Green Synthesis of Nanoceria (CeO_2) and Evaluation of Enzyme like Characteristics

Azize Alayli Gungor[1,2,*], Hayrunnisa Nadarglu[2,3], Aynur Babagil[2], Hicran Onem[2]

[1] *Ataturk University, Erzurum Vocational Training School, Department of Chemical Technology, 25240 Erzurum, Turkey.*
[2] *Ataturk University, Faculty of Engineering, Department of Nano-Science and Nano-Engineering, 25240 Erzurum, Turkey.*
[3] *Ataturk University, Erzurum Vocational Training School, Department of Food Technology, 25240 Erzurum, Turkey.*

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ABSTRACT

In this study, the synthesis of ceria (CeO_2) nanoparticles (NPs) was examined by the biosynthesis method. Then, enzyme-like features of synthesized nanoceria were examined. Peroxidase enzyme from fig (*Ficus carica*) was used as a synthesis and stabilizer reagent. Furthermore, it was investigated whether the obtained nanoceria has superoxide dismutase (SOD), catalase (CAT) and peroxidase (POX) activities. UV-VIS absorption spectroscopy was employed for monitoring of creation of ceria nanoparticles. The characteristics of the obtained ceria nanoparticles were studied with X-ray diffraction (XRD), scanning electron microscope (SEM) and transmission electron microscope (TEM). Also, cerium oxide NPs showed enzyme-like activities, and its activities were determined with specific enzyme activity measuring methods. Surface morphology and size of the synthesized ceria were investigated by chromatographic techniques. The diameter of the biosynthesized ceria nanoparticles was determined to be 14 nm using XRD chromatogram. Advantages of unique properties of nanoceria have been promising for being enzyme-like reagent in nano-biotechnological investigations. This research explored and discussed if ceria nanomaterial s different kinds of enzymes. We examined their kinetics, mechanisms and applications, including in superoxide dismutase (SOD), catalase (CAT), and peroxidase (POX) activities. The results showed that the ceria oxide nanoparticles exhibited catalase, peroxidase and superoxide dismutase activities.

1-Introduction

Recently, artificial enzymes have been regarded by researchers as extremely stable and low-cost choices in comparison to natural enzymes in a variety of applications. A variety of resources, such as metal complexes and biomolecules, have been discovered for simulating the creation of certain naturally occurring enzymes. A number of nanomaterials are known to create enzyme-like activity, and extensive improvements have been made in this area [1].
Metal nanoparticles have been used as artificial enzymes (nanozymes). The word “nanozymes” was first created by Pasquato et al. to describe

* Corresponding author:
E-mail address: aalayli@atauni.edu.tr

their thiol monolayer threatened gold clusters with outstanding ribonuclease-like action [2]. Wei and Wang adopted the term and also used it to describe nanomaterials with enzyme-like activities. Recently, nanoparticles of cerium, iron, copper, silver and cobalt have also been used as nanozymes [3]. These nanometal particles have shown antimicrobial and antibacterial activities [1-3]. Nanoceria (CeO_2) is used in catalytic agents, abrasives, dyes, solid electrolytes, and sensors. Additionally, ceria is used for catalytic nanozymes because of its unique redox properties. From the perspective of catalysis, ultra-high surface area materials provide the largest number of active area for catalytic reactions.

The most remarkable biological source for the production of nanoparticles is plants. Plants have a noteworthy amount of biomolecules, including carbohydrates and proteins that can reduce metals to nanoparticles in one easy step. Furthermore, this process can be simply carried out at room temperature and pressure without any expensive mechanical tools. Plant parts supply sterols, phenolic complexes, and alkaloids for reducing mediators. Another advantage of using plants for nanoparticle synthesis is it is a green process.

There are also technical and outcome-based advantages that make plants a superior source for nanoparticle synthesis. Experiments can be seen in the researches involving plant material for the synthesis of NPs [4,5]. On the other hand, the established methods for producing nanoparticles from plant materials are easy and environmental friendly [6]. Enzyme-like materials are very important branches of biomimetic chemistry. It is affected by nature and it objects to reflect the vital values of natural enzymes using different materials. For this reason, biomolecules (nucleic acids and proteins), polymers and metal complexes have been widely explored and found to replace the configurations and roles of enzymes [6-10]. Today, most of the experiments employ plant material for silver and gold nanoparticle synthesis. However, there has not been any research about the synthesis of nanoceria [3-4].

Ceria is well known for its highly catalytic performance in various applications due to the presence of mixed valence states (Fig.1) and the existence of oxygen positions.

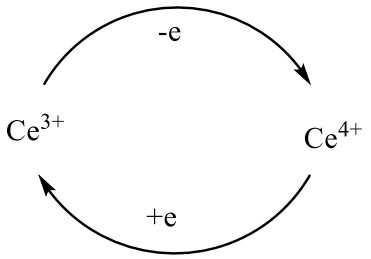

Fig.1. Transformation of cerium ion charges.

The defensive character of nanoceria was recognized due to the removal of radiation-induced free radicals through the regeneration procedure described in Fig. 1. Several studies have established that certain enzymes including peroxidase, super oxide dismutase, and catalase simulate properties of nanoceria and show promising biomedical applications for scavenging radicals [11-13].

Superoxide anion, one of the reactive oxygen species, was known to cause damage in tissues and related inflammation. Nanoceria is known as SOD simulator, which can be used to catalyze the dismutation of superoxide anions into hydrogen peroxide and molecular oxygen [14-17]. Nanoceria-based nanozymes exhibited anti-inflammatory effects due to the presence of mixed valence and oxygen defects, making them highly efficient catalysts. Nanoceria-based SOD mimics have also been investigated as antioxidants that promote stem cell growth. Nanoceria mimics catalase and exhibits neuroprotective activity also (Fig. 2). Nanoceria acts as an efficient antioxidant since it mimics either SOD or catalase activities [14-17].

