Ultrasonics - Sonochemistry 48 (2018) 453–462

Contents lists available at ScienceDirect

Ultrasonics - Sonochemistry

journal homepage: www.elsevier.com/locate/ultson

Synergistic effect of ultrasonication and co-immobilized enzymes on tomato peels for lycopene extraction

Mayur R. Ladole[a], Rajiv R. Nair[a], Yashomangalam D. Bhutada[a], Vinod D. Amritkar[b], Aniruddha B. Pandit[a,*]

[a] Department of Chemical Engineering, Institute of Chemical Technology, Mumbai, India.
[b] Abhay Nutrition Pvt. Ltd., India.

ARTICLE INFO

Keywords:
Ultrasonication
Co-immobilized enzymes
Lycopene
Amino functionalized magnetic nanoparticles
Tri-solvent extraction

ABSTRACT

In the present work, tomato peels were pre-treated using combination of ultrasound and enzyme co-immobilized amino-functionalized magnetic nanoparticles (AMNPs) for the efficient release of lycopene. To achieve maximum activity of enzymes in the co-immobilized form, optimization of several parameters were carried out. Moreover, the influence of ultrasound and enzyme co-immobilized magnetic nanoparticles on lycopene release was studied. Maximum lycopene release was obtained at 3% (w/w) enzyme co-immobilized AMNPs, pH 5.0, temperature of 50 °C, at 10 W ultrasound power and 20 min incubation time. After enzymatic pre-treatment, lycopene from the pre-treated mixture was extracted and separated using tri-solvent extraction method. Maximum recovery of lycopene using solvent extraction was obtained at 50 °C, 90 min of incubation time and agitation speed of 150 rpm. The presence of lycopene in the extract was confirmed by FT-IR, UV–vis spectroscopy and HPLC analysis. The co-immobilized bio-catalyst showed excellent reusability giving more than 50% lycopene yield even after 6th cycles of reuse.

1. Introduction

Tomatoes are one of the widely grown and the most preferred garden crops. Every year several million tons of tomatoes are produced and are processed for multiple tomato products consisting of pastes, purees, canned tomatoes, juices and sauces [1]. During this manufacturing process, a huge amount of waste called as tomato pomace which consist of pulp, tomato seeds, and peels are generated [2]. The tomato pomace is generally considered as a waste and has no viable importance and presently it is used as an animal feed or directly disposed off [3]. Although these waste have no commercial value but, it contains various valuable bioactive compounds such as β carotene, ascorbic acid, tocopherols and lycopene. Out of all these bioactive moieties, lycopene is of utmost important and is highly responsible for red color of tomatoes and is very expensive in terms of its commercial value [4]. Therefore, the waste tomatoes are considered as a cheap source of this value added carotenoid. Lycopene, is a tetraterpenic hydrocarbon with 13 carbon (C=C) double bonds, out of which 11 are conjugated [5]. Lycopene is considered as one of the most potent antioxidants due to its high degree of conjugation. The application of lycopene in several sectors has been significantly increased and hence it has now received considerable attention. The widespread applications of this value added compound includes pharmaceuticals, foods, cosmetics, and it is also utilized as an antioxidant, natural dye, antiaging, and anti-cancer agent [6]. Because, of its potential antioxidant property it is useful in the treatment of prostate cancer and cardiovascular diseases. In ripe tomatoes, lycopene is present in almost all parts such as pulp, water-insoluble fraction and in peels. The significant quantity of this red pigment (lycopene) (72–98%) is predominantly present in the peels [2].

For the past few years, ultrasonication which is also known for its versatile feature of green chemistry, is widely used in the area of chemical technology. The applications include in preservation, processing and enhanced extraction of various bioactive moieties from cellular compartments [7]. There are many benefits of ultrasonication over microwave, heating and supercritical methods of extractions for flavonoids. Recently, research is more focused on the development of extraction protocols for lycopene in which the use of enzyme assisted extraction is most favored [8,9]. Pectin and cellulose are the main constituents of plant cell wall matrix hence, enzymes viz. cellulase and pectinase are widely used for the enzyme aided extraction processes [4]. However, there are very few literatures which have described the

* Corresponding author.
E-mail address: ab.pandit@ictmumbai.edu.in (A.B. Pandit).

https://doi.org/10.1016/j.ultsonch.2018.06.013
Received 31 May 2018; Accepted 17 June 2018
Available online 27 June 2018
1350-4177/

successful use of enzymes for the release of lycopene from tomato tissues [1]. Recently, Konwarh et al., (2012) studied the mutual outcome of enzyme and sonication for the effective release of lycopene from tomato peels and got excellent results. But, the use of enzymes in their native form leads to the waste as it is very difficult to recycle them after completion of reaction. Several reports are there in which enzymes have been successfully immobilized on various supports viz. glass and sodium alginate beads, matrix entrapment, carbon nanotubes etc. Nowadays enzyme immobilization on magnetic nanoparticles (MNPs) has gain tremendous attention due to the capability of MNPs to bind more enzymes due to high-surface area available, high enzyme stability, and more importantly ease in separation using an external magnet after the completion of reaction [10]. There is not a single report in which immobilized enzymes has been successfully utilized for lycopene extraction from tomato peels or any other sources.

There are several reports in which mild sonication conditions were used to increase the activities of cellulase and pectinase [11,12]. And, in our previous work, we have reported that, under mild sonication conditions cellulase immobilized MNPs showed significant enhancement in the activity [13]. Also, it has been observed that under the influence of ultrasonic irradiation, there is increased in lycopene release from the tomato peels [14]. Recently, cellulase and lysozyme were successfully co-immobilized on AMNPs and was effectively utilized for an efficient release of lipids from micro-algae [15].

Considering all these aspects, in the present work, the aim was to study possible synergistic effects of sonication and co-immobilized enzyme for the efficient extraction of lycopene from waste tomatoes. Moreover, various parameters were optimized for the co-immobilization of both enzymes on AMNPs. Effect of time, pH, temperature and sonication power on the release of lycopene was extensively studied. Also, optimization of tri-solvent extraction was carried out to increase the overall yield of lycopene from the tomato peels.

2. Materials and methods

2.1. Materials and chemicals

The undetoriated fully ripened waste tomatoes were obtained from the local market of Matunga, Mumbai. APTES (3-aminopropyltriethoxysilane), pectinase, pectin, cellulose (CMC-medium viscosity) and fluorescein isothiocyanate (FITC) were procured from Hi-Media (Bangalore, India). Cellulase was obtained from Novozymes Pvt. Ltd. Glutaraldehyde 25% (w/v), sodium hydroxide (NaOH), were the product of Thomas bakers (Mumbai, India). $FeCl_3{\cdot}6H_2O$, $FeCl_2{\cdot}4H_2O$, ethanol, acetone, hexane (99.0%) and DNS (3,5-dinitrosalicylic acid) were obtained from S.D. fine chemicals Ltd. (Mumbai, India). All the other reagents were of analytical grade with their highest purity.

2.2. Amino functionalization of magnetic nanoparticles (AMNPs)

Proceeding to amino-functionalization, first step is the preparation of magnetic nanoparticles (MNPs). The MNPs were prepared as per our previous work by chemical co-precipitation method [10]. The prepared MNPs were then subjected for amino-functionalization using APTES as a coating agent [13]. For this, 100 mg MNPs were added in ethanol and water mixture (50:50) and were kept for sonication (5 min) for complete dispersion. The amino-functionalization reagent (APTES) was then added slowly into the mixture and solution was then kept for stirring using overhead stirrer for 8 h. Subsequent to stirring, the amino-functionalized MNPs were separated magnetically and washed with ethanol and deionized water (3 times each) for the removal of unbound reagent. The AMNPs were then dried and stored under moisture free condition.

2.3. Enzyme co-immobilization on AMNPs

For enzyme co-immobilization of pectinase and cellulase on AMNPs, glutaraldehyde (GA) was used a cross-linking reagent [17]. In brief, AMNPs (50 mg) were added in acetate buffer (30 mL, 0.1 M, pH 5.0) and enzymes (with known activities) followed by glutaraldehyde (40 mM) were added to the mixture. The entire solution then kept for stirring at ambient condition (30 ± 1 °C) for 2 h. After proper mixing, enzyme grafted AMNPs were separated using an external magnet and rinsed with buffer (pH 5.0, 0.1 M). The amount of enzymes (in the form of proteins) in the supernatant after each wash were quantified using Bradford method [18]. The quantity of enzyme co-immobilized on AMNPs was calculated using following Eq. (1).

$$\text{Percentage immobilization}(\%) = \frac{C_{int}V_{int} - C_{fin}V_{fin}}{C_{fin}V_{fin}} \quad (1)$$

where, C_{int} & C_{fin} = Initial and final protein content (mg/mL), V_{int} & V_{fin} = Initial and final amount of solution (mL).

