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Kinetic characterization of laccase from *Bacillus atrophaeus*, and its potential in juice clarification in free and immobilized forms[§]

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In the present study, a laccase gene (*BaLc*) from a lignin degrading bacterium, *Bacillus atrophaeus*, has been cloned and expressed in *Escherichia coli*. The optimal catalytic activity of the protein was achieved at 5.5 pH and 35°C temperature, measured by oxidation of ABTS. The K_m and V_{max} values were determined as 1.42 mM and 4.16 µmole/min, respectively. To achieve the enzyme recovery, the biocatalyst (BaLc) was covalently attached onto the functionalized iron magnetic-nanoparticles. The nanoparticles were characterized by zeta-potential and FTIR analyses. The immobilized BaLc enzyme was physico-kinetically characterized, exhibiting retention of 60% of the residual activity after ten reaction cycles of ABTS oxidation. The immobilized biocatalyst system was tested for its biotechnological exploitability in plant juice processing, achieving 41–58% of phenol reduction, 41–58% decolorization, 50–59% turbidity reduction in the extracts of banana pseudo-stem and sweet sorghum stalk, and apple fruit juice. This is the first study to demonstrate the use of nanoparticle-laccase conjugate in juice clarification. The findings suggest that *B. atrophaus* laccase is a potential catalytic tool for plant juice bioprocessing activities.

***Keywords*:** laccase, *Bacillus atrophaeus*, immobilization, magnetic nanoparticles, fruit juice clarification

Introduction

Laccases are multi-copper polyphenol oxidase enzyme, which catalyzes various oxidation reactions by using molecular oxygen as the electron acceptor (Yin *et al*., 2017). Laccase participates in catalyzing three main reactions – cross-linking of lignin monomers, degradation of polymers, and ring cleavage of aromatic metabolites (De Gonzalo *et al*., 2016). To catalyze the oxidation of non-phenolic group of compounds, laccase requires a mediator that behaves as 'electron shuttle' between laccase enzyme and its substrate. The mediator is catalytically oxidized by laccase into stable radicals, which can chemically interact with non-phenolic compounds (e.g., lignin), executing their non-enzymatic oxidation. The commonly used mediators are 2, 2′-azinobis (3-ethylbenzothiazoline-6-sulphonic acid) (ABTS), violuric acid (VLA), 1-hydroxybenzotriazole (HBT), and N-hydroxyacetanilide (NHA) (De Gonzalo *et al*., 2016; Chauhan *et al*., 2017).

Laccase acts on a broad range of substrates, therefore, possesses the potential for its applications in several bioprocesses, such as pulp-delignification, dye-decolorization, soil-bioremediation, sewage-treatment, phenols monitoring in food products, wine, and juice clarification, etc. (Chauhan *et al*., 2017). Fruit juices are the most widely accepted drink worldwide. Polyphenols in juices are considered as antioxidant biomolecules of enormous health benefits. However, during storage, the biochemical reaction among phenolic and protein molecules creates turbidity, haziness, color intensification, and alteration in flavor and fragrance of the juice (De Souza Bezerra *et al*., 2015; De Gonzalo *et al*., 2016). This severally affects the shelf-life, stability, and consumer acceptance of the juice. Application of the biocatalyst with laccase activity is a promising approach for fruit juice clarification, as it oxidizes most of the phenols in the juice, leading to minimized turbidity and haziness, and enhanced juice stability (Yin *et al*., 2017). Immobilization of the biocatalyst to a support matrix provides several advantages related to enzyme recovery from the treated sample and its recyclability (De Souza Bezerra *et al*., 2015; Chauhan *et al*., 2017).

Laccase enzyme is ubiquitously found in bacteria, archaea, and eukaryota. More than 100 laccases have been biochemically characterized, out of which most of them are from fungal species. However, the application of fungal laccases is often limited by their reduced catalytic efficiency under extreme conditions. The industrial importance of bacterial laccases is increasing due to its notable tolerance range of pH and temperature, and stability in the presence of many inhibitory agents (Chauhan *et al*., 2017). The most characterized bacterial laccases belong to the genera, *Bacillus* and *Streptomyces*. The biochemical properties of *Bacillus* laccases have been reported from many species, e.g., *B. subtilis*, *B. pumilus*, *B. licheniformis*, *B. halodurans*, *Bacillus* sp. HR03, *B. vallismortis*, *B. tequilensis*, *B. amyloliquefaciens*, *Bacillus* sp. ADR, *B. sphaericus*, *B. clausii*, *B. altitudinis*, *B. safensis*, and *B. cereus* (De Souza Bezerra *et al*., 2015; Chauhan *et al*., 2017; Guan *et al*., 2018).

In a study, *B. atrophaeus* has been found to efficiently degrade lignin (Huang *et al*., 2013). However, no characterization study has been done on the laccase gene from this organism. In the present investigation, the laccase gene from

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B. atrophaeus has been heterologously expressed and physicokinetically characterized. Further, the recombinant laccase was immobilized onto the magnetic nanoparticles, and the nano-biocatalyst was recruited for the removal of phenolics and clarification of the juice samples.

Materials and Methods

Materials

ABTS, syringaldazine, 2,6-dimethoxyphenol (2,6-DMP), isopropyl-β-D-thiogalactopyranoside (IPTG) were purchased from Sigma-Aldrich Chemicals Co. Nickel-chelating nitrilotriacetic acid (Ni-NTA) agarose gel was procured from Qiagen. Restriction enzymes were used from New England Biolabs. The cloning vector (pJET1.2), and DNA and protein ladders were obtained from Thermo-Fisher Scientific. *Escherichia coli* BL21(DE3) and pET28a expression vector were purchased from Novagen.

Sequence analysis of *B. atrophaeus* NRS 1221A

The gene sequence, annotated as laccase, was retrieved from NCBI databases using genome of *Bacillus atrophaeus* NRS 1221A (accession number: PRJNA273316). NCBI Blastx tool (http://blast.ncbi.nlm.nih.gov/Blast/) was used to examine sequence similarity between the *B. atrophaeus* laccase gene (*BaLc*) and other sources present in the NCBI database. The phylogenetic tree was constructed using the maximum likelihood method in MEGA 7 (https://www.megasoftware.net/). Molecular mass, isoelectric point (pI) and instability index were determined by Protparam tool hosted at Expasy web browser (http://web.expasy.org/protparam/). Secondary structure prediction for BaLc protein was done by using SOPMA tool (https://npsa-prabi.ibcp.fr/cgi-bin/npsa_automat.pl?page=/NPSA/npsa_sopma.html). The 3D homology-based model was generated by Phyre2 (http://www.sbg.bio.ic.ac.uk/phyre2), using CNF1/YfiH-like cysteine hydrolases (PDB: 1t8h) as the template, and it was further validated through RAMPAGE (http://mordred.bioc.cam.ac.uk/~rapper/rampage.php). The homology model of BaLc was superimposed to its template by using the UCSF Chimera package. The enzyme binding site was predicted by 3D ligand Site (http://www.sbg.bio.ic.ac.uk/~3dligandsite/). Subcellular localization prediction was analyzed by PSORT prediction tool (http://psort.hgc.jp/form.html).

