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Fenton process for Basic Red 9 degradation: immobilized apolaccase on a nanomagnetite system

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Abstract

The Fenton process was used for decolorization of Basic Red 9 (BR9) from industrial wastewater using nanomagnetite ($FeO \times Fe_2O_3$) immobilized on starch and modified with apolaccase. Changes on the starch surface were assessed with a scanning electron microscope, Fourier transform infrared spectroscopy, X-ray diffraction spectroscopy and energy dispersive X-ray spectroscopy. The parameters of the decolorization system, such as pH of the reaction (3 to 9), temperature (20 to 80 °C), contact time (0 to 180 min), initial BR9 concentration (0 to 50 ppm), and adsorbent concentrations (0 to 0.2 g) were investigated to understand their effects on the removal of BR9. The optimized parameters were found as follows: pH 6, temperature 40 to 60 °C, contact time 30 min, initial BR9 concentration 50 ppm, and adsorbent concentration 0.1 g. These results indicate that immobilized nanomagnetite (81.08 % yield) or apolaccase modified immobilized nanomagnetite (87.70 % yield) can be used for removing BR9 in industrial wastewater by the Fenton process. In addition, these experiments showed that the adsorbent was re-useable, cheap, biocompatible, easy to prepare, nontoxic (nanomagnetite, H_2O_2 and starch) and usable for Fenton reactions with and without apolaccase. Therefore, it is concluded that this adsorbent can be used for decolorization of toxic dyes from industrial waste water.

Key words: apolaccase, Basic Red 9, decolorization, Fenton, nanomagnetite, wastewater.
Abbreviations: ABTS, 2.2'azino-bis (3-ethylbenzylthiazoline-sulphanic acid); AOPs, advanced oxidation processes; BR9, Basic Red 9; EDX, energy dispersive X-ray spectroscopy; FTIR, Fourier transform infrared spectroscopy; Laccase, benzenediol: oxygen oxidoreductase, EC 1.10.3.2; SEM, scanning electron microscopy; UV, ultraviolet; XRD, X-ray diffraction spectroscopy.

Introduction

Plastic, tannery, container, nutrition, paint, paper, pulp and fabric industries abundantly use more than 10 000 synthetic dyes (Baskar, Sivarajasekar 2013). Textile dyes alone were produced more than 7×10^5 t annually (Gurtekin, Sekerdag 2008). About 10 to 15% of the used dyes are released as waste at the end of the production process (Guivarch et al. 2003). Dyes in wastewater are very dangerous for biota, health and the environment, and also can cause cancer. Nowadays, the decolorization of dyes from wastewater is an important and attractive technology, since they may have hazardous effects.

In a view of their chemical structures, dyes can be classified as azo dyes, triaryl methane dyes, anthraquinone dyes, heterocyclic dyes and phthalocyanine dyes. Dyes can also be classified according to their application methods, such as container, reactive, straight, acidic, dissolved and cationic dyes (Xu et al. 2004).

Basic Red 9 monohydrochloride is used to produce Solvent Blue 23 and is also a component of magenta dye (C.I. 42510). Magenta must contain at least 50% of C.I. Basic Red 9 in order to be a component of nutrient agar used in biological testing. Basic Red 9 monohydrochloride is also used as a biological dye and as a dye for textile products (silks and acrylics), leather, fur, paper, carbon paper, plastics, glass, waxes, polishes, soaps, cosmetics, drugs, toilet sanitary preparations, automobile antifreeze solutions, anodized aluminum, high-speed photoduplicating inks, photo-imaging systems, and ink-jet computer printers (Martins et al. 2006).

The use of large amounts of dye objects during the dyeing stages of the textile manufacturing process is the cause of environmental contamination (Georgiou et al. 2002). Apart from visual problems related to colored waste, dyes also block the sunlight, which causes reduced photosynthetic activity of aquatic plants. Therefore, they may become extremely hazardous for the whole environment (Kuo 1992). Most of the dyes disposed are non-decomposable in flora and have direct biological treatment of colored effluent, which is not effective in terms of decolorization (Uygur, Kök 1999). Therefore, physical and/or chemical management methods have to be used for decolorization, or to partially degrade the dyes in dye house effluent to make them more cooperative to secondary biological management (Tatli et al. 2003; Banat et al 1996; Kocaer, Alkan 2002; Midik 2011).

On the other hand, methods such as coagulation/

flocculation, activated carbon adsorption and reverse osmosis can only transfer the contaminants from one phase to another, leaving the pattern of color in dye industry effluent (Torrades et al. 2004).

