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RESEARCH ARTICLE



## Enhancing purification of $\alpha$ -amylase by superparamagnetic complex with alginate/chitosan/ $\beta$ -cyclodextrin/TPP

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### ABSTRACT

The main aim of this study was to synthesize the superparamagnetic nanoparticles coated by alginate/chitosan/ $\beta$ -cyclodextrin to purify  $\alpha$ -amylase. Isolated bacteria were identified by morphological, biochemical and taxonomic molecular studies. FTIR- spectrometer, VSM, X-ray instruments and Malvern Zetasizer were used to characterize nanoparticles characteristics. The morphological structures and the elemental composition of the nanoparticles were studied by using FESEM and EDS, respectively. The molecular weight of enzyme was determined using SDS-PAGE, and the enzyme activity detected by zymographic analysis. FTIR studies showed the presence of Fe–O–Fe in the  $\text{Fe}_3\text{O}_4$  and verified the interaction between chitosan,  $\beta$ -cyclodextrin and alginate. The saturation magnetization for superparamagnetic and coated superparamagnetic nanoparticles was indicated 39 and  $1.9 \text{ emu g}^{-1}$ , respectively. The maximum intensity of the XRD peak indicated the presence of the  $\text{Fe}_3\text{O}_4$ . FESEM and EDS analysis showed that the nanoparticles were regular and spherical in shape and corresponded to the Fe and O elements. Enzyme purification by synthesized nanoparticles was achieved  $13.84 \text{ U mg}^{-1}$ ; purification fold of 3.50. The molecular weight of  $\alpha$ -amylase was about 22 kDa. The highest activity of  $\alpha$ -amylase was observed at  $70^\circ\text{C}$ , pH 9.3 and  $\text{Ca}^{2+}$ -independent. As a conclusion, the coated superparamagnetic nanoparticles showed more applications in enzyme purification comparing to the conventional methods.

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### KEYWORDS

Superparamagnetic;  
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## Introduction

Amylases are enzymes that catalyse the hydrolysis of large polysaccharides, for example, starch and glycogen into smaller carbohydrate molecules such as glucose, maltose, maltotriose and dextrin (Chakraborty et al. 2014). They are applied in a wide number of industrial processes such as food, fermentation, paper, detergent, and pharmaceutical modification (Asoodeh et al. 2010; Chakraborty et al. 2011; Sharma and Satyanarayana 2013; Emtenani et al. 2015). Amylases are produced from different sources such as plants, animals and microorganisms (Maity et al. 2011). Microbial amylases sources are preferable compared with those of plants or animals because of the faster growth rate, facile manipulation and easier availability (Chakraborty et al. 2011). *Streptomyces* are the most widely used source of bacteria to produce amylases (Flärdh and Buttner 2009). Amylases produced by *Streptomyces* showed special characteristics, for example, temperature, pH and metals tolerance

(Manivasagan et al. 2015; Afrisham et al. 2016). Bacterial amylases are purified by precipitation, chromatography and liquid–liquid extraction methods (Asoodeh et al. 2010; Fincan et al. 2014). Because of these methods are very expensive, difficult and time-consuming; bio-separation by using magnetic particles embedded in gel forming polymers like alginate and chitosan are now focusing (Tang and Qian 2007). So the main aim of this study was the purification of  $\alpha$ -amylase produced from *Streptomyces lavendulae* strain FSHJ9 by new synthesized superparamagnetic nanospheres coated with alginate/chitosan/ $\beta$ -cyclodextrin/TPP.

## Materials and methods

### Materials

3, 5-Dinitrosalicylic acid (DNS), chitosan (CS),  $\beta$ -cyclodextrin ( $\beta$ -CD) and alginate (ALG) were purchased from Sigma (Sigma-Aldrich, USA). Nutrient broth (NB),

nutrient agar (NA), soluble starch, calcium chloride ( $\text{CaCl}_2$ ), ethylene diamine tetra acetic acid (EDTA), Triton X-100 and sodium-dodecyl sulphate (SDS) were purchased from Merck Chemicals (Darmstadt, Germany). All other applied materials were of analytical grade.

### **Screening of the amylase producer microorganisms**

Soil samples were collected from different part of Kerman, Iran. One gram of each sample was poured in a 50 ml volumetric flask and diluted with sterile solution of 0.9% NaCl up to volume. Then, the samples were filtered by using a filter paper (Whatman No. 1). Thereafter, 200  $\mu\text{l}$  of each sample was transferred to 1% nutrient starch agar (NSA) and casein starch agar (CSA) media containing (g/l) starch, 10;  $\text{KNO}_3$ , 2; NaCl, 2;  $\text{K}_2\text{HPO}_4$ , 2;  $\text{MgSO}_4$ , 0.5;  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.01 and  $\text{CaCO}_3$ , 0.02 using spread plating technique. Samples were incubated at 30 °C until the halo zone of amylase activity was formed around the related colonies. The selected positive colonies were then sub-cultured by streak-plate technique on 1% CSA. Furthermore, the produced halo plates were floated in Iodine solution. The ratio of coloured halo size (HS) around the colonies was then determined and used as an indicator to select suitable isolates (Ramesh et al. 2006; Kafizadeh and Dehdari 2015).

