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A co-immobilization of pectinase and cellulase onto magnetic nanoparticles for antioxidant extraction from waste fruit peels

Shamraja S. Nadar, Virendra K. Rathod*

Department of Chemical Engineering, Institute of Chemical Technology, Matunga E, Mumbai 400019, India

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ABSTRACT

The major obstruction in efficient extraction of intracellular bioactive components is highly complex cell wall structure which is made up of rigid polysaccharides. An enhancement in the release of bioactive compounds from plants and fruits can be facilitated by hydrolysing cellular cellulosic material along with pectin. In this context, co-immobilized magnetic nanobiocatalyst was prepared by simultaneous immobilization of Pectinex® and Celluclast® 1.5 L onto amino-functionalized magnetic nanoparticles (MNPs) by 75 mM cross-linker concentration (glutaraldehyde) with 12.5 h cross-linking time for efficient extraction. The prepared nanobiocatalyst was characterized by FT-IR, TGA, SEM and XRD. In thermal stability studies, magnetic nanobiocatalyst showed two folds increased half-life in the temperature range of 50–70 °C as compared to native form. Further, the magnetic biocatalyst retained up to 80% of residual activity even after ten successive cycles. At the end, the antioxidants were extracted from peel residues of orange, mango and banana by using co-immobilized magnetic nanobiocatalyst and mixture of individually immobilized enzymes. This showed more than two fold higher free radical scavenging activity as compared to conventional solvent based extraction.

1. Introduction

Bioactive compounds are extra-nutritional secondary metabolic components that naturally occur in small quantities in plants and food products. In the last decade, they have gathered significant attention from scientific and industrial communities due to an increased market demand and their potential application in functional foods, cosmetics and pharmaceutical industries (Acosta-Estrada et al., 2014; Ignat et al., 2011; Sun-Waterhouse, 2011). Among phytochemicals, phenolic compounds have been extensively researched due to their diverse health benefits with multiple biological effects including antibacterial, antiviral, anti-inflammatory, antiallergic, antithrombotic, and vasodilatory actions. For these reasons, significant efforts have been made to extract these natural bioactive compounds from plant materials (Dai and Mumper, 2010; Oroian and Escriche, 2015).

Mostly, phenolic compounds are covalently bound to structural components of cell wall such as cellulose, hemicellulose and pectin. The presence of these cell wall polysaccharides strongly reduces the extraction efficiency of classical extraction methods. Enzyme-assisted extraction is an efficient procedure gaining more attention as an eco-friendly extraction technology to augment the release of bioactive compounds from plants and fruits residues (Joana Gil-Chávez et al., 2013; Puri et al., 2012). Enzymes can effectively catalyse the degradation of cell walls, favouring the release of bioactive components trapped inside the cell walls (Chamorro et al., 2012). Thitiratsakul and co-workers extracted antioxidant from longan (*Dimocarpus longan Lour.*) with the help of pectinase. The longan extract treated by enzyme showed 4-fold enhanced release of antioxidants as compared to the untreated extract (Thitiratsakul and Anprung, 2014). Further, Margo and co-helpers determined the synergic effect of combination of pectinase and Lallzyme Beta (cellulase) for grape juice extraction from *Vitis labrusca* L. variety Concord. They found that combination of two enzymatic preparations improved the juice yield as well as bioactive compounds extraction when compared with each preparation individually (Dal Magro et al., 2016). The major challenges in enzyme assisted extraction are high cost, separation/recovery and reusability of the enzyme after the reaction. To overcome these challenges, while keeping the advantages of enzymes intact, immobilization of enzymes on a suitable prefabricated support has been considered as a graceful approach, because immobilization can offer several benefits, including repeated use of enzymes, ease of separation from a reaction mixture, improvement of the stability of enzymes (Nadar and Rathod, 2016a; Sojitra et al., 2016; Talekar et al., 2017).

Globally, significant amount of food processing wastes (such as the skins, peels and fibres) is produced during juice and food processing. This food waste needs to be immediately valorised by using clean

* Corresponding author.
E-mail address: vk.rathod@ictmumbai.edu.in (V.K. Rathod).

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technologies, compatible to obtain useful biomolecules (Girotto et al., 2015; Mirabella et al., 2014; Santana-Méridas et al., 2012). The valorisation of such food waste not only reduces the burden of bio-waste on industries and eco-system, but also positively influences industry's profits by extracting high value added products (Lin et al., 2013). Several studies showed that fruit peels are rich in biologically active compounds including natural antioxidants, such as flavonoids, phenolic acids, etc. (El-ghfar et al., 2016). In an attempt to valorise waste fruit peel residues, in the present work, we have simultaneously co-immobilized two different enzymes viz. Pectinex® and Celluclast® 1.5 L onto amino functionalized MNPs for extraction of antioxidants. The breakdown of polysaccharides like pectin, and cellulosic components facilitates better extraction yield of intra-cellular contents (Li et al., 2014; Nadar and Rathod, 2018a, 2018b; Tao et al., 2016). This multi-enzymatic approach can significantly enhance the production with short reaction time and efficient mass transfer during enzymatic reactions in one pot which translates to substantial environmental efficiency and economic viability (Ansari and Husain, 2012; Ricca et al., 2011; Santacoloma et al., 2011). In addition to that, the presence of MNPs helps in rapid and effective separation by simply applying an exterior magnetic field which enables ease of handling. Also, it prevents protein (enzyme) contamination in the reaction mixture after the extraction procedure (Jiang et al., 2016; Shi et al., 2016; Talekar et al., 2013c; Zhuang et al., 2017). For the preparation of multi-enzyme magnetic nanocatalyst for extraction, the processing parameters such as cross-linker concentration, cross-linking time and ratio of enzyme to magnetic support (w/w) were optimized. The prepared multi-enzyme magnetic nanocatalyst was analysed by Fourier transform infrared spectroscopy (FT–IR) and thermogravimetric analysis (TGA). The crystal structures and crystal size were determined by X–ray powder diffraction (XRD). The values of V_{max} and K_m were evaluated for co-immobilized magnetic nanobiocatalyst. Further, the thermal stability of prepared nanobiocatalyst was evaluated in the temperature range of 50–70 °C in terms of deactivation rate constants (k_d), half-life ($t_{1/2}$) and energy required for deactivation (E_d) which compared with free form of enzyme. Finally, recyclability of immobilized magnetic nanobiocatalyst was examined up to 8th cycle to determine its industrial feasibility. At the end, prepared nanobiocatalyst was used for the extraction of antioxidant from peel residues of orange (*Citrus sinensis*), mango (*Mangifera indica*) and banana (*Musa acuminate*) which were analysed for their extracted phenolic content (Folin-Ciocalteu method) and free radical scavenging capacity (DPPH assay).