Proposed reaction mechanisms were expressed by Wang [18]. Catalase mimicking activity of our synthesized cerium NPs was adapted to proposed mechanism Fig. 3.

In this research, nanoceria particles were synthesized using POX enzyme obtained from Fig plant *(Ficus carica)* by means of a new bio-reducing method. At easier environmentally friendly and economical synthesis is aimed with this method. The synthesized nanoceria particles are desired to be resistant to environmental conditions (heat, light and proteases etc.) Then, the characterization of obtained CeO_2 NPs were determined using UV-VIS spectroscopy, X-ray diffraction (XRD), scanning electron microscope (SEM) and transmission electron microscope (TEM).

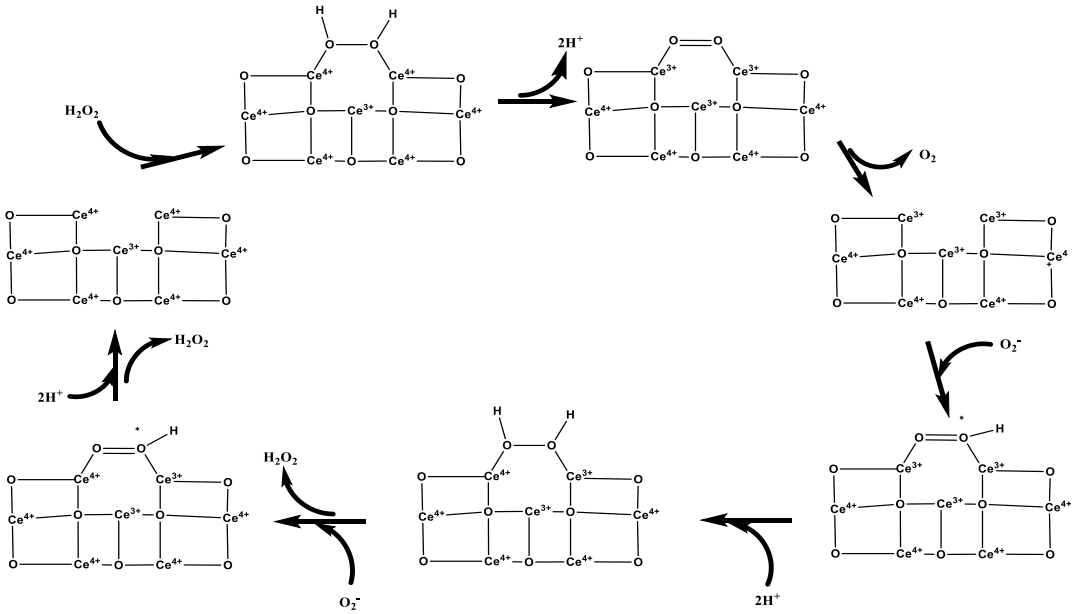

Fig.2. The reaction cycle of nanoceria, mimicking SOD, synthesized with partially purified peroxidase enzyme obtained from Fig plant (Ficus carica) [18-19].

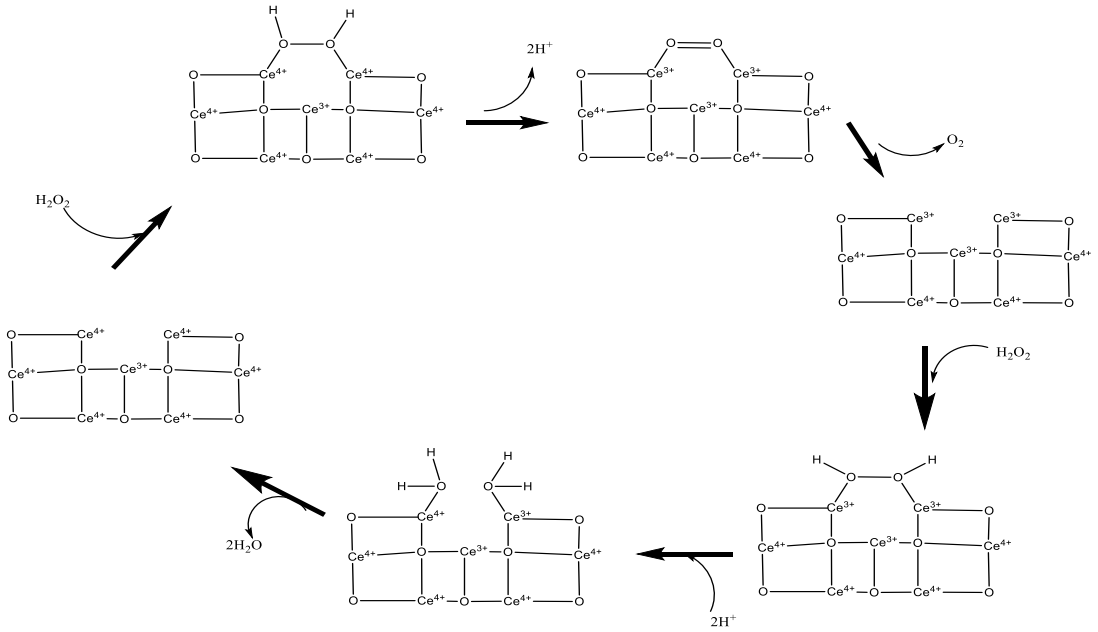

Fig.3. Catalase mechanism for the reaction of nanoceria (Wang (2016))[18-19].

We studied the nanoceria's enzyme mimicking activities by determining peroxidase (POX), catalase (CAT) and superoxide dismutase (SOD) activities. In this research, we tackled whether the nanoceria has enzyme mimicking activities or not, by means of peroxidase (POX), catalase (CAT) and superoxide dismutase (SOD) activities. In case nanoceria shows such an activity, it can be used in many fields, such as biosensor, health, drug development etc..

