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A laccase heterogeneous magnetic fibrous silica-based biocatalyst for green and one-pot cascade synthesis of chromene derivatives

Mehdi Mogharabi-Manzari,[a,b] Mohammad Hossein Ghahremani,[c] Tabassom Sedaghat,[a] Fatemeh Shayan,[a] and Mohammad Ali Faramarzi*[a]

Abstract: Green cascade approaches, which utilize sustainable and recyclable heterogeneous biocatalysts, can be used in the catalysis of multicomponent organic reactions to synthesize biologically important substances. Three magnetic nanoparticles, iron (II, III) oxide, cobalt ferrite, and nickel ferrite, were prepared by co-precipitation and functionalized with fibrous silica (KCC-1), and characterized by scanning electron microscopy (SEM), X-ray diffraction (XRD), and vibrating sample magnetometry (VSM). Laccase immobilized on $CoFe_2O_4$-KCC-1 was used as a heterogeneous biocatalyst for green and one-pot cascade synthesis of 2-amino-5-oxo-4-aryl-4H,5H-pyrano[3,2-c]chromene-3-carbonitriles. The reaction was performed in the presence of 2,2,6,6-tetramethylpiperidine-1-oxyl (TEMPO) as a synthetic redox mediator. The optimum reaction conditions were found to be as immobilized laccase (100 mg, 95 U) and TEMPO (2 mol%) in a 100 mM sodium citrate buffer (pH 4.5), 40 °C, and incubation time 17 h. About 80% of initial activity of the immobilized laccase was retained after 15 independent runs.

Introduction

Heterogeneous biocatalytic processes are considered an important route to green and sustainable organic synthesis.[1] Heterogeneous catalysts have attracted substantial attentions because of their various advantages, such as improved atom efficiency of reactions, enhanced turnover number, facile catalyst separation, recovery, and reusability.[2] Moreover, the simplicity of catalyst recovery in heterogeneous biocatalytic processes provides economic and green approaches for the production of biological substances.[3] Several types of nanocarriers have been developed to progress traditional immobilization methods leading to enhancement of enzyme loading, activity, and stability, thereby aiding the development of environmentally friendly synthetic applications.[4] A heterogeneous biocatalyst composed of immobilized enzymes provides a broader range of operating temperature and pH than that of free enzymes.[5] In the last decade, the use of nanoparticles in enzyme stabilization has attracted increased attention based on their very small size of particles and large surface area.[6] Magnetic nanoparticles show a high binding capacity to conjugated enzymes and can be used for enzyme immobilization.[7] The potential of magnetic nanoparticles as carriers for immobilized enzymes can be increased by the use of nanoparticles containing silica with an ordered structure, such as KCC-1, on the surface of magnetic supports. This has unique properties, including fibrous morphology, high surface-to-volume ratio, and good thermal and mechanical stability.[8] The potential application of heterogeneous biocatalysts in organic synthesis has increased in recent decades due to major advancements in enzyme immobilization techniques and efforts to imitate the production of organic compounds. Among the enzymes applied in organic synthesis, oxidoredactases are widely used as biocatalysts for oxyfunctionalization and oxidation reactions.[9] Laccases, copper-containing oxidases, can catalyze the oxidation of a wide range of phenolic and nonphenolic substrates.

Laccase-catalyzed oxidation of various phenolic compounds, such as diphenols, diamines, and benzenethiols, occurs by four-electron reduction of O_2 to HO_2.[10] The scope of laccase substrates can be extended to compounds with high redox potential using natural or synthetic mediators. The ability of laccase-mediator systems to catalyze multicomponent reactions provides a powerful and effective strategy for synthesis of complicated organic compounds. These reactions are considered pivotal in the production of several important natural products, such as coumarin and chromene derivatives. Chromenes belong to a main category of biologically active and natural oxygen-containing heterocycles, which are commonly found in flavonoids and alkaloids.[11] These compounds possess a range of biological activity and pharmacological properties, such as anti-inflammatory, antioxidant, antimalarial, antimicrobial, anticoagulant, antiallergenic, and anticancer activities.[11a,12] Chromene derivatives are also applied in laser dyes, liquid crystal display, optical brighteners, optical chemo-sensors, and fluorescence markers.[13] Amino-4H-chromene derivatives was previously prepared by Michael addition of an aromatic aldehyde to malononitrile, followed by cyclization with hydroxycoumarins. Although several synthetic approaches have been proposed for production of chromenes, these methods suffer from some limitations, including the usage of highly toxic and chlorinated solvents, transition metal catalysts, lengthy multistep work-up procedures, and low yields.[14] Elimination of these restrictions is essential for the development of an efficient, simple, and sustainable method for the synthesis of 2-amino-4H-chromenes.[15]

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The present study describes an approach for the preparation of a heterogeneous biocatalyst by laccase immobilization on the surface of KCC-1 functionalized magnetic nanoparticles. The immobilized laccase was applied in green and one-pot cascade synthesis of chromenes in the presence of TEMPO as a shuttle redox mediator.