Cloning of laccase from *B. atrophaeus* NRS 1221A

The gene sequence, annotated as laccase, was retrieved from NCBI databases using genome of *B. atrophaeus* NRS 1221A. *BaLc* gene was chemically synthesized with *Nde*I and *Eco*RI restriction sites at the up- and down-stream, respectively. *BaLc* gene was cloned in pET28a expression vector and transformed in *E. coli* BL21(DE3) cells.

Expression and purification of recombinant protein BaLc

E. coli BL21 cells containing pET28a-*BaLc* construct were cultured in Luria-Broth and induced by 0.8 mM IPTG at 16°C. An induced bacterial culture was harvested, and the pellet was resuspended in 10 ml extraction buffer (10 mM imidazole, 50 mM Tris-buffer; pH 7.0, 300 mM NaCl, and 10% glycerol). Afterward, the cells were disrupted by sonication (Qsonica Inc.). The lysate was centrifuged at 12,000 rpm for 30 min at 4°C, and the crude extract was subjected to Ni-NTA affinity chromatography. BaLc protein was purified using 50 mM sodium acetate buffer pH 5.5 following the manufacturer's instructions (Qiagen). The purified BaLc protein was dialyzed, and the salt-free protein was analyzed on 12% SDS-PAGE after Coomassie Blue-R250 staining. The protein concentration was estimated by the Bradford method using bovine serum albumin as a standard. Purified BaLc recombinant protein was stored at 4°C.

Laccase activity assay

The laccase enzyme activity was evaluated by using syringaldazine, 2,6-DMP, and ABTS as the substrate. ABTS exhibited significant activity, therefore, it was selected for further enzymatic assay. Laccase activity was determined by spectrophotometrically using ABTS as the substrate according to the method described by Yin *et al*. (2017). The reaction mixture (100 µl reaction volume), comprised of 1 mM ABTS, 1 mM $CuSO_4$, 50 mM sodium acetate buffer (pH 5.5), and 10 µg of the enzyme (free/immobilized), was incubated at 35°C for 30 min. The enzymatic reaction was terminated by adding 2% trichloroacetic acid of equal reaction volume. Further, the total reaction volume was made up to 1 ml with 50 mM sodium acetate buffer (pH 5.5). The rate of enzymatic reaction was estimated by measuring the change in absorbance at 420 nm (ε=36,000 M/cm). One unit of laccase activity was defined as the amount of enzyme that oxidized 1 µmol of ABTS per min.

Effect of pH and temperature on BaLc activity

The effect of pH on laccase activity was studied in the buffers of 2.0 to 10.0 pH range. Independent reaction assays were performed in the buffers of different pH. The reaction mix (100 µl reaction volume), containing 1 mM ABTS, 1 mM $CuSO_4$, and 10 µg of the enzyme, was incubated at 35°C for 30 min. The following buffer systems were used for optimum pH testing: 50 mM sodium acetate buffer (pH 2.0–6.0), 50 mM sodium-phosphate buffer (pH 6.0–7.0), and 50 mM Tris-HCl buffer (pH 8.0–10.0).

The effect of temperature on BaLc activity was investigated by performing reaction assay in 50 mM sodium acetate buffer (pH 5.5), containing 1 mM ABTS, 1 mM $CuSO_4$, and 10 µg of the enzyme, at the temperatures ranging from 20–70°C. The thermostability of the BaLc enzyme was determined by pre-incubating the enzyme in 50 mM sodium acetate buffer (pH 5.5) at various temperatures ranging from 20–70°C for 5 h. The heat-exposed enzyme was used to perform standard reaction assay, as described above. The residual enzymatic activity in the heat exposed enzyme was determined, taking the fresh enzyme fraction (not exposed to heat) as control. Each experiment was performed in triplicate.

Effect of heavy metal ions on BaLc activity

Chloride salt of different metals (e.g. $CuCl_2$, $CoCl_2$, $CaCl_2$, $CrCl_2$, $ZnCl_2$, $MnCl_2$, $NiCl_2$, NH_4Cl, LiCl, KCl, $FeCl_2$, $MgCl_2$,

and NaCl) was used to examine metal's effect on BaLc's catalytic activity. The metal salt (1 mM) was added in 50 mM sodium acetate buffer (pH 5.5) containing BaLc (10 µg) and incubated at 35°C for 30 min, and then enzymatic assay (100 µl reaction volume) was performed using 1 mM ABTS as the substrate. In the control sample, no metal salt was added. The activity of the control sample was taken as 100%.

Kinetic characterization of BaLc

The kinetic parameters were examined by performing enzymatic assays under the optimal conditions of pH and temperature, using ABTS as the substrate. The concentration of ABTS used in the enzymatic assay was ranged from 0.1–7.5 mM. The kinetic parameters (K_m, V_{max}, k_{cat}, and k_{cat}/K_m) of the recombinant BaLc enzyme were measured through the Michaelis-Menten equation and the Lineweaver-Burk plot.

Treatment of juice with free BaLc

Banana pseudo-stem juice (BSJ), sorghum stem juice (SSJ), and apple fruit juice (AFJ) were mechanically extracted, as described previously (Sharma *et al*., 2017; Jadaun *et al*., 2019). The purified recombinant BaLc enzyme was mixed at different concentrations (0, 5, 10, 20, and 50 U/ml) with the BSJ, SSJ, and AFJ, and incubated at 35°C for 1 h. The reaction was terminated by heating at 100°C for 5 min. The laccase-treated juices were centrifuged at 1,000 rpm for 2 min at 25°C and quantitatively analyzed for total phenolic content, color, and clarity of juices. The total phenolic content in the juice before and after BaLc enzyme treatment was determined by the method described by Folin-Ciocalteu assay (Zhang *et al*., 2013). Gallic acid was used as the standard for estimating phenolics contents in fruit juice. The total phenolic content in juices of the control (untreated juice) was taken as 100%. The clarity and color of the juice, before and after treatment with BaLc, were determined by measuring the absorbance at 650 and 420 nm using UV-visible spectrophotometer, respectively.