### **Identification of the amylase producer microorganisms**

The biochemical characteristics of the selected isolates were determined according to the Bergey's Manual of Determinative Bacteriology (Roy et al. 2012). In addition, salt tolerance and temperature resistance were also demonstrated after culturing of each isolate in NA basic culture medium supplemented by different concentrations of NaCl (0–10%) followed by incubation at temperature ranges of 50 °–70 °C. DNA extraction of the selected isolates was done by phenol-chloroform extraction methods (Sambrook and Russell 2006). Universal 16S rDNA PCR forward primer of 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and reverse primer of (5'-ACGGCTACCTGTTACGACTT-3') were used (Hasan-Beikdashti et al. 2012). Additionally, the sequence of amplified DNA was determined by automated sequencing (Bioneer, South Korea), and its similarity with GenBank was performed using the Basic Local Alignment Tool (BLAST) software

(Moshfegh et al. 2013). Then the related phylogenetic tree was constructed using MEGA4 software.

### **Enzyme production**

The isolated strains were cultured in casein starch broth (pH 7.2) in 500-ml flasks (containing 100 ml of liquid medium), incubated in the shaker incubator at 30 °C; 160 rpm for two weeks. Then fermented broths were harvested at different time (0, 96, 144, 216 and 288 h) intervals and the bacterial mass was removed by using a centrifuge (6000 rpm; 10 min). Then amylase activity was measured by spectrophotometric device at 540 nm.

### **Enzyme activity determination**

The activity of  $\alpha$ -amylase was determined according to the method of Bernfeld (1995). Briefly, 0.5 ml of each sample was mixed in the reaction mixture containing 0.5 ml of starch (1% w/v) dissolved in 0.1 M phosphate buffer (pH 6.8) (30 °C; 300 rpm; 30 min). Blank was contained all above mentioned materials without samples. The enzyme activities were stopped by adding the DNS solution to the tubes and centrifuged to separate the remaining insoluble starch (6500 rpm; 6 min). One ml of the obtained substance were heated till 95 °C for 15 min, cooled down to room temperature, and then 0.2 ml of the Rachel solution (DNS, 10 g/l; sodium sulphite, 0.5 g/l; sodium hydroxide, 10 g/l; potassium sodium tartarate, 400 g/l) was consequently added to the reaction. The concentration of reducing sugar was obtained by measuring the absorbance of the samples at 540 nm using a Shimadzu spectrophotometer (UV-1800, Japan) based on the standard curve of sugar. One unit of enzyme activity (U) was defined as the amount of enzyme that produced 1  $\mu\text{mol}$  of glucose in 1 min.

### **Protein estimation**

The protein concentration was determined according to the method of Bradford, using bovine serum albumin (BSA) as standard (Bradford 1976).

### **Preparation of superparamagnetic nanoparticles**

Suspensions of  $(\text{NH}_4)_2 \text{Fe} (\text{SO}_4)_2 \cdot 6\text{H}_2\text{O}$  (0.425 g) and  $\text{NH}_4 \text{Fe} (\text{SO}_4)_2 \cdot 12\text{H}_2\text{O}$  (0.762 g) was prepared in 100 ml deionized water (DW) separately. Two suspensions were then mixed together in a 250 ml three-neck flask, stirring in a water bath under nitrogen gas

(400 rpm; 45 °C). Then  $\text{NH}_4$  (1%) was added drop-wise to hydrolyze the whole volume of the reaction with continuous stirring until complete precipitation of the black magnetite was achieved (pH 10). The black magnetite precipitate was separated by using a permanent magnet and then freeze dried.

### **Preparation of superparamagnetic alginate nanospheres (SAN)**

Nanospheres were prepared by addition of 25 ml sodium alginate (2%) to the 25 ml superparamagnetic nanoparticles suspension (2.2%), and completely mixing for 4 h and then 1 ml of 100 mM  $\text{CaCl}_2$  solution drop-wise addition into 30 ml of the mixture under continuous stirring. Superparamagnetic sodium alginate nanospheres were obtained after drying at room temperature under vacuum for 12 h.

### **Preparation of superparamagnetic alginate/chitosan- $\beta$ -cyclodextrin TPP nanospheres (SAC $\beta$ TN)**

Chitosan $\beta$ -cyclodextrin-TPP nanoparticles were synthesized by ionotropic gelation as described in our previous work (Eslaminejad et al. 2016a). Briefly, volumes of the chitosan solution LMV (low molecular weight) and  $\beta$ -cyclodextrin with concentrations of 2 mg/ml and 6–12 mg/ml, respectively, were mixed by dissolving CS in 1% (v/v) glacial acetic acid solution at 3 ml under stirring for 24 h. In addition, the corresponding quantity of the TPP solution (1.5 mg/ml) was added at a final volume of 1 ml.

Superparamagnetic alginate suspension 27 ml was slowly mixed with chitosan/ $\beta$ -cyclodextrin/TPP complex 3 ml. Then 0.5 ml of 100 mM  $\text{CaCl}_2$  solution was added drop-wise into suspension 30 ml. The resultant mixture was filtered through a magnet in order to separate and washed with distilled water. SAC $\beta$ TN nanospheres were obtained after drying at room temperature under vacuum for 12 h.