2-Material and Method

2-1-Materials

Sodium phosphate, 2,2'-Azino-bis (3-etilbenztiyoazol-6-sulfonic acid) (ABTS), Cerium (IV) sulphate $Ce(SO_4)_2$, Sodium acetate ($NaCH_3COO$), Tris ($HOCH_2)_3CNH_2$, Sodium carbonate (Na_2CO_3), Sodium hydroxide (NaOH), Hydrochloric acid (HCl), Potassium phosphate $K_3(PO_4)$, Hydrogen peroxide (H_2O_2), Riboflavine, Methionine, Nitro-blue tetrazolium (NBT), EDTA and Sodium carbonate $Na_2(CO_3)$ were purchased from Sigma-Aldrich GmbH, (Sternhe, I. Germany) and Merck (Kenilworth).

2-2-Extraction of plants extract and partial purification of the peroxidase enzyme

The fig plant *(Ficus carica)* leaves used in the experiment were collected from the city of Sakarya, Turkey and defined by a taxonomist (Ataturk University, Faculty of Science, Department of Biology). Plants were rinsed with distilled water to clean dust and soil on the leaves. Then, these plants were cut into small pieces (100 g), which were then thoroughly shattered to form a homogeneous mixture in blender using 250 mL, 10 mM Na-phosphate buffer solution (pH 6.5). Finally, the mixture was centrifuged at 7.500 $\times g$ for 30 minutes and a supernatant was used for enzyme purification. Fig plant *(Ficus carica)* homogenate which was prepared earlier was saturated from 60 to 80% with solid $(NH_4)_2SO_4$, and the peroxidase enzyme was precipitated by centrifuging at 5.000 $\times g$ for 20 mins. The resulting precipitate was dissolved at 10 mM Na-phosphate buffer solution (pH 6.5) and was incubated at 4 ^oC for further analysis [20-21]. Peroxidase enzyme activity assay was carried out at 412 nm using ABTS as a substrate.

2-3-Synthesis and characterization of ceria (CeO_2) nanoparticles

Enzyme extract (500 μg/L) from fig plant *(Ficus carica)* was added into sample of cerium (IV) sulfate solution $Ce(SO_4)_2$ (10 mL, 10 mM) and was incubated in a closed space for 24 hours. The solution became light yellow, which indicates the presence of cerium nanoparticles. Then, water was removed using an evaporator and synthesized ceria nanoparticles were dried at 60 ^oC for 48 hours [22-23].

Synthesized ceria NPs were characterized using UV-VIS spectrophotometer in a range of 200-1000 nm. Topography determination of ceria NPs was carried out by using SEM. Images of CeO_2 NPs were magnified 5.000 times by using Metek, Apollo prime, Active area 10 mm^2, SE detector R580. Additionally, in order for the determination of ceria nanoparticle sizes; XRD (X-ray diffraction) and TEM (FEI Tecnai G2 Ruh BioTwin, 120 kV) analyses were done.

Contact time, optimum pH, optimum temperature and metal ion concentration were determined in order to optimize synthesized ceria NPs. Maximum synthesis of ceria NPs were measured between 0 and 240 min with 3 min intervals to determine the optimum contact time. Synthesis of Ceria NPs was carried out in Na-phosphate buffer (pH 2.0-3.0), Na-acetate buffer (pH 4.0-6.0), Tris-HCl buffer (pH 7.0-8.0) and Na-carbonate buffer (pH 9.0-11.0), and the absorbance values were measured by UV-VIS spectrophotometer. pH was adjusted to the demanded pHs using 0.1 N HCl and 0.1 N NaOH. Synthesis of Ceria NPs was separately performed at the range of 10 ^oC and 90 ^oC, and the absorbance changes of the samples were measured by UV-VIS spectrophotometer. Synthesis of Ceria NPs were performed by means of Cerium (IV) sulphate solution (1 mM, 5 mM, 7.5 mM and 10 mM), and the sample absorbance were measured by UV-VIS spectrophotometer.

2-4-Determination of peroxidase enzyme (POX), superoxide dismutase (SOD) activity and catalase (CAT) mimic activity

Peroxidase (POX) activity was carried out using guaiacol/H_2O_2 as substrate by spectrophotometrically [24]. The rise in the absorption due to the creation of the oxidized product (tetraguaiacol) was determined to be 470 nm. The reaction mixture contained 5 mM guaiacol and 0.5 mM H_2O_2 in 100 mM phosphate buffer (pH 6.0) and different concentration of ceria NPs (0-200 □g/mL) at room temperature. The measured absorbance was determined for 3 mins using a spectrophotometer (NanoDrop Epoch UV-VIS Spectrophotometer).

Superoxide dismutase activity was measured by nanoparticles recording the decreases in optical density of nitro-blue tetrazolium (NBT) dye

using Ceria NPs. The amount of enzyme necessary to inhibit the decrease of NBT by 50% in optimal conditions was found to be as one unit of SOD activity. The reaction mixture included 2 M riboflavine, 13 mM methionine, 75 mM NBT, 0.1 mM EDTA in 50 mM Na-phosphate buffer (pH 7.8), 50 mM Na-carbonate and different concentrations of Ceria NPs (0-600 mg/mL) samples. Experiments were initiated by adding 60 □L of 100 M riboflavine solution and performed the tubes under 30 W fluorescent lamps for 15 minutes. Then, the reaction was stopped and the changes in absorbance were measured at 560 nm [25]. Catalase activity (CAT) experiment was based on the absorbance changing at 240 nm in the presence of different concentrations of ceria NPs (1-200 mg/mL) using a spectrophotometer (NanoDrop Epoch UV-VIS Spectrophotometer). Change in absorbance was recorded for a while as described by Aebi [26].