Immobilization of enzyme

The magnetic-nanoparticles (MNP) were prepared by the co-precipitation method, as described previously (Patel *et al*., 2018). In this method, iron chloride solution was prepared by mixing $FeCl_2{\cdot}4H_2O$ (0.4 M) and $FeCl_3{\cdot}6H_2O$ (0.8 M) in 1:2 ratio, followed by incubation at 120°C for 15 min under stirring condition. This iron chloride solution (25 ml) was added, in the drop by drop manner, into 250 ml of 1.5 M NaOH solution at 80°C. Inter environment was maintained in this mixture by using N_2 gas. This mixture was incubated at 80°C for 30 min. Then the reaction mixture was cooled at room temperature, and magnetic-nanoparticles was retrieved by applying a magnetic field. These magnetic particles (MNPs) were sequentially washed with de-oxygenated water and 0.1 M tetramethylammonium hydroxide pentahydrate (TMAOH). The MNPs and 3-phosphonopropionic acid (3-PPA) solution were mixed in 1:1 molar ratio and pH was adjusted to 8. Then, ultra-sonication was performed at 30% amplitude (10 sec 'on' and 15 sec 'off') for 30 min. The magnetic-nanoparticles were recovered by keeping on a permanent magnet for ~30 h, and washed with de-oxygenated water, followed by suspension in 50 mM sodium acetate buffer (pH 5.5).

Sequentially, the carboxyl group of MNP-3PPA was activated by ~2 mM EDC [1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride] and ~5 mM NHS [N-hydroxysuccinimide] solution. After 15 min of incubation, the activated magnetic-nanoparticles were collected by applying the magnetic field. After that, BaLc enzyme and the functionalized magnetic-nanoparticles were mixed in 1:2 (w/w) ratio in 50 mM sodium acetate buffer (pH 5.5), following the protocol reported in the previous studies (Sahoo *et al*., 2011; Singh *et al*., 2016; Patel *et al*., 2018; Thanh *et al*., 2019). After incubation at room temperature for 2 h, the immobilized BaLc were retrieved by a magnate. The enzyme-linked nanoparticles were washed with 50 mM sodium acetate buffer (pH 5.5) and stored at 4°C.

The percentage of immobilized BaLc enzyme onto the magnetic nanoparticles was determined by comparing the initial protein concentration and protein obtained in unbound and washing fractions as follows:

Immobilized enzyme (%) = (BaLci−BaLcs/BaLci) × 100

BaLci = Initial BaLc protein concentration.

BaLcs = BaLc Protein concentration in unbound and wash fractions.

Nanoparticle characterization

The nanoparticles were characterized by recording the zeta potential and Fourier-Transform Infrared Spectroscopy (FTIR) spectra. The zeta potential of the MNP, MNP-3PPA, and enzyme immobilized nanoparticles was measured by using Malvern-Zetasizer (Nano-ZS) at 25°C. Further, the infrared spectroscopy was used for surface characterization of nanoparticles in the spectral range from 450–4000 cm^{-1} by using FTIR-ATR spectrophotometer (Spectrum two, Perkin-Elmer).

Enzyme assay and kinetic characterization of immobilized BaLc enzyme

The reaction assay conditions for immobilized BaLc were similar to that of the free form of the enzyme. The reaction mixture (1 ml reaction volume), comprised of 1 mM ABTS, 1 mM $CuSO_4$, 50 mM sodium acetate buffer (pH 5.5), and 100 µg of the immobilized enzyme, was incubated at 35°C for 30 min. The enzymatic reaction was terminated by adding 2% trichloroacetic acid. The activity was estimated by measuring the change in absorbance at 420 nm. The optimum pH and temperature for immobilized BaLc enzyme were determined as per the previously described method in case of the free form of the enzyme. Similarly, the kinetic parameters were examined by performing enzymatic assay with immobilized BaLc using ABTS as the substrate under optimal assay conditions. Consequentially, the kinetic parameters (K_m, V_{max}, k_{cat}, and k_{cat}/K_m) of the immobilized BaLc enzyme were determined through the Michaelis-Menten equation and the Lineweaver-Burk plot.

Reusability analysis

To check the reusability of immobilized BaLc enzyme, reaction assays were performed using ABTS as substrate. After

each reaction cycle, the immobilized enzyme was recovered by applying a magnet. It was then washed twice with 50 mM sodium acetate buffer (pH 5.5) to remove the contamination of any residual substrate on the immobilized enzyme surface. The washed enzymatic beads were reintroduced into a fresh reaction medium, and the laccase activity was analyzed. This was performed for multiple cycles to determine its residual activity.

Banana pseudo-stem, sorghum stem, and apple juices were treated with magnetic nanoparticle-immobilized BaLc for 1 h at 40°C, followed by the analysis of total phenolic content, color, and clarity of juices, as described above. The immobilized enzyme was recovered by applying the magnetic field and reused for multiple duty cycles.

Results and Discussion

BaLc sequence analysis and protein structure prediction

The open reading frame of *B. atrophaeus BaLc* gene of 837 bp encodes a protein of 278 amino acids. In BLASTx analysis, *BaLc* exhibited 77 to 99% sequence identity with the protein sequences, annotated as laccase in the NR database. The phylogenetic analysis indicated genetic relatedness among laccase genes from different sources of *Bacillus* species *viz.*, *B. atrophaeus*, *B. subtilis*, *B. subtilis* subsp. subtilis, *B. velezensis*, *B. amyloliquefaciens*, *Bacillus* sp. BSC154, and *Bacillus* sp. JS (Fig. 1A). The genetic relationship among these putative laccases more or less coincides with the phylogenetic association among these species (Wang *et al.*, 2008). Further, BaLc showed a maximum of 48.9% identity with a characterized laccase protein (AUM59988) from *Bacillus megaterium* (Ozer *et al.*, 2018). The theoretical molecular weight and pI of BaLc protein were computed as 30.94 kDa and 6.12 kDa, respectively. BaLc was predicted to follow cytosolic localization due to the absence of any signal sequence in the protein. Secondary structure prediction study revealed 26.26% alpha helix, 25.90% extended strand, 12.95% beta turn, and 34.89% random coil in BaLc protein. The secondary structure prediction results of BaLc protein are in accordance with the previous secondary structure analyses, reporting the highest percentage of random coils followed by α-helices and β-turns in bacterial laccases (Kurian and Kumar, 2015).

