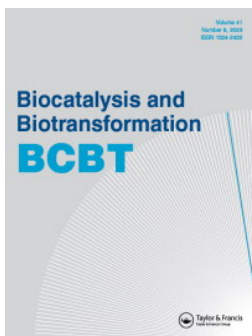

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RESEARCH ARTICLE

Thermo-active and alkaliphilic amalgamated laccase immobilized on sodium alginate for synthetic dye decolourization

Chiedu E. Edoamodu[a,b] and Uchechukwu U. Nwodo[a,b]

[a]Faculty of Science and Agriculture, University of Fort Hare, Alice, South Africa; [b]Applied and Environmental Microbiology Research Group (AEMREG), Department of Biochemistry and Microbiology, University of Fort Hare, Alice, South Africa

ABSTRACT

Purified crude laccase of *Enterobacter* sp. Kamsi and *Bacillus* sp. NU2 in the hybrid and combined form was immobilized on sodium alginate beads and applied to decolourize various textile dyes through several decolourization reaction cycles. The enzyme/alginate (E/A) loading efficiency and immobilization yield were evaluated. The SEM-EDX analysis, pH, and temperature effects of both forms of immobilized laccases were examined. The maximum condition for Kamsi and NU2 laccases into Na-alginate beads is 2.5% (w/v), which resulted to >83 and 61.71% immobilization and loading efficiency, respectively. The SEM-EDX analysis showed a rough-spherical surface attributable to significant entrapment of the laccase at the centre of the beads. The hybrid laccase in the free and immobilized form showed >52% and 80% decolourization effects, while the amalgamated laccase showed >63 and 83% decolourizing effect on Malachite Green (MG), Remazol Brilliant Blue R (RBBR), Reactive Blue 4 (RB4), Congo Red (CR), and Methyl Orange (MO), respectively. The immobilized laccase retained >50 and 81% activity of the hybrid and amalgamated laccases, respectively, after six successive treatment cycles. The results showed that the immobilization technique of the Kamsi and NU2 laccase holds the potential for textile dye effluents degradation, but the amalgamation of an enzyme from different species could improve decolourization potentials for various dyestuff treatments.

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Amalgamation; decolourization; hybrid; immobilization; laccase; textile-dye

Introduction

Despite enzyme non-hazardous and biological properties towards degrading environmental pollutants and reducing contaminants, enzymes still face some bottlenecks that restrict their pivotal functions. The hindrance in utilizing enzymes for an industrial and ecological scale is due to the high cost of isolation, purification, instability, low oxidation activity in varied pH and temperature conditions, and instability in organic medium. Several methods to overcome these drawbacks have been suggested. The immobilization techniques have been the most studied and applied methods for this cause (Popović et al. 2021; Rocha et al. 2021).

Enzyme immobilization provides outstanding enzyme durability towards varied substrates decolourization and environmental settings with better turnover for an extensive period of time (Datta et al. 2013). It is an effective and alternative process that offers various advancements for enzyme utilization in virtually all industrial processes (Zhang et al. 2021). Although the vast improvement in using different chemical applications of remediation, extensive attention has been turned towards applying immobilized enzymes for remediation purposes (Wang et al. 2018). Furthermore, the choice of the immobilized methods used, the carrier matrix, and the enzyme of interest to be immobilized are of crucial importance (Yavaşer and Karagözler 2021).

Various enzyme immobilization techniques have been proposed and utilized, such as entrapment/encapsulation, adsorption, covalent binding, and self-immobilization processes, but the entrapment/encapsulation seems the most promising applied techniques reported (Asgher et al. 2014). The nature of polymer that is majorly used in entrapment techniques is non-toxic and does not alter the native state of the enzyme, making it a good choice for immobilization (Bilal et al. 2016). Among the carrier support used, alginate matrix has been the most frequent support material applied bioscience for enzyme immobilization and micro-encapsulation, even in living cells. The

CONTACT Chiedu E. Edoamodu chieduuzoma@outlook.com

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alginate is an anionic polymer composed of α-L-guluronic acid and β-D-mannuronic acid that is cross-linked by calcium chloride ($CaCl_2$), barium chloride, or poly(L-lysine) (Daâssi et al. 2014). Enzyme entrapment in calcium alginate beads has been applied in several environmental matters and as a transport mechanism for split reagents, heavy metal absorbents, and the release of oxygen agents (Rodríguez Couto 2009; Kihun et al. 2014; Lee et al. 2014). This technique provides several advantages: simplicity in the applied process, cost-effectiveness, and its eco-friendly in nature (Osma et al. 2010; Olajuyigbe et al. 2019).

The active bioremediation process depends on the activity of the applied enzyme for the metabolic conversion of organic substrate to product. Oxidoreductases, such as laccases, monooxygenases, and dioxygenases have been chiefly used to biodegrade environmental pollutants (Bilal et al. 2021). However, laccase has received extensive attention due to its wide substrate variability, and it requires available molecular oxygen for activation (Imam et al. 2021).

Laccase (EC 1.10.3.2) is a class of compounds in the multicopper oxidoreductase family found in various organisms and plants. Laccase is evident in catalysing the oxidation of multiple arrays of organic pollutants, e.g. aromatic and phenolic compounds, etc., utilizing available molecular oxygen (Popović et al. 2021). Laccase catalytic mechanism entails oxidation of phenol and the release of molecular nitrogen without the formation of low weight aromatic amine. This mechanism of action makes laccase a suitable compound for detoxifying synthetic dyes and for bioremediation purposes (Imam et al. 2021).

Due to emerging pollutants in freshwater bodies, the health challenges involved, and the need for an enzyme with high redox potential to degrade diverse toxic and environmental effluents has motivated this research. In this study, laccase from *Enterobacter* sp. Kamsi and *Bacillus* sp. NU2 was purified and amalgamated together. The amalgamated (combine laccase from both bacteria species) and hybrid (uncombine extract laccase from each bacteria species) laccases were immobilized into carrier support. They were characterized and applied to treat five synthetic dyes.

Materials and methods

Materials

Bisphenol A (BPA), sodium alginate (Na-alginate), 2,2-azinobis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS), 1-Naphthol, 2,6-dimethoxyphenol (2,6-DMP), potassium ferrocyanoferrate (PFC), guaiacol, syringaldazine (SGZ), trichloroacetic acid (TCA), DEAE-Sephacyl S-200, DEAE-Sephacel, and Bradford reagent were purchased from Sigma-Aldrich, South Africa. Energy-dispersive X-ray spectroscopy (EDX; Thermo fisher scientific, Waltham, MA) with Scanning Electron Microscope (JEOL-JSM-6390LV) and UV/VIS spectroscopy microtitre reader (Synergy MX, Bio Tek, Winooski, VT) were used for this research.

Methods

The microbe

The bacteria used in this study; *Enterobacter* sp. Kamsi and *Bacillus* sp. NU2 were retrieved from the BioCat chest of Applied and Environmental Microbiology Research Group (AEMREG). The organisms were identified and deposited in the NCBI database with the assigned deposition numbers MN686603 and MN686607, respectively. The organism was resuscitated for further analysis.