3-Results and Discussion

3-1-Synthesis and Characterization of Ceria Nanoparticles

The color of the solution changed from dark yellow to light yellow, which indicates the presence of CeO_2 nanoparticles. Water in the reaction medium was removed by evaporation and synthesized ceria nanoparticles were dried at 65°C for 4 hours (Fig. 4).

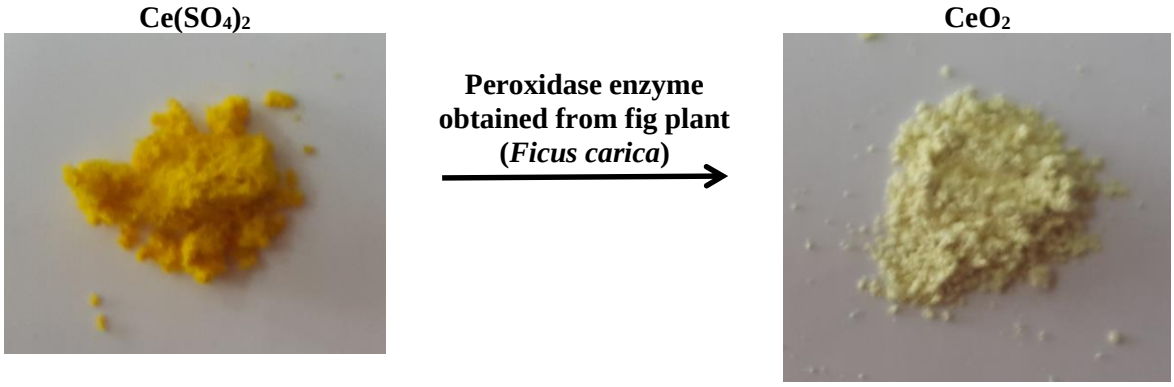

Fig. 4. Synthesis of nanoceria through bio-reducing approaches using peroxidase enzyme obtained from fig (Ficus carica) plant.

The obtained Ceria NPs were characterized by scanning at the range of 200-1.000 nm using a UV-VIS spectrophotometer (NanoDrop Epoch UV-VIS Spectrophotometer). Lovón et al. presented the UV-VIS diffuse reflectance spectra for their samples. In their research, pure CeO_2 showed three characteristic peaks at 226, 305, and 345 nm [27]. Our experiments also showed the same characteristic peaks for enzymatically-synthesized nanoceria. For further experiments, a sharp peak at 345 nm was chosen for recognizing ceria nanoparticles in Fig. 5.

Purity of the synthesized nanoceria was assessed by XRD examination and the result is given in Fig. 6. The nanoceria exposed diffraction peaks (2θ) at around 28.63°, 33.05°, 47.53°, 56.41°, 59.07°, 69.45°, 76.77°, and 79.19° which are recognized to the 111, 200, 220, 311, 222, 400, 331, and 420. These peaks are similar to those presented in the study by Ghomi et al. in 2015, and were found to be similar with the standard card they gave. Ghomi et al indexed their sample as a face centered cubic (fcc) phase using the CeO_2 standard (JCPDS Card No. 43- 1002)[28]. Using Scherrer's formula, the average size of the nanoparticle samples was calculated to be 14 nm from the full-width at half-maximum (FWHM) of 111 reflection as presented in Fig. 6.

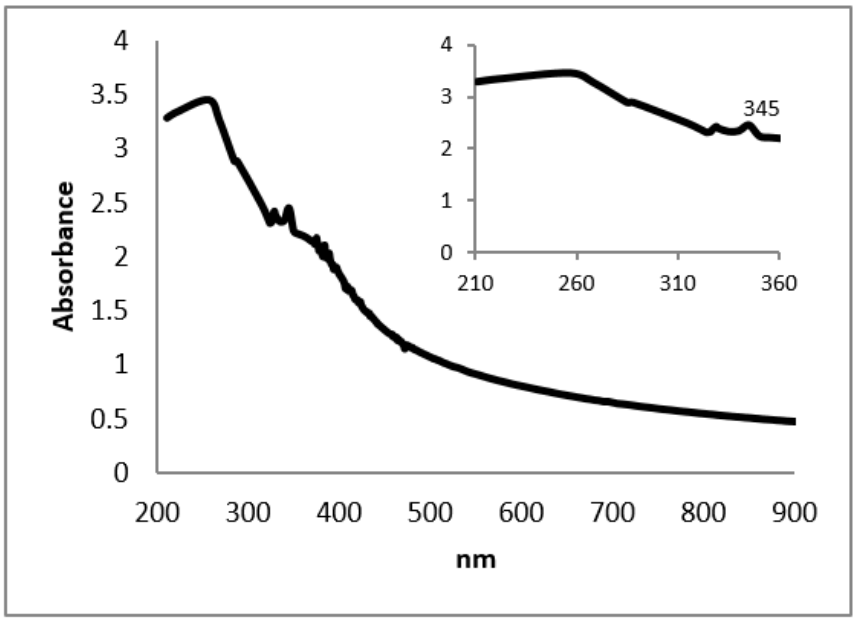

Fig. 5. UV-Vis absorption spectrum of nanoceria synthesized with $Ce(SO_4)_2$ (10 mM) solution and 500 µg/L peroxidase enzyme.