### **Characterization of the superparamagnetic nanospheres**

FTIR- spectrometer of superparamagnetic nanoparticles, chitosan-  $\beta$ -cyclodextrin TPP, SAN and SAC $\beta$ TN were recorded as KBr discs on Bruker Optics' Rock Solid<sup>TM</sup> design. Vibrating scanning magnetometer (VSM-7300) apparatus (Meghnatis Daghigh Kavir Co., Kashan, Iran) was used to characterize the magnetic characteristic of the samples (Eslaminejad et al.

2016b). The MNPs structure was investigated using X-ray in the range of 5° to 80° (2 $\theta$ ), and the diffraction result was measured at 4000 points. The qualitative and semi-quantitative diffraction measurements were performed in the laboratory using the software X'Pert high score methodology to compare the diffraction pattern measured with reference diffraction patterns (Xpert Philips, Holland) diffractometer (Cu K radiation,  $\lambda = 0.154056$  nm). Field emission scanning electron microscopy (FE-SEM) (SigmaVP, Carl Zeiss, Germany) at voltage of 20 kV was applied to study the morphological structures of samples. Elemental composition of the MNPs was determined by energy disperses spectroscopy (EDS, Map, Oxford Instrument, England). The mean particle size, size distribution and zeta potential of the nanoparticles, were measured by Malvern Zetasizer ZS series and Scattering Particle Size Analyser (Malvern Co., UK).

### **Enzyme purification by ammonium sulphate (AS) precipitation**

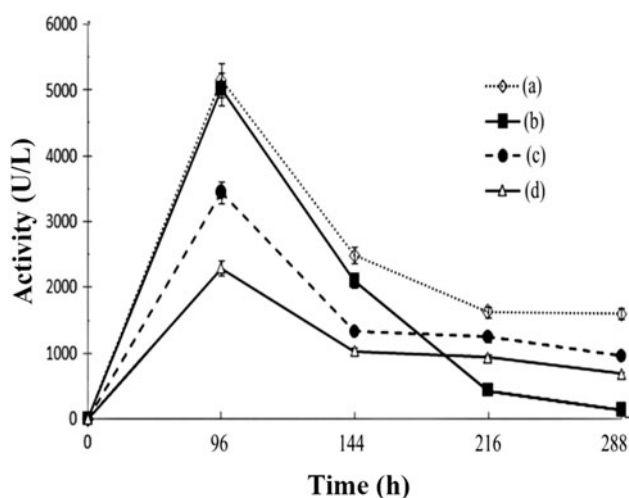
The 72 h culture broth was centrifuged (12,000 rpm; 25 min) and then supernatant was precipitated by gradually increasing the concentration of Ammonium Sulphate to 80% under stirring at 4 °C and left over night. The precipitation was centrifugation at 12,000 rpm for 15 min and dissolved in 0.1 M phosphate buffer (pH 8.0). Then enzyme was dialyzed against same buffer for 48 h.

### **Enzyme purification by SAN and SAC $\beta$ TN**

2 ml of culture medium supernatant containing  $\alpha$ -amylase was loaded on the 3 ml prepared nanospheres 1% which 1 ml of Tris buffer (pH 8.5) was added and incubated overnight. On the next day, nanospheres were separated by using a permanent magnet and then washed three times with 10 ml PBS buffer solution (Sodium phosphate buffer 0.2 M, pH 6.8) each time for 10 min to discard unattached enzyme. Amylase activity was measured as described previously.

To determine the molecular weight of the purified  $\alpha$ -amylase, SDS-PAGE (10%) was performed comparing to the molecular size marker (Sigma-Aldrich, USA) according to Laemmli (Laemmli 1970). After electrophoresis, the gel was stained by Coomassie brilliant blue R-250 and washed by 10 mM phosphate buffer (pH 6.8).

Zymographic was conducted in 10% acrylamide gel containing 1% starch (Lu et al. 2007). All buffers that



**Figure 1.** Time-course studies on amylase production; *Streptomyces lavendulae* strain FSHJ9 (a), *Streptomyces sp* FSHJ42 (b), *Streptomyces griseobrunneus* strain FSHH12 (c), *Streptomyces flavogriseus* strain J12 (d) strains.

were used for native-PAGE was made without SDS. Thereafter, the gel was stained using Iodine reagent solution. The quantitative of bands were analysed by using Image J software.

#### Characterization of the purified enzyme activity and its stability

The effect of different temperature on the enzyme activity and its stability were evaluated by measuring the  $\alpha$ -amylase activity at 4 °C, 25 °C, 30 °C, 50 °C and 70 °C in 0.1 M phosphate buffer (pH 6.9) incubated for 30 min.

The activity of enzyme in pH ranges from 5 to 9.3 at 42 °C was evaluated using three buffers of 50 mM acetate buffer (pH 5), 50 mM phosphate buffer (pH 6.8), and 50 mM Tris buffer (pH 8.5 and 9.3). The stability of enzyme was also examined by incubating the purified  $\alpha$ -amylase at 30 °C for 30 min in the mentioned buffers and measuring of the residual activity. The influences of detergents, for example, SDS, Tween-40 and Triton X-100 and inhibitor like EDTA on the activity of the enzyme were examined by addition of them separately into the reaction mixture, incubated at 30 °C for 30 min, and then measuring of the remaining amylase activity. In each test, the standard was used to compare the results.