Results and Discussion

Magnetic nanoparticles (Fe_3O_4, $CoFe_2O_4$, and $NiFe_2O_4$) were prepared via co-precipitation as described in the literature (Supporting Information) and then coated by a fibrous silica layer using sol-gel method. Morphology of all magnetic nanoparticles, KCC-1 coated nanoparticles, and the enzyme immobilized nanoparticles were studied and the porous surface of the silica-coated nanoparticles and the core-shell structure of the magnetic KCC-1 were observed in the scanning electron microscopy (SEM) and transmision electron microscopy (TEM) images, respectively (Figure 1a-f). The Fe_3O_4-KCC-1 nanoparticles had a uniform size, with an average diameter of 100 nm and good dispersity. The magnetization curves of the Fe_3O_4-KCC-1, $CoFe_2O_4$-KCC-1, and $NiFe_2O_4$-KCC-1 magnetic samples (Figure 1g) indicated saturation magnetization values of 76, 59, and 19 emu g^{-1}, respectively, which are in the range of reported values for amorphous silica coated magnetic nanoparticles.[16] Three nanoparticles, Fe_3O_4, $CoFe_2O_4$, and $NiFe_2O_4$, were examined for immobilization of laccase. The obtained results showed that $CoFe_2O_4$-KCC-1 nanoparticles were able to immobilize a higher amount of the enzymes than other two nanoparticles (see Supporting Information). Although iron oxide nanoparticles showed better magnetic saturation values than $CoFe_2O_4$ nanoparticles (Figure 1e), the obtained results from the kinetic parameters showed that $CoFe_2O_4$-KCC-1 was a suitable support for the enzyme immobilization (see Supporting Information).

The magnetic properties of the KCC-1 coated magnetic particles were measured and it was found that the saturation magnetization values of the fibrous silica-coated magnetic nanoparticles were decreased (*ca.* 10 emu g^{-1}) which was expected and could be explained by diamagnetic contribution of coated silica layer. However, the decrease showed no dramatic effect on the efficiency of the prepared catalyst.

The wide-angle X-ray diffraction (XRD) patterns of the prepared magnetic nanoparticles revealed that the nanoparticles embedded in the core had not changed, despite being fabricated with fibrous silica (Figure 1h). Elemental mapping of sulfur and copper atoms investigated by SEM image and color-coded maps revealed uniform distribution of the enzyme on the surface of the fibrous silica coated magnetic support (Figure 2).

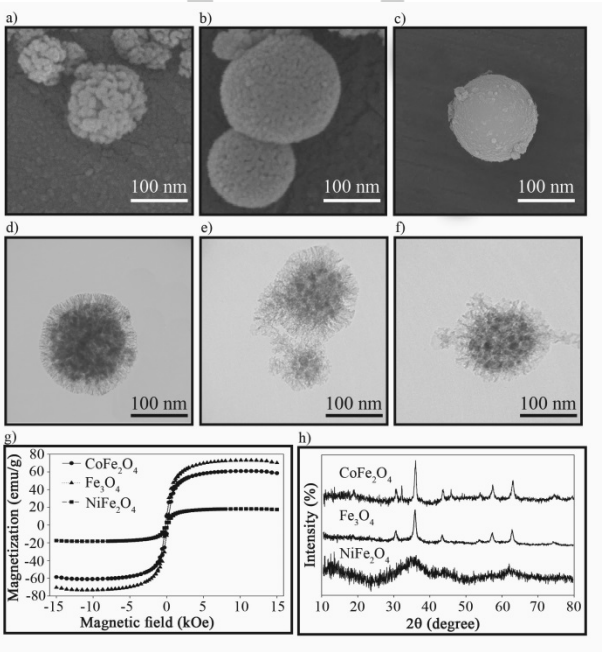


Figure 1. SEM images of a) $CoFe_2O_4$-KCC-1, b) Fe_3O_4-KCC-1, c) $NiFe_2O_4$-KCC-1, TEM images of d) $CoFe_2O_4$-KCC-1, e) Fe_3O_4-KCC-1, f) $NiFe_2O_4$-KCC-1, g) VSM of magnetic nanoparticles, and h) XRD pattern of magnetic nanoparticles.