The three-dimensional structure prediction generated a homology-based model of BaLc protein (Fig. 1B), using CNF1/YfiH-like cysteine hydrolase (PDB: d1t8ha) as the highest similarity template. YfiH is a laccase-like protein that displays polyphenol oxidase activity (Chai *et al.*, 2017). A total of 260 (94%) residues were modeled, with 100.0% confidence level, in the 3D homology-based protein structure. Ramachandran plot analysis endorsed the predicted model of BaLc protein by stipulating 95.3% residues under the favored region; whereas, only a small fraction of residues, 3.5%, and 1.2%, were computed as allowed and outlier region (bad), respectively (Supplementary data Fig. S1). The superimposition of the predicted BaLc protein model on its template exhibited substantial reliability of the structure with the Root Mean Square Deviation (RMSD) value of 0.000 (Fig. 1C). The protein structure analysis also predicted the amino acid residues, 43Lys, 44Pro, 45Pro, and 47Glu, involved in the substrate binding (Fig. 1D; Supplementary data Table S1). However, this needs to be experimentally validated by site-directed mutation strategy.

Recombinant expression of BaLc protein

Heterologous protein expression of BaLc was done in *E. coli* BL21 cells. The recombinant protein was purified through Ni-NTA affinity column chromatography. SDS-PAGE analysis specified the molecular mass of the BaLc as ~31 kDa

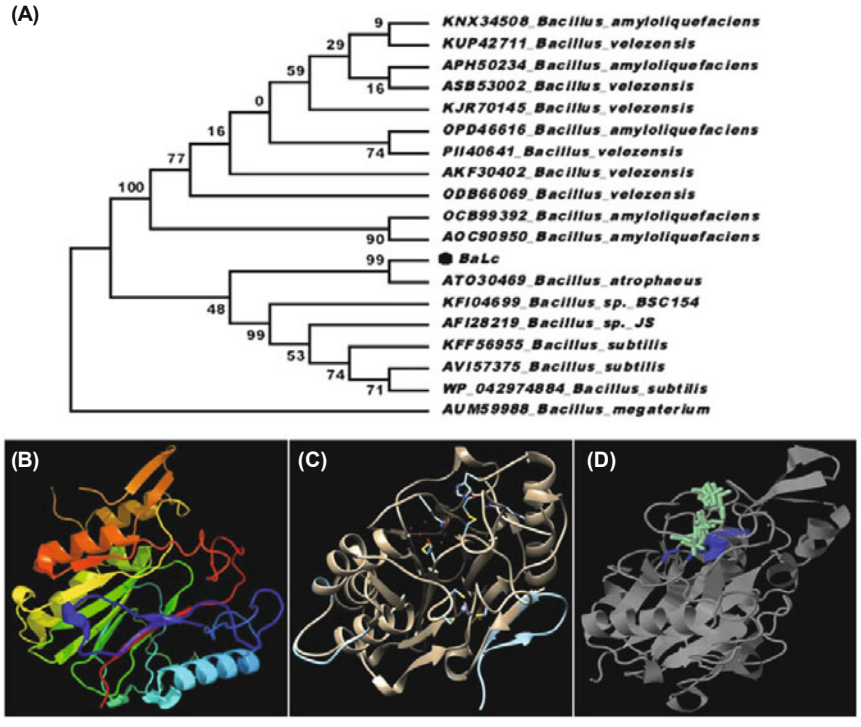

Fig. 1. Bioinformatic analysis of BaLc protein sequence. (A) Phylogenetic analysis of BaLc gene with other laccases genes from *Bacillus* species (B) 3D homology based modeled structure of BaLc predicted by Phyre2. CNF1/YfiH-like cysteine hydrolases/laccase (PDB: 1t8h) was used as template. (C) Superimposition of BaLc on template and (D) Ligand binding pocket (light green) predicted by 3DLigandSite, residue involve in active site are depicted in blue. Visualizations of modeled structures were performed by UCSF Chimera package. *BaLc*, *Bacillus atrophaeus* laccase.

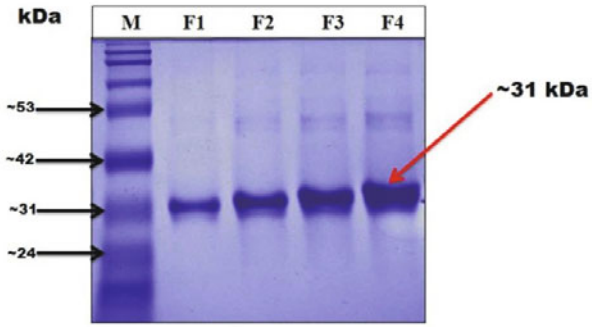

Fig. 2. SDS-PAGE analysis of BaLc protein. Different fractions of Ni-NTA purified BaLc protein was run on SDS-PAGE to estimate its molecular weight. M, Pre-stained protein molecular marker; F1 to F4, Ni NTA purified fractions.

(Fig. 2). It is a low-molecular-weight laccase, which is supposed to be more efficient in lignin degradation (Guo *et al.*, 2017).

Effect of pH and temperature

The effect of pH on enzymatic activity was investigated in the acidic to alkaline pH range of 2–10 using ABTS as the substrate (Table 1). The activity was found high at pH 5, but its activity diminished at 6 pH. Therefore, we checked the activity in the middle of pH 5 and pH 6, i.e., pH 5.5. Interestingly, at pH 5.5, the maximum activity of BaLc was noted. At acidic pH of 4, BaLc retained 70% of its activity, which indicates its suitability for application in acidic samples. At neutral pH, about 50% reduction in activity was observed, whereas, under extreme acidic and extreme alkaline pH (pH 2 and 10) the activity of BaLc was virtually nil (Table 1). The performance of BaLc in this pH range was more or less similar to laccase from *Bacillus* sp. A4 (Guo *et al.*, 2017), *B. subtilis* cjp3 (Qiao *et al.*, 2017), *Bacillus* sp. HR03 (Dalfard *et al.*, 2006), and *Abortiporus biennis* (Yin *et al.*, 2017).

The effect of temperature on BaLc activity was determined at different temperatures ranging from 20–70°C. The optimum temperature for BaLc was found to be 35°C. Severe loss in activity was observed at a temperature of 50°C or above (Table 2). The optimum temperature was similar to the laccases identified from *Bacillus* sp. A4 (Guo *et al.*, 2017), *Bacillus* sp. (Kaushik and Thakur, 2013), and *A. biennis* (Yin *et al.*, 2017). Laccases from *Lentinula edodes*, *Rigidoporus lignosus*, and *Streptomyces griseus* are reported to display 40°C as the optimal temperature (Endo *et al.*, 2003; Nagai *et al.*, 2009; Yin *et al.*, 2017).