Therefore, much attention has been paid to the development of water treatment techniques that lead to complete destruction of the dye molecules. Because of the high oxidative power of hydroxyl radicals, numerous processes based on this type of technology have been categorized under the name of advanced oxidation processes (AOPs). AOPs have been used for the reduction of harmless/refractory organic, as well as inorganic contaminants found in water or waste water. The most often used AOPs contain heterogeneous photocatalytic oxidation (Hoffman et al. 1995; Ollis 1970; Perez et al. 1997; Arslan, Balcioglu 2001; Lizama et al. 2001; So et al. 2002), ozonation jointly with hydrogen peroxide (H_2O_2), ultraviolet (UV) light, or both, H_2O_2/UV , Fenton and photo-Fenton reagents (Solmaz et al. 2006).

Fenton technology is extensively studied and described as a good method for the management of industrial wastewater with non-biodegradable organic contaminants (Deng et al. 2008).

The heterogeneous Fenton process and other similar processes have received abundant consideration. In heterogeneous Fenton reaction, iron salts are adsorbed on the surface of supported catalysts and in an appropriate aqueous intermediate; the reduction-oxidation reactions between Fe(II) and Fe(III) take place in presence of hydrogen peroxide, which promotes the formation of reactive components such as (•OH) and hydroperoxyl (•OOH) radicals (Daud, Hameed 2009). The radicals produced by the breakdown of hydrogen peroxide can oxidize organic compounds. The area of active iron ions includes both the reagent area and the loose liquid stage. Consequently, the Fe (III)/Fe(II) complexes are made on the support area that can react with H_2O_2 further allowing iron ions to join in the Fenton catalytic cycle (Gürtekin, Şekerdag 2008; Sum et al. 2004; Sun et al. 2006; Neyes, Baeyens 2003).

Laccase is a member of a wide group of enzymes, called polyphenol oxidases, which contain copper atoms in the catalytic area, so-called multi-copper oxidases (Alcalde 2007). Laccase refers to catalysis arising from reduction of oxygen to water accompanied by the oxidation of substrate. Laccases are oxidases that oxidize polyphenols, methoxy-substituted phenols, aromatic diamines and a variety of other compounds (Baldrian 2006). These enzymes are used for pulp delignification, pesticide or insecticide degradation (Riva 2006), organic synthesis, waste detoxification, textile dye transformation, food technological uses, and biosensor and analytical presentations (Zamorani 1989; Mahmood et al. 2007).

Iron atoms immoblized on inert surface, like starch, will increase the speed of Fenton reactions. The complex can be further modified by apolaccase, an enzyme, in which three copper atoms have been removed, resulting in enzyme molecules able to interact with iron atoms in the matrix material. Thus, a complex of laccase and nanomagnetite is formed and apolaccase might regain activity. It was thought that this complex could be prevented by removal from the aqueous medium resulting in immobilization of laccase to the starch surface, making it non-toxic and water-insoluble.

This study investigated the decolorization of the BR9 by heterogeneous Fenton process using immobilized nanomagnetite on starch and modified with apolaccase catalyst. The effects of different initial immobilized nanomagnetite concentration, initial pH, initial contact time, initial temperature and initial BR9 on the decolorization efficiency of the process are studied.

Materials and methods

Chemicals and reagents

DEAE-sephadex, ammonium sulphate, ABTS, Na_2HPO_4, CH_3COONa, dipiclonic acid, BR9 (Fig. 1, Table 1), magnetic nano particles Fe_3O_4 (Iron I, II oxide; De Castro et al. 2001), starch and hydrogen peroxide (30% w/w) were purchased from Sigma-Aldrich. The pH of the solution was adjusted by 0.1 M of HCl or 0.1 M of NaOH. All the chemicals used were analytical grade without any further purification. Distillated water was used for all the tests (GFL 2004).

Preparation of nanomagnetite immobilized on starch surface

For the synthesis of immobilized nano particles, 0.03 M of Fe_3O_4 was prepared by dispersing in 100 mL deionized water. Then, 25 g of starch was added into the reaction mixture. The resultant mixture was mixed for 24 h by using a stirrer (500 rpm, 25 °C). The mixture was centrifuged at 5 000 rpm for 20 min. Then, it was washed three times with distilled water to remove the residual Fe_3O_4. The wet paste was dried in an oven at 40 °C for 72 h. The immobilized surface was grinded before using it in experiments.

Purification of laccase enzyme

Lactarius volemus was collected in April-May from the region of Hasankale in the city of Erzurum, located in eastern

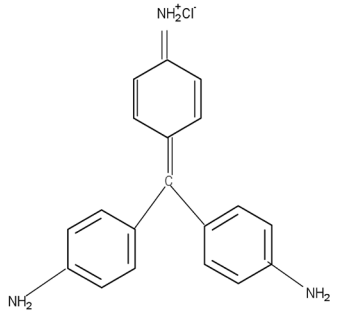

Fig. 1. Chemical structure of Basic Red 9 monochloride (BR9).