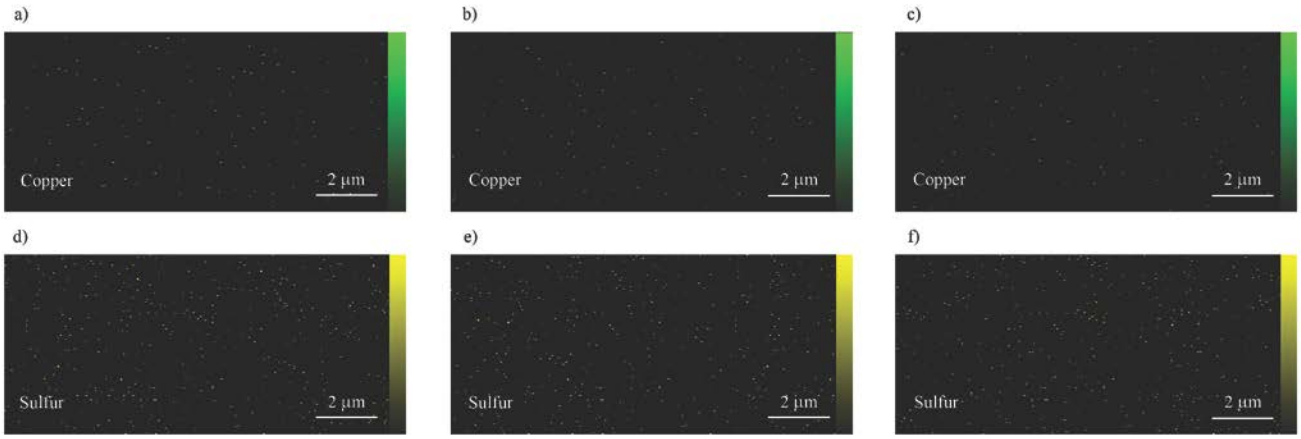


Figure 2. Color coded dot maps of copper atoms of immobilized laccase on a) Fe_3O_4, b) $CoFe_2O_4$, c) $NiFe_2O_4$, and sulfur atoms on d) Fe_3O_4, e) $CoFe_2O_4$, and f) $NiFe_2O_4$.

Radial-like morphology and large surface area helped the target enzymes attach to the fibrous structure quickly and efficiently, thereby decreasing the time of attachment. The enzyme was immobilized on the modified surface of the prepared magnetic fibrous silica KCC-1 activated by (3-aminopropyl)triethoxysilane and glutaraldehyde. The obtained results showed that laccase immobilization increased enzyme storage stability at 4 and 25 °C (Figure 3). The study of enzyme reusability showed that immobilized laccase on the surface of the $CoFe_2O_4$-KCC-1 magnetic nanoparticles could retain around 85% of its initial activity after 15 independent runs (Figure 4).

The activated nanoparticles and the immobilized laccase were investigated by Fourier transform infrared (FTIR) spectroscopy and it was found that carbonyl adsorption band (1578 cm^{-1}) of the attached glutaraldehyde was disappeared after the enzyme immobilization. Disappearing of the carbonyl band proves the reaction between carbonyl groups in the surface of nanoparticles with amine groups of the enzyme leading to conformational changes of laccase. Wang et al.[16b] showed that the carbonyl stretching vibration of carboxyl groups of modified mesoporous carbon was disappeared after immobilization and amide peaks were used to determine the conformational change of protein.

The yield and efficiency of immobilization were calculated as the percentage of total immobilized enzyme activity and the percentage of the bound enzyme, respectively. The yield and efficiency of immobilization were found to be 78% and 86%, respectively. Therefore, laccase immobilized on $CoFe_2O_4$-KCC-1 was selected as a heterogeneous catalyst for aerobic oxidation of some aromatic alcohols containing one, two, and three aromatic rings. The biocatalyst effectively catalyzed the oxidation reaction, with a yield up to 95% (Figure 5 & Table 1). An ionic mechanism has been proposed for laccase-induced oxidation of aromatic alcohols using a synthetic redox mediator. In this mechanism, the oxygen lone pair electrones of the aromatic alcohol attacks onto the laccase-catalyzed generated oxoammonium, which leads to a transient complex formation. Fainally, carbonyl-containing product is achieved by deprotonation of this adduct.[17]

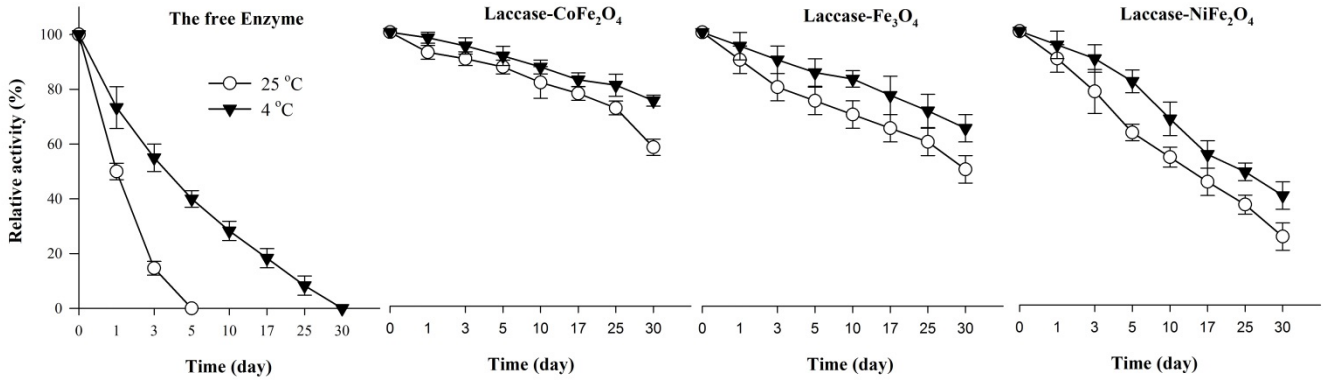


Figure 3. Residual activity of a) free enzyme, and immobilized laccase on b) $CoFe_2O_4$, c) Fe_3O_4, and d) $NiFe_2O_4$ at 4, and 25 °C.