The effect of metal ions, including  $\text{CaCl}_2$ ,  $\text{CdCl}_2$ ,  $\text{FeCl}_3$ ,  $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ ,  $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$ ,  $\text{AgNO}_3$ ,  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$  and  $\text{ZnCl}_2$  on amylolytic activity was also determined. About 2 mM of each ion was added to the standard assay mixture and the amylase activity was compared with the blank.

#### Statistical analyses

All mentioned experiments were performed in triplicate and results were presented as mean  $\pm$  SD. Statistical package for social sciences (SPSS) software 15 for windows (SPSS Inc., Chicago) was utilized for statistical analysis. Differences between the groups were determined using one-way analysis of variance (ANOVA) and  $p \leq .05$  were considered as significant.

#### Results and discussion

##### Isolation and identification of the amylase producer microorganisms

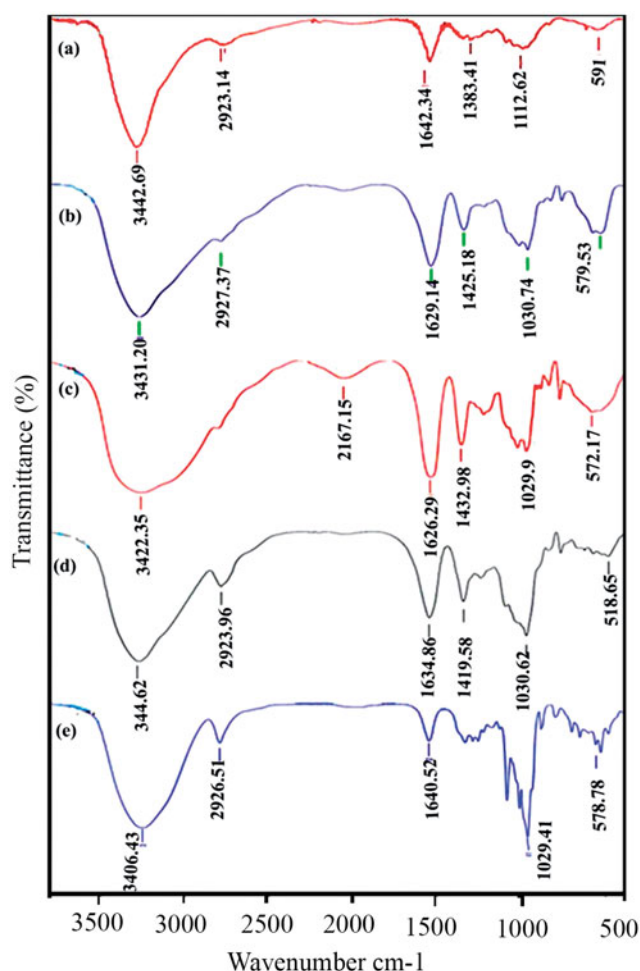
Out of 45 bacterial colonies, four isolates exhibited halo zone of starch hydrolysis on the NA culture plate, indicating the production of the  $\alpha$ -amylase. 16S rDNA gene sequencing observed with 1500 bp (*S. lavendulae* strain FSHJ9, *S. griseobrunneus* strain FSHH12, *Streptomyces sp.* FSHJ42, *S. flavogriseus* strain J12) which was deposited in the GenBank under the accession number of KC626003, KC626002, KC626005 and KP137824, respectively.

The maximum  $\alpha$ -amylase was produced by *S. lavendulae* strain FSHJ9 (5161 U/L) (Figure 1). Therefore this strain was selected for the later experiments.

##### Characterization of the superparamagnetic nanospheres

FTIR-spectrometer of superparamagnetic nanoparticles, chitosan-  $\beta$ -cyclodextrin TPP, SAN and SAC $\beta$ TN were shown in (Figure 2).

The peak of  $1642\text{ cm}^{-1}$  is related to the chitosan as a broad band appearing at  $3442.62\text{ cm}^{-1}$  (Figure 2(a)) (Khoobi et al. 2014). As it can be seen from the spectra a broad absorption band at  $579.53\text{ cm}^{-1}$  was observed in the Fe–O–Fe bond in  $\text{Fe}_3\text{O}_4$  (Figure 2(b)) and the major peak at  $3431.20\text{ cm}^{-1}$  corresponds to O–H stretching vibration. Thus, the peaks of chitosan and  $\beta$ -cyclodextrin after complication with alginate shifted to 1626 and  $1083\text{--}1295.34\text{ cm}^{-1}$  (Figure 2(c)) (Gazori et al. 2009). In addition, the pure alginate spectrum assigned at  $1419.58\text{ cm}^{-1}$  was illustrated in (Figure 2(d)). The broad band at the range of  $1079.64\text{--}1156.83\text{ cm}^{-1}$  is attributed to  $\beta$ -cyclodextrin (Figure 2(e)) (Fan et al. 2013), which are characteristics of C–O and C–O–C bands. The board adsorption band at  $2107.15\text{ cm}^{-1}$  can be recognized to C–H stretching vibration. Consequently, an ionic interaction between the carboxyl groups of alginate and amide groups of the chitosan formed the polyelectrolyte complex.