Selective media composition for extracellular laccase production

To selectively choose the best media composition for laccase production, *Enterobacter* sp. Kamsi and *Bacillus* sp. NU2 were subjected to three minimal salt media (MSM) for extracellular laccase production. Briefly, the overnight bacteria cells were washed with normal saline and standardized to 0.5 McFarland at 600 nm. Then, 3% of the standardized cultures were inoculated separately into 50 mL of three different MSM (MSM1, MSM2, and MSM3). The MSM1 consists of (g/L) of 0.8-KH_2PO_4, 0.004-$MnSO_4.H_2O$, 0.5-$MgSO_4.7H_2O$, 4-glucose, 0.003-$CuSO_4$, 1-Yeast extract, 0.006-$FeSO_4.7H_2O$, 0.5-KCl, 0.02-$(NH_4)_2SO_4$, and 1-peptone, 0.009-$ZnSO_4.7H_2O$, and 0.03-$KNaC_4H_4O_6.4H_2O$. MSM2 contained (g/L)1-KH_2PO_4; 0.8- KH_2PO_4, 0.003-$MnSO_4.H_2O$, 0.572-$MgSO_4.7H_2O$, 4-fructose, 0.006 -$CuSO_4$, 0.05-Vanillin, 0.5-Yeast, 0.003-$FeSO_4.7H_2O$, 0.5-KNO_3, and 1-$NaNO_3$. The MSM3 comprised (g/L): 0.92-KH_2PO_4, 0.008-$MnSO_4.H_2O$, 0.5-$MgSO_4.7H_2O$, 4-Sucrose, 0.009-$CuSO_4$, 2-Yeast extract, 0.006-$FeSO_4.7H_2O$, 0.05-$(NH_4)_2$ SO_4, 0.004-$ZnSO_4.7H_2O$, and 0.07-NH_4Cl, 0.006-$FeCl_2.6H_2O$, 0.5-$C_8H_5KO_4$, and 0.101-Tryptone. Each of the media was added with 0.003% (w/v) 2,6-DMP and ABTS. The various media were measured at pH 6 and were incubated at 150 rpm under 30 °C for 7 d.

Laccase activity

The assay for laccase activity was carried out using the method of Edoamodu and Nwodo (2021a). The media was harvested after incubation and centrifuged at 12,500 × *g* under 4 °C for 10 min using the Avanti Centrifuge Instrument (Beckman Coulter, Inc., Brea, CA). The cell-free supernatant was aseptically pipetted out and assayed for laccase activity. The 150 μL reaction mixture contains 100 μL of 2 mM ABTS in 100 mM potassium phosphate buffer (pH 6), reacted with 50 μL crude laccase at 30 °C for 10 min incubation time. Subsequently, the reaction was stopped using 30 μL of 15% TCA and absorbance was monitored at 420 nm. The enzyme activity was calculated using the molar extinction coefficient $\varepsilon = 36{,}000\ M^{-1}\,cm^{-1}$ by applying the formula:

$$\text{Enzyme activity} = \frac{\text{Absorbance/min.} \times \text{Total volume mixture}}{\text{Time} \times \text{Extinction coefficient} \times \text{Volume of enzyme}}$$

Furthermore, protein concentration was estimated using Bradford (1976) method, and bovine serum albumin (BSA) was prepared as standard. All experiments were done in triplicates.

Purification

All listed purification steps were conducted at 4 °C, applying Olajuyigbe and Fatokun's (2017) method with slight modifications. After removing the bacteria cells by centrifugation, the 200 mL cell-free supernatant retrieved was subjected to a two-step ammonium sulphate purification process. Forty percent (40%) ammonium sulphate was added into the 200 mL crude supernatant under the stirring condition at 4 °C overnight, and the precipitated protein was retrieved by centrifugation (12,500 × *g*, 15 min). Then the collected protein was brought up to 80% saturation with ammonium sulphate solution under the same condition and centrifuged (12,500 × *g*, 20 min). The precipitate was dissolved in 100 mM phosphate buffer and dialysed against the same buffer, and the buffer solution was changed every 6 h at four consecutive cycles. Then the precipitated protein collected was loaded into the Econo-column (1.5 × 10 cm, Bio-Rad, Sandton, South Africa) prepared with DEAE-Sephacel of particle size 40 − 160 μm (Sigma-Aldrich) equilibrated with the 100 mM phosphate buffer of the particle size 40–160 μm. Unbound protein eluents were collected by washing with the same buffer solution at a slow flow rate of 20 mL/mm. Then bound protein was eluted using NaCl gradient (0.2–1.2 M) at a slow flow rate of 0.8 mL/mm under room temperature, and the eluents were texted for laccase activity (420 nm: 2 mM ABTS), and protein content was measured at A_{280}. The fraction with laccase activity was pooled together and concentrated with 4 M sucrose solution and subjected to final purification in prepared DEAE-Sephacryl S-200 gel of particle size 27–75 μm (Sigma-Aldrich, Johannesburg, South Africa) column calibrated with potassium phosphate buffer (100 mM pH 6). The eluents (1 mL) were then collected and examined for laccase activity and protein concentration.

Laccase amalgamation and substrate specificity

The purified bacterial laccases from "*Enterobacter* sp. Kamsi and *Bacillus* sp. NU2" were amalgamated by combining the purified laccase from bacteria strain Kamsi and NU2 together at a ratio of 1:1 (Ba et al. 2014). Substrate specificity of the amalgamated (Kamsi/NU2) and hybrid (NU2 and kamsi) laccases were determined using ABTS, 2,6 DMP, PFC, 1-Naphthol, and guaiacol for the best substrate oxidizing activity applying the standard method of Edoamodu and Nwodo (2021b).

Laccase immobilization

The immobilization assay was conducted using the entrapment technique by entrapping the purified laccases in the sodium alginate beads using a standard method (Niladevi and Prema 2008), with slight modification. Concisely, sodium alginate (2.5% w/v) solution was prepared in a conical flask and stirred overnight on a rotary shaker using a magnetic stirrer at room temperature. Then, the purified laccases were added to the sodium alginate solution separately at a ratio of 1:1 and thoroughly mixed for about 15 min. The viscous alginate-laccase solution was withdrawn with a 50 mL syringe and gently added dropwise into 2% $CaCl_2$ (Sigma-Aldrich, South Africa) solution to produce the beads and was allowed to harden for two hours at room temperature. The beads were further filtered and washed repeatedly with distilled water until no laccase activity was found. The percentage of immobilized efficiency was calculated using the formula below as described by Wang et al. (2018).

$$\text{Loading efficiency } (\%) = \left[\frac{(C_i V_i - C_f V_f)}{C_i}\right] \times 100$$

where C_i = initial protein concentration, V_i = volume of the initial enzyme solution, C_f = Protein concentration of the total filtrate, and V_f = Overall volume of

the filtrate

$$\%\,\text{immobilization} = \left(\frac{A_i - A_{wash}}{A_i}\right) \times 100$$

where A_i = initial activity of the free enzyme, A_{wash} = enzyme activity detected in the wash solution

$$\%\,\text{Laccase recovered} = \frac{T_i}{T_f}$$

T_i = Activity of the total immobilized enzyme, T_f = Activity of the total free enzyme.

The immobilized laccases were kept in distilled water under 4 °C upon use.

Characterization of the laccase immobilized beads

Analytical techniques

The characterization of the laccase beads was demonstrated using scanning electron microscopy (SEM) coupled with EDX spectroscopy. The laccase beads were air-dried for 5 d at room temperature to remove moisture content. The air-dried beads were attached to the stubs with two-sided adhesive tape, and a sputter coater for SEM was used in applying an ultra-thin aluminium gold on the beads. The coated beads were visualized using the SEM-EDX spectrum instrument.

pH and temperature effects on the free and immobilized laccases

The optimum pH and temperature of the free and immobilized laccases were evaluated using varying pH (3.0, 4.0, 5.0, 6.0, 7.0, 8.0, 9.0, and 10.0) and temperature conditions (30, 40, 50, 60, 70, 80, 90, and 100 °C) as described by Edoamodu and Nwodo (2021b). The laccase immobilized beads were subjected to various pH solution adjusted using sodium citrate (pH 3.0, 4.0, and 5.0), potassium phosphate (pH 6.0–7.0), Tris-HCl (pH 8.0), and glycine-NaOH (pH $9.0-11.0$). Likewise, the enzyme immobilized beads were also subjected to a preheated buffer solution with a temperature of $30-100$ °C. The substrate ABTS was used to monitor the relative activity at each consecutive cycle.

Laccase kinetics

The hybrid and amalgamated laccase both in the free and immobilized form were assayed for kinetic studies applying standard method (Olajuyigbe and Fatokun 2017) with slight modification. The Michaelis constant K_m V_{max} K_{cat}/K_M, were assayed utilizing ABTS substrate at a concentration ranging from 0.2 to 2 mM in a phosphate buffer of pH 6.0. Laccase kinetics was calculated from the Lineweaver–Burk plot drawn from the excel sheet.