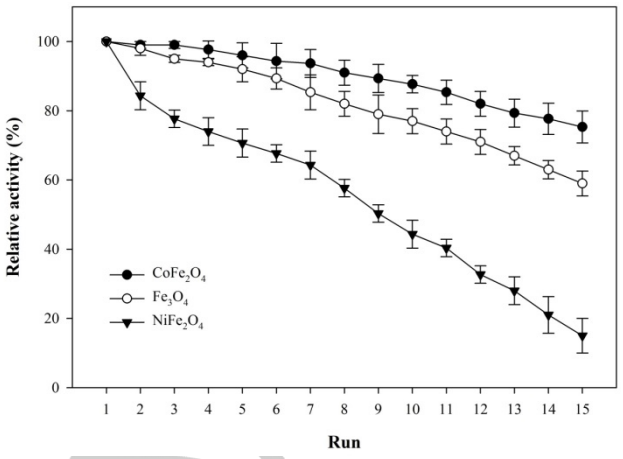


Figure 4. Relative activity of laccase after 15 independent cycles (pH 4.5, and 40 °C).

Proper control experiments were carried out in the absence of laccase to explore the possible catalytic activity of the nanoparticles. The magnetic nanoparticles did not exhibit significant catalytic activity in the reaction conditions. Although laccase is used extensively as a biocatalyst in simple organic reactions, such as oxidation, dimerization, and polymerization, only a few studies have applied this enzyme in multicomponent organic reactions for the synthesis of heteroatom-containing polycyclic compounds.[18]

Therefore, in the present study, to investigate the capability of this heterogeneous biocatalytic system in the catalysis of multicomponent organic reactions in aqueous solution and under slight reaction conditions, the system was applied in the green synthesis of 2-amino-5-oxo-4-aryl-4H,5H-pyrano[3,2-c]chromene-3-carbonitriles. Aromatic alcohols were oxidized to corresponding aldehyde via an enzyme-catalyzed reaction. Chromenes were obtained by the addition of malononitrile and 4-hydroxycoumarin to the in situ generated aldehydes. Alcohol oxidation was completed after 10 h. The optimum amounts of the heterogeneous catalyst and TEMPO were 100 mg (95 U)

and 2 mol%, respectively (Figure 6). Study of the effect of pH (3.5-7.5) and temperature (25-50 °C) on the yield of 2-amino-5-oxo-4-phenyl-4H,5H-pyrano[3,2-c]chromene-3-carbonitrile demonstrated that the optimal reaction conditions were pH 4.5 at 40 °C (Figure 7). The biocatalyst effectively catalyzed the synthesis of chromenes, with a yield up to 88% (Table 2).

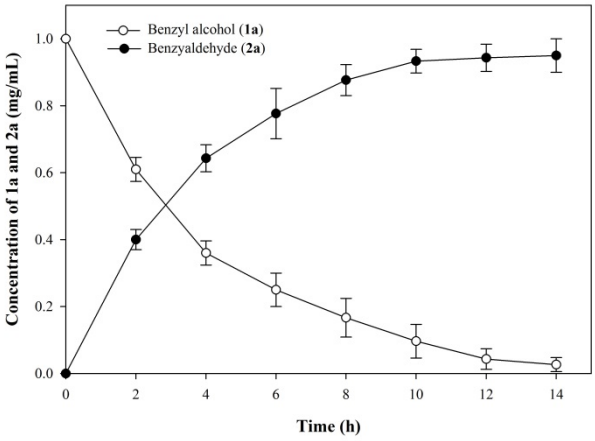
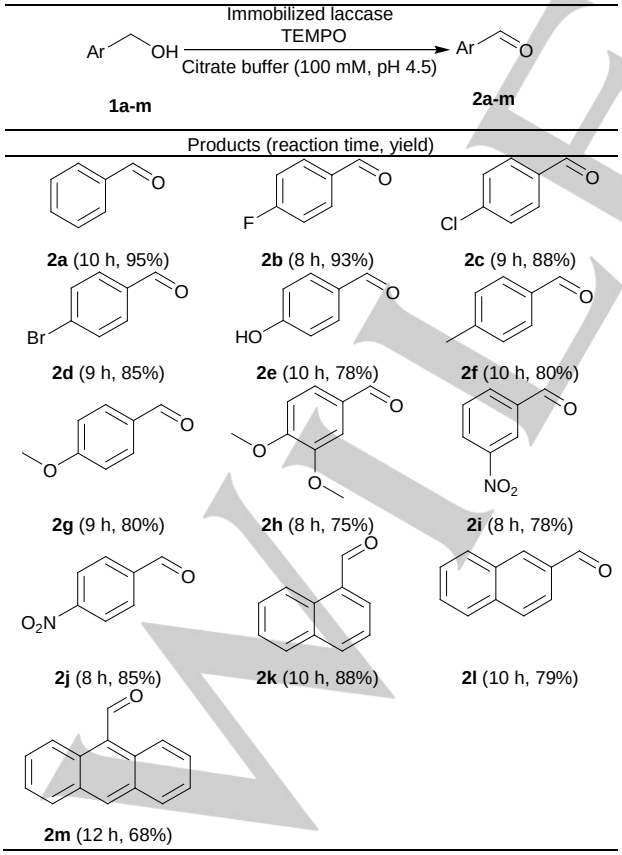


Figure 5. Time-course study of consumption of benzyl alcohol and production of benzaldehyde in citrate buffer (100 mM, pH 4.5) under air at 40 °C.

Table 1. Aerobic oxidation of aromatic alcohols using immobilized laccase in the presence of TEMPO (2 mol%) at pH 4.5 and 40 °C.