**Figure 2.** FTIR- spectrometer of coated magnetic nanoparticles; (a)  $\text{Fe}_3\text{O}_4$ , (b) CS, (c) ALG, (d)  $\beta$ -CD, (e) SAC $\beta$ TN.

The VSM graphs of the superparamagnetic nanoparticles and SAC $\beta$ TN were done (Figure 3(a,b)). The saturation magnetization for superparamagnetic nanoparticles and SAC $\beta$ TN was indicated 39 and 1.9 emu  $\text{g}^{-1}$ , respectively. Due to the presence of hysteresis loops these nanoparticles were superparamagnetic. As a result, the saturation magnetization of SAC $\beta$ TN was less than superparamagnetic nanoparticles which was similar to results were obtained by (Zarnegar and Safari 2015).

XRD results of the superparamagnetic nanoparticles were illustrated in (Figure 3(c)). The maximum intensity of the peak was obtained as  $2\theta = 35.9$  according to the result from the peaks; 20.1, 30.1, 35.6, 43.3, 53.5, 57, 63, 74 and 76. The peak list was also compared with 79-0417 and 08-0097 codes, and it was determined to be more similar to the peaks of the peak list of 79-0417 code peaks which indicated the presence of the  $\text{Fe}_3\text{O}_4$ . The crystalline structure of the superparamagnetic nanoparticles was demonstrated using XRD diagram owing to the several

sharp peaks as shown in Figure 3(c) (Zarnegar and Safari 2015).

The positive zeta potential value is attributed to the presence of the  $\beta$ -cyclodextrin/chitosan and superparamagnetic nanoparticles. The average of the particles size  $\beta$ -cyclodextrin/chitosan and superparamagnetic nanoparticles was 337.87 and 463.85 nm which were in agreement with previous findings (Valerio et al. 2013). Zeta potential was 2.87 and 26.81 mV, respectively. The morphological structures of samples were investigated by FESEM was shown in Figure 4. It was indicated that the nanoparticles were regular and spherical in shape. The small spherical shapes were observed to be the superparamagnetic nanoparticles and chitosan particles (Figure 4(a,b)), and the particles size was recorded above 35 nm, respectively. The size of nanospheres was near to 282 nm (Figure 4(c)). Superparamagnetic nanoparticles were also analyzed by an EDS spectrometer, data were showed in Figure 4(d). The observed peaks correspond to the Fe and O elements (the presence of the Au is due to the coating of particles with gold).

### Enzyme purification

Superparamagnetic nanospheres were applied for separation, purification and immobilization of enzyme. For example superparamagnetic carboxymethyl chitosan/sodium alginate nanospheres were used for immobilizing  $\alpha$ -amylase (Jiang et al. 2016).

Results from the activity of the purified enzyme were shown in the Table 1. Purification was achieved 7.59, 6.33 and 13.84 U  $\text{mg}^{-1}$ , which were related to the purification fold of 1.92, 1.60 and 3.50, respectively. Somehow, some of the physical interaction could form between hydroxyl, carboxyl and amino groups of alginate, chitosan and  $\beta$ -cyclodextrin (carriers), and enzyme (substrate). They also have the potential to increase absorption and bio-adhesion of the enzyme. Indeed, alginate and chitosan biopolymers are potential candidates for developing purification of the enzyme, due to their chemical and physical properties (Valerio et al. 2013). Purification of the bacterial amylase by magnetic alginate particles, such as micro-particle and macro-particle have been recently employed (Teotia and Gupta 2002; Safariková et al. 2003). Alginate can be used on macro affinity ligand for starch degrading enzymes (Teotia and Gupta 2002). Smaller-size particles are significant for higher binding capacity. However, to purify  $\alpha$ -amylase, the alginate magnetic micro particles are better compared with alginate magnetic macro-particles. Because of the positively charged chitosan nanoparticles attached to  $\beta$ -cyclodextrin, it may have a higher binding affinity.