To achieve maximum enzyme loading and to recover maximum activity, various parameters viz. concentrations of GA, time for cross-linking, ratio of enzymes to AMNPs were studied [17]. The activity (%) retained in the co-immobilized form was determined as (Eq. (2)):

$$\text{Activity recovery}(\%) = \frac{\text{Activity of immobilized enzyme(U)} \times 100}{\text{Activity of free enzyme used for enzyme immobilization(U)}} \quad (2)$$

2.4. Enzyme activity assays

The activities of both enzymes in free and co-immobilized form were calculated using standard enzyme assay protocol. For pectinase activity, pectin was used as the substrate. In detail, pectin (1% w/v) was dissolved in acetate buffer (0.1 M, pH 5.0) followed by pectinase addition [19]. The solution was then incubated in water bath (40 °C), after 20 min, DNSA (1 mL) was added into the solution and kept in boiling water bath (10 min) and cooled. The change in the color of solution was then measured at 470 nm using UV spectrophotometer. The unit activity of pectinase (1U) is defined as the amount of pectinase necessary to release a μmole of D-galactouronic acid from pectin under optimized assay conditions.

The activity of cellulase enzyme was measured using CMC as the substrate. In this, enzyme (cellulase-1 mL) was added in CMC solution (1% w/v, 0.1 M sodium acetate buffer, pH 5.0) and was kept in water bath for incubation (40 °C, 30 min) [13]. After 30 min, DNSA was added into the solution and kept for boiling in water bath for 10 min. The amount of glucose produced was then estimated spectrophotometrically at 540 nm. The unit activity of cellulase (1 unit) is defined as the amount of cellulase necessary to release one μmole of glucose from CMC under optimized conditions. For co-immobilized enzyme same protocols were used, with only the difference of the magnetic decantation of co-immobilized enzyme before DNSA addition.

2.5. Thermal deactivation studies

The kinetics of thermal deactivation for both enzymes before and after co-immobilization was studied by incubating enzymes (free and co-immobilized) at 50, 60 and 70 °C in acetate buffer (0.1 M, pH 5.0) separately. After every 30 min, till 120 min of incubation time, the samples were removed, cooled and analyzed for the presence of any residual activity [20]. The thermal deactivation rate constant (k_d) was then calculated by plotting a semi-log plot of residual activity vs. time. Using $0.693/k_d$, the half life ($t_{1/2}$) of enzyme was calculated. Half life of enzymes is the time necessary for activity to decrease to half of its initial activity. Further, Arrhenius plot was used to calculate the deactivation energy (E_d) of enzymes in both free and immobilized form.

2.6. Preparation of tomato peels powder and determination of total lycopene content

The lycopene content of tomato peels was determined as per our previous work [19]. Briefly, tomatoes were washed with fresh water and kept in water bath (60 °C, 15 min) for easy peeling. The peels were then dried in vacuum oven (40 °C) and powder was prepared using mortar pestle, then sieved and kept in an air tight polythene bags (high-density) at cold condition [2]. The lycopene content in the extract was determined spectrophotometrically [9]. The tomato peels mixture with and without pretreatment was extracted using tri-solvents viz. acetone, ethanol and hexane in the ratio of 1:1:2 (v:v:v). After this, water (deionized) was used for the phase separation. The upper layer of solvent was collected and the absorbance was measured at 503 nm. The amount of lycopene present in the extract was calculated as 1 μg/g and then converted to mg/100 g.

$$\text{Lycopene}(\mu g/g) = \frac{A_{503} \times 31.2 \times \text{Diltution}}{\text{amount of sample}} \quad (3)$$

2.7. Characterization of MNPs, AMNPs, enzyme co-immobilized AMNPs and lycopene

The surface modification of MNPs, enzymes binding on AMNPs and presence of lycopene in the extract was confirmed by Fourier transform infrared (FT-IR) spectroscopy (IRPrestige-21, Shimadzu) in the range of 400–4000 cm^{-1}. The binding of enzymes on AMNPs was also confirmed by confocal scanning laser microscopy (CLSM) (DMi8 microscope and SP8 scanner Leica, Germany) by tagging enzymes using fluorescein isothiocyanate (FITC) [21]. The crystalline structure and phase changes in MNPs before and after modifications were studies by XRD analysis (XRD D-8 advance, Bruker Axs GnbH, Berlin Germany). The surface morphologies of MNPs before and after enzyme co-immobilization were observed using field emission gun scanning electron microscopy (FE-SEM) analysis (TESCAN MIRA 3 model). The magnetic behavior of bare MNPs and enzyme co-immobilized AMNPs were evaluated using vibrating sample magnetometry (VSM analysis, Lake-Shore-7407, USA) at room temperature. The absorbances were recorded on UV-double beam spectrophotometer (UV-1800, Shimadzu). The presence of lycopene in the extract was confirmed by HPLC analysis (Thermo Fischer, ultimate 3000).

2.8. Pre-treatment of tomato peels using ultrasound and co-immobilized enzyme on AMNPs

The main focus of this study is to study the effect of ultrasound and enzyme co-immobilized AMNPs for the pre-treatment of tomato peels. For ultrasound irradiation, temperature controlled ultrasonic bath operated over power dissipation range of 2–120 W at fixed frequency of 20 kHz was used throughout the experiment (Dakshin, Mumbai (India)). The major constituent of tomato peels constitutes of pectin network followed by cellulose therefore, both pectinase and cellulase were used together for the pre-treatment studies [4]. For maximum release of lycopene using enzymatic pre-treatment, parameters viz. concentration of co-immobilized enzyme, sonication power, pH, temperature, and incubation time were optimized.

2.9. Extraction of lycopene using tri-solvent method

After optimization of tomato peels pre-treatment, next step is to recover lycopene using tri-solvent extraction (ethanol, acetone and hexane) as per the protocol previously given by Ranveer et al., (2013). Enzyme co-immobilized AMNPs were magnetically decanted from the reaction mixture and tomato peels were then mixed with tri-solvents consisting of ethanol (12 mL), acetone (12 mL with 0.05% BHT), hexane (24 mL), deionized water (12 mL) and incubated at 120 rpm, at 35 ± 2 °C for 90 min [1]. For better extraction of lycopene, optimization of various parameters was carried out. For temperature optimization, the reaction mixture was incubated at a wide range of temperature (25–55 °C), 120 rpm and incubated for 90 min. The incubation time was further optimized by incubating reaction mixture at 120 rpm for a range of incubation time (0.5–3 h and at optimized temperature). Further, the agitation speed was optimized by shaking the reaction mixture at different agitation speed (50–300 rpm and at optimized temperature and time) [19]. The lycopene content in the extract was spectrophotometrically detected as before.

2.10. Reusability and storage stability of enzyme co-immobilized AMNPs

One of the main applications of enzyme immobilization is its reusability after completion of reaction. To study this, the prepared enzyme co-immobilized magnetic nano-biocatalyst were subjected for reaction under all optimized parameters and after each reaction, the co-immobilized biocatalyst was separated magnetically (washed with buffer) and reused again for the next batch of reaction. The first yield of lycopene using co-immobilized enzyme was considered as 100% and was compared with all reusability study reactions. Further, the storage stabilities of co-immobilized enzymes were calculated by measuring their activities over a period several days. The co-immobilized enzymes were stored in acetate buffer (pH 5.0, 0.1 M) at room temperature and the activities of both enzymes were determined after an interval of five days each up to 30 days [17].

3. Results and discussion

3.1. Co-immobilization of pectinase and cellulase on AMNPs

The process of enzyme co-immobilization involves the binding of enzymes onto AMNPs using a suitable cross-linker (glutaraldehyde). The process of binding more than one enzyme on AMNPs is called as co-immobilization method [22]. In the co-immobilization process, glutaraldehyde reacts on the amino groups of both enzymes and AMNPs to form a strong covalent bond [16]. The optimization of this cross-linking agent is very crucial as at its low concentration, lesser amount of enzymes get immobilized to give lesser activity recovery while exceeding the concentration will form a more rigid structure hence hindering the enzyme active sites.