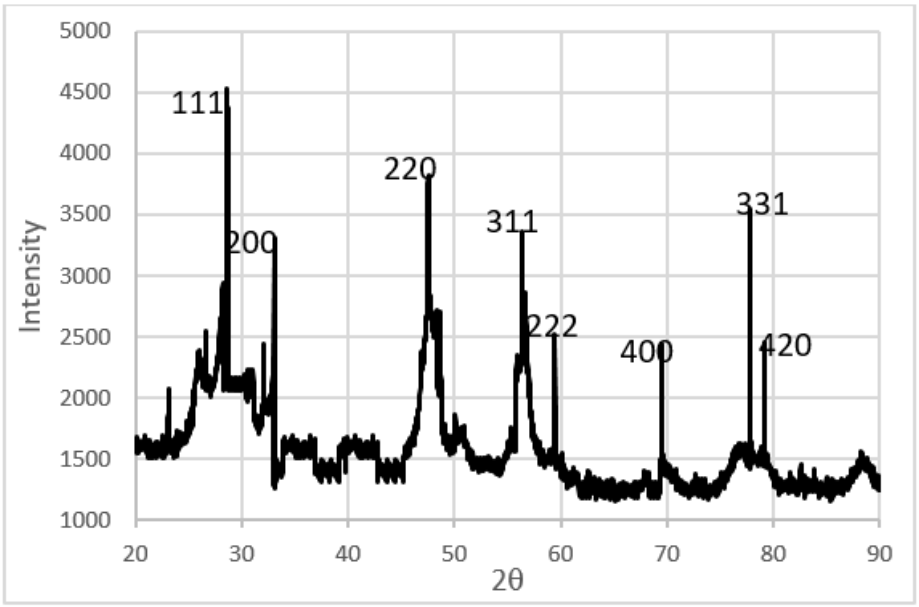

Fig. 6. XRD analysis of nanoceria, synthesized with $Ce(SO_4)_2$ (10 mM) solution and 500 µg/L peroxidase enzyme.

Absorbance changes, determined based on the period of synthesis reaction of the Ceria NPs, were shown in Fig.7. The absorbance values monitored at 345 nm were increased over time. The ceria nanoparticle synthesis reaction had a completion rate of 97.6% after an hour. The results indicate that CeO_2 nanoparticles could be prepared with a green synthesis method, and they did not aggregate. In addition, continuous seven week follow-up showed that NP were in stable position without any decay. UV-VIS spectra of the CeO_2 nanoparticles were given at different pHs in Fig. 8. The effects of different pHs on the synthesis of CeO_2 NPs were investigated from pH 3.0 to 11.0.

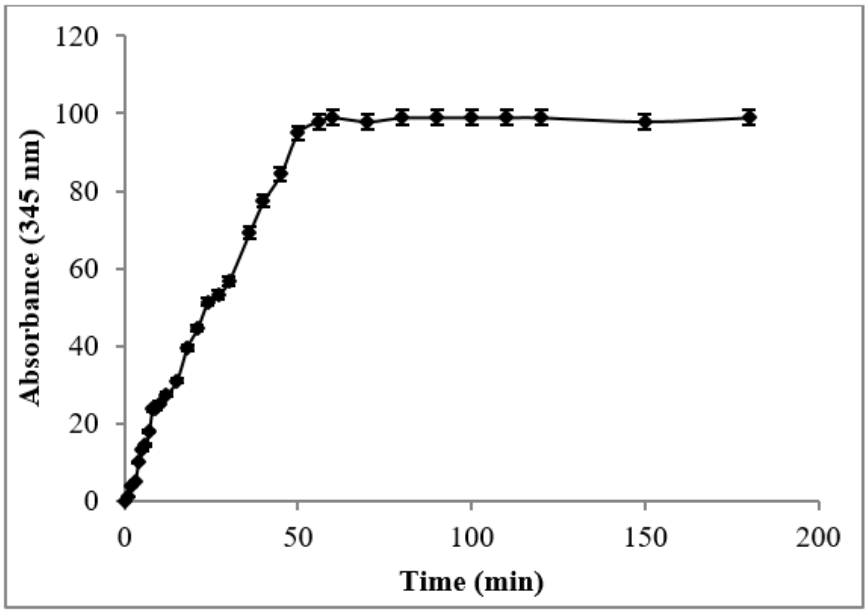

Fig.7. The effect of time on the synthesis of nanoceria with $Ce(SO_4)_2$ (10 mM) solution and 500 µg/L of purified peroxidase enzyme for 3 h.

Suitable buffers, such as Na-phosphate buffer (pH 2.0-3.0), Na-acetate buffer (pH 4.0-6.0), Na-phosphate buffer (pH 7.0–8.0), and carbonate buffer (9.0-11.0) were used. it is seen a high level of synthesis of CeO_2 NPs was observed at pH:7.0-8.0. Considering the findings, it can be concluded that realized in peroxidase enzyme catalysis NPs synthesis, performed at the highest rate in enzyme's optimum pH 7.0. UV-VIS spectra of CeO_2 NPs synthesized at different temperatures are given in Fig. 9. CeO_2 NPs were synthesized with the help of peroxidase enzyme obtained from fig plant (*Ficus carica*) for 1 hour between 10 °C and 90 °C.

The absorbance increased over time at both 20°C and 30°C, with a the maximum absorbance occurring at 20°C, as shown in Fig. 9. The highest rate of CeO_2 nanoparticle synthesis was observed at room temperature (22ºC) by using proteins from peroxidase as a catalyst. Synthesizing nanoceria at room temperature would reduce heating cost and prevent the activity loss of the peroxidase enzyme, but it is denatured at high-temperatures. Structure of the peroxidase enzyme could change at high temperatures and the enzyme activity might decrease. It could provide great advantages in the synthesis nanoceria performed at room temperature instead of spending extra energy. The effects of $Ce(SO_4)_2$ concentrations on the synthesis of CeO_2 NPs were evaluated using purified peroxidase enzyme obtained from fig plant *(Ficus carica)* as indicated in Fig.10. Different concentrations of $Ce(SO_4)_2$ (1, 5, 7.5, and 10 mM) with the same amount of peroxidase enzyme solutions were also used for the synthesis of CeO_2 NPs. It was observed that CeO_2 NPs synthesis increased with the increase of $Ce(SO_4)_2$ concentration over a period of an hour. A 10 mM concentration of $Ce(SO_4)_2$ was appropriate for measuring the absorbance and following the reaction.

