Characterisation and optimisation of a novel laccase from *Sulfitobacter indolifex* for the decolourisation of organic dyes

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Abstract

Laccases are multi-copper oxidases that possess the potential for industrial wastewater treatments. In this study, a putative laccase from *Sulfitobacter indolifex* was recombinantly produced and characterised. The enzyme was found to be stable and active at low to ambient temperature and across a range of pH conditions. The ability of the putative bacterial laccase to catalyse the decolourisation of seven common industrial dyes was also examined. Our results showed that the putative laccase could efficiently decolourise Indigo Carmine, Coomassie Brilliant Blue R-250, Congo Red, Malachite Green and Alizarin in the presence of 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) as a redox mediator. Furthermore, the use of enzyme immobilisation technology to improve the operational stability and reusability of the putative laccase was also investigated. We found that immobilising the enzyme through the cross-linked enzyme aggregates (CLEA) method significantly improved its tolerance towards extreme pH as well as the presence of organic solvents. This work expands the arsenal of bacterial laccases that are available for the bioremediation of dye-containing wastewater.

Keywords

Bacterial laccase; Bioremediation; Dye decolourisation; Enzyme immobilisation; Enzyme technology

1 Introduction

Since the discovery of mauveine by the English chemist William Henry Perkin in 1856, synthetic dyes have found applications in almost all everyday items of our lives, from plastics to papers, foods to cosmetics, textiles to pharmaceuticals [1,2]. The heavy use of synthetic dyes in industry has created a huge pollution problem. For example, significant amount of dyes, with reported concentration ranges from 10 mg/L to 7000 mg/L, have been found in textile effluents around the world [3]. Much of these dyes contain toxic properties, causing significant negative impact on the aquatic biota, human health and the environment [4,5].

The most common ways to remove dyes from wastewater include physical methods, such as filtration, coagulation and adsorption, and chemical methods, such as ozonation, photochemical and electrochemical degradation, and chemical oxidation [5–10]. However, these methods are far from ideal. For example, filtration and coagulation often generate a large amount of sludge that may require complicated and costly secondary treatments, and chemical oxidation methods typically involve the use of heavy metal catalysts, which may suffer from undesirable drawbacks such as the generation of hazardous by-products [5–10]. In contrast, the use of microorganisms and their enzymes for dye decolourisation may offer advantages over conventional physico-chemical approaches [9–11]. Biocatalysts are not only bio-renewable, they avoid the use of heavy metals and enable the reaction to be performed at relatively mild conditions [12,13]. Thus, bioremediation of dye-contaminated wastewater holds the potential to be a more sustainable alternative to the existing physico-chemical approaches.

Laccases (benzenediol:oxygen oxidoreductase, EC 1.10.3.2) belong to a group of enzymes that have found increasing interest as biocatalysts for bioremediation in the last decade [14–16]. Laccases are multi-copper oxidases found in a diverse range of organisms including plants, fungi and bacteria. They catalyse single electron oxidation of substrates containing phenolic or aromatic amine groups accompanied by the reduction of O_2 to water [16–19]. Laccases typically contain four copper ions in

their active site. The copper ions occupy three distinct binding sites, which can be classified as Type 1 (T1), Type 2 (T2) or binuclear Type 3 (T3) based on their spectroscopic characteristics. T1 and T3 coppers absorb light at 600 and 330 nm respectively, while T2 copper lacks significant absorption features [16–19]. Substrate oxidation takes place at the T1 site and the electrons get rapidly transferred to the tricopper T2/T3 ensemble, where O_2 is reduced [16–19]. The redox potential of the T1 site usually varies from 0.4-0.5 V in plants and bacterial laccases to 0.7-0.8 V in fungal laccases [20]. As such, fungal laccases were the initial focus for industrial and bioremediation applications [21–23]. Interest on the use of bacterial laccases for bioremediation has increased significantly in recent years as bacterial laccases can be efficiently expressed in prokaryotic hosts and many bacterial laccases have shown to exhibit stability over a wide range of operational conditions [24–27]. Hence, the adaptability and versatility of bacterial laccases may give them an edge over fungal laccases.