Table 1. Effect of pH values on activity of the recombinant enzyme (BaLc) in free and immobilized forms

pH	Free BaLc Relative activity (%) ± SD	Immobilized BaLc Relative activity (%) ± SD
3	9.43 ± 0.42	ND
4	63.14 ± 2.81	25.46583851± 0.178
5	85.61 ± 3.77	69.56521739 ± 0.277
5.5	100 ± 3.34	100 ± 0.393
6	66.96 ± 1.071	93.7888198 ± 0.170
7	40.67 ± 0.35	59.62732919 ± 0.635
8	22.24 ± 0.22	36.64596273 ± 0.120
9	13.48 ± 0.2	3.726708075 ± 0.070
10	7.41 ± 0.15	ND

ND, not detected.

Table 2. Effect of temperature on activity of the recombinant (BaLc) enzyme in free and immobilized forms

Temperature (°C)	Free BaLc Relative activity (%) ± SD	Immobilized BaLc Relative activity (%) ± SD
20	22.1 ± 0.63	18.05 ± 0.039
25	41.47 ± 0.85	25.00 ± 0.013
30	88 ± 1.13	55.55 ± 0.065
35	100 ± 0.78	79.16 ± 0.013
40	53.68 ± 0.69	100 ± 0.026
50	8.42 ± 0.84	67.36 ± 0.058
60	3.36 ± 0.14	18.75 ± 0.019

To investigate thermostability, BaLc enzyme was pre-incubated at different temperature (20–70°C) for 5 h, and then its catalytic activity was measured using ABTS as substrate. BaLc protein was found stable at 20–40°C by retaining about 84–95% activity. However, a noteworthy loss in the activity was observed when exposed to the temperature of 50°C or above (Fig. 3). Similar thermal tolerance was noticed in case of laccases reported from other bacterial sources, such as *Abortiporus biennis*, *Bacillus* sp., *Trametes sanguinea*, *Shigella dysenteriae*, *Haloferax volcanii*, *Cyathus bulleri*, and *Pyrobaculum aerophilum* (Endo *et al.*, 2003; Niladevi *et al.*, 2008; Nagai *et al.*, 2009; Shao *et al.*, 2009; Uthandi *et al.*, 2010; Kaushik and Thakur, 2013; Yin *et al.*, 2017).

Effect of metal ions

The activity of the recombinant BaLc enzyme was tested in the presence of various metals. In the presence of copper, the enzyme's activity was found increased by more than two folds, as compared to the control (Fig. 4). Predominantly, laccase enzymes belong to the multi-copper oxidase family, therefore, its catalytic activity can be induced by the presence of Cu^{++} in the reaction mixture. Several studies have conveyed Cu^{++} induced enhancement in the catalytic activity of laccases from different bacterial sources (Shao *et al.*,

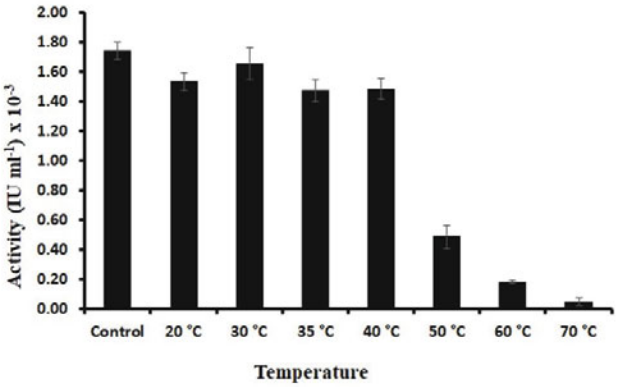

Fig. 3. Thermostability analysis of recombinant BaLc enzyme. The BaLc protein was exposed to heat for 5 h, and thereafter, enzymatic assay was performed to examine activity. In the control sample, the enzyme was not given any heat exposure.

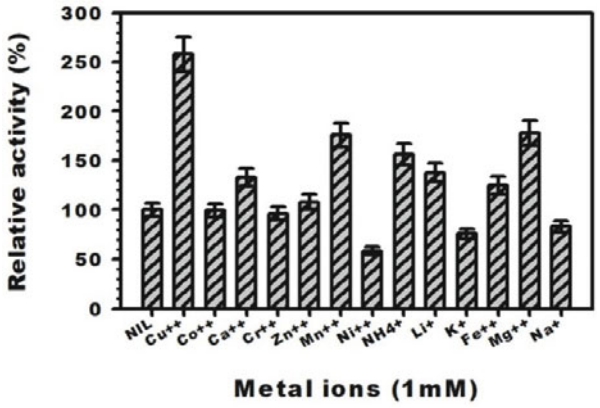

Fig. 4. Effect of different metal ions on the catalytic activity of BaLc enzyme. All the metal salts were used in 1 mM concentration. The enzymatic activity of BaLc with different metal salt was compared.

2009; Nakade *et al.*, 2010; Uthandi *et al.*, 2010). Therefore, copper was used as a co-factor in all the enzymatic reactions. The enzymatic activity was found similar in the presence of two copper compounds, $CuCl_2$ and $CuSO_4$. This suggests that the anions from the two metal compounds ($CuCl_2$ and $CuSO_4$) do not exert any notable effect on BaLc's activity. Further, the effect of different concentrations of $CuSO_4$ on BaLc enzyme activity was measured. A continuous increment was recorded in BaLc's activity from 100 µM to 1 mM $CuSO_4$ in the reaction (Supplementary data Fig. S2). Thus, the results suggest that BaLc is a metal (mainly Cu^{++}) dependent enzyme.

In case of other metals like Fe^{++}, Ca^{++}, Li^{+}, NH_4^{+}, Mg^{++}, and Mn^{++} 14–71% enhancement was experienced in the enzymatic activity (Fig. 4). The presence of Ni^{++}, K^{+}, and Na^{+} in the reaction assay gave a negative upshot to the activity, while Co^{++}, Cr^{++}, and Zn^{++} did not exhibit any obvious effect. The results are in agreement with the previous findings of the stimulatory influence of Mg^{++}, Mn^{++}, NH_4^{+}, and Li^{++}, and the inhibitory effect of Ni^{++}, and K^{+} on the bacterial laccases (Niladevi *et al.*, 2008; Yin *et al.*, 2017).