Immobilization studies on five synthetic dye decolourizations

The ability for Na-alginate immobilized *Enterobacter* sp. Kamsi and *Bacillus* sp. NU2 laccases in the hybrid and amalgamated form to decolourize five synthetic dyes *viz:* Congo Red (CR), Remazol Brilliant Blue R (RBBR), Reactive Blue 4 (RB4), Malachite Green (MG), and Methyl Orange (MO) were studied over a 4 h treatment period (Daâssi et al. 2014). The stock solution of the dye (50 mg/L) was kept in a dark environment at room temperature before use. The 5 mL reaction mixture contains 10% (w/v) immobilized beads in pH 6 potassium phosphate buffer (100 mM), and the dye solution in an Erlenmeyer flask and was placed at 30 °C in the dark incubator. The control reaction mixture contains Na-alginate beads without laccase with the dye prepared under the same condition and assayed for dye colour removal by adsorption into the Na-alginate beads. Dye decolourization treatment was performed in triplicates, and treated dye solutions were aseptically withdrawn every 1 h, and the concentration was examined at their respective wavelengths using the UV/VIS spectroscopy microtitre plate reader (SynergyMX, Bio Tek, Winooski, VT). Decolourization of the synthetic dye was calculated using the following formula:

$$\%\,\text{Decolourization} = \frac{(A_{i-}A_f)}{Ai \times 100}$$

where A_i represents initial absorbance, A_f represents final absorbance.

Reusability studies

The Na-alginate immobilized beads were applied several times for different dye decolourization reactions to ascertain the reusability potentials of the laccase entrapped beads using a standard method (Bilal and Asgher 2015). In brief, the laccase immobilized Na-alginate beads were incubated in 10 mL of 50 mg/L synthetic dye solution and incubated in the dark for 30 min each. At the end of each cycle, the beads were withdrawn, washed with 100 mM potassium phosphate buffer (pH 6), and replaced in a fresh dye solution. The analysis was investigated on seven consecutive cycles, and the activity of the initial run was taken as 100%. All analyses were done in triplicate.

Result and discussion

Selective media for laccase production

The selection of salt components is a crucial path for the production of extracellular laccases in a quantifiable amount, as organisms respond differently to various molecular and inducer compounds regarding their source of isolation (Chauhan et al. 2017). This relates the influence of salts, trace metal, and pH variation as dependent factors that could scale up laccase yield on a commercial scale for industrial application. In this study, from the three MSM used for laccase production, NU2 and Kamsi MSM3 media gave laccase activity at the range of 1.4 and 1.7 U/mL, while MSM1 showed a considerable laccase yield of 28.52 and 29.51 U/mL on both bacteria producers (Figure 1). The MSM2 media optimally favoured laccase production with laccase activity of 54.47 and 32.1 U/mL (Figure 1) for NU2 and Kamsi strain, respectively. The remarkable laccase yield displayed by the isolates might be attributed to the conformation of the salt media containing all essential amino acids required for extracellular laccase biosynthesis (Deepa et al. 2020).

Furthermore, the high laccase yield could result from the unaltered conformational structure of the catalytic sites that are involved in laccase secretion. The lack of suitable fixation of an inducible compound at an early stage of commercial laccase bio-project might cause changes to the conformational structure of the microbes in the media, leading to the shutting down of the gene responsible for laccase biosynthesis.

Laccase purification

This study presents the four sequential purification steps applied to laccase from *Bacillus* sp. NU2 and *Enterobacter* sp. Kamsi, as shown in Table 1. The resulting protein from the second ammonium sulphate experiment resulted in 1.28 and 1.33-fold purification factors and total recovery protein of 28.69 and 24.74% for NU2 and Kamsi laccase, respectively. The concentrated dialysates loaded separately into the DEAE-Sepharose column gave an increased specific activity of 108.98 and 174.4 U/mg or NU2 laccase and 108.98 U/mg and protein yield of 3.35 and 4.69% for Kamsi laccase, respectively. Finally, the eluted fractions

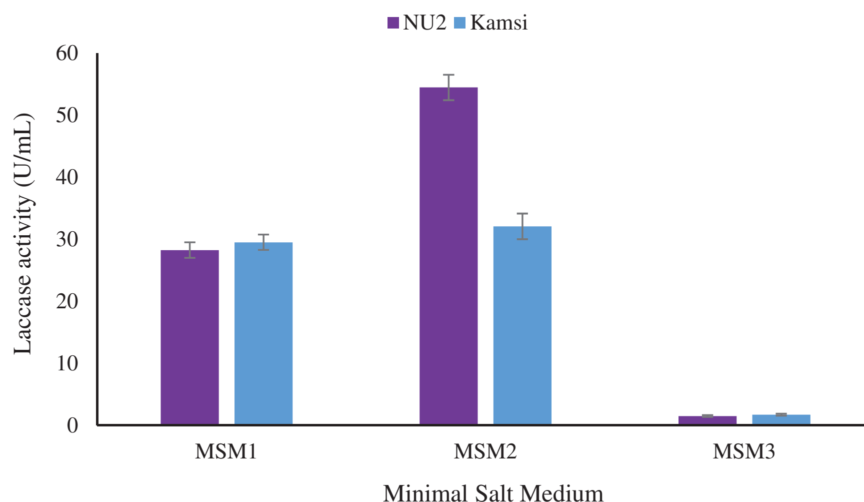

Figure 1. Quantified report for laccase produced under the selective minimal salt composition.

Table 1. Purification steps for *Bacillus* sp. NU2 and *Enterobacter* sp. Kamsi laccases.

Purification steps	Total volume (mL)	Total activity (U/mL)	Total protein content (mg)	Specific activity (U/mg)	Recovery (%)	Purification fold
		Bacillus sp.	NU2 laccase			
Crude enzyme	200	2500.86	53.991	46.32	100	1
Precipitation	19	717.495	12.059	59.49	28.69	1.28
DEAE-Sepharose	2.9	83.818	0.77	108.98	3.35	2.35
Sephacryl S-200	11.1	59.49	0.17	349.94	2.38	7.55
		Enterobacter sp.	Kamsi laccase			
Crude enzyme	200	3157.46	63.488	49.73	100	1
Precipitation	20	781.144	11.818	66.1	24.74	1.33
DEAE-Sepharose	5.2	148.214	0.85	174.4	4.69	3.51
Sephacryl S-200	14	85.08	0.39	218.15	2.69	4.39

loaded into DEAE-Sephacryl S-200 gel resulted in 2.38 and 2.69% laccase yield for NU2 and Kamsi, respectively, with an increased specific activity of 349.94 and 218.15 U/mg for NU2 and Kamsi laccases, respectively (Table 1). The high-specific activities of each bacteria species at the end of each purification step suggest their capability to secrete viable laccase commercially. The low laccase yield and purification fold could be due to the loss of enzyme activity at every purification step. It could also result from manipulation of the procedure step, leading to denatured proteins. Also, it could be due to the type of buffer used as some buffer contains some detergent that could denature protein (Dako et al. 2012). Moreover, potassium phosphate buffer entails the ability to inhibit enzymatic activity. However, the appropriate buffer solutions of desired ionic strength will be considered in future studies. In comparison to our study, Olajuyigbe and Fatokun (2017) reported a low enzyme yield of 3.9% and a purification fold of 2.84 of laccase from *Sporothrix carnis* CPF-05 of an initial crude enzyme of 1700 mL. However, the initial crude enzyme of 200 mL used in this study could have resulted in the low laccase yield obtained after a series of purification steps. Following our findings, Neelkant et al. (2020) reported an overall specific activity of 377.2 U/mg and a 9.69% yield from *Sphingobacterium* sp. Ksn-11.