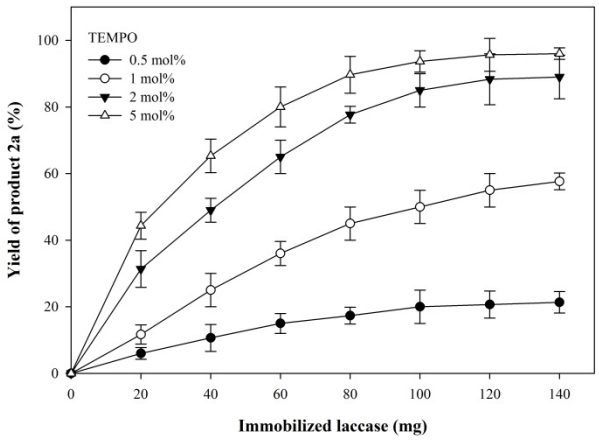


Figure 6. Effect of immobilized laccase and TEMPO on the yield of oxidation reaction in citrate buffer (100 mM, pH 4.5) under air at 40 °C.

Although, rising the temperature up to 40 °C improved the yield by more than 90%, the yield was decreased with further increases in reaction temperature that caused by enzyme deactivation. The efficiency of laccase in organic synthesis depends on its tendency toward time-dependent inactivation under reaction conditions. There is a belief that thermal inactivation is mainly caused by denaturation of the tertiary structure of protein which leads to unfolding and disruption of the active site.[19] As shown by a literature survey, several homogeneous or heterogeneous catalysts have been applied for the production of chromene derivatives, such as piperidine, cetyltrimethylammonium chloride (CTAB), triethyl amine, tetrabutylammonium bromide (TBAB), triethylbenzylammonium chloride, N,N-dimethyl aminoethyl benzyl dimethyl ammonium chloride, hydrotalcite, chitosan, heteropolyacid, ionic liquids, and methane sulfonic acid.[20, 21]

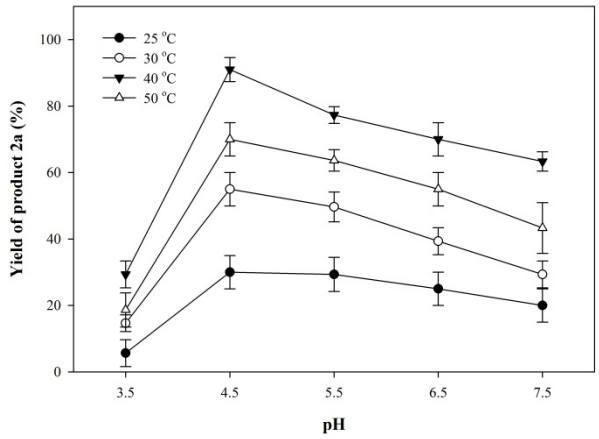
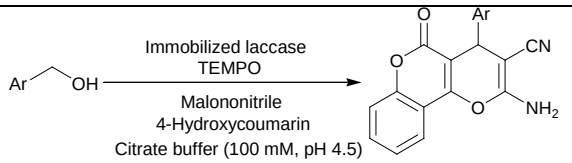


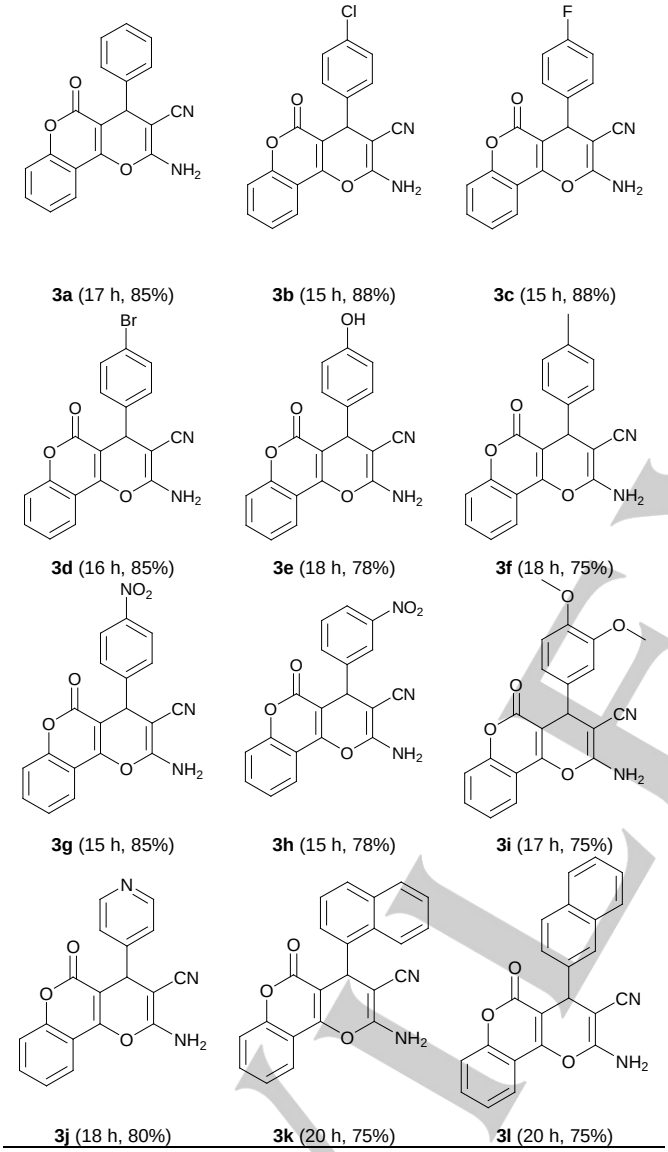
Figure 7. Effect of reaction conditions (pH and temperature) on the yield of laccase-catalyzed oxidation reaction.