Table 1. General characteristics of magnetic nanoparticles Fe_3O_4 and BR9 (Gessner, Mayer 2002)

Chemical formula	Molar mass	Surface area	Particle size	Color index number	λ max
Fe_3O_4	231.53 g mol^{-1}	>60 m^2 g^{-1}	50-100 nm	–	–
$C_{19}H_{18}ClN_3$	323.82 g mol^{-1}	–	–	42500	545 nm

Turkey. It was identified by a botanist and maintained in a deep-freezer at –40 °C. *Lactarius volemus* (10 g) was then powdered in liquid N_2 and it homogenized in a blender with 50 mL of 1 M of KCl, and centrifuged at 5 000 × g for 60 min. The homogenates was centrifuged and precipitates were removed. Then, the enzyme was purified (Nadaroglu, Tasgin 2013) by $(NH_4)_2SO_4$ precipitation, anion exchange chromatography and gel filtration chromatography.

The protein content of chromatographic eluates was measured by a spectrophotometer (Beckman Coulter DU 730 Life Science UV/VIS) at 280 nm by the Bradford method (Bradford 1974).

The reagent ABTS was used as a substrate for spectrophotometric determination of laccase activity. One activity unit (U) was the amount of enzyme that oxidized 1 μmol of ABTS min^{-1} and the activities were expressed in U L^{-1} (Niku-Paavola et al. 1990; He et al. 2003).

Preparation of apolaccase enzyme

An aliquot (100 mg) of laccase was dissolved in 5 mL 0.2 M phosphate buffer (pH 7.0), containing 0.075 M dipiclonic acid. The solution was dialyzed in 1 L of the same buffer in a dialysis tube for 5 h. Then it was dialyzed for 18 h using deionized water, changing the solution 5 to 6 times, and then for dialysis for 5 h against 0.01 M of acetate buffer (pH 5.0), which was used for the measurement of activity. Almost 100% pure apoenzyme was obtained (Kazuko 1970; Demir et al. 1993). Laccase (100 mg) was used to produce apolaccase enzyme, in which, the dipiclonic acid was used as a chelation compound. Estimation of activity indicated almost 100% pure apoenzyme (Demir et al. 1993).

Fe_3O_4-immobilized starch surface characterization

Chemical and mineralogical compositions of Fe_3O_4-immobilized starch samples were determined by scanning electron microscopy. SEM was used to examine the surface of the adsorbent. Images of the nanomagnetite-starch, nanomagnetite-starch-BR9 and nanomagnetite-starch-apolaccase-BR9 were magnified 5000 times by Metek, Apollo prime, active area 10 mm^2, microscope inspect S50, SE detector R580.

Before the examination of SEM, sample surfaces were coated with a thin layer (20 nm) of gold to obtain a conductive surface and to avoid electrostatic charging during examinations. The same machine was also used for the EDX spectra analysis in order to determine the elemental composition of the powdered Fe_3O_4 immobilized starch.

In addition, FTIR analysis was carried out to identify functional groups and molecular structure in the Fe_3O_4 immobilized starch and the modified enzyme. FTIR spectra were recorded with a Mattson 1000 FTIR spectrometer. The spectrum of the adsorbent was measured within the range of 4000 to 400 cm^{-1}.

The XRD pattern of the adsorbent was determined by Rigaku D-Max 2000 and was analyzed with CuKα (λ = 0.154 nm) radiation with 2θ, 5 to 100° (with a step size of 0.1).

Decolorization study

The assay was carried out in 100-mL Erlenmeyer flasks filled with BR9 (50 mg L^{-1}) and 1 g Fe_3O_4 immobilized starch added. The pH was adjusted to the desired value by using 0.1 M of NaOH or 0.1 M of HCl. Reactions were initiated by adding 3% (w/w) of H_2O_2 solution to the flaks. Then, the glasses were placed in a shaker at room temperature and 200 rpm. The samples were taken out from the flaks periodically by using a micropipette. Then the reaction mixtures were centrifuged at 5000 rpm for 10 min. Supernatant was filtered on a 0.45-nm filter. The concentration of BR9 was measured using a Beckman Coulter Du 730 Life Science UV/VIS spectrophotometer at 545 nm.

In all the runs of the study, as described above, other parameters were kept stable and samples were taken at regular times for analysis.

The decolorization efficiency of BR9 was defined as follows:

$$\text{Decolorization efficiency (\%)} = (C_0 - C_t) / C_0,$$

where C_o is the initial concentration of Basic Red 9 C (mg L^{-1}), and C_t is the concentration of Basic Red 9 C (mg L^{-1}), at reaction time, t (min).