2. Materials and methods

2.1. Materials

Pectinex® (from *Aspergillus aculeatus*, 117 U/mL) and Celluclast® 1.5 L (from *Trichoderma reesei*, 36 U/mL) were procured from Sigma Aldrich (Bangalore, India). Carboxy methyl cellulose (CMC) and apple pectin (degree of esterification 85–90%, galacturonicacid min. 65%, M_w 100–120 kDa) were purchased from Sisco Research Laboratories (SRL) Pvt. Ltd. (Mumbai, India). 3,5–dinitrosalicylic acid (DNSA) and 3-Aminopropyl triethoxysilane (APTES) were purchased from HiMedia Laboratories Pvt. Ltd. (Mumbai, India). Glutaraldehyde (25% v/v) was purchased from S. D. Fine Chemicals (Mumbai, India). All other chemicals and reagents were of analytical grade used without any further purification and procured from reliable sources.

3. Methods

3.1. Synthesis of magnetic nanoparticles and their functionalization

Initially, the magnetic nanoparticles (MNPs) were synthesized by using conventional co-precipitation method with slight modifications (Xia et al., 2016). The ferric ions ($FeCl_3{\cdot}6H_2O$, 1.0 M) and ferrous ions ($FeCl_2{\cdot}4H_2O$, 0.5 M) were completely dissolved in the sonicated deoxygenized distilled water (45 mL) with overhead high speed mechanical agitation (1250 rpm) at 70 °C. After one hour of mixing, NaOH solution (30% w/v, 25 mL) was added drop wise to the reaction mixture which turned into brown coloured solution into black precipitate. The precipitated black magnetic nanoparticles were separated with external magnet and washed with distilled water for five times. Further, MNPs were dried overnight at room temperature (30 ± 2 °C). The prepared MNPs were functionalized with amino group (–NH_2) by silanization using 3–aminopropyl triethoxysilane (APTES) as reported by Feng and co-workers (Feng et al., 2016). Briefly, magnetic nanoparticles (1.5 g) were dispersed in alcoholic aqueous solution (1:1 v/v ethanol/water, 200 mL) mixture using sonication for 30 min to produce a homogeneous suspension mixture. APTES (10 mL, 5% v/v) was added slowly to the suspension with vigorous mechanical stirring (250 rpm) for 5–6 h at 70 °C. At the end, the amino functionalized MNPs were separated magnetically, washed five times with alcoholic water solution and dried at room temperature (RT ~ 30 ± 2 °C) for overnight. The prepared anino functionalized MNPs were further analysed by FT– IR and XRD to determine their functionalization and crystalline size respectively.

3.2. Enzyme activity assay and protein estimation

Pectinex® (pectinase) activity was determined by mixing enzyme with pectin (1% w/v in 100 mM sodium phosphate buffer, pH 6.5) solution (Sojitra et al., 2017). The reaction mixture was incubated for 10 min at 50 °C in a water bath. Then, the reaction was stopped by DNSA reagent (1 mL) and heated (90 °C) for 15 min. Finally, the absorbance of brown coloured solution was measured spectroscopically at 540 nm by using UV/Vis spectrophotometer (Jasco V-630, Japan) (Miller, 1959). One unit of Pectinex® activity (U) was defined as the amount of the enzyme required to release one µmole of D-galacturonic acid from pectin in one minute at optimum temperature and pH.

Similarly, Celluclast® 1.5 L (cellulase) activity was determined by DNSA method as described above in terms of released reducing sugar (glucose) from CMC. One unit (U) of enzyme activity is defined as the amount of enzyme required to release one µmole of reducing sugar (glucose equivalents) from CMC at optimum conditions.

Protein content was determined by Bradford reagent using bovine serum albumin (fraction V) as the standard (Bradford, 1976).

3.3. Co-immobilization of Pectinex® and Celluclast® 1.5 L onto magnetic nanoparticle

The enzyme solution of Pectinex® (with total protein solution of 9 mg/mL, 117 U/mL) and Celluclast® 1.5 L (with total protein solution of 3 mg/mL, 36 U/mL) was mixed with functionalized MNPs in varying ratio of 1:1–1:6 (w/w in 100 mM sodium phosphate buffer, pH 6.5). The suspension was mixed for 30 min at RT. After proper mixing, cross-linking agent glutaraldehyde was added in different concentration (15–120 mM) and shaken for different incubation time (2.5–17.5 h) with constant shaking at 175 rpm. After cross-linking time, magnetic nanobiocatalyst was separated using magnet, washed for five times by sodium phosphate buffer (pH 6.5) and stored in same buffer at 4 °C (Sojitra et al., 2016). For comparison purpose, Pectinex® and Celluclast® 1.5 L was immobilized separately on functionalized MNPs covalently by glutaraldehyde.

The activity recovery (%) of enzymes after immobilized form was determined as:

$$\text{Activity recovery}(\%) = \frac{\text{Activity of immobilized enzyme}\,(U) \times 100}{\text{Activity of free enzyme used for immobilization in}\,(U)}$$

3.4. Characterization of magnetic co-immobilized nanobiocatalyst

The MNPs, amino functionalized MNPs and co-immobilized magnetic nanobiocatalyst were analysed by FT–IR spectroscopy (Shimadzu

IR Affinity-1 FT–IR Spectrophotometer). Powder XRD was performed to get crystal structure and crystal size of amino functionalized MNPs before and after immobilization which was investigated by Philips PW 1830 ×–ray Diffraction. The morphological study of prepared co-immobilized magnetic nanobiocatalyst was done by scanning electron microscopic (SEM) which was captured using field emission–SEM, S4800 Type-II (Hitachi high technologies Corporation). The percentage immobilization of enzyme was determined by thermogravimetric analysis (TGA) by using DTG-60H EME instrument. It was calculated by considering the weight loss in co-immobilized magnetic nanobiocatalyst with 10 °C/min heating rate over the temperature range of 30–450 °C in nitrogen atmosphere.