Table 2. One-pot synthesis of 2-amino-4-(aryl)-5-oxo-4H,5H-pyrano[3,2-c]chromene-3-carbonitrilein aqueous medium using immobilized laccase.



1a-m **3a-m**

Products (reaction time, yield)[(a)]



[(a)]Reaction condition: benzyl alcohol (1 mmol), 4-hydroxy coumarin (1.2 mmol), malononitrile (0.5 mmol) immobilized laccase (100 mg, 95 U), and TEMPO (2 mol%) in a citrate buffer (100 mM, pH 4.5) at 40 °C.

Kaur et al.[20] reported a series of 4-aryl/heteroaryl-4H-fused pyrans that were produced in a microwave synthesizer via a multicomponent reaction and showed remarkable in vitro inhibition of xanthine oxidase.[20] Another study reported the synthesis of 2-amino-4H-chromene derivatives using $H_{14}[NaP_5W_{30}O_{110}]$ as a heteropolyacid catalyst under reflux conditions for 3–5 h.[21]

In the present study, the cascade one-pot reaction was performed in various aqueous organic media. Some ratios of organic and aqueous solution including 1:10, 2:10, and 3:10 were examined. The obtained results showed that activity of the enzyme and the yield of the product decreased by increasing the ratio of organic solvents. Therefore, the ratio of 1:10 was selected as the optimal ratio. The reaction proceeded in a mixture of both organic and aqueous solutions (1:10, v/v) and the product was achieved with moderate yields (55–85%).

The obtained results showed that the citrate buffer (100 mM, pH 4.5) was the appropriate reaction medium (Table 3). Water molecules are bonded to the surface of enzymes and act as a lubricant or plasticizer, thereby enabling these enzymes to exhibit the conformational mobility required for optimum enzyme structure and activity. Hydrophobic solvents are more suitable than hydrophilic ones for promoting enzyme activity due to their reduced ability to strip essential water from enzymes and to restrict conformational flexibility. The most important driving force for enzyme activity is the bonding energy between the substrate and active site.

Hydrophobic active site of some enzymes provides a large energetic barrier that restricts movement of hydrophilic substrates from water into the active site and decreases rate of catalytic reaction. On the other hand, when the reaction is performed in the presence of organic solvent, the energetic advantages of partitioning decrease, and the ground state energy of the hydrophobic molecule is more stabilized in organic solvents than water.[22] Therefore, the increased activation barrier slows down the enzymatic reaction.[22] Kinetic parameters (V_{max} and K_m) were calculated using the Lineweaver–Burk curve and applying ABTS as substrate. Calculated values of V_{max} for free and immobilized laccase was found to be 61 and 56 mM min^{-1}, respectively.

Table 3. Synthesis of 2-Amino-5-oxo-4-phenyl-4H,5H-pyrano[3,2-c]chromene-3-carbonitrile (5a) in the presence of organic solvent (1:10, v/v) after 17 h incubation at pH 4.5 and 40 °C.

Entry	Solvent	log *P*	Time (h)	Yield (%)
1	water	-	17	85
2	Citrate buffer	-	15	85
3	DMSO	-1.3	17	60
4	Dioxane	-1.1	18	75
5	Hexanone	1.3	20	78
6	Toluene	2.5	17	80
7	Octanol	2.9	20	75
8	hexane	3.5	20	55
9	Heptane	4.0	17	80
10	Octane	4.5	17	80

A slight declining trend of V_{max} occurred due to conformational changes, which reduced the availability of the active site of laccase after immobilization (Supporting Information). The K_m values of the immobilized and free laccase were estimated as 1.4 and 2.2 μM, respectively. Kinetic parameters of an enzyme usually change significantly after immobilization on a solid support. These parameters can be improved by applying a suitable support and an appropriate immobilization method. Some conformational changes may be occurred on the tertiary

structure of the enzyme during the immobilization procedures which affect the kinetic behavior of the immobilized enzyme.[22a] Covalent bonds between the enzyme and the support may increase accessibility of the active site and lead to improved kinetic constants. In addition, catalytic properties are also influenced by diffusion of substrate and product from porous matrix of the support. The found values showed an increase in affinity of the immobilized enzyme toward the substrate.[22b] The most key effecting factors on the affinity of an immobilized enzyme toward a substrate are the loss of required flexibility of enzyme that is necessary for substrate.[23]

Conclusions

In summary, laccase was immobilized on $CoFe_2O_4$-KCC-1, and the prepared catalyst was characterized. This heterogeneous catalytic system was used to yield 2-amino-5-oxo-4-aryl-4H,5H-pyrano[3,2-c]chromene-3-carbonitriles via a one-pot cascade enzymatic reaction. The optimal reaction conditions, including pH, temperature, incubation time, and amounts of heterogeneous biocatalyst and mediator, were investigated.
The prepared catalytic system described herein can be used to enable improvements in future biocatalytic systems that utilize heterogeneous biocatalysts in cascade organic reactions. Moreover, the cascade and enzymatic approach described in the present study can be considered as an effective and straightforward method for advancement of applying simple biotransformations in the targeted synthesis of valuable compounds via green multicomponent reactions.