The relationship between the amount of dye removed and contact time was determined.

The removal of BR9 in the pH range of 3 to 9 was investigated in order to determine the optimal pH for the process. Concentration of BR9 was measured as above using a spectrophotometer at 545 nm.

Temperature is known to have a profound effect on various chemical processes. The effect of temperature on adsorption was studied in the range of 20 to 80 °C. Reduction of the concentration of BR 9 was measured by using a spectrophotometer at 545 nm.

The decolorization effect of the nanomagnetite-starch and apolaccase that modified nanomagnetite starch was examined using different starch and dye concentrations. The dye measurements were conducted as described above.

Results

The enzyme used was obtained from *Lactarius volemus* collected in April-May from the city of Erzurum in eastern Turkey. The enzyme was purified using a method developed by Nadaroglu and Tasgin (2013), based on $(NH_4)_2SO_4$ precipitation, anion exchange chromatography and gel filtration chromatography (Fig. 2). Gel filtration chromatography showed a good purification efficiency for the enzyme. For each purification step, the protein amount and enzyme activities were calculated. The purification fold was by 146.13 times (Table 2).

To adsorb enzyme, Fe_3O_4 was immobilized on a starch surface. Chemical and mineralogical composition of the immobilized Fe_3O_4 with starch samples were determined. EDX, SEM, and FTIR were used to examine the surface of adsorbent. EDX and SEM spectra were analyzed in order to determine the elemental composition and other features of the powdered Fe_3O_4 immobilized starch, starch-nanomagnetite-BR9 and starch-nanomagnetite-apolaccase-BR9. The elemental composition was also observed after Fenton and enzymatic Fenton reactions. Increased amount of carbon and iron showed that enzyme and nanomagnetite was bonded to the surface of starch matrix (Fig. 3 and Table 3).

SEM images of starch-nanomagnetite, starch-nanomagnetite-BR9 and starch-nanomagnetite-apolaccase-BR9 showed some differences at the surface after Fenton and enzymatic Fenton reactions (Fig. 4). It was clear that starch-nanomagnetite-apolaccase had considerable numbers of pores where dyes could be adsorbed. Before the dye Fenton process, high heterogeneous pores within starch-nanomagnetite-apolaccase particles were observed, which after BR9 dye treatment were packed with dyes.

In addition, FTIR analyses were carried to identify functional groups and molecular structure of starch, nanomagnetite-immobilized starch and enzyme modified

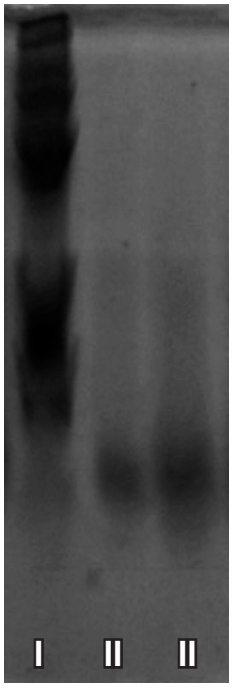

Fig. 2. SDS-PAGE electrophoretic pattern of laccase. I, standard proteins: bovine serum albumin, 66 kDa; egg ovalbumin, 45 kDa; pepsin, 34 kDa; trypsinogen, 29 kDa; carbonic anhydrase. II, purified laccase enzyme from Russulaceae (*Lactarius volemus*).

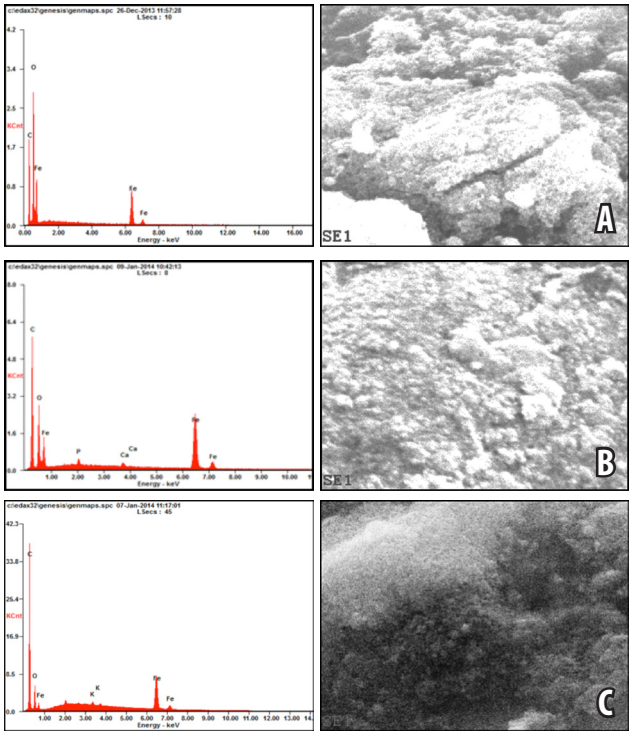

Fig. 3. Energy dispersive X-ray spectroscopy spectra (left) and images (right) of starch-nanomagnetite (A), starch-nanomagnetite-BR9 (B), starch-nanomagnetite-apolaccase-BR9 (C). Most intensive peaks of spectra are indicated by CK, OK, FeK, FeL, Pk, respectively.