Substrate specificity of hybrid and amalgamated laccases

The substrate specificity studies of the hybrid (NU2 and Kamsi) and amalgamated (Kamsi/NU2) laccase showed that both laccases elicited high catalytic activity against the non-phenolic substrates (ABTS and PFC) than other phenolic counterparts (Figure 2). Laccase activity of 50.16, 66.08, and 68.102 (U/mL) for ABTS and 45.21, 20.12, and 58.22 (U/mL) was evaluated for PFC (Figure 2). However, traced activity towards other tested substrates like the guaiacol and 2,6-DMP was observed, and a very low activity was examined on SGZ and 1-naphthol. Bacteria laccase is known for its wide substrate specificity (Unuofin et al. 2019), which was also observed in this study. Nevertheless, it was observed that the amalgamated laccase displayed wider catalytic activity on the selected substrates used than the hybrid laccases displaying activity of $>28.92 - 37.33$ (U/mL) on the phenolic substrates. This might result from the combined laccases' increased redox catalytic potential. Reiss et al. (2013) reported that the substitution model of phenol could be linked to laccase. The oxidation reaction rate can also be attributed to the disparities in the redox or ionization potentials of the laccase (Xu 1996; Medina et al. 2013).

Additionally, the catalytic activity of an enzyme differs amongst different substrates irrespective of the host of secretion; this could also be the response obtained from our result. Also, solubilities, the structural conformation of the laccase, substitutional pattern, pH, temperature, and other conditional factors may result in the high catalytic activity or reduced laccase activity observed (Medina et al. 2013). A comparative study using *Bacillus* sp. strain WT laccase showed high affinity towards ABTS than SGZ examined (Siroosi et al. 2016). Correspondingly,

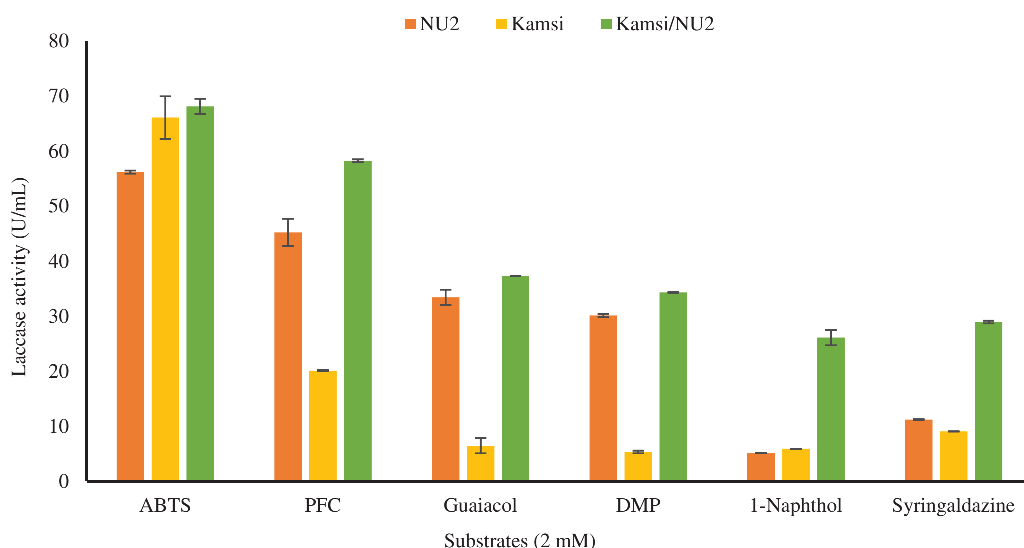

Figure 2. Substrate specificity studies of various phenolic and non-phenolic substrates on the hybrid and amalgamated laccase.

Stenotrophomonas sp. BIJ16 laccase substrate specificity assay conducted on phenolic and non-phenolic compounds displayed high catalytic efficiency on the non-phenolic compounds (ABTS and PFC) than other investigated substrates (Unuofin et al. 2019). The substrate specificity assay investigated suggests Kamsi and NU2 laccases may be suitable for treating phenolic effluents. The application of laccase in dye debasement has been studied (Bagewadi et al. 2017).

Immobilized laccase

A microcapsule having a rough spherical shape of the laccase immobilized beads was produced (Figure 3(a)) with the help of $CaCl_2$ cross-linkers as hardeners. The percentage loading efficiency (% value of the total entrapped enzyme) for Kamsi, NU2, and Kamsi/NU2 was 61.71, 85.90, and 86.13%, respectively (Figure 3(b)). The loading efficiency values of 60% and above are considered a desirable fit (Unuofin 2020), which was a continuous value in these reports. The optimal loading efficiency of the enzyme-alginate (E/A) and desirable fit examined on all laccase immobilized beads might be due to the high affinity of the $CaCl_2$ as a cross-linker, forming a polymeric complex with the beads (Ouwerx et al. 1998; Olajuyigbe et al. 2019). Unuofin (2020) reported a maximum loading efficiency of 88 and 90.11% at 2.5% (w/v) for *Achromobacter xylosoxidans* HWN16 and *Citrobacter freundii* LLJ16 laccases, respectively. Daâssi et al. (2014) presented a

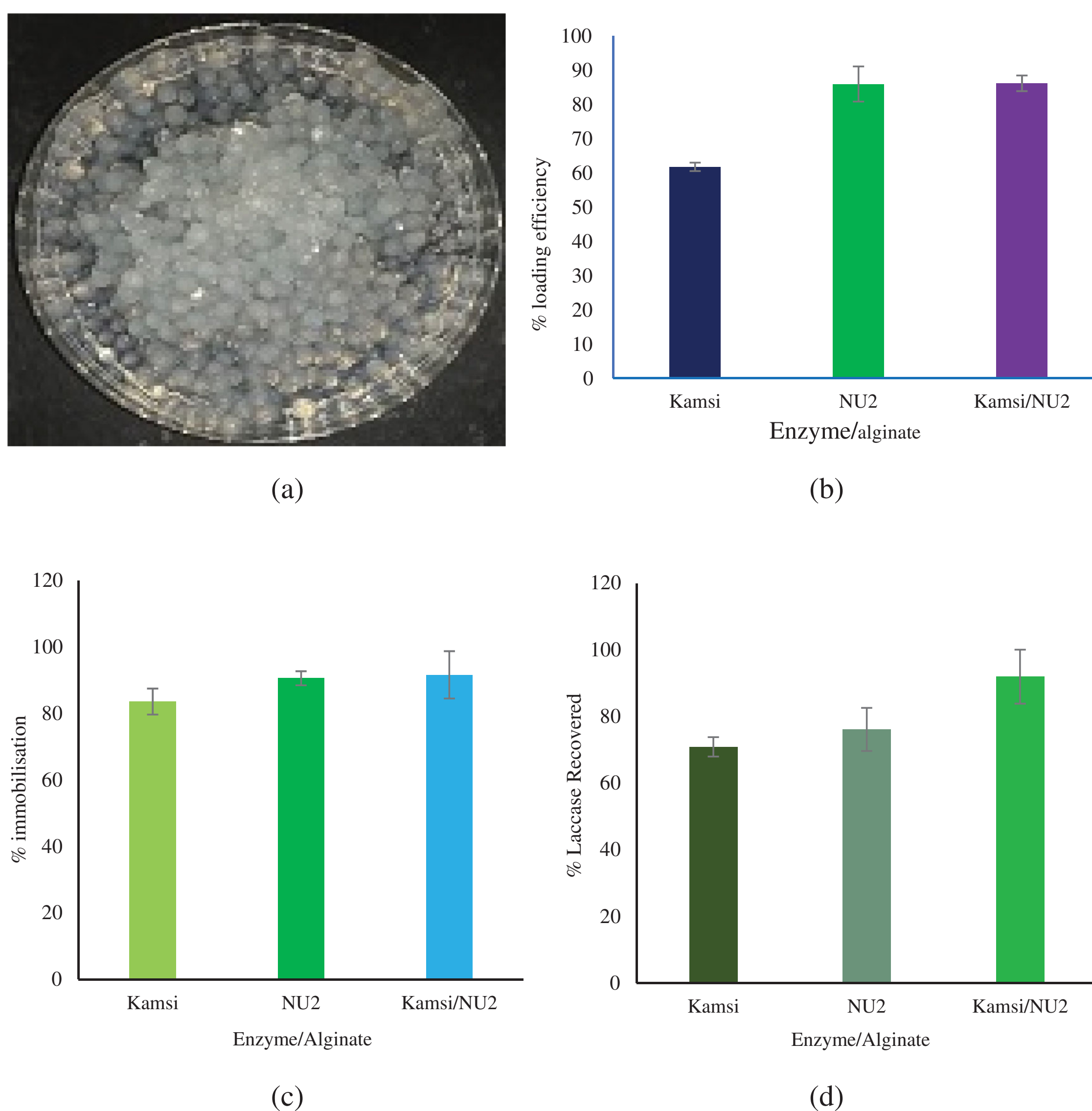

Figure 3. a–d: The immobilization assay of (a): the spherical display of Na-alginate/laccase immobilized beads formed, (b): % loading efficiency (c) % immobilization, and (d) % laccase recovered of hybrid (Kamsi, NU2) and amalgamated (Kamsi/NU2) laccases at 2.5% w/v Na-alginate solution.