Optimization study revealed that there was an increase in the activity recoveries of both enzymes by increasing the concentration of glutaraldehyde up to 30 mM however; when the concentration exceeds above 30 mM, it resulted in decreased activity (Fig. 1A). This illustrates that at lower concentration of this cross-linking agent there must be the leaching of enzymes into the reaction mixture hence poor activity recovery. The leaching of enzymes was also detected in the supernatant obtained after the completion of reaction. However, at higher concentration, there were some conformational changes in the enzymes which were as a result of excessive interaction of both enzymes and AMNPs with excessive aldehyde group to form a more rigid structure which hinders the active sites of the enzymes [23].

Second important parameter while preparation of co-immobilized AMNPs is the cross-linking time. It is the time necessary to capture maximum initial activity of the enzymes on AMNPs. For this, the reaction mixture containing enzymes, AMNPs and cross-linker were subjected to cross-linking reaction at different intervals of time. From the results it has been found that, time period of 2 h was the optimum cross-linking period to get maximum activity recoveries of (pectinase 85% and cellulase 80%) both enzymes (Fig. 1B). However, time period than 2 h, gave less activity recoveries of both enzymes which were attributed to insufficient cross-linking of enzymes and AMNPs using glutaraldehyde. Moreover, time period of above 2 h resulted in lesser activity recoveries, this might be due the fact that, prolonged exposure to cross-linking resulted in restriction of enzymes flexibility eliminating

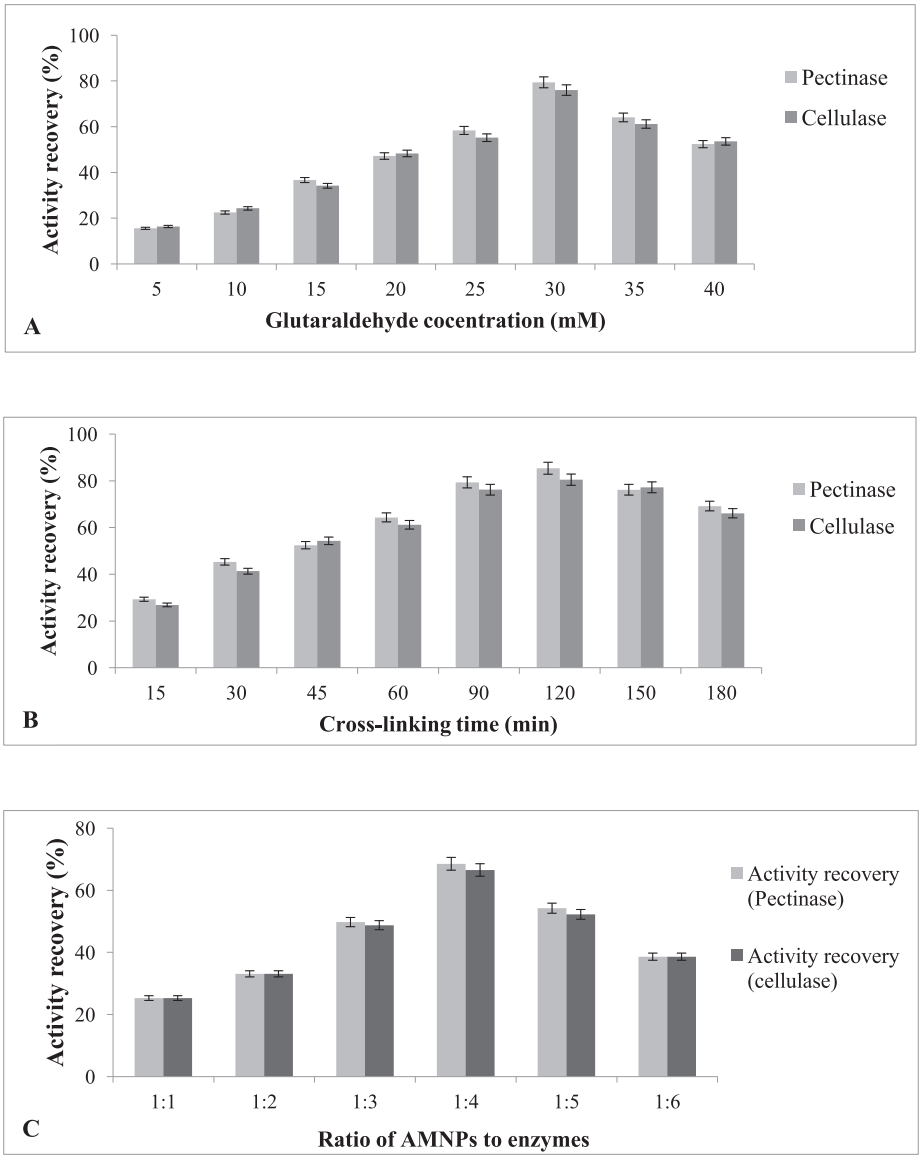

Fig. 1. Effect of glutaraldehyde concentration (A); cross-linking time (B); and ratio of AMNPs to enzymes (C) on the activity recovery of co-immobilized enzyme on AMNPs. The measurements were performed in triplicate and the error bar represents the percentage error.

enzyme activity due to intensive cross-linking [17].

Ratio of AMNPs to enzymes (w/v) is also an important factor which has to be considered during the co-immobilization process. For this, AMNPs (of known quantity) were subjected to cross-linking by varying the enzymes concentration (w/v). Results showed that enzymes showed maximum activity recovery at the ratio of 1:4 (w/v) with enzyme loading of more than (Fig. 1C). However, at lower ratio, the recovered activity was less which might be due to lesser availability of amino groups in enzymes to cross-link on AMNPs. Similarly, at higher enzyme concentration the activity recovery got reduced even though higher enzyme loading of more than 80% was observed. This could be due to excess of enzyme present in the reaction mixture to form a more rigid co-immobilized structure hence hindering the active site of enzymes [13].

3.2. Physicochemical characteristics of co-immobilized enzymes on AMNPs

FTIR spectroscopy is one of the important tools to reveal the confirmation and binding of enzymes on AMNPs. The appearance of peak at 579 cm^{-1} on the spectra of enzyme co-immobilized AMNPs were contributed to absorption of Fe-O group of magnetic nanoparticles (Fig. S1A). Characteristic peaks subsequent to the amide bond (C═O and C—N) appeared at 1448 and 1625 cm^{-1} which confirms the surface functionalization of MNPs. Finally, occurrence of bands at 1043 and 1396 cm^{-1} confirmed the successful binding of enzymes on AMNPs (Fig. S1B) [24].

The surface morphology and size distribution of MNPs and enzyme co-immobilized AMNPs were observed by scanning electron microscopy (SEM) (Fig. S2A and B). Results clearly indicated that bare AMNPs and enzyme co-immobilized AMNPs are smooth and spherical and have size below 50 nm. It has been also observed that, AMNPs after enzyme co-immobilization remained distinct without forming agglomeration and have almost the same characteristics as that of unbound AMNPs [25]. Hence, the process of co-immobilization indicated no change in the morphologies of MNPs even after enzyme co-immobilization.

The crystallinity and phase changes of the AMNPs before and after enzyme co-immobilization were studied by XRD analysis (Fig. S3). It has been observed that the indices of both before and after enzyme immobilized AMNPs were same. These results clearly indicated that, even after enzyme co-immobilization there was no significance difference in the phase change [26]. Also, the appearance of sharp diffraction peaks before and after enzyme co-immobilization on AMNPs showed

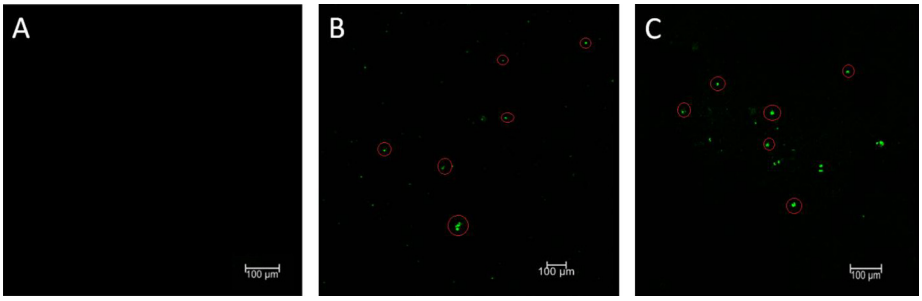

Fig. 2. Confocal laser scanning microscopy of FITC labeled MNPs (A); APTES grafted MNPs (B); and enzyme co-immobilized AMNPs (C).

that the particles are crystalline in nature with no impurity peaks.