3.5. Thermal kinetic studies

Thermal stability of Pectinex® and Celluclast® 1.5 L in co-immobilized and free form were estimated by keeping at different temperature in the range of 50–70 °C. The sample was incubated at respective temperature and collected after every ten min intervals till 1 h. The activity of each enzyme was determined by enzyme activity assay as described above section. A semi-log plot of residual activity of enzyme against time was plotted to determine inactivation rate constant (k_d). The half-life ($t_{1/2}$) is time required to reduce the activity to half of the original activity and was evaluated as $0.693/k_d$. Further, activation energy for deactivation (E_d) was determined from linear Arrhenius plot for free form and immobilized form of enzymes.

3.6. Optimal temperature for free and immobilized enzyme

The effect of temperature on activity in the range of 30–75 °C was studied for free form and immobilized form of Pectinex® and Celluclast® 1.5 L. For comparison purpose, both these i.e. free and co-immobilized enzymes with same activities were used. The relative activity of enzyme at each temperature was determined by considering highest activity as100%.

3.7. Kinetic parameters

The Michaelis–Menten kinetic parameters; maximum velocity (V_{max}) and Michaelis constant (K_m) were determined for Pectinex® and Celluclast® 1.5 L in free and immobilized form by varying the amount of substrate pectin and CMC in the range of 0.0–4.0 mg/mL respectively, in phosphate buffer (pH 6.5, 100 mM). The K_m and V_{max} were evaluated from non-linear regression fitting of the initial reaction rates with respect to different substrate concentrations by using Graph Pad Prism software version 7.0.

3.8. Reusability studies

The co-immobilized magnetic nanobiocatalyst was recycled upto eight cycles in batch operation mode at optimum conditions. The reaction was carried out by mixing known amount of co-immobilized enzyme for a specified time and residual activity was measured using respective substrate considering the activity of first run to be 100%. After each cycle, magnetic nanobiocatalyst was separated using external magnet, washed with phosphate buffer several times and to start a new reaction cycle, re-suspended in fresh substrate solution.

3.9. Application of co-immobilized magnetic nanobiocatalyst

The co-immobilized magnetic nanobiocatalyst, mixture of immobilized Pectinex®, and Celluclast® 1.5 L was employed to extract antioxidants from peel waste residues of orange (*Citrus sinensis*), mango (*Mangifera indica*) and banana (*Musa acuminate*). The peel residues were dehydrated in a hot-air oven at 60 °C until complete drying and then pulverized to 30 mesh particle size. Antioxidant extraction was carried out by mixing dry peel residue (100 mg) with appropriate amount of co-immobilized magnetic nanobiocatalyst in phosphate buffer (pH 6.5) at 50 °C. After 6 h of extraction, the obtained mixture was centrifuged (8000 rpm) and supernatant was diluted with ethanol (1:10 v/v). The hydrolysis level was monitored by determining the reducing sugar and total phenolic contents in the extracts by dinitrosalycilic acid (DNSA) method (Miller, 1959) and Folin–Ciocalteu method (detail procedure in Supplementary information) (Kim and Lim, 2016) respectively. Further, the anti-oxidant activity of extract was determined by using DPPH (2,2-diphenyl-1-picryl-hydrazyl-hydrate) free radical savaging activity (detail procedure in Supplementary information) (Oszmiański et al., 2011).

4. Results and discussion

4.1. Synthesis and characterization of magnetic nanoparticles

Initially, the MNPs were successfully synthesized by the co-precipitation of ferrous and ferric ions taken in 1:2 M ratio in alkaline condition. The synthesized MNPs were characterized by using FT–IR and XRD measurements. The FT–IR spectrum of bare MNPs showed characteristic peaks at 585.5 cm^{-1} corresponding to Fe–O stretching from magnetic nanoparticles which confirms the presence of Fe and O atoms (Fig. 1a-b). Further, amino functionalization of MNPs was done by APTES silanization via condensation reaction. It formed monolayer of amino group on the surface of MNPs by the formation of Fe–O–Si covalent bonds (Liu et al., 2013). The new bands appear at 893.8 and 1016.3 cm^{-1} after silanization correspond to stretching vibrations of Fe–O–Si and N–H from NH_2 respectively (Nadar et al., 2016). Further, the crystalline structure and size were determined by using Powder XRD (Fig. 1d-e). The typical XRD pattern of as-synthesized MNPs is as shown in Fig. 1d. Powder XRD profile exhibited six diffraction peaks at $2\theta = 30.31^\circ$ (2 2 0), 35.67° (3 1 1), 41.18° (4 0 0), 57.24° (5 1 1), 62.84° (4 4 0) which were characteristic peaks of standard Fe_3O_4. These peaks are in good agreement with joint committee on powder diffraction standards (JCPDS) database with reference code 01–075–0449. The characteristic peak at $2\theta = 35.61^\circ$ represents the inverse spinel crystal structure of Fe_3O_4 with 100% purity (Ladole et al., 2017). The average crystallite diameter of magnetic nanoparticles and amino functionalized MNPs was 19.3 nm and 22.1 nm respectively, on the basis of full width half maximum (FWHM) by using Debye-Scherrer's equation. The increase in size of functionalized MNPs was attributed by silanization by APTES onto Fe_3O_4.

4.2. Preparation of co-immobilization on magnetic nanobiocatalyst

The enzyme mixture of Pectinex® and Celluclast® 1.5 L were covalently bounded onto amino functionalized MNPs through cross-linking agent. Prior to covalent attachment of enzyme to surface of amino functionalized MNPs, glutaraldehyde a bi-functional cross-linking reagent was employed to react with both the amino groups of functionalized MNP and enzymes (free amino acid residues present in terms of lysine) to form Schiff base (Wang et al., 2015). Cross-linking parameters i.e. concentration and cross-linking time are directly related to activity recovery, enzyme loading and operational stability (temperature and pH stability) of prepared magnetic nanobiocatalyst (Talekar et al., 2013b). In order to attain higher activity of multi-enzyme magnetic nanobiocatalyst, it was necessary to optimize the degree of chemical cross-linking between amino functionalized MNPs and enzyme. If lower concentration of cross–linker is used during immobilization procedure, the enzyme may not get linked onto functionalized MNPs, while, higher concentration of cross–linker can result into loss of conformational flexibility required to maintain catalytic activity of enzyme. Therefore, effect of cross–linker concentration on immobilization of enzyme was carried out by varying glutaraldehyde concentrations in the range of