maximal loading efficiency of 83% at 2.5% (w/v) sodium-alginate but inaccurately stated maximal efficiency at a lower concentration. However, at low concentrations (0.5% and 1% w/v), a larger pore size in the bead would be generated, leading to enzyme leakage (Daâssi et al. 2014). Also, an increase in Na-alginate concentration increases the viscosity of the homogenate leading to difficult enzyme entrapment (Geethanjali and Subash 2013). On the other hand, Riaz et al. (2008) found that immobilized beads form a larger pore size at lower Na-alginate concentrations and cause laccase efflux. Olajuyigbe et al. (2019) presented an optimum loading efficiency of 82% at 2.5% w/v alginate solution. Moreover, the optimum value from the concentration stated in this study is in line with that of Geethanjali and Subash (2013), Olajuyigbe et al. (2019), and Unuofin (2020).

The percentage immobilization, percentage loading efficiency, and laccase recovery of Kamsi, NU2, and the amalgamated (Kamsi/NU2) laccases is depicted in Figure 3(c,d). The calculated parameters showed that percentage immobilization for kamsi, NU2, and Kamsi/NU2 were 83.6, 90.63, and 91.66 (%), respectively (Figure 3(c)), while laccase recovered were 70.91, 76.13, and 91.99 (Figure 3(d)), respectively. Daâssi et al. (2014) reported a percentage immobilization of 93.3%. Unuofin (2020) stated a 91.78 and 91.062% immobilization and laccase yield of 90.09 and 89.43 on the two bacteria species used in his study. This resulted in the optimum values depicted in this study and may result from the 2.5% w/v alginate solution used.

SEM-EDX instrumental studies

The immobilized laccase Na-alginate beads and the control (Na-alginate beads) were characterized using the Emission SEM with Electron Dispersive X-ray (EDX). The sizes of the encapsulated beads fall between 1.14 and 2.34 mm, while the control falls within the range of 1.22 − 2.83 mm. Won et al. (2005) reported that enzyme activity increases with reduced microcapsule size due to less mass transfer resistance. In addition, the smaller the immobilized beads, the higher potential of the enzyme adsorption with regards to increased surface/volume area ratio, which could be more efficient in the wastewater treatment process (Lu et al. 2007). Furthermore, the cross-sectional surface area of the laccase/Na-alginate beads showed a different structural image from the control. Laccase/Na-alginate beads displayed a uniformly distributed surface with evidence that the $CaCl_2$ was homogeneously circulated, forming a cross-link or bonds with the alginate monomers as observed by SEM (Figure 4(a–d)). In addition, the SEM image showed a very rough surface on the beads as compared to that of the Na-alginate beads. The occurrence of the rough surface signifies the existence of a physical barrier on the immobilized beads, indicating the Na-alginate beads' enhanced potential to retain and prevent efflux of the entrapped laccase. It also showcases proper transport of laccase to the centre of the beads, thereby maintaining the enzyme stability and activity (Gülay and Şanli-Mohamed 2012). Also, the fractures on the surface of the enzyme immobilized beads may well enhance the mass

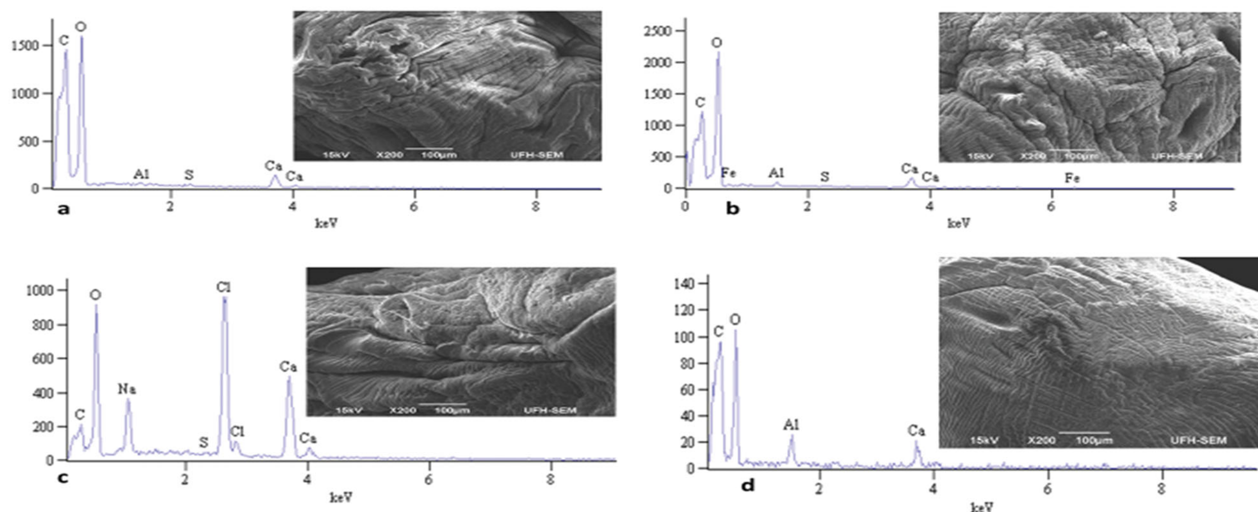

Figure 4. a–d: SEM-EDX micrograph of sodium alginate beads (a) Kamsi-laccase/Na-alginate immobilized analysis; (b) NU2-laccase/Na-alginate immobilized (c) amalgamated (kamsi/NU2) laccase/Na-alginate beads and (d) The control (Na-alginate) prepared in calcium chloride solution.