Table 2. The purification process of laccase from Russulaceae (*Lactarius volemus*)

Enzyme fraction	Volume (mL)	Activity (EU mL^{-1})	Total activity (EU)	(%)	Protein (mg mL^{-1})	Specific activity (EU mg^{-1})	Purification (fold)
Crude extract	100	121.5 ± 1.31	1.22×10^4	100	195.2 ± 0.32	0.62	–
$(NH_4)_2SO_4$	95	93.2 ± 0.11	8.85×10^3	72.5	45.1 ± 1.25	2.07	3.34
DEAE-Sephadex	40	72.1 ± 1.11	2.88×10^3	32.5	6.1 ± 2.31	11.82	19.10
Sephacryl S 200	30	65.2 ± 0.33	1.96×10^3	68.1	0.72 ± 1.10	90.60	146.13

Table 3. Percentage of most intensive peaks of adsorbents in energy dispersive X-ray spectroscopy spectra

	Starch-nanomagnetite	Starch-nanomagnetite-BR9	Starch-nanomagnetite-apolaccase-BR9
Percentage by atomic (Wt%)			
CK	25.59	36.81	38.83
OK	30.43	16.31	5.10
FeK	43.98	49.42	55.16
Percentage by weight (At%)			
CK	44.20	60.92	70.85
OK	39.46	20.26	6.99
FeK	16.34	14.03	21.69

as nanomagnetite immobilized starch. At 3352 cm^{-1}, a shallow peak belonging to the –OH band of starch structure was identified. The observed peaks at 2928 and 2895 cm^{-1} were due to the symmetric and asymmetric vibration of $–CH_2$ groups. The fingerprint region of the starch molecule occurred in peaks just below 1500 cm^{-1}. The bands located in this section showed binding of water to starch, C–C and C–O vibrations belonging to glycoside molecules. The magnetite nano structure of the starch and apolaccase, when bound to the starch surface structure, showed different patterns in FTIR. In particular, adsorption of BR9 completely changed the structure of the fingerprint region of the starch-nanomagnetite-apolaccase-BR9 structure, indicating absorption of the paint. When nanomagnetite and apolaccase were bound to the starch surface, some changes occurred on the surface (Fig. 5) as described by De Giacomo et al. (2008).

The XRD diffractograms of starch-nanomagnetite, starch-nanomagnetite-BR9 and starch-nanomagnetite-apolaccase-BR9 are shown in Fig. 6. The diffractogram recorded shortly after extrusion predominantly showed a diffuse pattern of a typical starch-nanomagnetite system with a sharp peak centered at around $2\theta < 22.5$. The starch-nanomagnetite-BR9 and starch-nanomagnetite-BR9-apolaccase systems had a sharp peak centered at around ($2\theta < 36$) which was related to adsorption of BR9.

The effect of nano-Fe_3O_4 immobilized on starch was examined in relation to surface material concentration (0.0125 to 0.2 mg). In addition, the ability of this catalyst to decolorize BR 9 was tested in the pH range of 3 to 9, temperature 20 to 80 °C, and initial concentration of Basic Red 9 from (3.25 to 50 ppm; Fig. 7).

It was observed that the removal of BR9 increased during the first 30 min of the contact time. The removal of BR 9 was fast at the beginning, and then gradually decreased to the equilibrium point. These results indicated that the concentration of BR9 in the solution decreased rapidly within 15 min and the removal was virtually completed within 30 min of contact time.

pH of the aqueous solution was found to be an important operational parameter in the decolorization process as it had effected solubility of the dye, concentration of counter ions on the functional groups of adsorbent and degree of ionization of adsorbate during reaction (Amuda et al. 2007). In other words, the uptake and percentage removal of dyes from the aqueous solution were strongly affected by pH of the solution (Benhammou et al. 2005; Ghazy, Ragab 2007; Onundi et al. 2010). Normally, Fenton reaction is resistant to lower pH (Kuo 1992; Lin, Lo 1997; Meriç et al. 2004; Modirshahla et al. 2007); however, in the present study, it was determined that for dye removal the optimum was pH 6. The higher pH stability of Fenton reaction was due to immobilized nanomagnetite and modified apolaccase. Thus dye removal process should operate at neutral pH for optimum elimination of harmful effects (Fig. 8; Nadaroglu et al. 2014). pH affected the adsorption rate by altering molecular interactions and solubility of adsorbate, BR9, enzyme and nanomagnetite. The effect of temperature on the decolorization by Fenton reaction with immobilized nanomagnetite and immobilized nanomagnetite and modified apolaccase are shown in