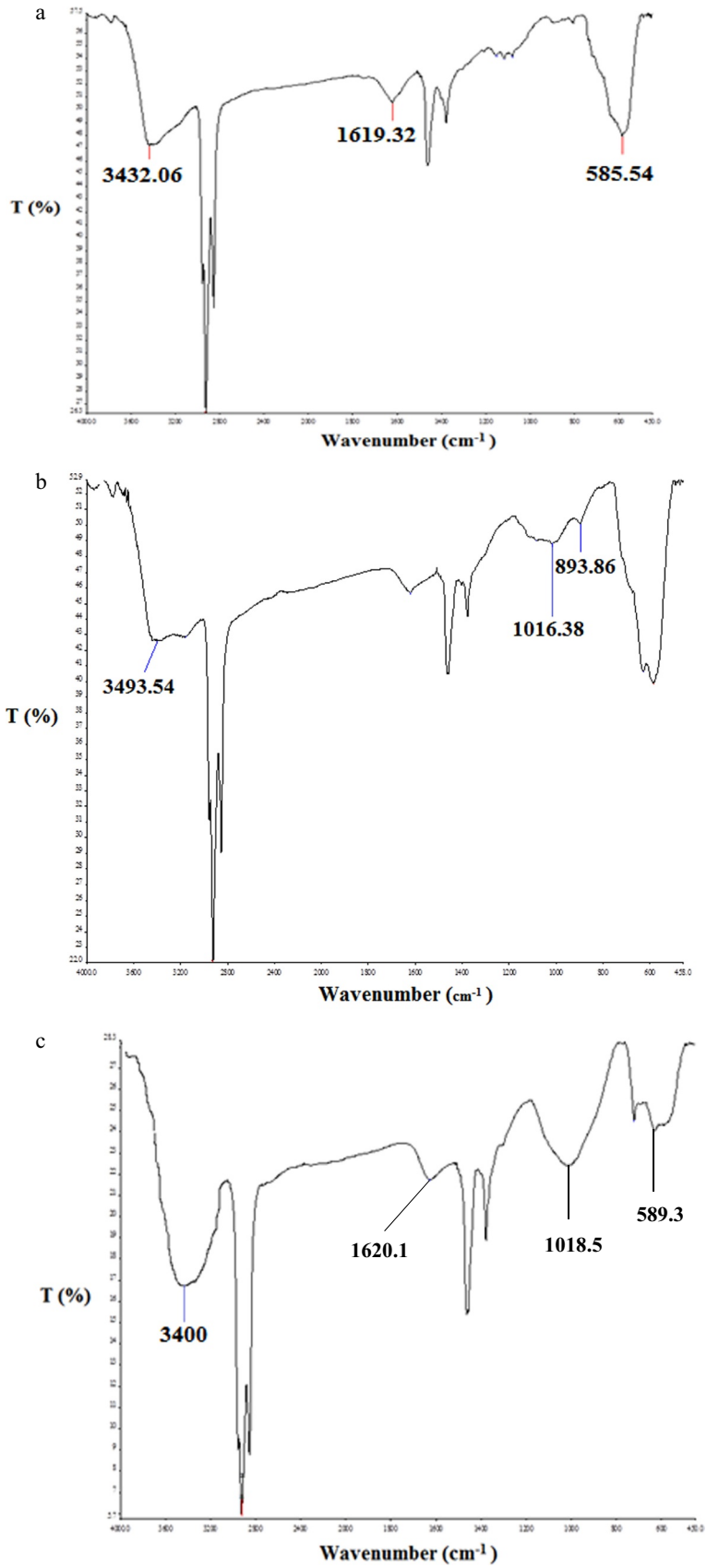

Fig. 1. FT-IR spectrums of (a) prepared MNPs, (b) amino functionalized MNPs and (c) co-immobilized magnetic nanobiocatalyst. XRD patterns of the prepared (d) MNPs, (e) amino functionalized MNPs and (f) co-immobilized magnetic nanobiocatalyst. TGA curves of amino function MNPs and co-immobilized magnetic nanobiocatalyst in nitrogen atmosphere (g). SEM image of co-immobilized magnetic nanobiocatalyst (h).