Recently, the surface grafting using aminosilane and successful enzyme biding on various supports was confirmed by FITC labeling protocol. The dye FITC binds to the amino-functional groups of AMNPs and enzyme co-immobilized AMNPs and observed under CLSM under excitation and emission wavelengths of 488 and 535 nm [21]. From the results, it has been observed that the bare MNPs showed no fluorescence (Fig. 2A) however, AMNPs and enzyme co-immobilized AMNPs showed emission of a clear green fluorescence which visually confirms the successful APTES coating (Fig. 2B) and enzymes loading on AMNPs (Fig. 2C).

One of the main properties of MNPs is being magnetic in nature and it was evaluated using Vibrating sample magnetometry (VSM) analysis. Results indicated that, the magnetization saturation value of bare MNPs was 58.39 emu/g, however after modification of MNPs with aminosilane group and enzyme co-immobilization, the magnetization saturation value decreased to 38.44 emu/g (Fig. 3). This decrease in saturation values of MNPs before and after modification confirmed the successful alteration of MNPs with enzymes and APTES. Grafting of both enzymes and APTES decreased the strength of magnetic field on MNPs which obstructs the arrangement of magnetic power in material hampering the lower saturation values than the native MNPs [27].

3.3. Thermal deactivation studies

The stability of enzymes (before and after co-immobilization) towards temperature was evaluated as rate constant k_d (thermal deactivation) and half life ($t_{1/2}$) [28]. It has been found that the k_d values of both the enzymes viz. pectinase and cellulase before immobilization (free form) is lower than after co-immobilization (Fig. 4A and B). Also, there was an enhancement in half life of co-immobilized enzymes almost by 3 folds compared to their native form. This increase in half life could be due to the formation of covalent bonds between enzymes and AMNPs using glutaraldehyde. This covalent cross-linking was helpful to maintain the active tertiary structure of enzymes in the co-immobilized form hence better thermal stability.

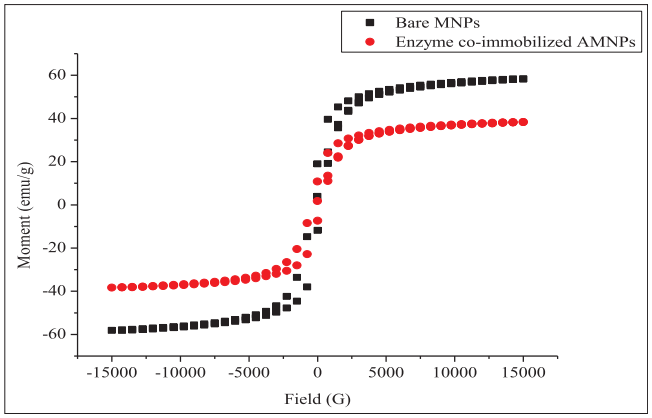

Fig. 3. Vibrating Sample Magnetometry (VSM) analysis of bare MNPs and enzyme co-immobilized AMNPs.

Additionally, the deactivation energy (E_d) was calculated using Arrhenius plot (Fig. 4C and D). Higher the value of deactivation energy higher will be the thermal stability of enzyme molecules [29]. From results it has been found that E_d value of free and co-immobilized pectinase was 15.41 and 46.32 kJ/mol respectively. However, for free and immobilized cellulase the E_d values were 23.84 and 41.08 kJ/mol respectively. This clearly indicated that additional energy was needed to rupture the active substantiation of enzymes after co-immobilization which was formed due to the rigid bond formation using glutaraldehyde.

3.4. Lycopene content of market waste tomato

The waste tomato peels were first evaluated for the lycopene content without any pre-treatment method (using conventional method) and the lycopene content was found to be 96.4 ± 3.3 mg/100 g on dry basis. Many researchers have also reported a wide range of lycopene content in tomato peels which is ranging from 90 to 190 mg/100 g on dry basis. The reported lycopene content values are based on conventional extraction methods. Considering these reported values, the lycopene content of tomato peels selected for this work is in the given range. However, it is very difficult to extract maximum lycopene content from the tomato peel tissue using conventional methods of extraction. The possible reason for this is the presence of lycopene in tomato peels. Lycopene is primarily originated in chromoplasts of the peel, and is deeply rooted inside the membrane structures; this is the region where the synthesis enhances rapidly during the ripening process [1]. Thus, due to complex location of lycopene, solvents used for the extraction process are not able to penetrate inside the tissues, which consist of complex materials along with a variety of polysaccharides (cellulose, pectin, and hemi-cellulose). The extraction efficiency can't be improved even with high amount of solvents due to oxidative degradation of lycopene. Therefore, enzymes were utilized to degrade these polysaccharides for enhanced release of lycopene.

3.5. Optimization of co-immobilized enzyme and ultra-sonication assisted release of lycopene

Tomato peels were pre-treated using only ultra-sonication (5–30 W), only enzyme co-immobilized AMNPs (0–5%) and combination of both ultra-sonication and co-immobilized enzymes. Initially, the ratio of tomato peels powder to co-immobilized enzymes (w/w) was optimized. For this, mixture of tomato peels powder and co-immobilized enzymes were mixed in acetate buffer (pH 5.0, 0.1 M) and kept for shaking for the period of 30 min without sonication. The highest release of lycopene (110.82 mg/100 g) was obtained at the ratio of 3% (w/w) enzyme co-immobilized AMNPs and tomato peels powder (Fig. 5A). The lycopene yield was significantly less at concentration of co-immobilized enzymes below and above 3% ratio. This might be due to the fact that, lesser availability of enzymes to carry out the hydrolytic reaction at

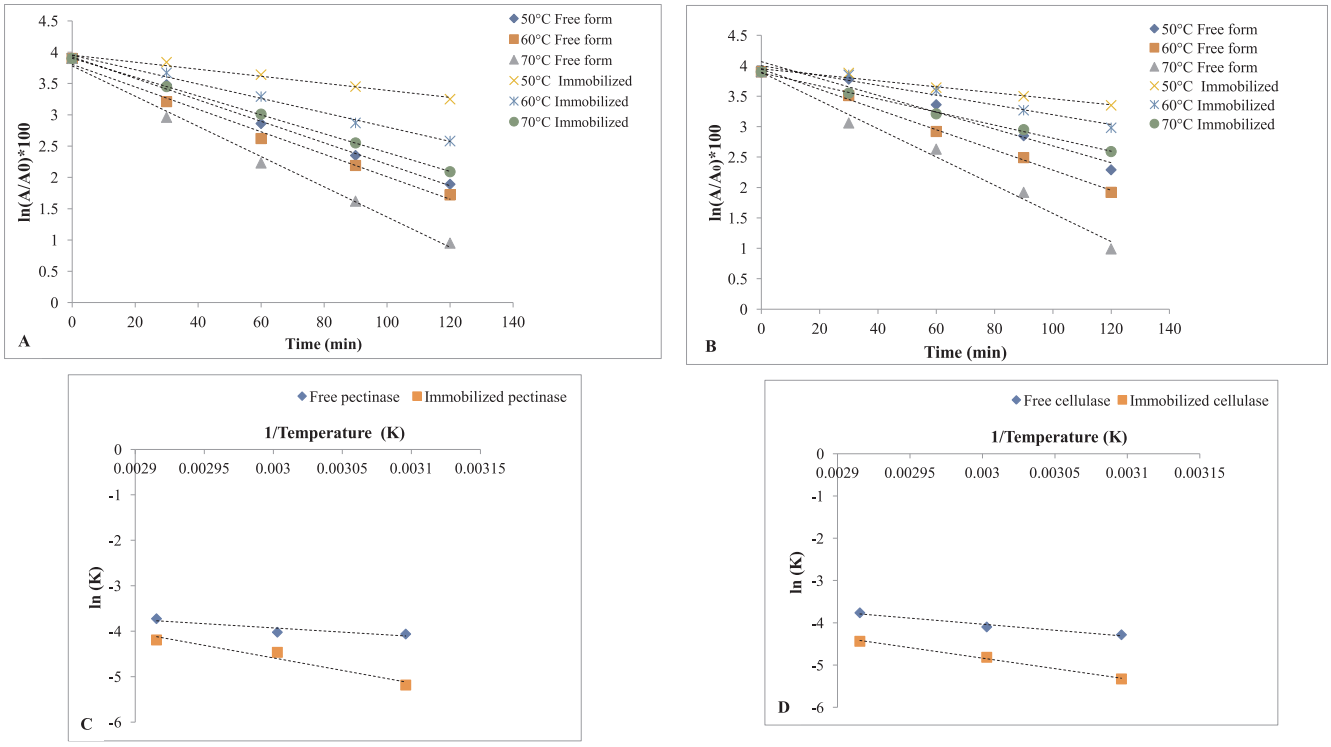

Fig. 4. Thermal inactivation kinetics study of free and immobilized pectinase (A); and free and immobilized cellulase (B); and Arrhenius plot for inactivation of free and pectinase immobilized AMNPs (C); and free and cellulase immobilized AMNPs (D).