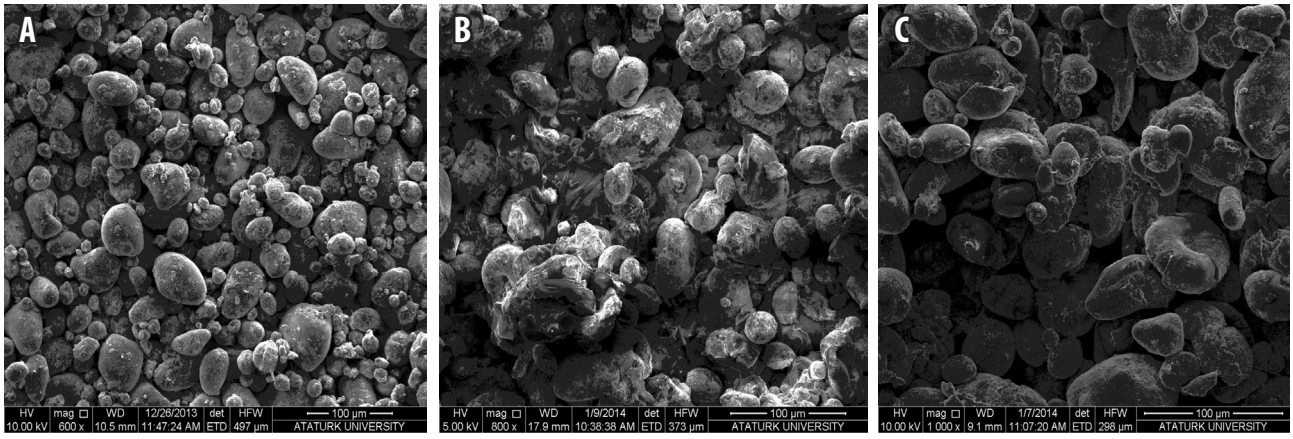

Fig. 4. Scanning electron microscopy images of starch-nanomagnetite (A), starch-nanomagnetite-BR9 (B), starch-nanomagnetite-apolaccase-BR9 (C).