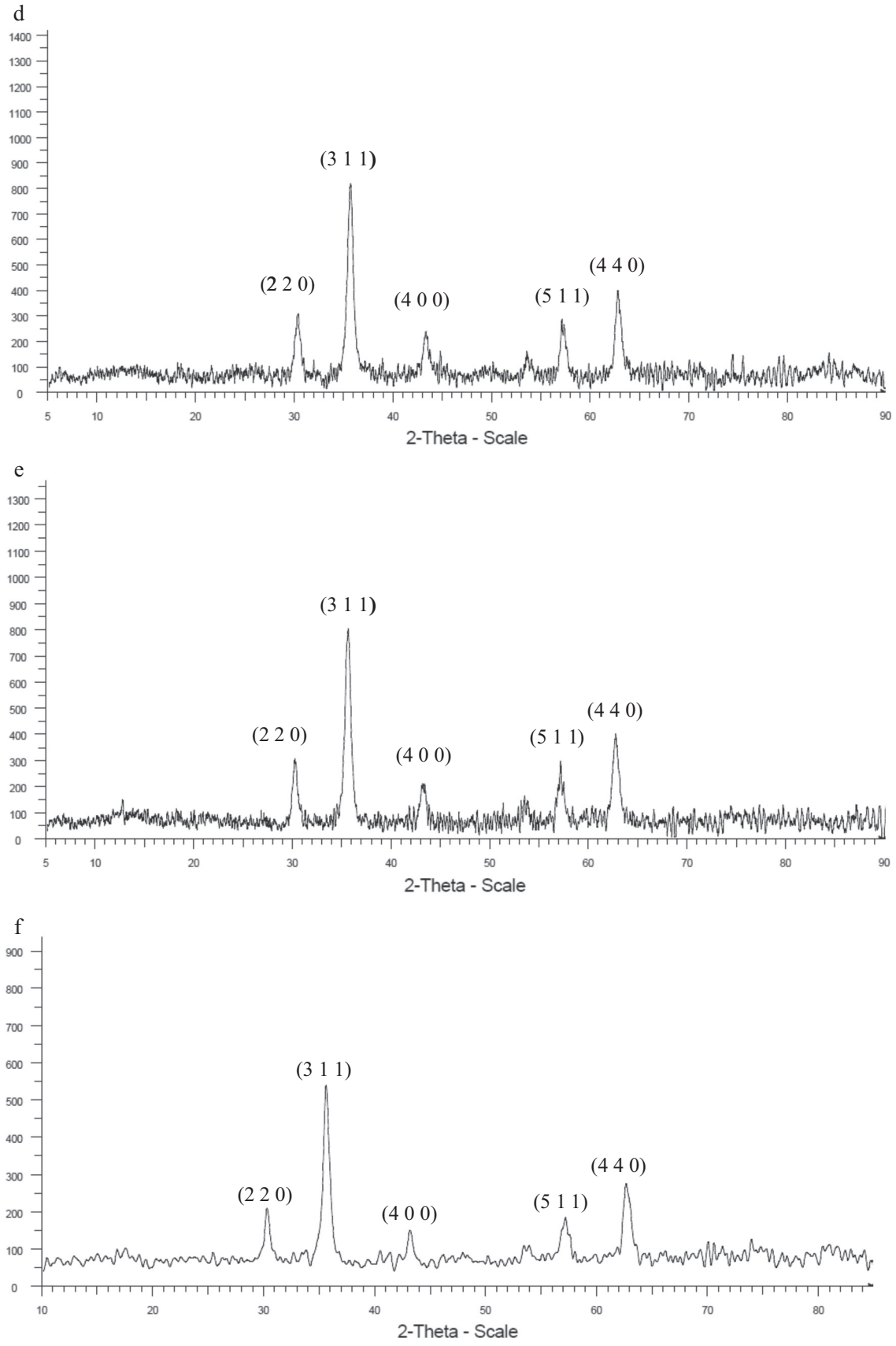

Fig. 1. (*continued*)

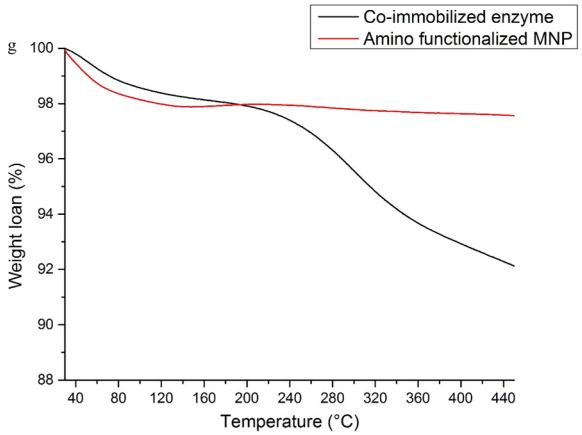

Fig. 1. (*continued*)

15–120 mM. As shown in Fig. 2a, activity recovery of enzymes increased with an increase in glutaraldehyde concentration up to 75 mM at which pectinase and cellulase exhibited the highest activity recoveries of 87% and 82%, respectively. The low concentrations of glutaraldehyde could probably have poor mechanical strength, easily leading to leaching of enzymes from magnetic biocatalyst which resulted in less activity recovery of enzymes in co-immobilized magnetic nanobiocatalyst (Yu et al., 2013). Indeed, Pectinex® and Celluclast® 1.5 L activities were detected in supernatants collected after immobilization onto MNPs conjugated with lower glutaraldehyde concentrations. With an increase of glutaraldehyde concentration, more amount of enzyme would bind onto MNPs which ultimately increase the activity recovery and also prevent the enzyme leaching in co-immobilized magnetic biocatalyst (Hu et al., 2015). However, as the concentration of glutaraldehyde was more than 75 mM, the activity recovery of enzymes in co-immobilized magnetic nanobiocatalyst reduced. This decreasing activity recovery might be caused due to conformational changes and chemical modification of enzymes resulting in the deactivation and rigidification of immobilized enzyme (Chang and Juang, 2007; Jiang et al., 2005; López-Gallego et al., 2005; Salgaonkar et al., 2018).

The cross-linking time is another important factor which affects the activity recovery of enzymes in co-immobilization. As shown in Fig. 2b, the activity recovery was found to be increased with cross–linking time. The maximum activity recoveries were achieved for Pectinex® and Celluclast® 1.5 L, at cross-linking time of 12.5 h. This phenomenon was associated with amount of reactive glutaraldehyde cross-linking with amino functionalized MNP and enzyme which reached its maximum after 12.5 h incubation. Further increase in cross linking time upto 15 h resulted in decrease in activity recovery of enzymes. It could be because of dimerization and over cross-linking of glutaraldehyde due to prolonged incubation time (Bolivar et al., 2009; Nadar and Rathod, 2017a, 2017b; Pan et al., 2009).

Furthermore, the ratio of enzyme and amino functionalized MNPs (non-catalytic support) is needed to be optimized which is shown in