Table 3. Kinetic parameters of free and immobilized BaLc enzyme, using ABTS as substrate

Kinetic parameter	Free BaLc	Immobilized BaLc
K_m	1.42 mM	5.53 mM
V_{max}	4.16 µmole/min	1.24 µmole/min
K_{cat}	21.46 sec^{-1}	6.41 sec^{-1}
K_{cat}/K_m	15.1 mM/sec	1.16 mM/sec

Kinetic parameters

Although BaLc displayed activity with different substrates like syringaldazine, 2,6-DMP, and ABTS, the maximum enzymatic activity was noted with ABTS substrate (Supplementary data Table S2). Therefore, ABTS was used as a substrate in the concentration range of 0.1–7.5 mM for the kinetic characterization of BaLc. The steady-state kinetics was performed using the Michaelis–Menten equation and the Lineweaver-Burk plot (Supplementary data Fig. S3). The observed K_m, V_{max}, k_{cat}, k_{cat}/K_m values for BaLc enzyme were 1.42 mM, 4.16 µmole/min, 21.46 sec^{-1}, and, 15.1 mM/sec, respectively (Table 3). In the literature, laccases are reported with the K_m values ranging from 0.001–48 mM using ABTS

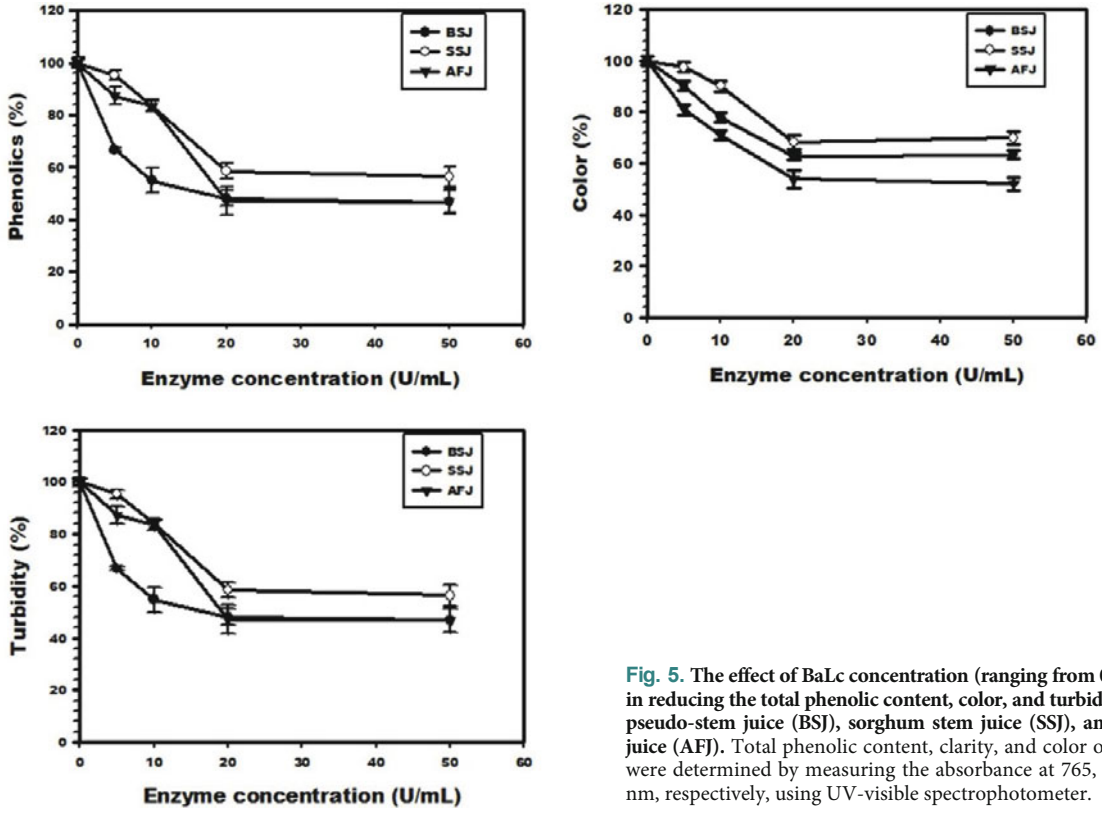

Fig. 5. The effect of BaLc concentration (ranging from 0 to 50 U/ml) in reducing the total phenolic content, color, and turbidity in banana pseudo-stem juice (BSJ), sorghum stem juice (SSJ), and apple fruit juice (AFJ). Total phenolic content, clarity, and color of these juices were determined by measuring the absorbance at 765, 650, and 420 nm, respectively, using UV-visible spectrophotometer.

as substrate. The K_m value for laccases isolated from *Trametes hirsute* and *Cyathus bulleri* was observed to be 0.001–48 mM, respectively (Vasdev *et al*., 2005; Schroeder *et al*., 2006). The previous reports suggest that laccases of the *Bacillus* group have lower value of K_m, for example the K_m value of *Bacillus pumilus*, *Bacillus* sp., *Bacillus subtilis* is 0.08 mM, 0.535 mM and 0.049–2.027 mM, respectively (Durao *et al*., 2006; Mohammadian *et al*., 2010; Reiss *et al*., 2011). The turn over number of laccase from different *Bacillus* species such as *B. subtilis*, *B. licheniformis*, *Bacillus* sp., and *B. pumilus*, has been reported to be in the range of 0.26–291 sec^{-1} (Durao *et al.,* 2006; Mohammadian *et al*., 2010; Reiss *et al*., 2011). The kinetic parameters suggested BaLc protein to be a laccase of fairly good catalytic attributes.

Application of BaLc in juice processing

Laccases have an old history of its uses in the food industry (Osma *et al*., 2010). The phenolic compounds present in the beverages prompt haze formation and browning during storage. Laccases are considered to be a promising catalytic tool for the oxidation of phenols, providing stabilization to beverages such as wine, beer, and fruit juices. To examine the usefulness of BaLc in the food industry, it was employed for enzymatic treatment of BSJ, SSJ, and AFJ. The effectiveness of the enzymatic activity was assessed by examining the total phenolic content, color, and clarity/turbidity in the treated juice samples. The total phenolic content in BaLc treated BSJ, SSJ, and AFJ was reduced by about 44–53%. Nonetheless, increasing the enzyme concentration beyond 20 U/ml did not lead to a further reduction in the phenol content (Fig. 5). Thus, BaLc catalyzed the oxidation of phenolic compounds present in the juice samples. The performance of BaLc was comparable to the previously reported laccases, e.g., *Lactarius volemus*, and *Pleurotus ostreatus* have been shown to reduce the total phenolic compounds in apple juice by about 36% and 45%, respectively (Nadaroglu and Tasgin, 2013; Lattera *et al*., 2016).