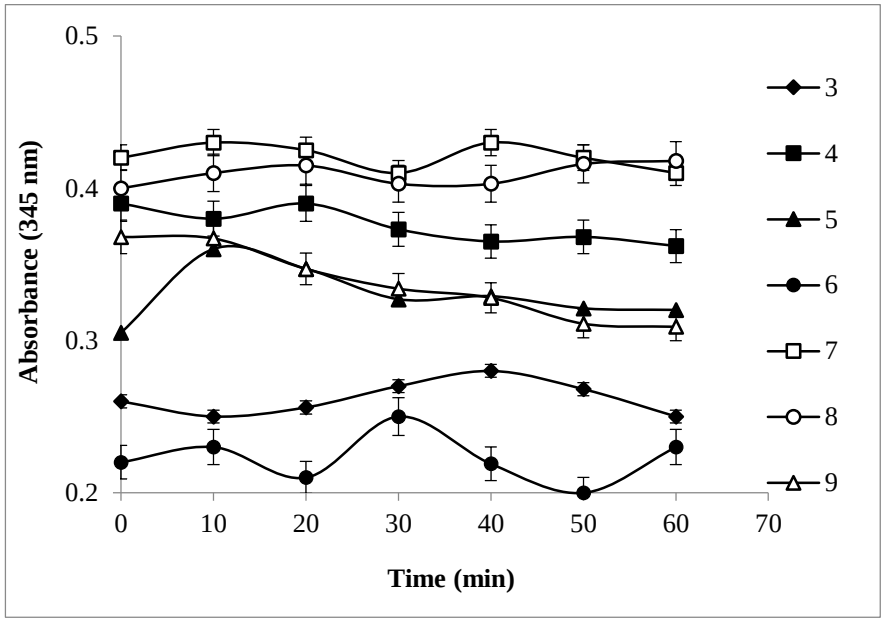

Fig. 8. The effect of pHs on the synthesis of nanoceria with $Ce(SO_4)_2$ (10 mM) solution and 500 µg/L of purified peroxidase enzyme for 1 h.

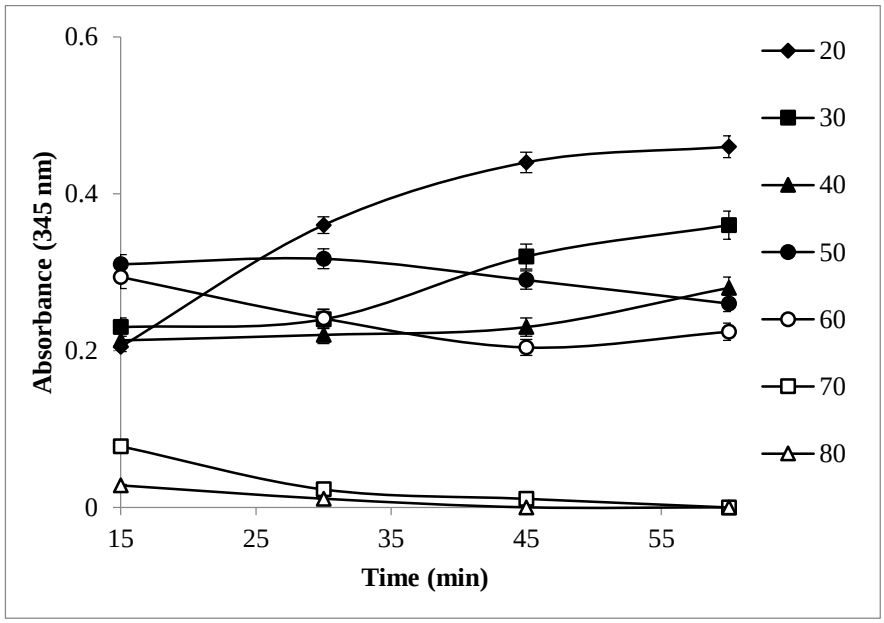

Fig. 9. The effect of temperature on the synthesis of nanoceria with $Ce(SO_4)_2$ (10 mM) solution and 500 µg/L of purified peroxidase enzyme for 1 h.

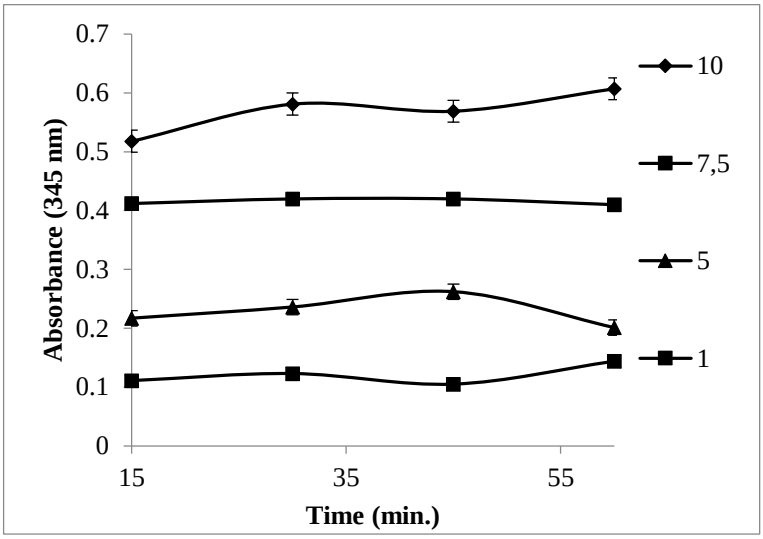

Fig. 10. The effects of $Ce(SO_4)_2$ concentrations on the synthesis of nanoceria with $Ce(SO_4)_2$ (1, 5,7.5 and 10 mM) solution and 500 µg/L of purified peroxidase enzyme for 1h.