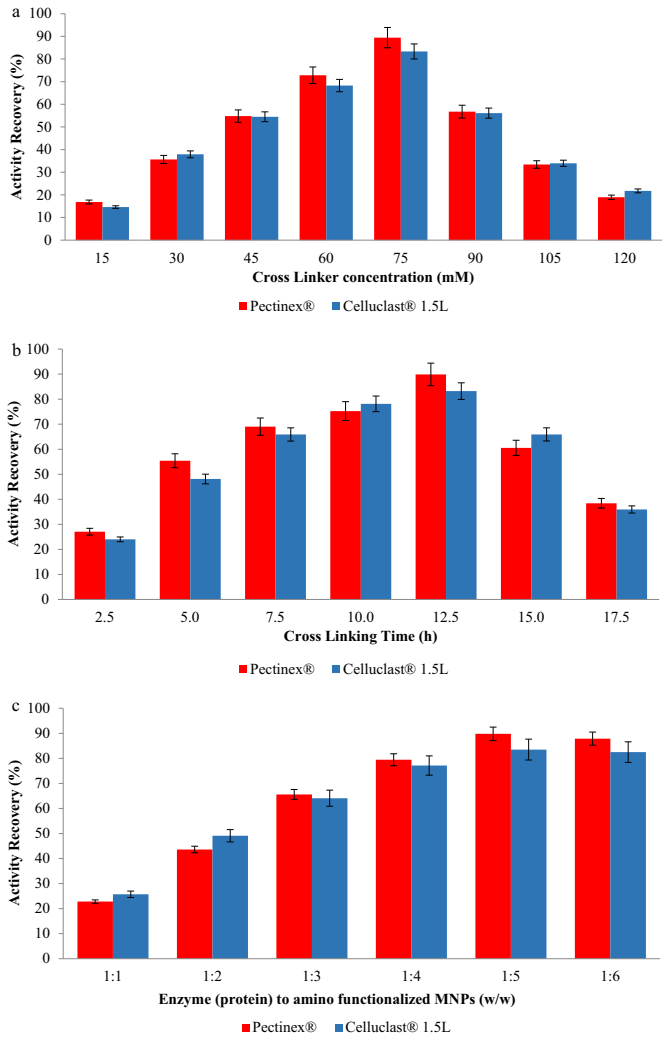

Fig. 2. Effect of (a) cross-linker concentration, (b) cross-linking time on activity recovery and (c) ratio of enzyme (protein) to amino functionalized MNPs (w/w) on activity recovery of Pectinex®, and Celluclast® 1.5 L in magnetic nanobiocatalyst. The 100% activity recovery corresponds to Pectinex® (117 U), and Celluclast® 1.5 L (36 U). The measurements were performed in triplicate and the error bar represents the percentage error.

Fig. 2c. An increase in amount of MNPs resulted in increased enzyme loading which subsequently showed higher activity recoveries to 92% and 87% for pectinase and cellulase, respectively. After 1:5 ratio of enzyme to MNPs, there was no further change in activity recoveries. It seems that most of enzyme was bound onto the functionalized MNPs surface through glutaraldehyde.

4.3. Characterization

The prepared co-immobilized enzymes onto MNPs were confirmed by using FT–IR analysis and crystalline structure was determined by using XRD (Fig. 1c and f). In FT–IR spectra of immobilized enzyme, the characteristic peak at 1621 cm^{-1} indicates amide–I region (stretching vibrations of N–H in $–NH_2$) from free enzyme along with the presence of stretching vibration at 901.4 and 595.6 cm^{-1} which corresponds to Si–O and Fe–O respectively, of amino functional MNPs (Sojitra et al., 2016). It confirmed anchoring of multiple enzymes onto amino functionalized MNPs through covalent bonding. Further, XRD patterns of the co-immobilized enzyme onto magnetic nanoparticles were evaluated. They showed consistent Fe_3O_4 diffraction pattern as that of bare and

functionalized MNPs (JCPDS card No. 01–073–0603) indicating that the nature and phase of crystalline structure of MNPs did not alter chemical/physical properties of MNPs after immobilization of enzymes. This confirmed that enzyme immobilization occurs only at the surface of MNPs (Nadar and Rathod, 2016b; Talekar et al., 2017). Further, the amount of enzyme immobilized onto amino functionalized MNPs was determined by TGA. It was calculated by considering percentage loss of weight of the functionalized MNPs and enzyme immobilized MNPs in the temperature range of 30–450 °C. The thermogram profile of functionalized MNPs and co-immobilized magnetic biocatalyst is shown in Fig. 1g. The weight loss of co-immobilized magnetic nanobiocatalyst was about 9.86% in a broad temperature range between 100 and 450 °C, while, there was a slight weight loss (approx. 1.6%) in functionalized MNPs in same temperature range. This weight loss was observed particularly due to presence of enzyme onto MNP which confirms the immobilization of pectinase and cellulase onto amino functionalized MNPs (Nadar and Rathod, 2018a, 2018b; Sahu et al., 2016). Surface morphology of co-immobilized magnetic nanobiocatalyst was studied by FE-SEM (Fig. 1h). After covalent binding of bulky enzyme molecules onto MNPs, co-immobilized biocatalyst remained spherical with smooth surface after the covalent binding of enzyme molecules onto functionalized MNPs.

4.4. Optimal temperature for free and immobilized enzyme

The activity of free Pectinex® and Celluclast® 1.5 L was increased with temperature and maximum activity was observed at 50 °C (Figs. S1 and S2). This optimum temperature of Pectinex® and Celluclast® 1.5 L in co-immobilized form was shifted to 55 °C and 60 °C respectively, after immobilization of enzymes. This shift in optimum temperature may be due to covalent bond formation between proteins caused by glutaraldehyde during co-immobilization on the surface of functionalized MNPs which might decrease the conformational flexibility of the enzymes. The reduction in conformational flexibility of enzymes protect them from distortion or damage by heat exchange leading to higher thermal resistance as compared to free enzyme (Li et al., 2015; Nadar and Rathod, 2017a, 2017b; Talekar et al., 2013a).

4.5. Thermal stability

The thermostability of Pectinex® and Celluclast® 1.5 L in immobilized form and free form was determined by incubating them at different temperatures in the range of 50–70 °C for 60 min. The enzyme activities were measured as residual activity described previously. The temperature dependent activity loss of free enzyme mixture and co-immobilized enzyme nanobiocatalyst are plotted in Fig. 3a-b. From the thermal deactivation kinetic plot, deactivation constant (k_d) and half-life ($t_{1/2}$) were calculated for each form of enzyme, and is summarized in Table 1. The rate of inactivation of enzymes in free form was considerably higher as compared to enzymes in co-immobilized enzyme nanobiocatalyst. Upon immobilization, on an average, there was 2.76-fold and 2.38-fold increase in half lives of Pectinex® and Celluclast® 1.5 L, respectively, in the temperature range of 50–70 °C. From the temperature dependent activity profiles, the Arrhenius plot was obtained for Pectinex® and Celluclast® 1.5 L in free as well as co-immobilized form (Fig. 3c). The slope of Arrhenius plot was used to calculate deactivation energy (E_d). The results listed in Table 1 show that Pectinex® and Celluclast® 1.5 L in co-immobilized nanobiocatalyst required higher deactivation energy than in free enzyme mixture in the temperature range of 50–70 °C. The increase in deactivation energies (ΔE_d) of enzymes in magnetic nanobiocatalyst was 17.59 and 19.08 kJ mol^{-1} for Pectinex® and Celluclast® 1.5 L, respectively. These results indicated the excellent thermal stabilization of pectinase and cellulase in co-immobilized form. This was due to the covalent bonding between enzymes and MNPs which might stabilize the configuration inevitably and restricts molecular flexibility, which is essential to