Experimental Section

Materials and instruments

Laccase from *Trametes versicolor* (≥ 10 U mg^{-1}), 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS), and 2,2,6,6-tetramethylpiperidine-1-oxyl (TEMPO) were obtained from Sigma-Aldrich (St. Louis, MO, USA). All other chemicals and solvents were purchased from Merck (Darmstadt, Germany) and applied without further purification. A high-performance liquid chromatography (HPLC) apparatus was employed from Knauer (Berlin, Germany). The apparatus was equipped with a smart line pump 1000, PDA detector 2800, and degasser 5000. Activity of the enzyme was measured using a double-beam PC scanning UV-vis spectrophotometer (UVD 2950, Labomed, Culver City, USA). Scanning electron microscopy was performed on a Hitachi S-4700 (Tokyo, Japan) instrument, with beam energy of 4 kV. The ^{1}H and ^{13}C nuclear magnetic resonance (NMR) spectra were recorded on a Bruker DRX-500 Avance instrument (Bruker Biospin AG, Faellanden, Switzerland) at 500 and 125 MHz, respectively. The spectra were measured in deuterated dimethyl sulfoxide (DMSO-d6) relative to tetramethylsilane (TMS). The chemical shifts and coupling constant (*J*) are given in *δ* and Hz, respectively. The splitting patterns of spectra are presented as singlet (s), doublet (d), triplet (t), and multiplet (m). Mass spectra were recorded by electrospray ionization technique using a micromass LCT spectrometer (Waters, Milford, MA, USA) using. Thin-layer chromatography (TLC) was carried out using precoated aluminum-backed plates (Kieselgel 60 F254, Merck, Darm-stadt, Germany) and visualized by ultraviolet irradiation at 254 nm. Flash chromatography was conducted using silica gel (SiO_2 60, 230–400 mesh, Fluka, Buchs, Switzerland).

Laccase assay

The activity of laccase was measured using a method previously reported by Johannes & Majcherczyk.,[24] using 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS). Briefly, 0.5 ml laccase solution was added to equal volume of freshly prepared ABTS (2 mM) in 100 mM citrate buffer pH 4.5. Then the reaction mixture was incubated for 10 min at 37 °C and 120 rpm and the absorbance was recorded at 420 nm (ε_{420} = 36,000 M^{-1} cm^{-1}). One unit of laccase activity was considered as the amount of enzyme that oxidized 1 µmol of ABTS per one minute.

Synthesis of Fe_3O_4 nanoparticles

The magnetic Fe_3O_4 nanoparticles were prepared by ultrasound-assisted reverse co-precipitation method, as previously reported by Wei et al.[25] In brief, an aqueous solution of iron (II) chloride (5.4 g) and iron (III) chloride (2 g) in 25 ml hydrochloric acid (2 M) was sonicated at 25 °C until the salts dissolved. A solution of aqueous ammonia (25%, 40 ml) was slowly added over 20 min to the above mixture under a N_2 atmosphere at at 25 °C, followed by 30 min stirring with a mechanical stirrer. The prepared Fe_3O_4 nanoparticles were separated using an external magnetic field. Then the magnetic nanoparticles were washed three times with distilled water and three times with ethanol.

Preparation of magnetic $CoFe_2O_4$ nanoparticles

Cobalt ferrite ($CoFe_2O_4$) nanoparticles were prepared by co-precipitating a mixtures of cobalt (II) chloride and iron (III) chloride aqueous solutions in an alkaline medium.[3] Mixed solutions of 100 ml $CoCl_2.6H_2O$ (1.0 M) and 100 ml $FeCl_3.6H_2O$ (2.0 M) were prepared and kept at 60 °C for half an hour. The mixture was abruptly added to a boiling solution of NaOH (1,200 ml, 0.63 M). Then the solution was stirred vigorously and maintained at 85 °C for one hour. At this stage, fine particles were collected using an external magnetic field. The prepared nanoparticles were washed several times with distilled water and dried at room temperature.[26]

Preparation of magnetic $NiFe_2O_4$ nanoparticles

Nickel ferrite ($NiFe_2O_4$) nanoparticles were produced via the autocombustion assisted sol-gel approach, as previously reported by Feng et al.[6] Briefly, $Fe(NO_3)_3.9H_2O$ (2 mol) and $Ni(NO_3)_2.6H_2O$ (1 mol) were dissolved in deionized water and in the presence of citric acid (3 mol) as a chelating agent. Ammonia solution (28 %) was added dropwise to control the pH value at 7. Then the solution was evaporated at 60 °C to form a sticky gel and followed by increasing the temperature up to 80 °C to form a thick gel. The gel was combusted by hold on a hot plate (200 °C) to release large amounts of gases, such as CO_2, H_2O, and N_2. A dark brown ferrite powder was washed three times with distilled water and collected using an external magnetic field.[27]