lower concentration and when the ratio was high the excessive enzyme leads to the oxidation of the released lycopene by substrate inhibition [30].

Another important parameter during pre-treatment using ultra-sonication is the sonication power. From the results, it was found that at a sonication power of 10 W, maximum lycopene release has occurred (114.98 mg/100 g). Fig. 5B, clearly indicates that below 10 W of sonication power, the lesser extraction could be due to insufficient generation of cavitation activity to release more lycopene from the peels. Above 10 W of ultrasound power, the lesser extraction was might be due to the fact that, at high ultrasound power the high energy waves might denature the enzymes along with degradation of released lycopene in the reaction medium due to the generation of oxidative OH* radicals ([13,19].

The incubation time is another important factor which has to be considered during the enzymatic treatment of tomato peels. It has been observed that, maximum release of lycopene (118.4 mg/100 g) was occurred at 20 min of incubation time (Fig. 5C). Below and beyond 20 min of incubation time resulted in less lycopene recovery. The probable reason for lesser yield might be that, time below 20 min is not sufficient to hydrolyze pectin and cellulosic material hence, poor release of lycopene. Time above 20 min resulted in lesser extraction, which could be due to oxidation of released lycopene to the surrounding atmosphere when exposed to a longer reaction period.

Results indicated that the release of lycopene using only co-immobilized enzyme and sonication both alone was less compared to the synergistic effect. The reason could be that, uses of only co-immobilized enzymes were not sufficient to hydrolyze the cell wall of tomato cells due to mass transfer limitations. Same was the case with sonication as the low power ultrasonic irradiations were not sufficient to release the maximum lycopene content of the tomato peels and difficulty of ultrasonic waves to penetrate beneath the chromoplast structure. However, the synergistic effect of both co-immobilized enzyme and sonication was significantly higher [30]. The reason for this synergistic effect is described in the later discussion part.

3.6. The mechanism of ultrasound and enzyme co-immobilized AMNPs

The synergistic effect of sonication and enzyme co-immobilized AMNPs has resulted into enhanced extraction of lycopene (Fig. 6). The probable mechanism for this could be that during ultrasonication, the generation of ultrasonic waves in the solvent leads to the formation of small vapor filled bubbles. This phenomenon of generation of cavities inside the solvent is known as cavitation. The process of cavitation includes the formation of small gas bubbles called nuclei, their growth and eventual collapse [32]. The dynamics of cavity growth and collapse depend upon the frequency and amplitude of oscillation, type of liquid, existence of nucleating agents (like dissolved gases) and the temperature of liquid. The cavitation nuclei are often observed as gas compartments which are steady in the crevices of boundary walls of liquid, solid surfaces and inside the bubbles. These nuclei grow into cavities when the surrounding pressure of the liquid drops below its vapor pressure and they continue to expand till the surrounding pressure remains low. At the end of expansion phase, the cavities violently collapse and create microscopic shock waves. A huge amount of energy in the form of localized high pressure and temperature pulse is released due to the implosion of the cavities when they reach a size above a critical point. The implosion is asymmetric when it takes place close to a solid surface. After implosion, the solvent rushes to fill the voids (at speed near 400 m/s) generating shock pressures of 1–5 kPa [30]. During the compression cycle, cavitation bubbles collapse with the formation of a microjets which is directed towards the solid surface that is the plant cell matrix. These microjets are responsible for the enhanced release of lycopene from tomato peels. During this cascade, the increase in the localized pressure and temperature opens the cell walls and results in the release of its content into the medium.

Regarding the effect of enzyme co-immobilized AMNPs, in our previous study it has been found that under the influence of ultrasonic irradiation cellulase immobilized MNPs activity was hyperactivated. Using this concept of enzyme hyperactivation using ultrasonication, in the present study, the enzyme co-immobilized (Pectinase and cellulase)

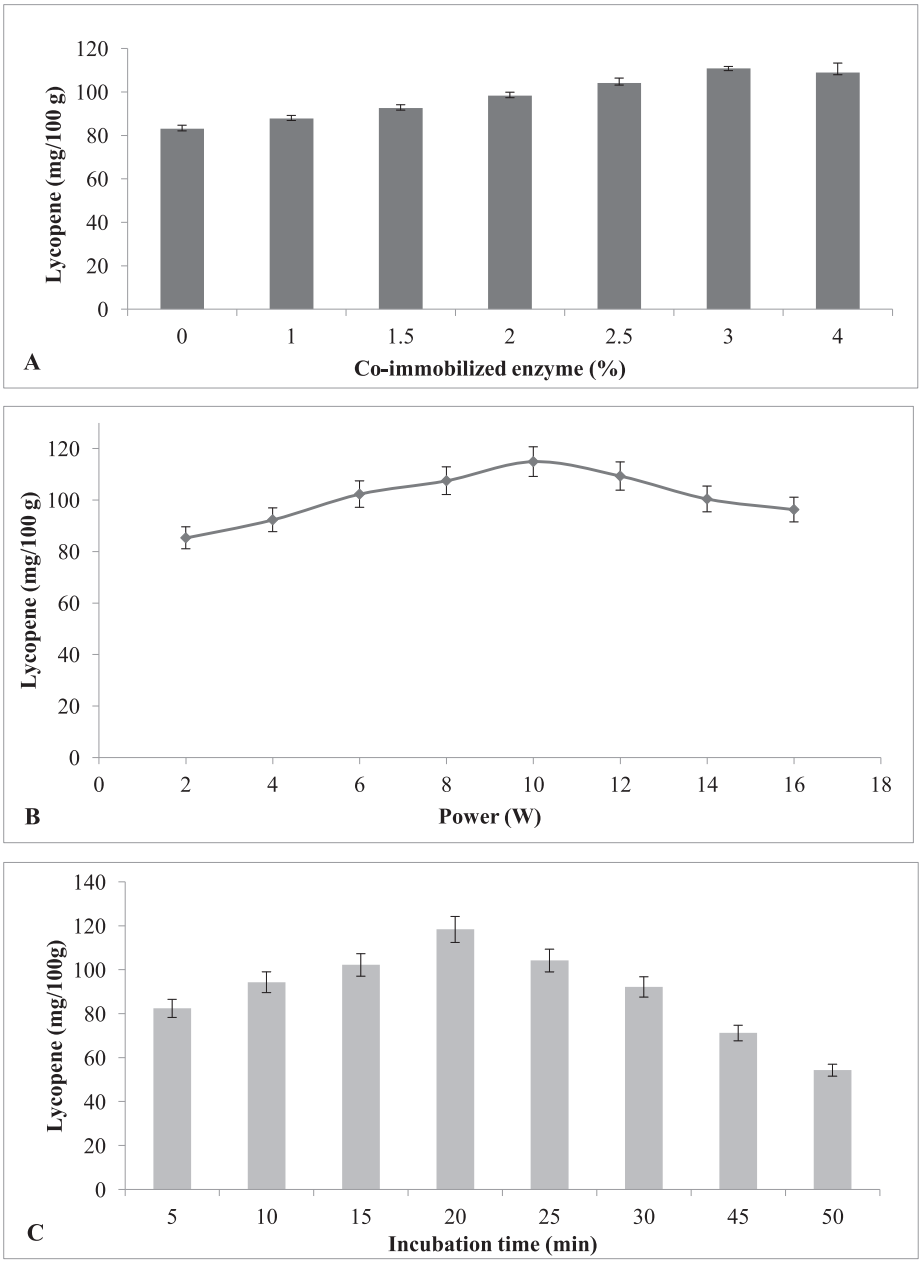

Fig. 5. Effect of enzyme co-immobilized AMNPs concentration (A); sonication power (B) and incubation time (C) on lycopene release. The measurements were performed in triplicate and the error bar represents the percentage error.