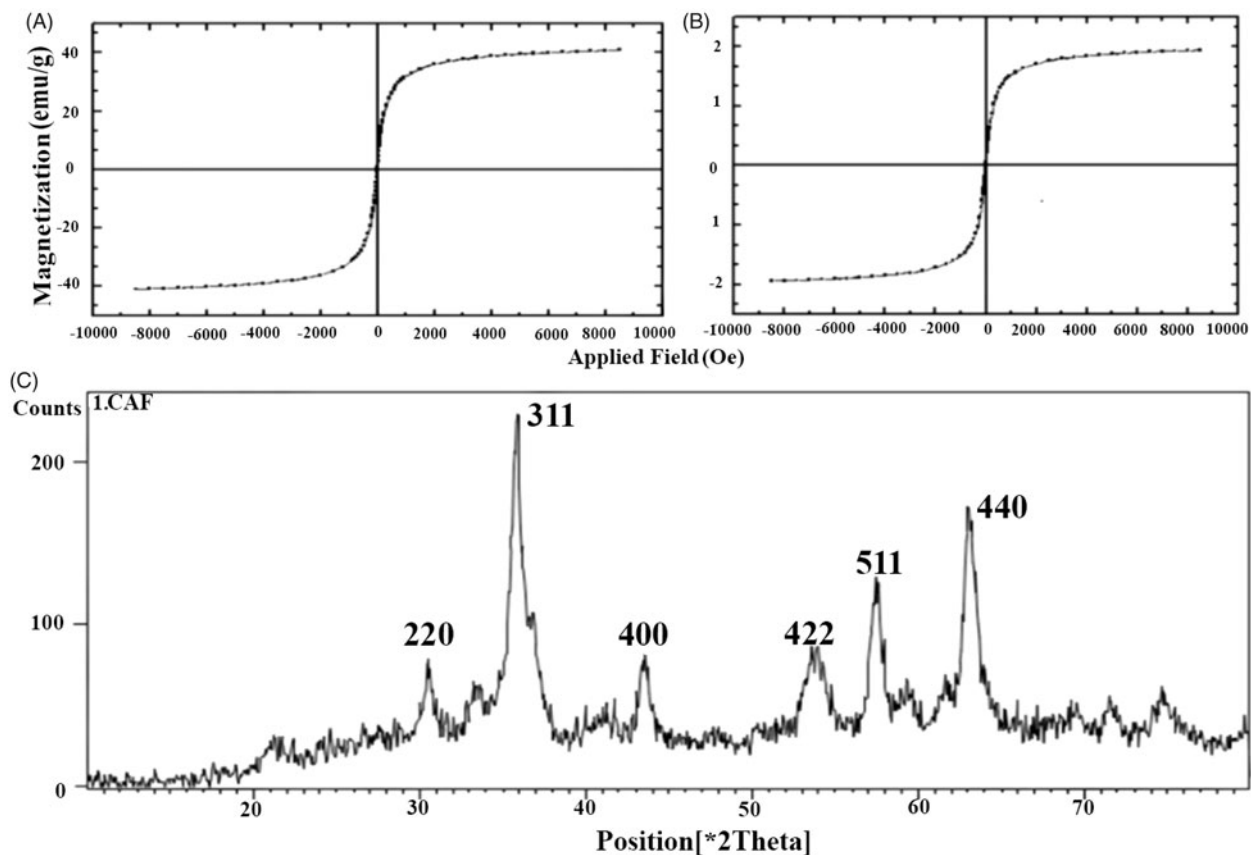


Figure 3. Magnetization curves and XRD pattern of the synthesis nanoparticles; (a)  $\text{Fe}_3\text{O}_4$ , (b) SACBTN, (c) XRD of  $\text{Fe}_3\text{O}_4$ .

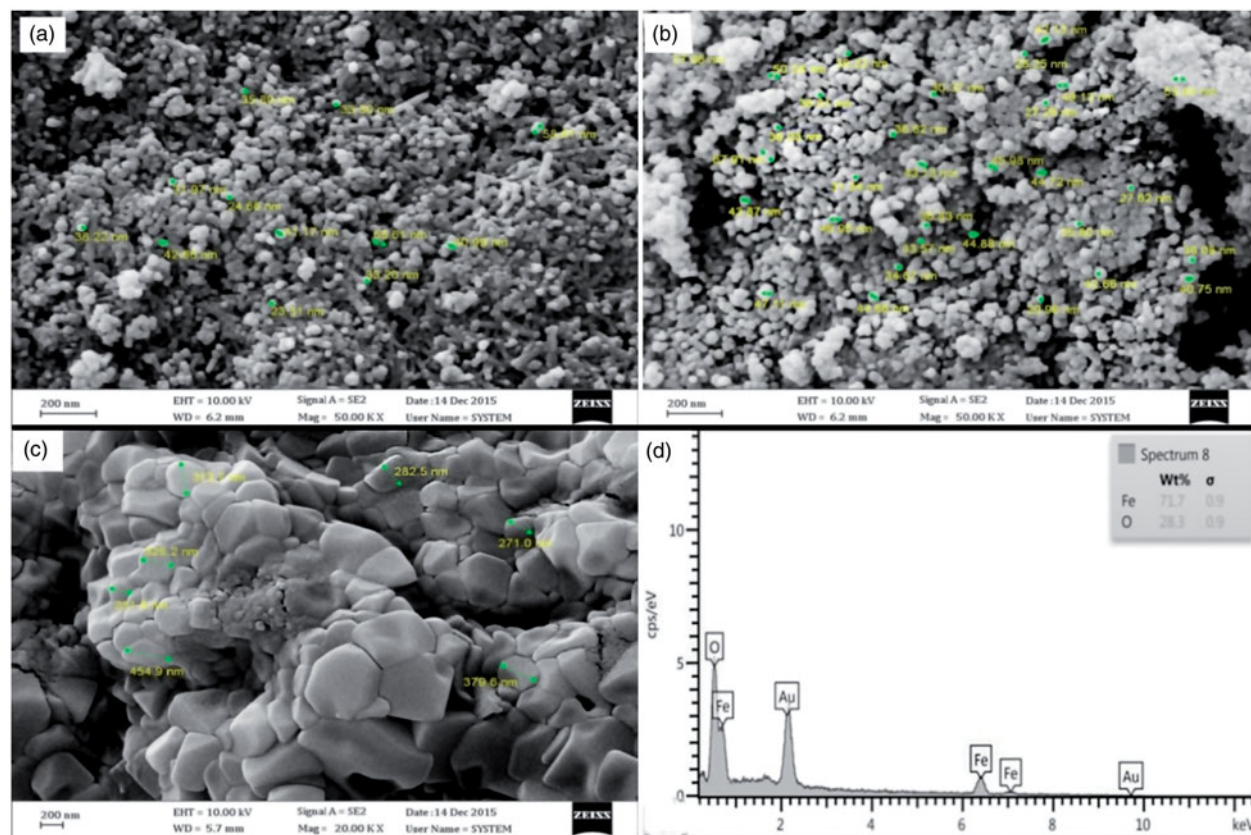
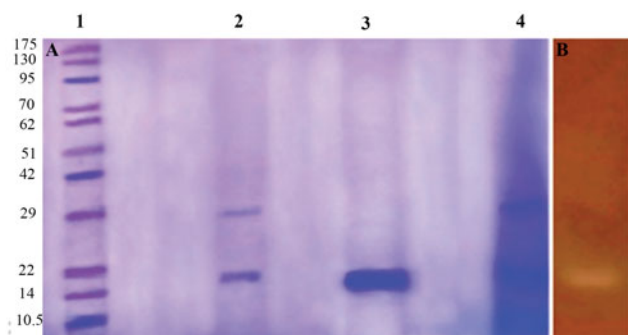