Preparation of silica-coated nanoparticles

The prepared magnetic nanoparticles were suspended in a mixture of ethanol (35 ml) and distilled water (6 ml). the mixture was sonicated for 15 min. Then 1.5 ml tetraethyl orthosilicate (TEOS) was dropwise added to the solution and placed in an ultrasonic water bath for 15 min. Subsequently, 1.4 ml aqueous ammonia (10%) was dropwise added over 10 min, while the mixture is stirred mechanically. The mixture was kept at 40 °C for an overnight. The iron oxide nanoparticles were washed three

times with ethanol. Finally, the silica coated nanoparticles were collected by an external magnetic field and dried over vacuum condition.[28]

Preparation of Amin functionalized magnetite nanoparticles

A mixture containing 10 g of silica-coated magnetite nanoparticle was dispended in dry toluene (200 ml) to form a uniform suspension. The mixture was sonicated for 30 min. After the dropwise addition of 2.5 ml (3-aminopropyl) triethoxysilane (APTES), the mixture was stirred mechanically and then the temperature was slowly increased up to 105 °C and remained for 20 h. Finally, the amine functionalized magnetic nanoparticles were washed three times with ethanol and collected by an external magnet. The prepared particles were dried under vacuum.[29]

Immobilization of laccase

Magnetic carrier (10 mg) was suspended in citrate buffer (100 mM, pH 4.5), 1.6% glutaraldehyde was added and the mixture slightly stirred at room temperature for 2 h. After attachment of glutaraldehyde, the nanoparticles were washed with the same buffer solution. The activated particles (0.16 g) were dispersed in a solution of the enzyme (20 mg) and the prepared mixture was slightly shacked at room temperature for 12 h (Supporting Information). The immobilized enzymes were collected by an external magnetic field and non-covalently-bounded enzymes were removed by washing with citrate buffer. The immobilized biocatalysts were dispersed in buffer and stored at 4 °C. The enzyme activity was measured using a colorimetric procedure. The yield and efficiency of immobilization were calculated as bellow:

Immobilization yield (%) = (A0−A1)/A0 × 100; where A0 and A1 are the activity of laccase before and after immobilization, respectively.

Immobilization efficiency (%) = (B0−B1)/B0 × 100; where B0 is the initial amounts of introduced protein for immobilization and B1 is amount of retained protein in the supernatant after immobilization.

Storage stability and reusability studies

In order to evaluation of storage stability of both free and immobilized laccase, residual activity was measured during 30 days incubation at 4 and 25 °C in acetate buffer (100 mM, pH 4.5). The reusability of the immobilized laccase was examined by carrying out oxidation reaction of the substrate over several independent runs. After completion of each cycle, the catalyst was collected by an external magnet. The immobilized laccase were washed three times with the same buffer and the oxidation of the substrate was repeated at the same reaction conditions.

Kinetic studies

The kinetic parameters (V_{max} and K_m) were estimated applying a Lineweaver–Burk curve, by measuring the initial rates of the reaction with a wide range of concentrations of the substrate (ABTS, ranging from 0.1–10 mM) in 100 mM citrate buffer (pH 4.5) at 40 °C. The data obtained by plotting the initial velocity against the substrate concentrations and using free laccase (0.1 mg) and immobilized laccase (1 mg) were fitted to the Lineweaver–Burk plot to estimate the kinetic parameters.

Oxidation of aryl alcohols

A solution of aromatic alcohol (1.0 mmol) and catalyst (1 mol%) in 10 ml citrate buffer (100 mM, pH 4.5) was magnetically stirred at 40 °C in a flask filled with air (balloon filled). Thin layer chromatography (TLC) was used to monitor the progress of the reaction and the yields were determined by HPLC. After completion, the reaction mixture was worked up and the catalyst was separated by an external magnet. Excess solvent was removed under reduced pressure to yield the product.

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Keywords: Laccase • Immobilization • Cascade reaction • Green synthesis • Biocatalysis

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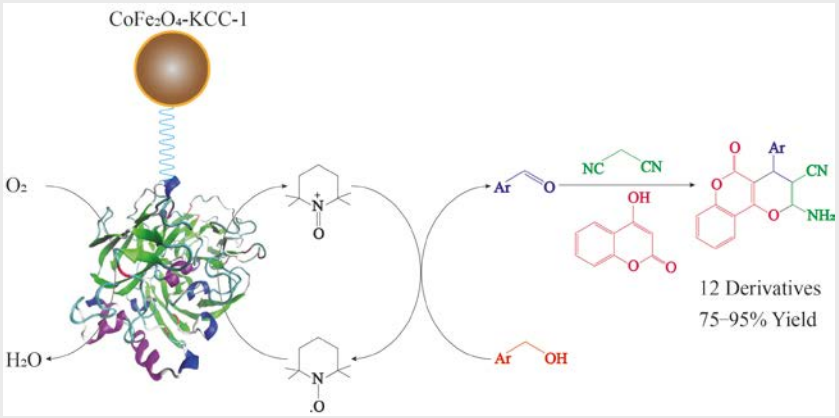

Biocatalysis

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A laccase heterogeneous magnetic fibrous silica-based biocatalyst for green and one-pot cascade synthesis of chromene derivatives

A heterogeneous biocatalyst was prepared by laccase immobilization on the surface of KCC-1 functionalized magnetic nanoparticles. The catalyst was applied in green and one-pot cascade synthesis of chromenes in the presence of TEMPO as a shuttle redox mediator.