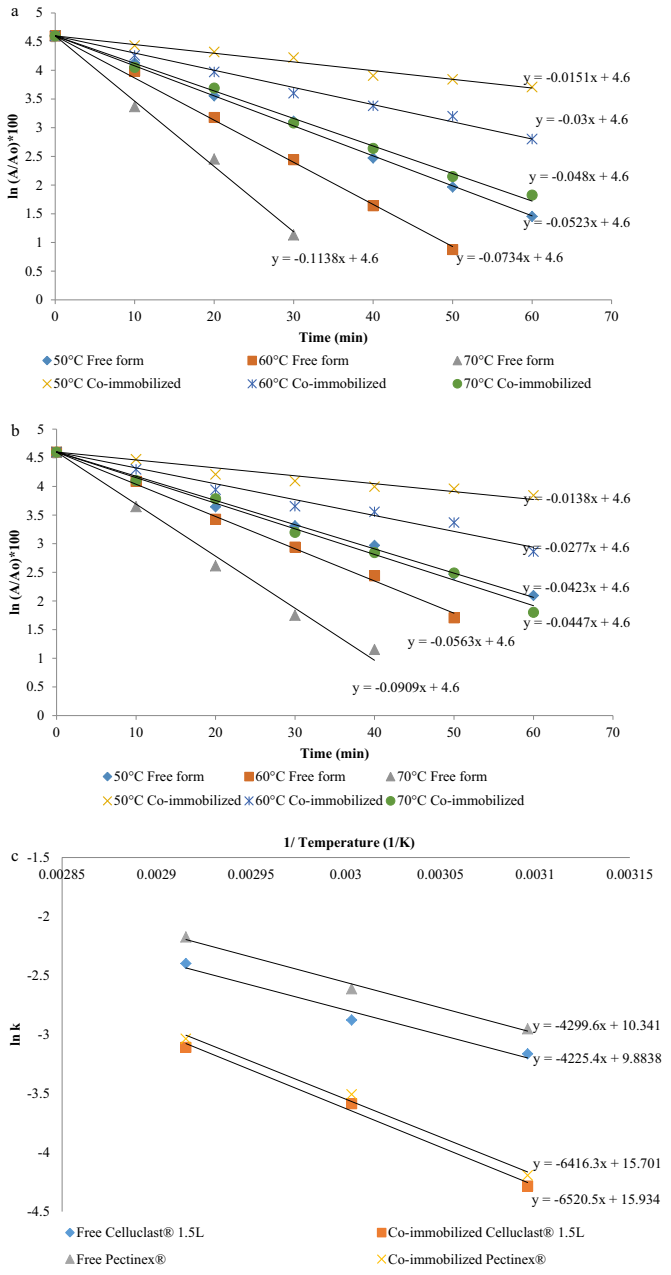

Fig. 3. Thermal kinetics profile of Pectinex® (a) and Celluclast® 1.5 L (b). Arrhenius plot for inactivation of free and immobilized form (c).

catalytic activity (Arnold et al., 2001; Nadar et al., 2018a; Soozanipour et al., 2015).

4.6. Kinetics parameters

The Michaelis constant (K_m) and maximum velocity (V_{max}) were determined to investigate the enzymatic activity of the co-immobilized form and free enzyme mixture. The K_m and V_{max} were determined for Pectinex® and Celluclast® 1.5 L in co-immobilized nanobiocatalyst and free enzyme mixture by measuring initial reaction rates of each enzyme with varying concentration of pectin and CMC, respectively. Experimental values were fitted using nonlinear regression procedures by Graph Pad Prism 7 software which are summarized in Table 2. The K_m value of an enzyme reflects its affinity for the substrate. The slightly higher values of K_m have been observed for Pectinex® and Celluclast® 1.5 L in co-immobilized form in comparison with that observed in free

Table 1
Kinetics of thermal deactivation parameter of free and immobilized form of Celluclast® 1.5 L and Pectinex®.

Forms	k_d (min^{-1})			$t_{1/2}$ (min)			E_d ($kJ\ mol^{-1}$)
	50 °C	60 °C	70 °C	50 °C	60 °C	70 °C	50–70 °C
Free celluclast® 1.5 L	0.0423	0.0562	0.0909	16.38	12.33	7.62	32.13
Immobilized celluclast® 1.5 L	0.0138	0.0277	0.0447	50.22	25.02	15.50	54.21
Free pectinex®	0.0523	0.0734	0.1138	13.25	9.44	6.09	36.75
Immobilized pectinex®	0.0151	0.03	0.048	45.89	23.10	14.44	53.35

Table 2
Michaelis–Menten kinetic parameters of co-immobilized form and free form of Celluclast® 1.5 L and Pectinex®.

Forms	K_m (mg/mL)	V_{max} (μmol/min)
Free celluclast® 1.5 L	0.5462 ± 0.023	5.8029 ± 0.4618
Immobilized celluclast® 1.5 L	0.5871 ± 0.019	5.4331 ± 0.4714
Free pectinex®	0.6526 ± 0.027	4.8214 ± 0.3810
Immobilized pectinex®	0.6990 ± 0.031	4.6916 ± 0.3112

enzyme mixture. This indicated that the affinity of magnetic nanobiocatalyst was lower after immobilization procedure which might be due to rigidification of enzyme molecular conformation after binding onto functionalized MNPs (Wu et al., 2004). Moreover, the V_{max} values of Pectinex® and Celluclast® 1.5 L in co-immobilized form were higher than that in free enzyme mixture, which means that a higher reaction rate for immobilized enzymes could be obtained at a similar concentration of substrate in the solution. It indicates that the catalytic velocity was increased in co-immobilized nanobiocatalyst. This observed phenomenon might be due to high surface area-to-volume ratio and decreased diffusion within co-immobilized magnetic nanobiocatalyst (Bi et al., 2009; Jiang et al., 2005).