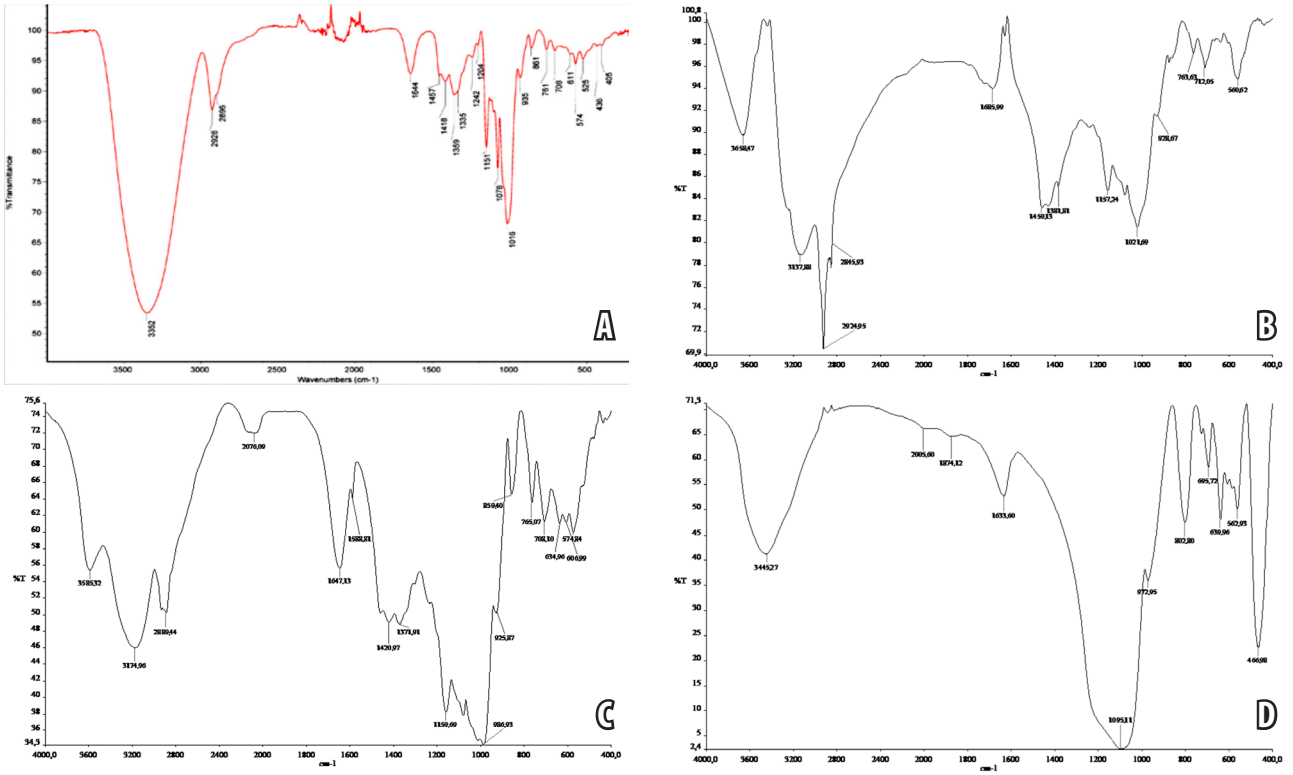

Fig. 5. Fourier transform infrared spectroscopy spectra of starch (A), starch-nanomagnetite (B), starch-nanomagnetite-BR9 (C), starch-nanomagnetite-apolaccase-BR9 (D). Graph A is modified from Kizil et al. 2002.

Fig. 9. It was observed that the degree of decolorization increased with temperature. The maximum decolorization of BR9 was obtained at 40 to 60 °C for the two processes. Effectiveness of the processes at higher temperatures is an advantage for industrial discharge.

The decolorization effects in Fenton reaction without apolaccase and with apolaccase-modified process are shown in Figs. 10 and 11. The removal of BR9 in the Fenton process without apolaccase occurred at a rate of 81% and with apolaccase at a rate of 87%. The rate of decolorization of BR9 increases with concentration of these adsorbents. Decolorization reached equilibrium at 0.1 g adsorbent concentration (Fig. 11).

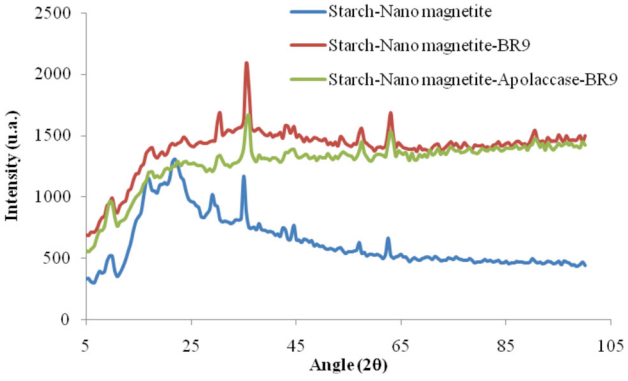

Fig. 6. X-ray diffraction spectroscopy spectra of starch-nanomagnetite, starch-nanomagnetite-BR9, and starch-nanomagnetite-apolaccase-BR9.

Effect of pH on the removal of BR9 with/without apolaccase modified Fe_3O_4 immobilized starch is shown in Fig. 12. It was observed that decolorization was increased with the increasing dye concentration. This optimization of process conditions can be used to ensure low cost and the efficiency of removal of the dyes. Therefore, the optimum time, pH, temperature, dosages reagents were calculated (dye and adsorbent).

Discussion

The enzyme laccase is a commercial enzyme that is widely used for the removal of toxic dyes in different types

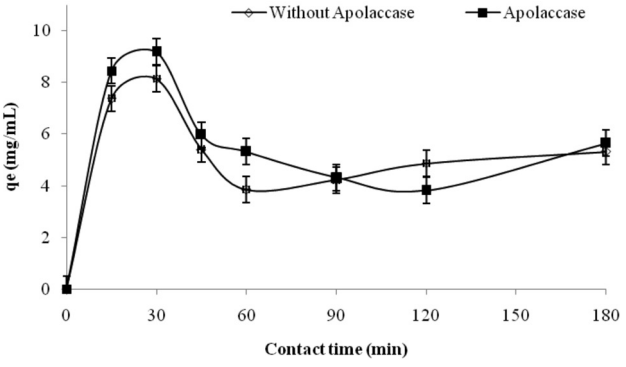

Fig. 7. Effect of contact time on the removal of BR9 by nano-Fe_3O_4-immobilized and apolaccase-modified starch.