Figure 4. FESEM of synthesis nanoparticles; (a)  $\text{Fe}_3\text{O}_4$ , (b)  $\beta\text{-CD/CS}$ , (c) SACBTN, (d) EDS analysis of  $\text{Fe}_3\text{O}_4$ .

**Table 1.** Comparison of the purification of alpha-amylase by AS, SAN and SACβTN.

| Purification method | Total protein (mg) | Total activity (U) | Specific activity (U mg <sup>-1</sup> ) | Purification (fold) | Yield (%) |
|---------------------|--------------------|--------------------|-----------------------------------------|---------------------|-----------|
| NB                  | 96.9               | 383.3              | 3.9                                     | 1                   | 100.0     |
| AS                  | 48.8               | 165.3              | 7.6                                     | 1.9                 | 49.3      |
| SAN                 | 18.7               | 148.1              | 6.3                                     | 1.6                 | 38.6      |
| SACβTN              | 23.4               | 259.3              | 13.8                                    | 3.5                 | 67.6      |


**Figure 5.** SDS-PAGE and zymography of the  $\alpha$ -amylase enzyme. (A) SDS-PAGE, (1) size marker (kDa), (2) SAN, (3) SACβTN, (4) AS and (B) zymographical image.

For this reason polymers used for bead formation can also be prevented from the aggregation behaviour of the magnetic nanoparticles (Khoobi et al. 2014; Paul et al. 2015). The purification of amylase were also obtained using starch-SPION with the specific activity of 3800 U mg<sup>-1</sup> and yield (%) of 93.22 in 12.27-fold (Paul et al. 2015). Also the micro particles of magnetic alginate beads were utilized for purification of the amylase (Teotia and Gupta 2002). Accordingly, results showed that specific activity was increased from 63.87 to 70 (Safariková et al. 2003). Also, superparamagnetic Fe<sub>3</sub>O<sub>4</sub>@Alginate/Chitosan nanospheres were used to immobilize the lipase that was extracted from *Candida rugosa* (Liu et al. 2012).

### Characterization of the purified $\alpha$ -amylase SDS-PAGE and zymography activity staining

The molecular weight of  $\alpha$ -amylase was estimated to be 22 kDa (Figure 5). Then, the zymography of  $\alpha$ -amylase was obtained in 10% gel. SDS-PAGE and zymography activity staining for different amylases were reported by Asoodeh et al. (2010). The molecular weight of  $\alpha$ -amylases from different microbial resources was reported to be from 22.5 to 184 kDa (Mollania et al. 2010).

The purified  $\alpha$ -amylase by SAN method showed a non-specific band at SDS-Page (29 kDa) in addition to 22 kDa, while SACβTN method showed a specific band of enzyme at 22 kDa. The band at 29 kDa is probably related to other enzymes or proteins in supernatant. According to results reported by (Karbalaei-Heidari et al. 2007), molecular weight of the protease is

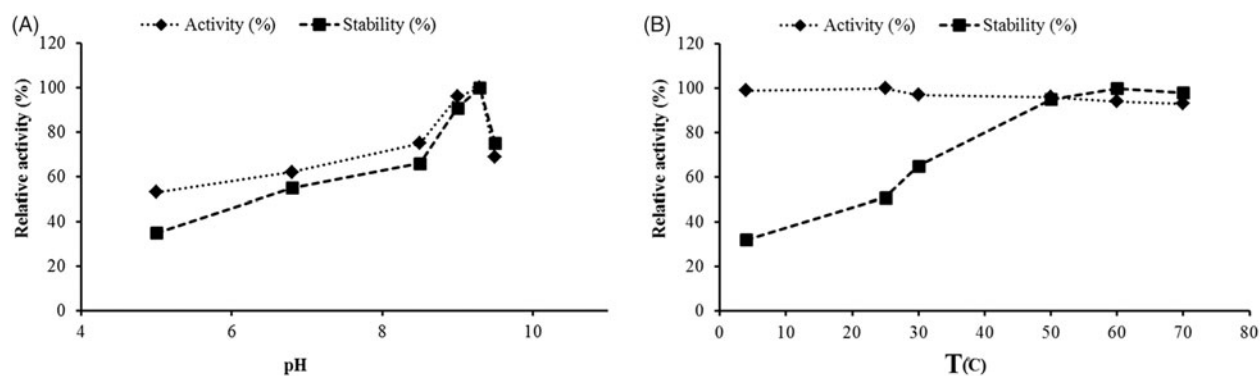
approximately 29 kDa. Result from analyses bands using Image J software indicating intensity of the enzyme bands on the gel that were 152.8, 112.7 and 75.7, for SACβTN, AS and SAN methods, respectively.