4.7. Recycling and reusing of magnetic nanobiocatalyst

It is necessary to study the recyclability of the magnetic nanobiocatalyst to check its industrial and economic viability. To study recyclability, co-immobilized magnetic biocatalyst was employed to conduct hydrolysis of respective substrate for certain time in batch mode. After each cycle, magnetic nanobiocatalyst was magnetically separated, washed and added to the fresh reaction medium. The residual activity of Pectinex® and Celluclast® 1.5 L in bi-enzyme nanobiocatalyst in each cycle is shown in Fig. 4. After eighth consecutive cycles, the immobilized Pectinex® and Celluclast® 1.5 L were still retaining 80% of their original activity. This outstanding reusability can

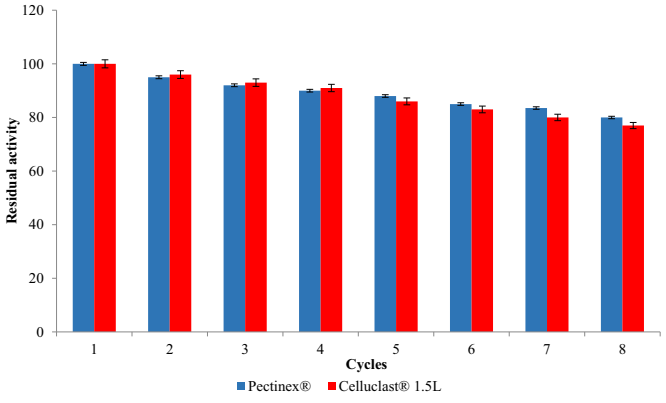

Fig. 4. Percentage residual activity (upto tenth cycle) of Celluclast® 1.5 L and Pectinex® in magnetic nanobiocatalyst. The 100% residual activity of respective enzyme corresponds to activity at first cycle. The measurements were performed in triplicate and the error bar represents the percentage error.

be attributed to the strong retention of stability of the enzymes in co-immobilized nanobiocatalyst (Sojitra et al., 2017; Talekar et al., 2012).

4.8. Application of co-immobilized magnetic nanobiocatalyst

Most of the phenolic compounds are entrapped within cell wall polysaccharides like cellulose, hemicelluloses, lignin and pectin which are linked by hydrogen bonds and hydrophobic interactions. The enzymatic hydrolysis of cell wall components (pectin and cellulose) could improve the cell walls permeability and porosity, thus readily leaching out the intracellular components including phenolic compounds (Nadar et al., 2018b). The co-immobilized magnetic biocatalyst was employed to extract an antioxidant from peel waste residues of orange (*Citrus sinensis*), mango (*Mangifera indica*) and banana (*Musa acuminate*). The extraction of phenolics was monitored by determining the reducing sugar content and total phenolic content after enzymatic treatment (Table 3). After synergistic Pectinex® and Celluclast® enzymatic treatment to fruit peel waste, the reducing sugar content increased which showed that immobilized enzyme degrades the polysaccharide present in the cell wall of fruit waste. Also, it helped to release phenolic contents from waste peels which was confirmed by analysing total phenolic contents. The total phenolic content of enzyme-treated samples was higher than total phenolic content of control. Further, the ability of antioxidants to scavenge DPPH was evaluated to determine their hydrogen donating activity and the free radical scavenging activity. DPPH scavenging activities were found to be two folds higher than untreated peel residual extraction. The increased DPPH scavenging activity after enzymatic treatment was due to the increased extractability of phenolic compounds from cell wall matrices (M'hiri et al., 2015; Oszmiański et al., 2011; Štambuk et al., 2016). The mixture of individually immobilized Pectinex® and Celluclast® 1.5 L showed slight lower extraction of polyphenolics from waste fruit peels. From these results, it is clear that co-immobilized magnetic nanobiocatalyst helps to liberate polyphenolic compounds from waste fruit peels with higher efficiency. Also, presence of magnetic nanoparticles helps to recover biocatalyst easily from reaction mixture after the extraction process.

5. Conclusion

In conclusion, we simultaneously co-immobilized Pectinex® and Celluclast® 1.5 L onto amino functionalized magnetic nanoparticle for extraction of antioxidants. The superior thermal stability as $t_{1/2}$ augmented by average of 2.4 folds in the range of 50–70 °C was exhibited by magnetic nanobiocatalyst as compared to free enzymes. The K_m and V_{max} values were found to be higher for Pectinex® and Celluclast® 1.5 L in immobilized form. Additionally, magnetic co-immobilized nanobiocatalyst showed upto 80% residual activity even after 8th cycle of reusability which emphasizes its industrial feasibility. Furthermore, co-immobilized magnetic nanobiocatalyst and mixture of individually immobilized enzymes was employed for extraction of antioxidant from real fruit waste peels; orange (*Citrus sinensis*), mango (*Mangifera indica*) and banana (*Musa acuminate*) which showed higher excitation of total phenolic contents as compared to control. Though further development is still needed, the implementation of immobilized enzyme treatment is very simple and readily applicable for the extraction of natural

Table 3
Extraction of antioxidant for peel residues of orange (*Citrus sinensis*), mango (*Mangifera indica*) and banana (*Musa acuminate*).

Fruit peel residues		Reducing sugar (mg/g)	DPPH radical scavenging activity (mg GAE/g)	Total phenolic contents (mg GAE/g)
Orange peel (*Citrus sinensis*)	Control	67 ± 2.16	12 ± 1.43	6.3 ± 0.6
	Co-immobilized enzyme treated	258 ± 4.13	21 ± 1.33	11 ± 1.2
	Individual immobilized enzyme	246 ± 3.09	18 ± 1.87	9 ± 1.4
Mango peel (*Mangifera indica*)	Control	59 ± 3.19	10 ± 1.53	8 ± 0.9
	Co-immobilized enzyme treated	234 ± 3.08	25 ± 2.02	15 ± 1.8
	Individual immobilized enzyme	225 ± 2.69	22 ± 1.95	13 ± 1.4
Banana peel (*Musa acuminate*)	Control	43 ± 3.15	7 ± 1.92	4 ± 0.5
	Co-immobilized enzyme treated	159 ± 2.25	19 ± 2.85	9 ± 1.4
	Individual immobilized enzyme	153 ± 1.53	17 ± 1.99	8 ± 1.8

antioxidants and other phenolic compounds from various fruit as well as food wastes.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.bcab.2018.12.015

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