AMNPs were also subjected under the influence of ultrasonication. The purpose of using two enzymes is described in the previous part. The rate of enzymatic reaction is dependent upon the diffusivity of the enzyme and that of the substrate. Ultrasonication of enzymatic reaction under specific conditions could provide an efficient mass transfer mechanism for enzyme macromolecule to react with the substrate. Under sonication condition the rapid collapse of cavitation bubbles generates considerable shear forces in the liquid medium. These shear forces provides a mechanical stirring effect which significantly increases the mass transfer to the surface of substrate by disturbing the boundary layer [31]. This decrease in mass transfer limitations along with enzymatic hyperactivity is the main factor responsible for the enhanced release of lycopene into the medium. The optimized sonication parameters for lycopene release were found to be appropriate for increasing extraction of lycopene without disturbing the structural integrity and bioactivity of the extracted lycopene.

3.7. Optimization of processing parameters

The effect of processing parameters viz. pH and temperature on the release of lycopene was studied. From the results, it has been found that maximum lycopene (120.07 mg/100 g) was released at pH of 5.0 (Fig. S4A). The enzymes, cellulase and pectinase have their optimal pH of 5.0 [13]. Results indicated that lycopene release was lower at both sides of optimum pH, which could be due to superior strength of the both enzymes at slightly acidic surroundings than towards more acidic and neutral environment [17].

It was also observed that lycopene release was increased gradually with increase in temperature till 50 °C (122.33 mg/100 g) and decreased gradually with further rise in temperature (Fig. S4B). The decrease in the lycopene content at elevated temperatures could be due to enzymatic denaturation caused by the destruction of strong covalent linkages formed between enzymes and AMNPs ([19,10]. On the other hand, at high temperature conditions the lycopene released gets

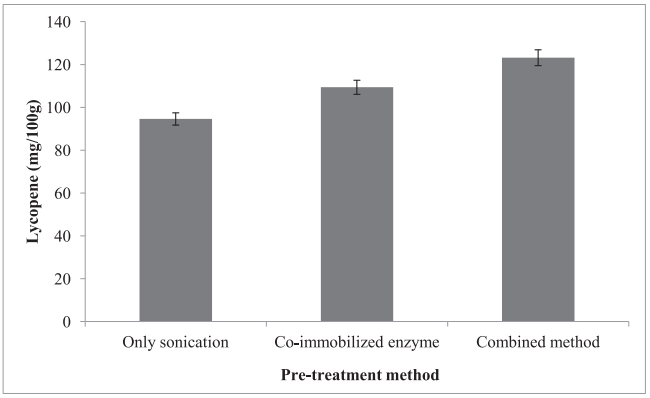

Fig. 6. Comparative study of lycopene release using only sonication, co-immobilized enzyme and combined method. The measurements were performed in triplicate and the error bar represents the percentage error.

oxidized eventually giving lower yield [33].

3.8. Optimization of tri-solvents extraction

After enzymatic pre-treatment, next step is to recover maximum lycopene using tri-solvent extraction technique. For this, the co-immobilized enzyme pre-treated tomato peels were mixed with tri-solvents, namely acetone, ethanol and hexane (1:1:2). This method of extraction using tri-solvents is proved as one of the easy bio-separation methods and was successfully utilized for the separation of various pigments and lipopholic biomolecules from several biological sources. In this method of tri-solvent extraction, development of three phases are observed under optimal conditions, in which the precipitated sugars and proteins are present in the middle layer and extracted pigments and lipids are extracted in upper most organic layer [34]. Various parameters viz. temperature, time and agitation speed were also optimized for higher extraction yield.

For temperature optimization, the co-immobilized pre-treated tomato peels were subjected for different temperature conditions. Results indicated that, maximum recovery of lycopene (111.8 mg/100 g) was obtained at a temperature of 40 °C (Fig. S5A). Temperature plays an important role in penetration of solvent inside the matrix of tomato peels. At temperature, lower and higher than the optimum temperature there was reduced recovery of lycopene. The reason for lower extraction at lower temperature could be due to lesser diffusion and the solubility of solvents. However, at elevated temperature there might be a degradation of the recovered product in spite of higher penetration and solubility [2].

In order to extract more lycopene, the contact between solvents and tomato peels plays an important role. The lycopene recovery was found to be higher with an increase in the extraction time till 90 min (146.6 mg/100 g) and decreased subsequently with subsequent increase in the time (Fig. S5B). The decrease in the lycopene recovery above 90 min could be due to the oxidation and development of transitional products like apo-lycopene and apo-carotenoids of lycopene due to the pro-longed exposure to the surrounding medium [35,36].

During tri-solvent extraction process, proper mixing is required which enhances the product yield. For this, the enzyme pre-treated tomato peels with tri-solvents were subjected for mixing at various speeds. During the reaction, the process of agitation not only facilitates a good contact between solvents and solid particles but also retains special uniformity for mass and heat transfer. At the time of extraction, the solvents moves within the solid to the surface of tomato peel by the process of diffusion. The moment lycopene comes to the surface; it is removed by the convective mass transfer. From the preset results, agitation speed of 150 rpm was found to be optimal for the maximum recovery (172.2*g*/100*g*) (Fig. S5C). However, the recovery was less, below and above the optimum value of the processing time. This probably is due to the fact that, at lower agitation speed, improper mixing of solvents and tomato peels leads to lower recovery. The exact opposite case was observed during the higher levels of agitation. At higher levels of agitation, the formation of vortex, squish effect and geometry effect could have been responsible for the lower yield [37].

3.9. Spectroscopic studies of extracted lycopene

The lycopene extracted from the tomato peels has to be confirmed and this was carried out using FT-IR spectroscopy and the λ_{max} was determined by UV–Vis spectrophotometry. The lycopene extracted in the upper most solvent layer showed three absorbances peaks at 446, 471 and 508 nm (Fig. S6A). This method of lycopene detection using UV–vis spectrophotometer is considered as one of the rapid and easiest methods [9].

Further, from FTIR spectra, appearance of vibrational peak around 864.61 cm^{-1} corresponds to stretching of C—C—H_2 and C—OH groups of lycopene. A characteristic peak at 1516 cm^{-1} and 1377 cm^{-1} validates the deformation vibration of —CH_2 and —CH_3 in lycopene molecule respectively. The asymmetric stretching of C—H groups was occurred by the presence of peaks at 2852, 2920 cm^{-1}. The bending of —CH_2 groups was observed at absorbances of 1101 and 1213 cm^{-1} (Fig. S6B). These results of FTIR spectrum were in accordance with the spectrum of standard and extracted lycopene from tomato products [38]. However, along with FT-IR analysis HPLC was also used for the confirmation of presence of lycopene in the extract.

HPLC is considered as one of the widely used technique for the detection of various compounds. It is known for its precise and accurate results. Hence, for the confirmation of lycopene present in the extract, HPLC method was used. As lycopene gets oxidized very rapidly when exposed to the surrounding environment, therefore it is very important to do the quick analysis for the more accurate results. The lycopene was appeared at 4.2 min which is the retention time of the extracted product and the appearance of spiky peak confirmed the presence of lycopene in the extract (Fig. S6C) [1,39].

3.10. Reusability and storage stability of enzyme co-immobilized AMNPs

Considering the economical value of biocatalysts, reusability is an important factor to study. To determine this, the enzyme co-immobilized AMNPs were subjected to the reusability test after each cycle of its use. The extent of magnetic property was helpful for the easier removal of the prepared bio-catalyst from the reaction mixture. The yield of lycopene for first cycle was considered to be 100%. From the reusability study, it has been found that the prepared enzyme co-immobilized magnetic biocatalyst could be successfully reused up to 6th cycle giving more than 50% lycopene yield at every subsequent trial (Fig. 7A). The decrease in the lycopene yield could be due to excessive reuse of co-immobilized AMNPs which could have resulted in the denaturation of the active sites of enzymes [13,17].