Reduction in phenolic content curtails turbidity and improves clarity in the juice (Gassara-Chatti *et al*., 2013). The decrease in absorbance value at 420 and 650 nm after enzyme (BaLc) treatment indicates a reduction in color and cloudiness of juices by about 28–55% and 30–48%, respectively (Fig. 5). The results signify the aptness of the biocatalyst, BaLc, to clarify different kinds of juices, e.g., BSJ, SSJ, and AFJ. Our group intends to bioprocess the abundant agro-industrial biomasses for catalytic biosynthesis of functional molecules (Sharma *et al*., 2017; Patel *et al*., 2018; Jadaun *et al*., 2019). As the phenolic molecules engender haziness and color intensification in plant extracts, affecting their shelf-life, application of BaLc will improve the stability and quality of the plant juice samples.

Immobilization of BaLc and its operational stability

The BaLc enzyme molecules were covalently immobilized onto the activated magnetic particles of iron oxide (Fe_3O_4). By comparing the initial protein concentration (before immobilization) and protein obtained in unbound and washing fractions, the immobilization yield of BaLc enzyme was estimated as about 50%. The unbound fraction of the protein was completely washed-out.

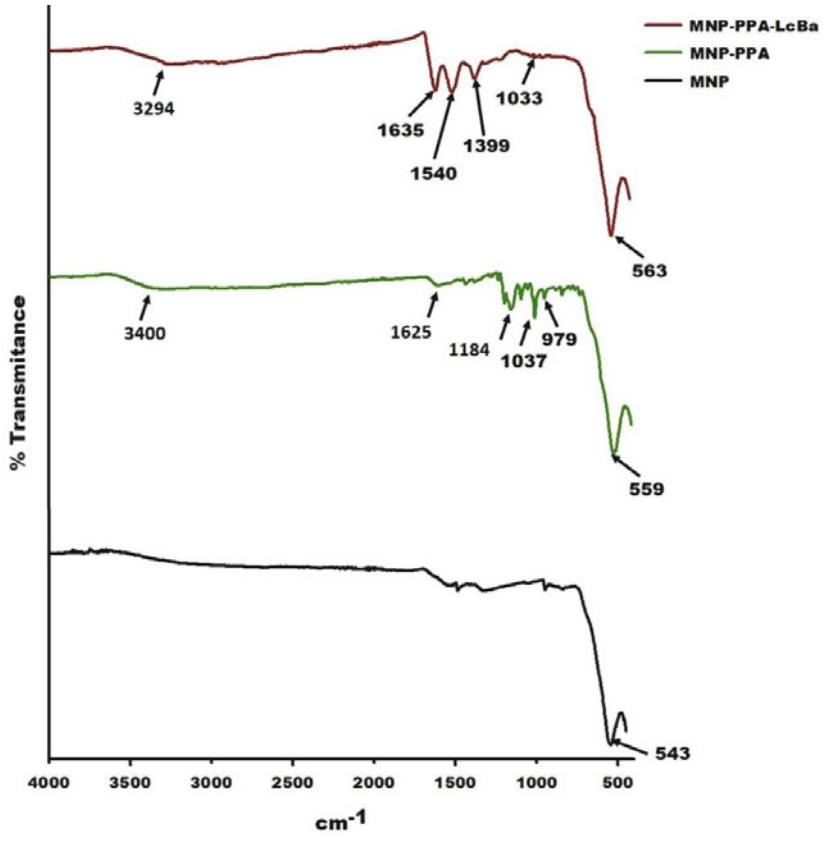

Fig. 6. FTIR spectra of Fe_3O_4 (MNP), Fe_3O_4-PPA, and Fe_3O_4-PPA-BaLc. MNP, Fe_3O_4; PPA, 3-phosphonopropionic acid; BaLc, *Bacillus atrophaeus* laccase. The characterization of surface modified nanoparticles was done in the spectral range of 450–4000 cm^{-1} by using FTIR-ATR spectrophotometer.

The zeta potential values were determined for Fe_3O_4 and Fe_3O_4-PPA nanoparticles as −46.9 mV and −47 mV, respectively. The negative zeta potential is due to the negatively charged hydroxyl and carboxyl groups at the surface of bare Fe_3O_4, and 3PPA functionalized Fe_3O_4, respectively. After immobilization of BaLc recombinant enzyme onto the functionalized nanoparticle, Fe_3O_4-PPA, the zeta potential was altered positively as -29.2 mV. The positive shift in the zeta potential of the nanoparticles indicates grafting of the enzyme on its surface (Patel *et al*., 2018).

FTIR spectra were recorded in the range of 450–4000 cm^{-1} to spot the functional groups in the naked and the surface modified Fe_3O_4 nanoparticles (Fig. 6). The FTIR peaks featured at around 550 cm^{-1} are ascribed to the Fe–O vibration. The bands located at around 3300 cm^{-1} and 1625 cm^{-1} correspond to the OH stretching and H–O–H bending vibration. The FTIR signals appeared in the spectral range of 1100–1000 cm^{-1} in the functionalized Fe_3O_4 nanoparticles are indicative of P-O and P-O-Fe stretching (Kalska-Szostko *et al*., 2013). The results confirmed 3-PPA grafting over Fe_3O_4 magnetic particles. The sharp peaks observed at around 1399 cm^{-1}, 1540 cm^{-1}, 1635 cm^{-1} can be owed to amide groups (Ulu *et al*., 2018). These step-wise changes in IR spectra, notified after the surface transformation of the iron nanoparticles, confirmed conjugation of the protein on the functionalized iron nanoparticles.

The optimum pH of the nanoparticle immobilized BaLc was similar to that of its free form (Table 1). However, the optimum temperature was altered by an increase of 5°C after immobilization (Table 1). This is in agreement with the previous studies demonstrating the enhancement in the enzyme's thermal tolerance after its attachment to the surface of a support matrix (Patel *et al*., 2018).

The kinetic parameter analysis specified an increase and decrease in K_m and k_{cat} values of BaLc (K_m 5.53 mM; k_{cat} 6.41 sec^{-1}), respectively, after emzyme's immobilization (Supplementary data Fig. S3 and Table 3). Similar changes in kinetic properties have been experienced in the previous studies performing laccase immobilization (Fortes *et al*., 2017; Salami *et al*., 2018; Wu *et al*., 2018).

The operational stability was examined by performing repeated catalytic reactions executed by the immobilized BaLc enzyme fraction, using ABTS as substrate. The nano-biocatalyst retained about 73% and 60% of its initial activity after 5 and 10 consecutive reaction cycles, respectively (Supplementary data Fig. S4). After 20 cycles, the residual activity was 26%. The recycling efficiency of BaLc was akin to the recent studies on covalently immobilized laccase, reporting the residual activity of about 75%, 61%, and 65–50% after 6, 8, and 10 cycles of its reuse, respectively (Xia *et al*., 2016; Fortes *et al*., 2017; Salami *et al*., 2018; Wu *et al*., 2018). The gradual decrease in the residual activity of the covalently linked enzyme could be due to inactivation of the enzyme during recycling (Wang *et al*., 2013).