The chemical and mineralogical compositions of green synthesized CeO_2 NPs were studied through SEM analysis used to examine the surface. Images of CeO_2 NPs were magnified as given in Fig. 11 using SEM instrument. It was observed that most CeO_2 NPs were in spherical shape. The nanoparticles are homogenous and fluffy with porous morphology. TEM image shows well-dispersed CeO_2 NPs, which range nanoparticles from 10.22–19.15 nm in size in Fig. 12 [29-33].

3-2-Enzyme mimic activities of CeO_2 NPs.

Peroxidase enzyme mimic activity was determined using ABTS as the substrate for CeO_2 NPs. The presence of H_2O_2 produced green colored ABTS radicals which could be measured by UV-absorption spectra or even visible to eye as blue-green color. Then, significant amount of studies were devoted to H_2O_2 detection by exploring peroxidase mimicking activities of various nanomaterial.

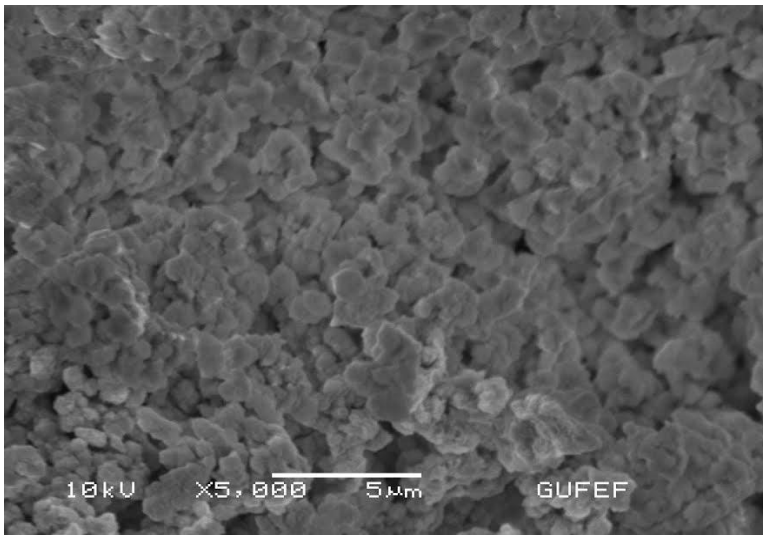

Fig. 11. The SEM photographs of prepared nanoceria.

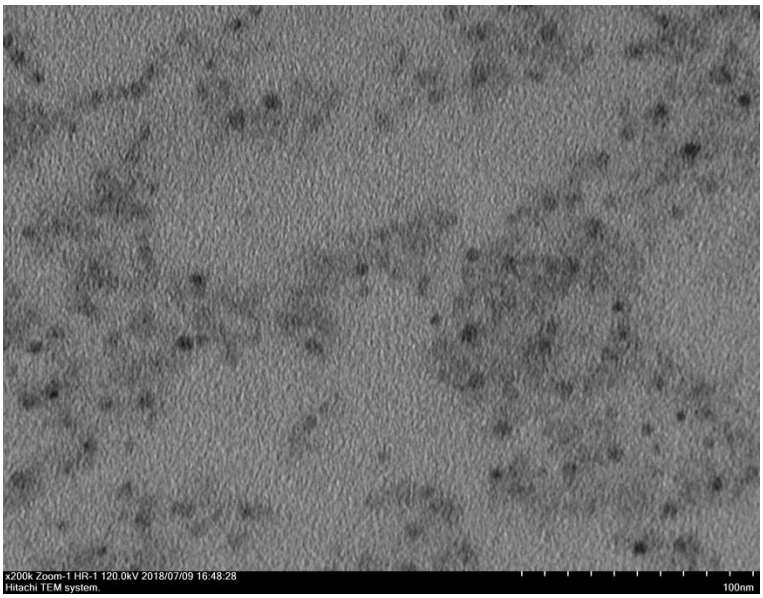

Fig. 12. TEM micrograph of synthesized nanoceria with good size uniformity.

Hui and Wang used Fe_3O_4 magnetic nanoparticles (MNPs) as the peroxidase mimic and ABTS as the substrate for signaling [10]. Also, our researches showed that CeO_2 NPs had maximum peroxidase mimic activity at 100-200 µg/mL ceria NPs concentration (Fig.13).

Our experiments showed that nanoceria exhibited remarkable and promising SOD activity [13, 30, 33]. The SOD mimetic activity of nanoceria was measured by recording the decrease in optical density of nitro-blue tetrazolium (NBT) dye. The data showed that CeO_2 NPs had maximum peroxidase mimic activity at 300-600 µg/mL concentration (Fig.14). Suggested reaction cycle was as expressed by Wang [18]. In our research, CAT trial was to determine the decreasing of absorbance at 240 nm using a UV spectrophotometer. The measurements showed that CeO_2 NPs had maximum peroxidase mimic activity at 100-200 µg/mL concentrations. It was observed that synthesized CeO_2 NPs showed catalase mimetic activity as seen in Fig 15.