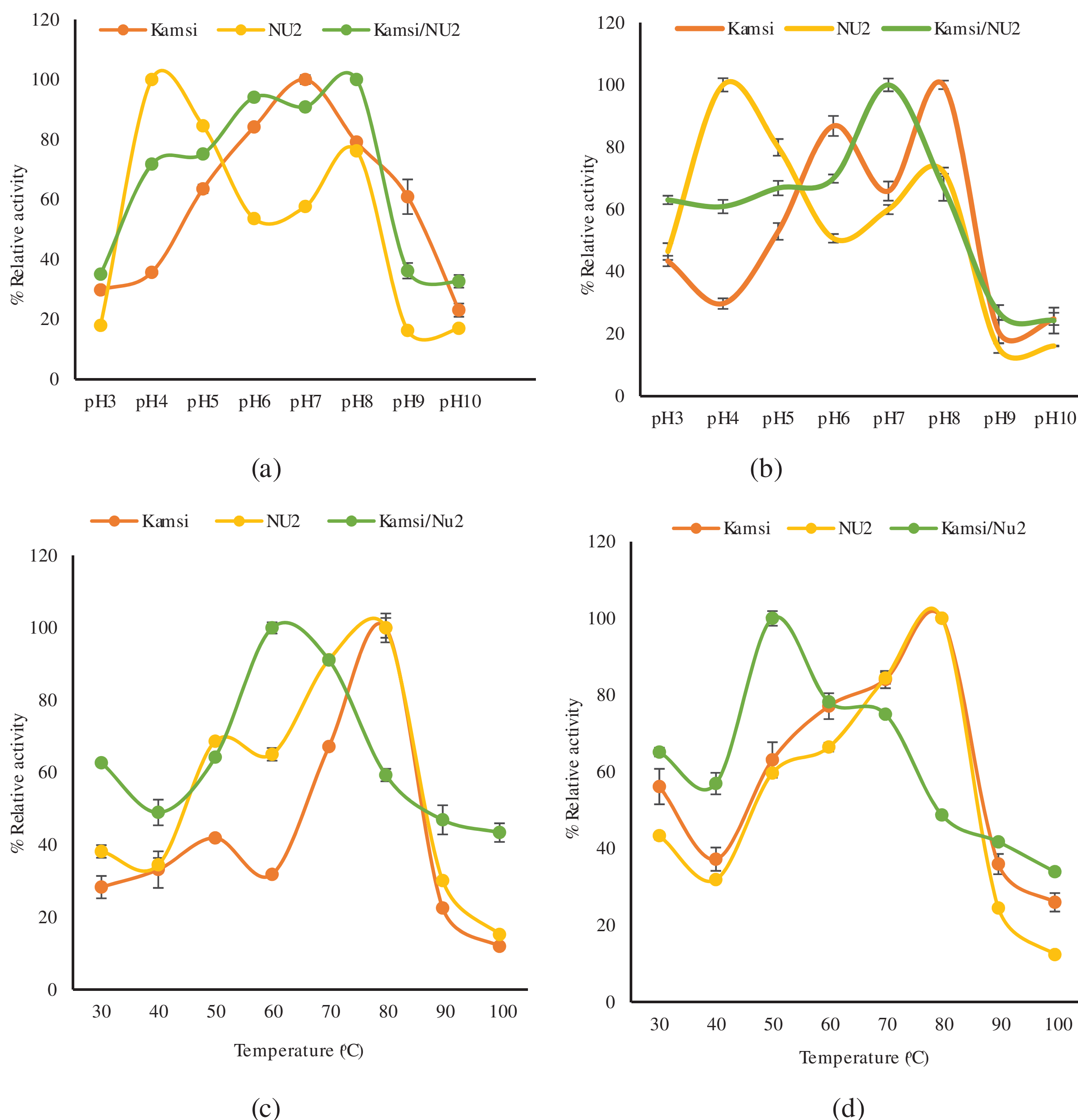

Figure 5. a–d: The effect pH variation and temperature parameters on (a and c) free laccases and (b and d) immobilized laccases.

transfer rate of the substrate and stimulate the elimination of toxic waste from contaminated environments (Wang et al. 2019). This structural formation might be due to the concentration (2.5% w/v) of the alginate used and the type of cross-linker agent ($CaCl_2$) used.

Furthermore, EDX analysis on the immobilized beads conducted shows the presence of SiO_2 and Al_2O_3, which are major components of Na-alginate. The EDX result revealed various trace salts and metals (Figure 4(a,b)). Through this characterization, one could demonstrate the structural properties of laccase/Na-alginate beads for bioprocess applications.

Effects of pH and temperature on laccase activity

Figure 5(a–d) demonstrates the enzyme relative activity of the free and immobilized laccases in varied pH and temperature conditions. The optimum enzyme activity free purified laccase was observed at pH 4, 7, and 8 for NU2, Kamsi, and Kamsi/NU2, while optimum activity was denoted at pH 4, 7, and 8 for NU2, Kamsi/NU2, and Kamsi immobilized laccases (Figure 5(a–d)). In the free and immobilized form of the amalgamated laccase, a remarkable resistance in acidic and alkaline (pH $4-8$) conditions was observed, with enzyme activity >47% showing the ability to withstand certain

acidic and alkaline regimes (Figure 5(a,b)) than the hybrid laccases. Laccase behaves differently in varying conditions and most times with regards to their host of secretion (Olajuyigbe et al. 2019). However, this might have resulted in the significant attributes of the combined laccases (Kamsi and NU2) in response to their increased redox potentials and observed catalytic strength. Also, it could be due to the charge supports that attract or repel substrate and products (Olajuyigbe et al. 2019). In addition, the increased relative activity of the immobilized laccases compared to free laccases observed might be due to reduced autolysis of the enzyme. Also, it might be due to conceivable stabilization of the entrapped laccase in the Na-alginate material, which must have resulted in the improved substrate binding capacity at the laccase active sites (Almulaiky and Al-Harbi 2022). Furthermore, the decreased enzyme activity detected by the free and immobilized laccase at a higher regime could be ascribed to enzyme inhibition resulting from hydroxide ion bonding to the copper centre of the laccase. Narayanan et al. (2015) reported a maximal relative activity on pH 9 for *Bacillus subtilis* 2414. Maximal pH activity at pH 5 and 6 on *Cyberlindnera fabianii* laccase has been reported (Olajuyigbe et al. 2019).

Temperature parameters of the free laccases showed maximal activity at 60 °C for amalgamated laccase (Kamsi/NU2) and 80 °C for the hybrid laccases (Kamsi and NU2), while the immobilized laccases were optimum at 50 °C (Kamsi/NU2) and 80 °C (Kamsi and NU2). The amalgamated laccase displayed a considerable thermal resistance at varying temperature regimes in the free and immobilized form. However, optimum thermal resistance was denoted on the hybrid laccases (Figure 5(c,d)). Nonetheless, at 90 − 100 °C amalgamated laccase showed an activity >46–43% and 33 − 41% in the free and immobilized form of the enzyme. In addition, the hybrid laccases displayed 15 − 30% and 11 − 25% in the free state for NU2 and Kamsi, while NU2 and Kamsi immobilized laccase gave 26 − 35% and 12 − 24% relative activity for NU2 and Kamsi laccases. However, the measurable enzyme activity exerted by the immobilized laccases at varying temperature conditions might be related to the Na-alginate matrix's resistance, resulting in an unaltered or denatured protein structure at a high-temperature regime. The decreased enzyme activity observed at the free and immobilized laccase might have occurred from protein denaturation upon continuous heating leading to structural deterioration of the active sites (Aldhahri et al. 2021). Likewise, an increase in the enzyme activity of the immobilized laccase than free laccase has been reported (Narayanan et al. 2015). Free and immobilized laccase from *C. fabianii* showed considerable enzyme activity at 40, 50, and 60 °C (Olajuyigbe et al. 2019). The thermotolerant and broad pH resistance showcased by the free and immobilized laccase suggests its suitability in biotech control. However, the remarkable enzyme activity displayed by the amalgamated laccases over broad pH and temperature factors suggest a potential technique to overcome limitations and promote industrial bioeconomy globally.

Laccase kinetics

Table 2 shows the laccase kinetics of the free and immobilized laccase studied at varying ABTS concentrations. The *Km* and *V*max values of the free and immobilized laccases are 0.19, 0.38, and 0.69 (mM^{-1}) for NU2; Kamsi, and the combined laccases (Kamsi/NU2), while 0.239, 0.401, 0.763 (mM^{-1}) were denoted for the immobilized laccases *viz:* amalgamated (Kamsi/NU2), and hybrid (Kamsi, NU2) laccases. The *V*max value calculated was 50, 50.76, 50 (μM/min/mL) for the free laccases (Kamsi/NU2; Kamsi, and NU2), while 23.47, 33.16, 29.06 (μM/min/mL) was denoted for immobilized laccases (Kamsi/NU2; Kamsi, NU2). The value of *Km* and *V*max expressed by the free laccases followed by the immobilized amalgamated laccases and the hybrid laccases, shows the enzyme affinity for the substrates (ABTS). The higher *Km* value expressed by the immobilized laccase over the free laccase might be due to the modification of the functional group at the catalytic site of the enzymes (Bilal et al. 2016). Furthermore, the lower *Km* value expressed by the

Table 2. Hybrid and amalgamated laccase kinetics on varying ABTS concentration.

