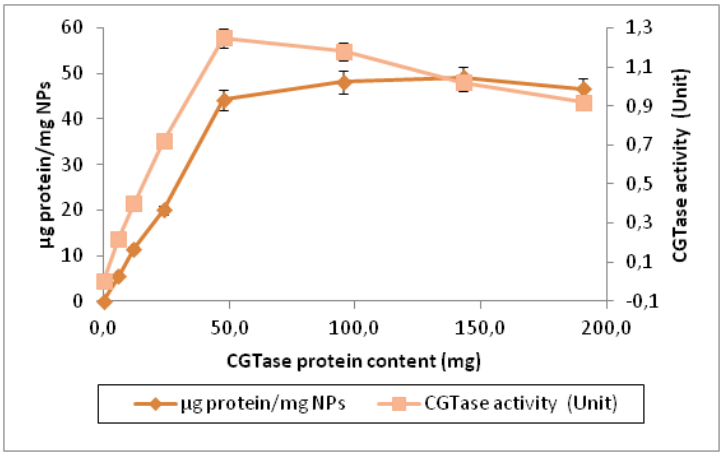

Fig. 8 Effect of the initial amount of CGTase on the amount and activity of immobilized enzyme. Results represent the mean of three separate experiments, and error bars are indicated.

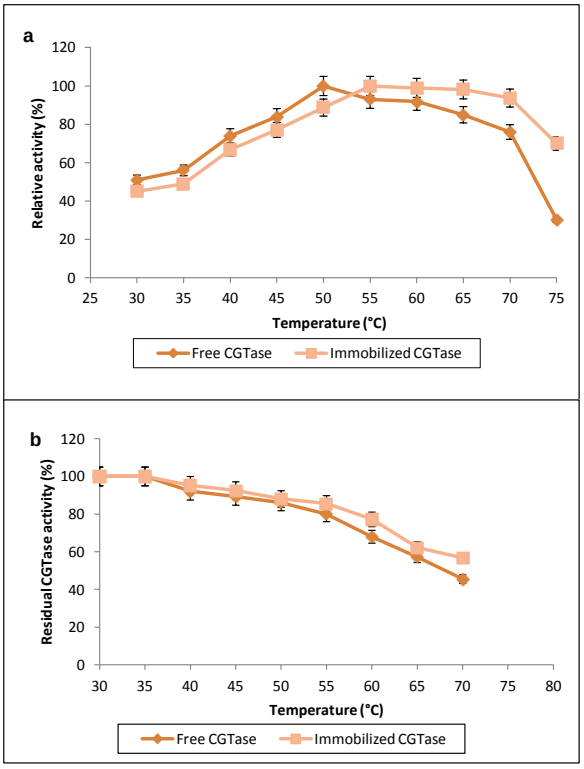

Fig. 9 Effect of temperature on the activity (a) and stability (b) of free and immobilized CGTase. Results represent the mean of three separate experiments, and error bars are indicated.

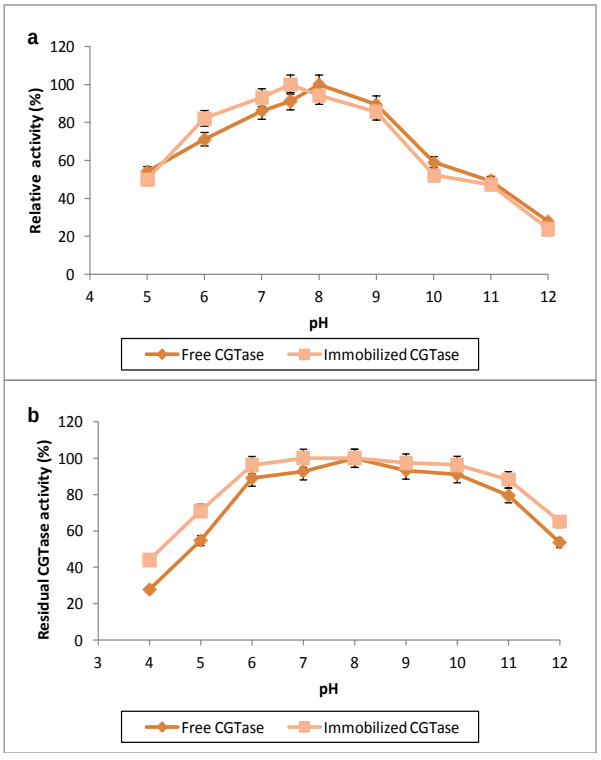

Fig. 10 Effect of pH on the activity (a) and stability (b) of free and immobilized CGTase. Results represent the mean of three separate experiments, and error bars are indicated.

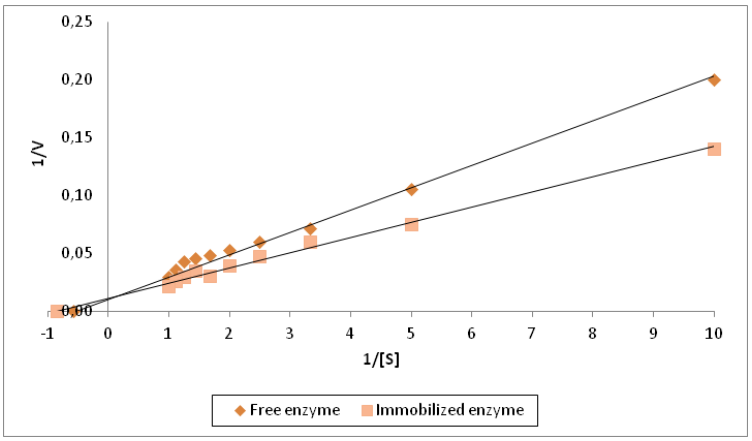

Fig. 11 Lineweaver-Burk plots of free and immobilized CGTase. Results represent the mean of three separate experiments.

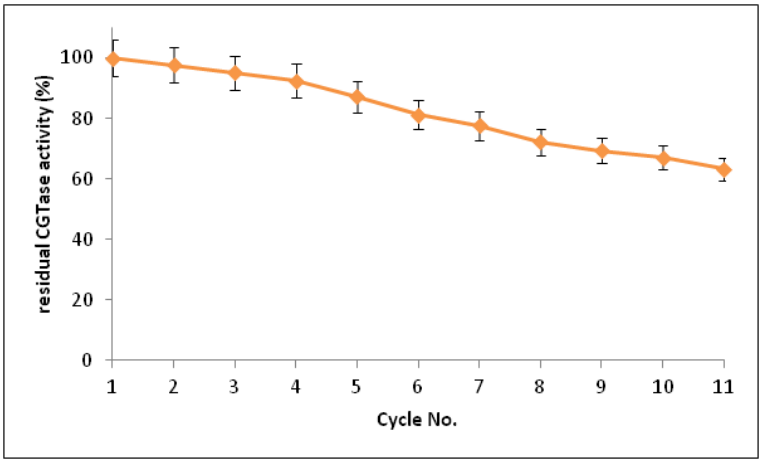

Fig. 12 Reusability of CGTase immobilized on NH_2-SiO_2 coated magnetic nanoparticles. Results represent the mean of three separate experiments, and error bars are indicated.