Storage stability is also an important part to study the extent stability of prepared co-immobilized biocatalyst. The co-immobilized enzymes on AMNPs were stored in the buffer solution (acetate buffer pH 5.0, 0.1 M) at room temperature (30 ± 2 °C) for 30 days. The residual activity was measured after every five days and found that both pectinase and cellulase retailed there residual activities of more than 90% even after 30 days (Fig. 7B). This proves that the prepared magnetic biocatalyst is durable and can be preserved at room temperature without slight reduction in the initial activity. The formation of strong covalent bond (between enzymes and AMNPs) during the preparation of this magnetic nano-biocatalyst helps to decrease the deformation effects on active sites of enzymes resulted due to buffering action [40].

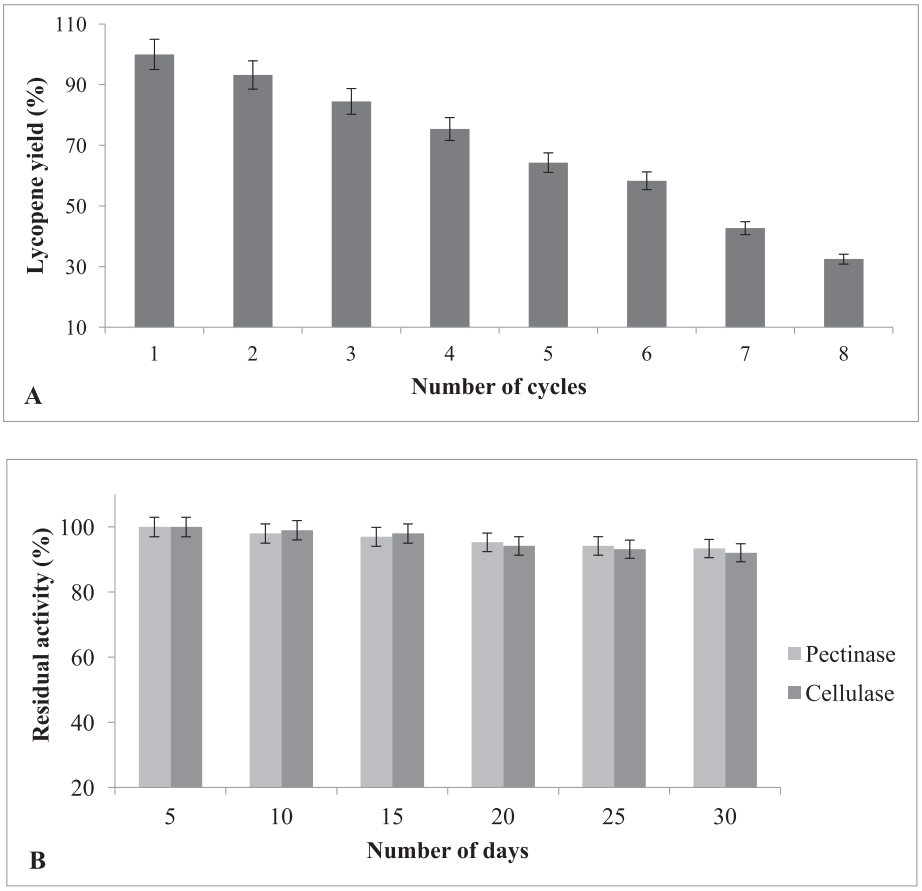

Fig. 7. Reusability of immobilized enzymes upto 8 cycles (A); and storage stability of co-immobilized enzyme on AMNPs (B). The measurements were performed in triplicate and the error bar represents the percentage error.

4. Conclusion

In this study, the synergistic effect of enzyme co-immobilized AMNPs and sonication for enhanced extraction of lycopene were successfully established. Optimization of various parameters further enhances the lycopene release by increasing the catalytic activity at optimized pH and temperature conditions. There was an enhancement in the extraction yield of up to 1.3 folds compared to single process of extraction. In addition, optimization of tri-solvent extraction enhances the overall lycopene yield. The reusability and storage stability study revealed an efficient use of co-immobilized form of enzymes on AMNPs which can be potentially used for industrial applications.

Acknowledgments

Authors would like to gratefully thank Abhay Nutrition Pvt. Ltd. and Prime Minister's Fellowship Scheme for Doctoral Research, a public-private partnership between Science & Engineering Research Board (SERB), Department of Science & Technology (DST), Government of India (GOI) and Confederation of Indian Industry (CII).

Conflict interest

The authors declare no conflict of interest.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.ultsonch.2018.06.013.

References

[1] S. Cuccolini, A. Aldini, L. Visai, M. Daglia, D. Ferrari, Environmentally friendly lycopene purification from tomato peel waste: enzymatic assisted aqueous extraction, J. Agric. Food Chem. 61 (2013) 1646–1651, https://doi.org/10.1021/jf3027815.
[2] R.C. Ranveer, S.N. Patil, A.K. Sahoo, Effect of different parameters on enzyme-assisted extraction of lycopene from tomato processing waste, Food Bioprod. Process. 91 (2013) 370–375, https://doi.org/10.1016/j.fbp.2013.01.006.
[3] R. Lavecchia, A. Zuorro, Improved lycopene extraction from tomato peels using cell-wall degrading enzymes, Eur. Food Res. Technol. 228 (2008) 153–158, https://doi.org/10.1007/s00217-008-0897-8.
[4] A. Zuorro, M. Fidaleo, R. Lavecchia, Enzyme-assisted extraction of lycopene from tomato processing waste, Enzyme Microb. Technol. 49 (2011) 567–573, https://doi.org/10.1016/j.enzmictec.2011.04.020.
[5] E.H. Papaioannou, A.J. Karabelas, Lycopene recovery from tomato peel under mild conditions assisted by enzymatic pre-treatment and non-ionic surfactants, Acta Biochim. Pol. 59 (2012) 71–74.
[6] A.L.S. Eh, S.G. Teoh, Novel modified ultrasonication technique for the extraction of lycopene from tomatoes, Ultrason. Sonochem. 19 (2012) 151–159, https://doi.org/10.1016/j.ultsonch.2011.05.019.
[7] P.B. Subhedar, P.R. Gogate, Enhancing the activity of cellulase enzyme using ultrasonic irradiations, J. Mol. Catal. B Enzym. 101 (2014) 108–114, https://doi.org/10.1016/j.molcatb.2014.01.002.
[8] L.S. Vaughn Katherine, C. Clausen Edgar, W. King Jerry, R. Howard Luke, C.D. Julie, Extraction conditions affecting supercritical fluid extraction (SFE) of lycopene from watermelon, Bioresour. Technol. 99 (2008) 7835–7841, https://doi.org/10.1016/j.biortech.2008.01.082.
[9] S.M. Choudhari, L. Ananthanarayan, Enzyme aided extraction of lycopene from tomato tissues, Food Chem. 102 (2007) 77–81, https://doi.org/10.1016/j.foodchem.2006.04.031.
[10] A. Sahu, P.S. Badhe, R. Adivarekar, M.R. Ladole, A.B. Pandit, Synthesis of glycinamides using protease immobilized magnetic nanoparticles, Biotechnol. Reports. 12 (2016), https://doi.org/10.1016/j.btre.2016.07.002.
[11] X. Ma, D. Wang, M. Yin, J. Lucente, W. Wang, T. Ding, X. Ye, D. Liu, Characteristics of pectinase treated with ultrasound both during and after the immobilization process, Ultrason. Sonochem. 36 (2017) 1–10, https://doi.org/10.1016/j.ultsonch.2016.10.026.
[12] Z. Wang, X. Lin, P. Li, J. Zhang, S. Wang, H. Ma, Effects of low intensity ultrasound