Laccase strain	K_m (mM)	V_{max} ($\mu M/min^{-1}/mL^{-1}$)	K_{cat} (min^{-1})	K_{cat}/K_m (mM^{-1}/min^{-1})
		Free laccases		
Bacillus sp. NU2	0.19	50	4.53	23.92
Enterobacter sp. Kamsi	0.3858	50.76	4.61	11.95
Kamsi/NU2	0.695	50	4.55	6.55
		Immobilized		
Na-Alginate/NU2	0.239	23.47	3.12	14.72
Na-Alginate/Kamsi	0.401	33.16	4.53	20.14
Na-Alginate/Kamsi/NU2	0.763	9.06	4.09	7.85

combined laccases (Kamsi/NU2) could be due to negligible diffusion of substrate and improved aptitude of Na-alginate microspheres to capture a high product concentration. On the contrary, *Bacillus* sp. strain WT showed a lower *Km* and high *V*max catalytic activity towards SGZ than ABTS reaction. Correspondingly, *Bacillus licheniformis* displayed a lower *Km* and high *Kcat/Km* kinetic value on ABTS (Koschorreck et al. 2008). The lower *Km* values expressed by free and amalgamated laccase demonstrate the enzyme higher affinity on ABTS substrates. A lower *Km* value on the free laccase than immobilized laccase with a higher *V*max value of free laccase than the immobilized laccase from *C. fabianii* was reported by (Olajuyigbe et al. 2019). The variation in *Km* and *V*max values of laccase from diverse microbial species could be due to genetic diversity among the species (Bonugli-santos et al. 2010; Janusz et al. 2015).

Synthetic dye removal studies by free and immobilized laccase

To investigate the catalytic potential of the free and immobilized laccase from *Enterobacter* sp. Kamsi and *Bacillus* sp. NU2 as a biocatalyst to efficiently decolourize five synthetic dyes (MG, RBBR, RB4, MO, and CR) was studied over a four-hour incubation period. From the data depicted (Figure 6(a–e)), the decolourization rate was gradually enhanced with an increase in the treatment period. The decolourized catalytic oxidation rate was maximum at 4 h of incubation on all laccase treated dye solutions, maintaining a removal level of >81.29% on the free and immobilized laccases. However, the free laccase showed a disparity in their decolourization pattern across the treatment time. The variation in the dye removal potentials displayed by the free laccase might result from low redox potentials since the dye was not a direct substrate for the laccases (Qiao and Liu 2019). Hence, the amalgamated (Kamsi/NU2) free and immobilized laccases elicited maximum decolourization activity of 77.89%, 93.95% (MG); 74.69%, 91.12% (CR); 63.16%, 89.68% (RBBR); 86.04%, 90.61% (RB4); and 77.96%, 83.61% (MO) at 4 h treatment period (Figure 6(a–e)). In control, Na-alginate beads were able to eliminate >11.02, 12.87, 13.24, 20.21, and 29.03% of MO, RBBR, CR, MG, and RB4, synthetic dyes, respectively, while the control on RB4 displayed a significant decolourization rate >36.34–69.37% compared to the other counterpart reaction (Figure 6(c)). Laccase from *Coriolopsis gallica* immobilized in Ca-alginate beads efficiently decolourized RBBR, Reactive Black 5 (RB5), Bismark Brown R (BBR), and Lanaset Grey (LG) to a maximum extent (Daâssi et al. 2014). The enhanced dye removal pattern attributed by the immobilized laccase over the free laccase might be a result of the synergistic effects between the enzyme and the carrier matrix. Nonetheless, the varied decolourization level shown by both free and immobilized laccase might be due to the enzyme source of extraction, carrier support, immobilization process, and treatment system (Daâssi et al. 2014). In addition, significant decolourization effects with an increase in treatment time on MG by immobilized *Bacillus subtilis* laccase has been reported (Qiao and Liu 2019). Furthermore, the remarkable degrading potential effect of the laccase/Na-alginate on the various dyes might be due to the enzymatic biodegradation or bioaccumulation or biosorption of the synthetic dyes into the alginate beads (Rodríguez Couto 2009; Daâssi et al. 2014).

A control reaction containing Na-alginate beads without laccase was prepared to determine the possibility of colour removal regarding dye adsorption into the immobilized beads. The dye removal pattern was investigated by accessing the colour of the beads up to the treatment dye process. It was then detected by the colour produced by the alginate beads after coming in contact with the synthetic dyes (picture not shown).

Successive batch decolourization cycles

Unlike the free enzyme, immobilized laccase can be reclaimed from a treatment process and reapplied, which is cost-effective for industrial settings. The reusability pattern of laccase/Na-alginate beads was evaluated through eight consecutive batches at 12 h each for the biodegradation of five synthetic dyes (MO, RB4, RBBR, CR, and MG), and to determine its stability with the residual activity as depicted in Figure 7(a–e). It was evaluated that the immobilized laccases showed remarkable decolourization potentials on the five synthetic dyes (MO, RB4, RBBR, CR, and MG) through eight successive circles applied (Figure 7(a–e)). After the initial decolourization step at 100%, a result >46% residual activity was retained on all laccase/Na-alginate immobilized beads. Additionally, it was observed that after the 6th cycle, the relative decolourization levels maintained were >66%, >53%, >50%, and >47%, for MO, RB4, RBBR, and CR with the immobilized amalgamated laccases giving a maximum decolourization value >81% (Figure 7(a–d)). In addition, a maximum relative dye removal potency was depicted at the 5th cycle maintaining a relative activity of >60% with the immobilized amalgamated laccases showing dye