### Effect of temperature and pH on enzyme activity and stability

The highest activity was observed at pH 9.3 as reported by other researchers (Fincan et al. 2014). However, there exist some reports which showed maximum activity of the enzyme in various pH ranges of 3.0–9.5 at 42 °C (Asoodeh et al. 2010). The thermal activity of the amylase was evaluated by incubation at 4 °C, 25 °C, 30 °C, 50 °C and 70 °C for 30 min and the results were reported. It was obvious that the highest activity was obtained at 70 °C. It was same as what was previously reported about *B. licheniformis* and *Thalasso bacillus* sp. LY18 (Božić et al. 2011; Li and Yu 2012). The optimum temperature for enzyme stability was examined using incubation at different temperature for 30 min at a pH of 6.9. Results showed that the enzyme was stable at 70 °C (Figure 6).

### Effects of detergent, inhibitors and metal ions on amylase activity

Triton X-100 was able to decrease the activity of starch-degrading enzyme of *S. Lavendulae* strain FSHJ9 to 20% (Table 2), which our results were in contrast with Wang et al. (2011). SDS and EDTA strongly inhibited activity of amylase and also Tween-40 did not effect. The amylase relative activity was achieved by effect of inhibitors such as ZnCl<sub>2</sub> (92%), MgSO<sub>4</sub> (92%), CdCl<sub>2</sub> (80%) and MnCl<sub>2</sub> (82%) on enzyme activity, so they had weak effect and FeCl<sub>3</sub> (40%) was approximately inhibitor an effective. It was activated by CoCl<sub>2</sub> (103%) and AgNO<sub>3</sub> (115%). In this study, AgNO<sub>3</sub> (115%) increased the amylase activity (Table 2). The results of the present study were in accordance with the previously reported (Jana et al. 2013; Fincan et al. 2014). The  $\alpha$ -amylase of *S. Lavendulae* strain FSHJ9 was shown to be Ca<sup>2+</sup>-independent, which was similar to the reports of (Han et al. 2013; Xian et al. 2015).



**Figure 6.** Effect of various pH and temperatures on amylase activity and stability. (A) Effect of pH, (B) effect of temperatures.

**Table 2.** Effect of the detergents, inhibitors and metal ions on the relative activities of the purified  $\alpha$ -amylase.

| Substance                            | Final concentration (mM) | Relative activity (%) |
|--------------------------------------|--------------------------|-----------------------|
| <b>Detergents; inhibitors</b>        |                          |                       |
| Control                              | 0.0                      | 100                   |
| SDS                                  | 5.0                      | 15*                   |
| Tween 40                             | 0.5                      | 100*                  |
| TritonX-100                          | 0.5                      | 20*                   |
| EDTA                                 | 5.0                      | 13*                   |
| <b>Metal ions</b>                    |                          |                       |
| Control                              | 0.0                      | 100                   |
| MgSO <sub>4</sub> ·7H <sub>2</sub> O | 2.0                      | 92*                   |
| CaCl <sub>2</sub>                    | 2.0                      | 100                   |
| ZnCl <sub>2</sub>                    | 2.0                      | 92*                   |
| FeCl <sub>3</sub>                    | 2.0                      | 40*                   |
| CdCl <sub>2</sub>                    | 2.0                      | 80*                   |
| CoCl <sub>2</sub> ·6H <sub>2</sub> O | 2.0                      | 103                   |
| MnCl <sub>2</sub> ·4H <sub>2</sub> O | 2.0                      | 82*                   |
| AgNO <sub>3</sub>                    | 2.0                      | 115*                  |

\*Significant means ( $p$ -value < .05) were obtained after ANOVA analysis with Dunnett's T3 post hoc test.

## Conclusion

The molecular weight of the purified enzyme by SAC $\beta$ TN was 22 kDa, while SAN method could purify the 22 kDa and nonspecific band of 29 kDa. The highest activity was obtained at 70 °C. SDS and EDTA showed a weak effect on the inhibition of the  $\alpha$ -amylase activity and Tween-40 did not have any significant influence on the relative activity. Ag<sup>+</sup> ions increased the amylase activity and it was Ca<sup>2+</sup>-independent. Therefore, according to the achieved results it can be concluded that superparamagnetic alginate/chitosan/ $\beta$ -cyclodextrin/TPP nanospheres (SAC $\beta$ TN) were more useable in enzyme purification.

## Disclosure statement

No potential conflict of interest was reported by the authors.

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