Treatment of juices with immobilized BaLc

The BaLc magnetic beads were employed to treat BSJ, SSJ, and AFJ. The nano-biocatalyst was found proficient in reducing the phenolic content by 41–58%, lightening of the color by 49–59%, and removing the turbidity by 50–59%. Although, immobilization was observed to reduce the kinetic performance of BaLc, when tested with the standard substrate, ABTS (Table 3), however, as evident from the results above, it did not lead to the negative effect on juice clarification potential of the biocatalyst. Fortes *et al.* (2017) have

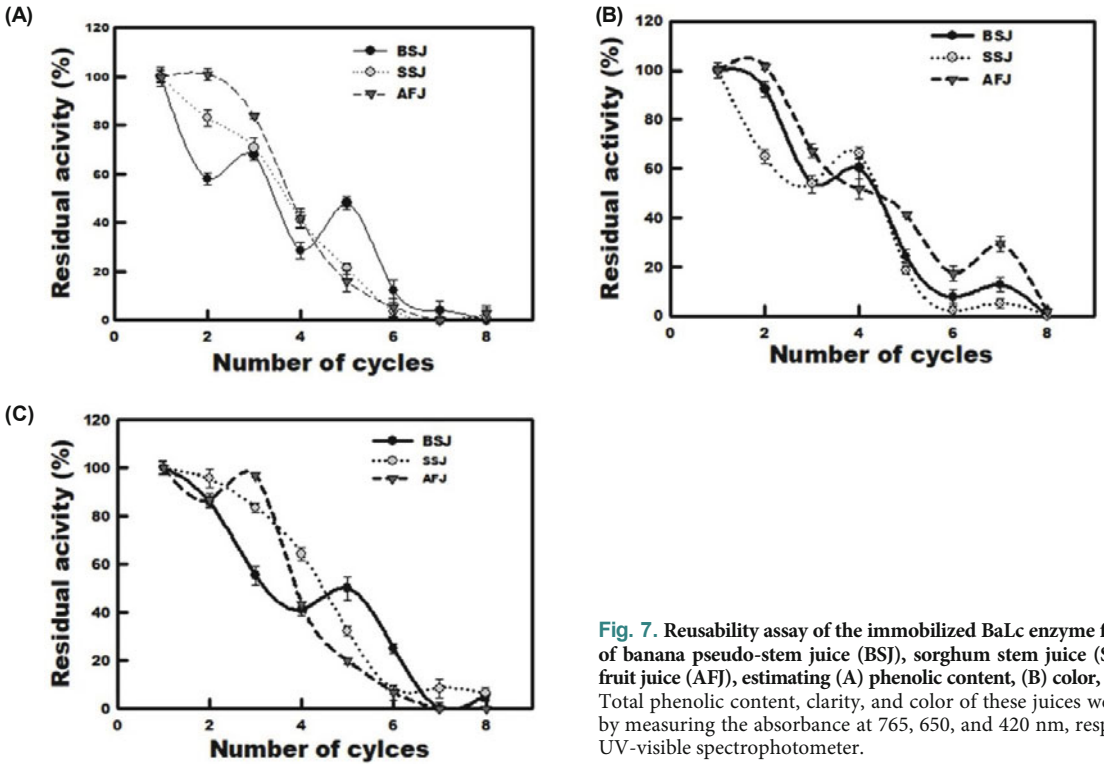

Fig. 7. Reusability assay of the immobilized BaLc enzyme for clarification of banana pseudo-stem juice (BSJ), sorghum stem juice (SSJ), and apple fruit juice (AFJ), estimating (A) phenolic content, (B) color, and (C) clarity. Total phenolic content, clarity, and color of these juices were determined by measuring the absorbance at 765, 650, and 420 nm, respectively, using UV-visible spectrophotometer.

demonstrated laccase immobilization on magnetic nanoparticles, but it was not employed for juice-clarification studies. Nevertheless, the performance of the nanoparticle immobilized catalyst was comparable with that of laccase immobilized on epoxy-activated poly-(methacrylate) matrix, which yielded 45% phenol reduction in fruit juice sample (Lettera *et al.*, 2016). In a study, the *Trametes versicolor* laccase was immobilized on magnetic bimodal carbon-based mesoporous composites and was demonstrated to remove about 80% phenol and p-chlorophenol from a solution (Liu *et al.*, 2012). However, this immobilized system was not employed to treat fruit juice sample.

Unlike free BaLc enzyme, the magnetic nanoparticle immobilized laccase can be recovered from the reaction system by applying the magnetic field, which provides the opportunity for reuse of the biocatalyst. Herein, the immobilized laccase was repeatedly used on juices for multiple duty cycles. After the first cycle of laccase treatment, the enzyme was washed with buffer and then reused to treat the fresh juice samples. The immobilized laccase was found to retain the residual activity of 29–42% for phenolic reduction, 52–66% for decolorization, and 41–64% for turbidity reduction, after 4 consecutive reaction cycles of its reuse during the juice treatment (Fig. 7). Recently, *T. versicolor* laccase immobilized to polyethylenimine (PEI) modified amine-functionalized iron nanoparticles has been demonstrated to retain 85% activity after six operational cycles of oxidation reaction of catechol (Xia *et al.*, 2018). However, it was not demonstrated to process any fruit juice. To the best of our knowledge, the present study is the first report on the use of magnetic particle immobilized laccase on plant part extracts or fruit juices.

Conclusion

In this study, for the first time, the laccase gene from *B. atrophaeus* was cloned and characterized. The use of enzyme for plant juice processing exhibited encouraging results of phenolic compound's oxidation, lightening of juices, and cloudiness removal. The iron nanoparticle was found compatible carriers for BaLc. The nanoparticle-enzyme conjugates were characterized by zeta-potential measurements and IR-spectra analysis. The immobilized enzyme molecules were kinetically characterized, and its reusability assay with ABTS suggested that 60% residual activity of the magnetic nanoparticle immobilized BaLc could be retained after 10 duty-cycles.

Furthermore, the covalently anchored enzyme molecules were recruited to treat the extracts from banana pseudo-stem, sweet sorghum stem, and apple fruits, which reduced the phenolic content, color, and turbidity at a considerable extent. To the best of our knowledge, this is the first report on the application of magnetic particle immobilized laccase in the processing of plant juices. The study concludes that magnetic nanoparticle-anchored laccase (BaLc) immobilized systems can be a potential catalytic tool for plant juice bioprocessing.

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Conflict of Interest

The authors declare no conflict of interest.

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