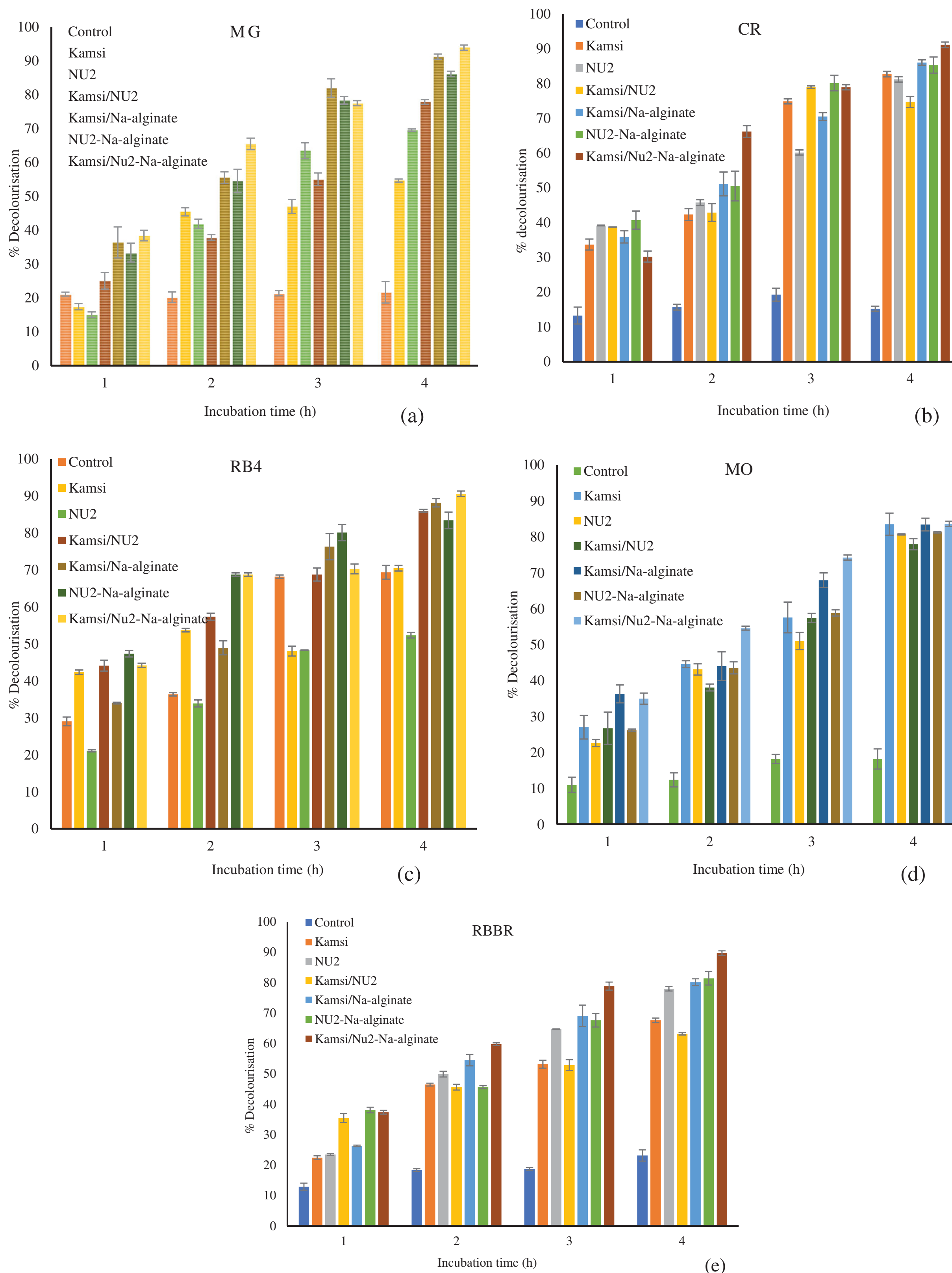

Figure 6. a–e: Synthetic dye decolourization time course study, (a) Malachite green (MG); (b) Congo Red (CR); (c) Reactive Blue 4 (RB4); (d) Methyl Orange (MO); and (e) Remazol Brilliant Blue R (RBBR), by free and immobilized laccases.

decolourization ability of >82% (Figure 7(e)). However, a substantial increase in synthetic dye's decolourization activity on further reaction cycles was observed. At the end of the five-reaction cycle, the immobilized beads were able to retain >55% residual activity on all immobilized laccases. This study observed that the

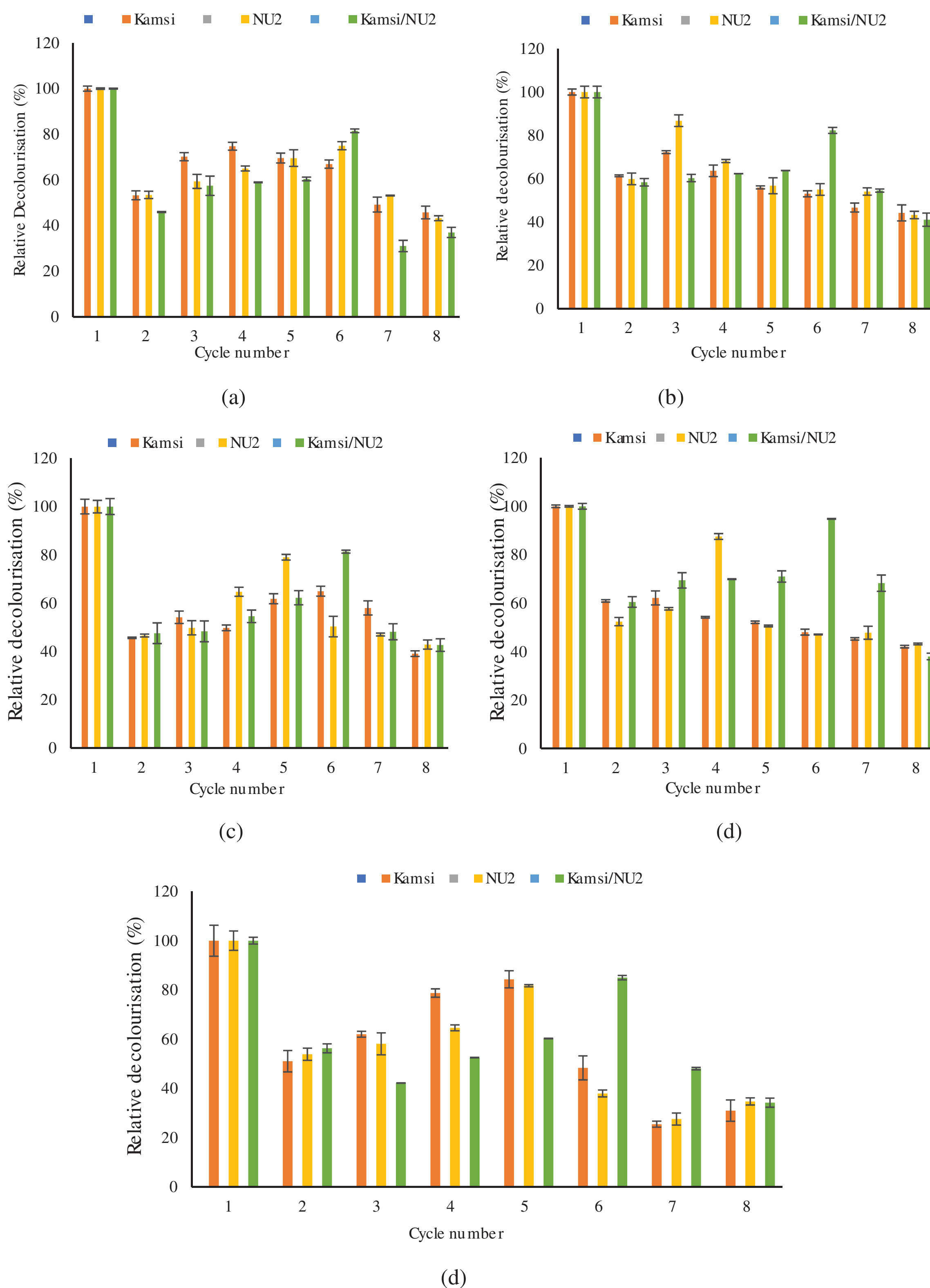

Figure 7. a–e: Successive decolourization cycles by Na-alginate immobilized laccases in (a) Methyl Orange, (b) Reactive Blue 4, (c) Remazol Brilliant Blue R, (C) Congo Red, and (D) Malachite Green reaction conditions through eight (8) decolourization cycles, 12 h each.

decolourization rate increases with an increase in the decolourization process, which might have occurred as a result of enzyme stability and activation on successive reuse. However, the gradual declination observed could be due to enzyme inactivation. Also, the continuous reuse and the reoccurring enzymatic

catalytic reactions might have resulted in decreased pore size, leading to difficult diffusion of the substrate and product in the Na-alginate matrix. Anwar et al. (2009) highlighted that leakage could occur during continuous reuse of an immobilized enzyme, causing reduced activity in the decolourization effects. An immobilized laccase was able to retain about 70% activity after four successive cycles in a report by Daâssi et al. (2014). Mogharabi et al. (2012) reported 85% retained activity on laccase alginate after five reaction cycles.

Conclusion

In this study, hybrid and amalgamated laccase from *Enterobacter* sp. Kamsi and *Bacillus* sp. NU2, both in the free and immobilized form, elicited tremendous enzyme activity displaying thermo-alkaliphilic properties through varied pH and temperature parameters tested. Furthermore, the hybrid laccase denoted a remarkable enzyme activity at pH 4, pH 7, and thermal activity at 80 °C. The free and immobilized hybrid laccase showed a decolourizing effect of >54% on all textile dyes after 4 h treatment period. The amalgamated laccase displayed maximum decolourization effect both on the free and immobilized laccase with >63% and >83% oxidizing activity. Consequently, after six reaction cycles, the relative decolourization levels were maintained at >66%, >53%, >50%, and >47%, for MO, RB4, RBBR, and CR with the immobilized amalgamated laccases giving a maximum decolourization value of >81%. In addition, the immobilized laccases elicited an efficient synthetic dye removal pattern through the number of succeeding batches. However, the amalgamated laccase could provide more catalytic oxidation potential in bioprocess control. This might help enhance the bioeconomy and offer a cost-effective approach in large-scale applications.

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Author contributions

CEE executed the research and prepared the first manuscript draft. UUN conceived and planned the research, funded and supervised the research. Prepared the final draft of the manuscript.

Disclosure statement

The authors declare no conflicts of interest.

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