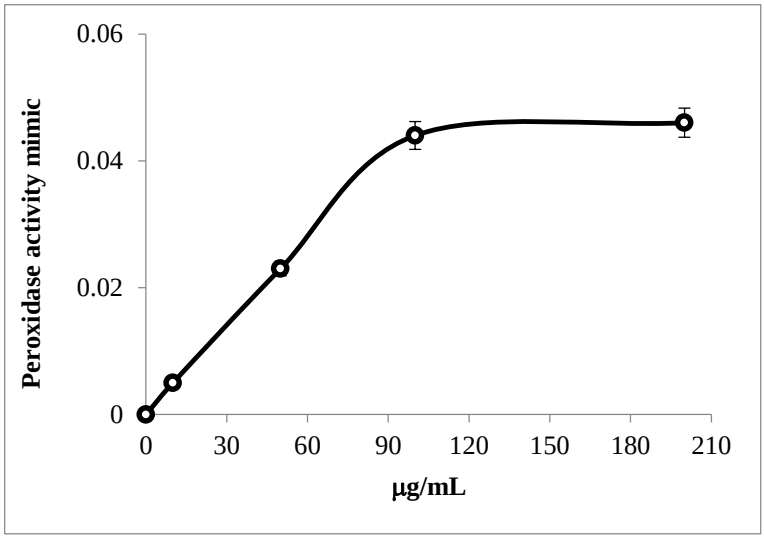

Fig. 13. Peroxidase enzyme activity of nanoceria was measured with ABTS substrate at 412 nm.

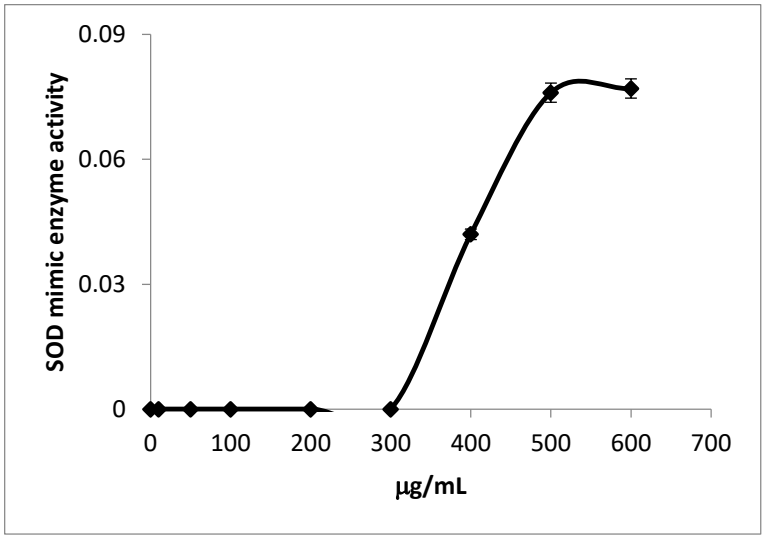

Fig. 14. Superoxide dismutase enzyme activity measurements of nanoceria with NBT dye at 550 nm.

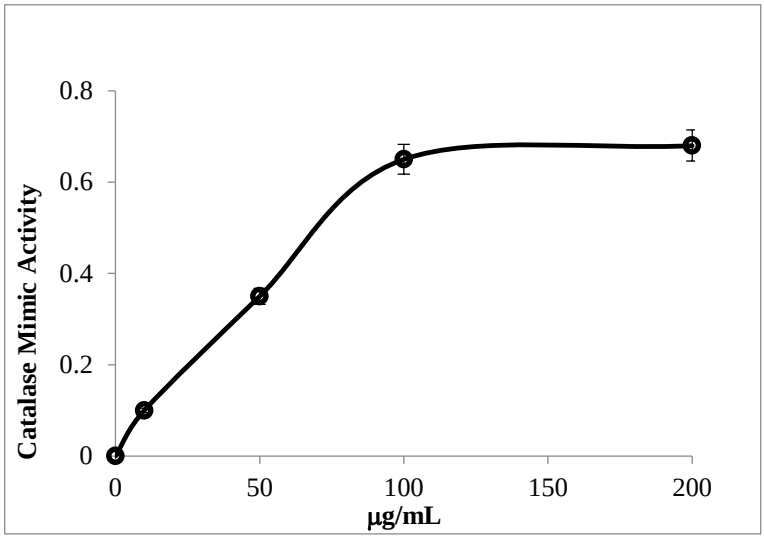

Fig.15. Catalase enzyme activity measurement ofnanoceria with a reaction mixture consisting of H_2O_2 at 240 nm.

4-Conclusions

It has been demonstrated that peroxidase enzyme obtained from fig (*Ficus carica*) is capable of producing Ceria NPs which shows good stability in solution. Success of such a rapid time scale for synthesis of nanoceria is an alternative to chemical synthesis protocols and low cost reductant for synthesizing nanoceria. Obviously, the synthesis of nanoceria in peroxidase from fig (*Ficus carica*) plant has a significant potential and offers a number of substantial advantages compared to traditional methods for nanoparticle synthesis. Our research showed that the reducing power of enzymes could be transferred to stable nano-composite structure and this reducing power was kept in nanoceria structure, then this power was used as enzyme mimic (SOD, POX and CAT). As a result of our experiments, cerium oxide nanoparticles were synthesized using reducing power of the enzyme and the obtained cerium oxide nanoparticles showed very high enzyme-mimic activity. In addition, synthesized cerium oxide nanoparticles showed superoxide

dismutase (SOD), peroxidase (POX) and catalase (CAT) activities. Consequently, the synthesized nanoceria can be used artificial enzymes (nanozymes). Today, (SOD), (POX) and (CAT) are used in many fields including medicine, biosensors, and diagnosis and analysis kits. Enzymes are easily degraded by environmental influences because of their protein structures. Therefore, thanks to their superior properties, the synthesized Ceria NPs can be used instead of POX and SOD enzymes in the aforementioned fields. Moreover, synthesized Ceria NPs may be used for developing biosensors. This antioxidant enzyme activity shown by synthesized nanoceria indicates that the green synthesis is a potential route for synthesis of metal nanoparticles employed in biomedical sector.

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