on cellulase pretreatment, Bioresour. Technol. 117 (2012) 222–227, https://doi.org/10.1016/j.biortech.2012.04.015.
[13] M.R. Ladole, J.S. Mevada, A.B. Pandit, Ultrasonic hyperactivation of cellulase immobilized on magnetic nanoparticles, Bioresour. Technol. 239 (2017) 117–126, https://doi.org/10.1016/j.biortech.2017.04.096.
[14] V.S. Oliveira, S. Rodrigues, F.A.N. Fernandes, Effect of high power low frequency ultrasound processing on the stability of lycopene, Ultrason. Sonochem. 27 (2015) 586–591, https://doi.org/10.1016/j.ultsonch.2015.04.016.
[15] Q. Chen, D. Liu, C. Wu, K. Yao, Z. Li, N. Shi, F. Wen, I.D. Gates, Co-immobilization of cellulase and lysozyme on amino-functionalized magnetic nanoparticles: an activity-tunable biocatalyst for extraction of lipids from microalgae, Bioresour. Technol. 263 (2018) 317–324, https://doi.org/10.1016/j.biortech.2018.04.071.
[16] S. Talekar, V. Ghodake, T. Ghotage, P. Rathod, P. Deshmukh, S. Nadar, M. Mulla, M. Ladole, Novel magnetic cross-linked enzyme aggregates (magnetic CLEAs) of alpha amylase, Bioresour. Technol. 123 (2012) 542–547.
[17] S. Talekar, A. Joshi, S. Kambale, S. Jadhav, S. Nadar, M. Ladole, A tri-enzyme magnetic nanobiocatalyst with one pot starch hydrolytic activity, Chem. Eng. J. 325 (2017) 80–90, https://doi.org/10.1016/j.cej.2017.05.054.
[18] M.M. Bradford, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding, Anal. Biochem. 72 (1976) 248–254, https://doi.org/10.1016/0003-2697(76)90527-3.
[19] P.J. Munde, A.B. Muley, M.R. Ladole, A.V. Pawar, M.I. Talib, V.R. Parate, Optimization of pectinase-assisted and tri-solvent-mediated extraction and recovery of lycopene from waste tomato peels, 3, Biotech. 7 (2017), https://doi.org/10.1007/s13205-017-0825-3.
[20] S.S. Nadar, A.B. Muley, M.R. Ladole, P.U. Joshi, Macromolecular cross-linked enzyme aggregates (M-CLEAs) of α-amylase, Int. J. Biol. Macromol. 84 (2016) 69–78, https://doi.org/10.1016/j.ijbiomac.2015.11.082.
[21] A.A. Kadam, J. Jang, D.S. Lee, Supermagnetically Tuned Halloysite Nanotubes Functionalized with Aminosilane for Covalent Laccase Immobilization, ACS Appl. Mater. Inter. (2017), https://doi.org/10.1021/acsami.7b02531.
[22] S. Talekar, A. Pandharbale, M. Ladole, S. Nadar, M. Mulla, K. Japhalekar, K. Pattankude, D. Arage, Carrier free co-immobilization of alpha amylase, glucoamylase and pullulanase as combined cross-linked enzyme aggregates (combi-cleas): a tri-enzyme biocatalyst with one pot starch hydrolytic activity, Bioresour. Technol. 147 (2013) 269–275.
[23] C. Pan, B. Hu, W. Li, Y. Sun, H. Ye, X. Zeng, Novel and efficient method for immobilization and stabilization of ??-d-galactosidase by covalent attachment onto magnetic Fe3O4-chitosan nanoparticles, J. Mol. Catal. B Enzym. 61 (2009) 208–215, https://doi.org/10.1016/j.molcatb.2009.07.003.
[24] A. Panek, O. Pietrow, J. Synowiecki, Characterization of glucoamylase immobilized on magnetic nanoparticles, Starch/Staerke. 64 (2012) 1003–1008, https://doi.org/10.1002/star.201200084.
[25] M.R. Ladole, A.B. Muley, I.D. Patil, M. Talib, V.R. Parate, Immobilization of tropizyme-P on amino-functionalized magnetic nanoparticles for fruit juice clarification, J. Biochem. Technol. 5 (2015) 838–845.
[26] C. Wu, C. Xu, H. Ni, Q. Yang, H. Cai, A. Xiao, Preparation and characterization of tannase immobilized onto carboxyl-functionalized superparamagnetic ferroferric oxide nanoparticles, Bioresour. Technol. 205 (2016) 67–74, https://doi.org/10.1016/j.biortech.2016.01.032.
[27] Y. Wu, Y. Wang, G. Luo, Y. Dai, In situ preparation of magnetic Fe3O4-chitosan nanoparticles for lipase immobilization by cross-linking and oxidation in aqueous solution, Bioresour. Technol. 100 (2009) 3459–3464, https://doi.org/10.1016/j.biortech.2009.02.018.
[28] A.B. Muley, S.A. Chaudhari, R.S. Singhal, Non-covalent conjugation of cutinase from Fusarium sp. ICT SAC1 with pectin for enhanced stability: process minutiae, kinetics, thermodynamics and structural study, Int. J. Biol. Macromol. 102 (2017) 729–740, https://doi.org/10.1016/j.ijbiomac.2017.04.072.
[29] A.B. Muley, S.A. Chaudhari, K.H. Mulchandani, R.S. Singhal, Extraction and characterization of chitosan from prawn shell waste and its conjugation with cutinase for enhanced thermo-stability, Int. J. Biol. Macromol. 111 (2018) 1047–1050, https://doi.org/10.1016/j.ijbiomac.2018.01.115.
[30] R. Konwarh, S. Pramanik, D. Kalita, C.L. Mahanta, N. Karak, Ultrasonication - a complementary “green chemistry” tool to biocatalysis: a laboratory-scale study of lycopene extraction, Ultrason. Sonochem. 19 (2012) 292–299, https://doi.org/10.1016/j.ultsonch.2011.07.010.
[31] N.S. More, P.R. Gogate, Intensified degumming of crude soybean oil using cavitational reactors, J. Food Eng. 218 (2018) 33–43, https://doi.org/10.1016/j.jfoodeng.2017.08.029.
[32] N.S. More, P.R. Gogate, Ultrasound assisted enzymatic degumming of crude soybean oil, Ultrason. Sonochem. 42 (2018) 805–813, https://doi.org/10.1016/j.ultsonch.2017.12.031.
[33] R. Ciriminna, A. Fidalgo, F. Meneguzzo, L.M. Ilharco, M. Pagliaro, Lycopene: emerging production methods and applications of a valued carotenoid, ACS Sustainable Chem. Eng. 4 (2016) 643–650, https://doi.org/10.1021/acssuschemeng.5b01516.
[34] S. Varakumar, K.V. Umesh, R.S. Singhal, Enhanced extraction of oleoresin from ginger (Zingiber officinale) rhizome powder using enzyme-assisted three phase partitioning, Food Chem. 216 (2017) 27–36, https://doi.org/10.1016/j.foodchem.2016.07.180.
[35] S. Xianquan, J. Shi, Y. Kakuda, J. Yueming, Stability of lycopene during food processing and storage, J. Med. Food 8 (2005) 413–422, https://doi.org/10.1089/jmf.2005.8.413.
[36] C. Caris-Veyrat, A. Schmid, M. Carail, V. Böhm, Cleavage products of lycopene produced by in vitro oxidations: characterization and mechanisms of formation, J. Agric. Food Chem. 51 (2003) 7318–7325, https://doi.org/10.1021/jf034735+.
[37] M. Mohamad, M.W. Ali, A. Ripin, Ahmad, Effect of extraction process parameters on the yield of bioactive compounds from the roots of eurycoma longifolia, J. Teknol. (Sciences Eng) 60 (2013) 51–57, https://doi.org/10.11113/jt.v60.1441.
[38] M.M. Kamil, G.F. Mohamed, M.S. Shaheen, Fourier transformer infrared spectroscopy for quality assurance of tomato products, J. Am. Sci. 27 (2011) 253–260.
[39] A.I. Olives Barba, M. Cámara Hurtado, M.C. Sánchez Mata, V. Fernández Ruiz, M. López Sáenz De Tejada, Application of a UV-vis detection-HPLC method for a rapid determination of lycopene and β-carotene in vegetables, Food Chem. 95 (2006) 328–336, https://doi.org/10.1016/j.foodchem.2005.02.028.
[40] M.A. Rahman, U. Culsum, A. Kumar, H. Gao, N. Hu, Immobilization of a novel cold active esterase onto Fe3O4∼cellulose nano-composite enhances catalytic properties, Int. J. Biol. Macromol. 87 (2016) 488–497, https://doi.org/10.1016/j.ijbiomac.2016.03.016.