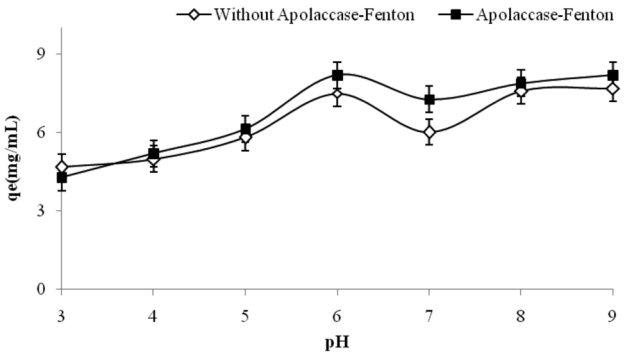

Fig. 8. Effect of pH on the removal of BR9 by nano-Fe_3O_4-immobilized and apolaccase-modified starch.

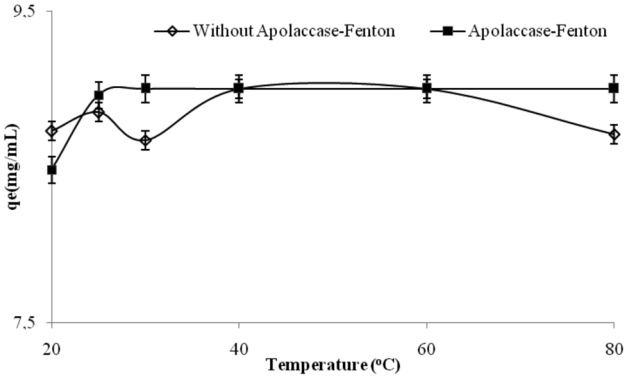

Fig. 9. Effect of temperature on the removal of BR9 by nano-Fe_3O_4-immobilized and apolaccase-modified starch.

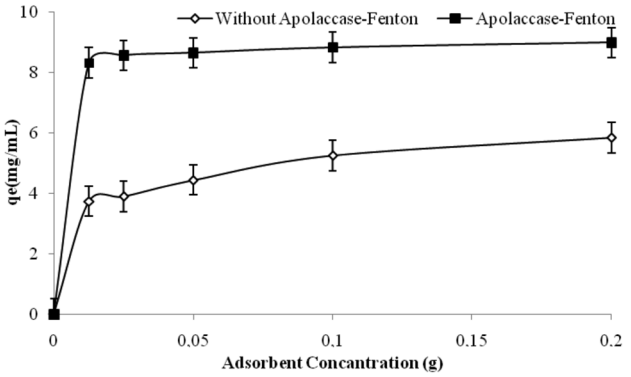

Fig. 11. Effect of adsorbent dosage on the removal of BR9 by nano-Fe_3O_4-immobilized and apolaccase-modified starch.

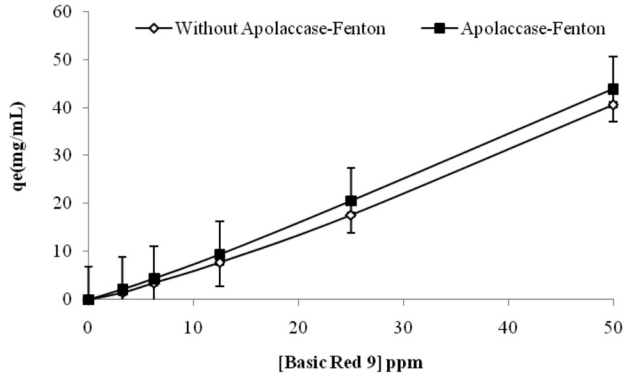

Fig. 12. Effect of BR9 dye concentration on the removal of BR9 by nano-Fe_3O_4-immobilized and apolaccase-modified starch.

of manufacturing including in the textile industry. In our study, apoenzyme of laccase was attached to starch matrix to promote the Fenton reaction activity, and it was reactivated with nano Fe (II/III) ions. In this way the yield of the Fenton reaction was increased. The removal rate of BR9 achieved by the Fenton process without apolaccase was 81% and when modified with apolaccase at a rate of 87%.

The Fenton process could be used to obtain high yield of decolorization. Being inexpensive and widely available, starch represents a convenient substrate for nanomagnetite immobilization (Lin et al. 2004). Iron lacks toxic effects and it can be found everywhere. Therefore, many industries use metallic iron to remove waste before it is released to the environment (Midik 2011).

The Fenton process has been used for the removal of toxic organic substances for a long time; but the use for decolorization of dye from wastewater is a relatively a new method. The reaction rate of the Fenton process increases with temperature. However, the degradation of peroxide to water and oxygen led to decrease of the reaction rate at a temperature higher than 50 °C. Our research showed

Fig. 10. Decolorization of BR9 by Fenton and apolaccase enzyme-modified Fenton reaction.

that higher temperatures did not affected the reaction rate for nanomagnetite-immobilized starch and apolaccase modified nanomagnetite immobilized starch. This is an excellent advantage for fabrics, which are released into wastewater in high temperatures (Malik, Saha 2003; Meric et al. 2005).

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