To date, a number of bacterial laccases, such as those from *Escherichia coli, Streptomyces ipomoeae, Bacillus subtilis, Bacillus pumilus* and *Klebsiella pneumoniae*, have been characterised [28–31]. However, most of the bacterial laccases that have been studied to date operate at above moderate to high temperatures [28–31]. Laccases from bacteria that can operate at low to ambient temperatures remain underexplored. The frigid environments of some marine ecosystems can moderate enzymes to acclimatise to colder working conditions as has been observed with some marine fungal enzymes [32,33]. Recently, a cold-adapted enzyme isolated from an uncultured bacterium extracted from the South China Sea exhibited laccase activity with high tolerance for pH, temperature and organic solvents [34]. Inspired by this study, we set out to identify putative bacterial laccases that may allow stability and activity in cold to ambient environments. Herein, we report our work in the production and characterisation of a putative laccase ($pLac_{Si}$) from a marine bacterium, *Sulfitobacter indolifex* [35,36]. $pLac_{Si}$ was recombinantly expressed in *E. coli* BL21 (DE3) competent cells. Its pH, temperature and solvent tolerance profiles were characterised. By using Alizarin, Acid Red 27, Congo Red, Bromophenol Blue, Coomassie Brilliant Blue R-250, Malachite Green and Indigo Carmine as

model dyes, the ability of pLac$_{Si}$ to decolourise these organic dyes was then explored. Finally, the immobilisation of the enzyme to enhance its stability and reusability was also studied.

2 Materials and methods

2.1 Materials

All chemicals were purchased from Sigma-Aldrich, AK Scientific, Thermo Fisher Scientific and Bio-Rad unless otherwise stated. Plasmid (pET-28a) encoding pLac$_{Si}$ was purchased from GenScript **(Fig. 1 and Supplementary Table S1)**, which allows the recombinant production of pLac$_{Si}$ with an N-terminal polyhistidine tag (MHHHHHH) along with a thrombin cleavage site (LVPRGS).

2.2 Sequence alignment search

The National Center for Biotechnology Information (NCBI) Basic Local Alignment Search Tool (BLAST) was used for the protein sequence alignment search. The input sequence was Lac1326 [34] **(Fig. 1)**.

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Sulfitobacter indolifex   MNRRQFLASASALAILPLQASAAADPLKLTAEAVTRQLLPKSEGTTAMLGFNGSMPGPEL   60
Lac 1326                  MLAFDFFLQASAGSILPLQALAKADPLEV-AEPVTAQILPEGEGTTAMLGFMGSTPGPEL   59

Sulfitobacter indolifex   RLKRGEQISIDVENRLEEGTAVHWHGIRLENRMDGVPVLTQDLINPGDSKTYSFAPPDAG  120
Lac 1326                  RARQGEQFDIDVQNRIEEGSAVHWHGARRDDNMDGVPVMTQDLIEPGDEKEYSFAAPDAA  119

Sulfitobacter indolifex   TYWYHSHYISQEQVARGMMGPLIVEDDMPPDVDH-DITVVLSDWIINEDGSLSDDFTDMH  179
Lac 1326                  TIWYHSHQGSAEQVARGTRGPLIVEMG-PPDVDHGDLTVLPDWWIIHEDGVLSDEFTNMS  178

Sulfitobacter indolifex   SVAHAGYMGNFARAFLSQAEVKEG-DRVRFRIINAATNRIFPLTVAGVAGSVVALDGMAL  238
Lac 1326                  GDAHRGYLGNFARASLSPVEEVAGDDRVRSRVINAATDRIFPLGRQGVDGTVVGLDGMTL  238

Sulfitobacter indolifex   GQPRGLSDLTLAPAQRADLIVDITG--PVSFDMHSRQGSYRLADVAVSGSNTDRKPEPIA  296
Lac 1326                  VSPRQPSDLTLAPAQRADHIMDATSPDPVGFGLISRDGRYFLPAHDVFGSNTARWPFWIV  298

Sulfitobacter indolifex   PLAAPNLPVPGDPTQHLTLTMMGGAM------GGRHGGANIWSFNDVSDLPDAPFASMKR  350
Lac 1326                  PL-LPNLADEPDAKGHVTLTMGTEDTPLHPLPQAGMMSANIWAFNFLEILPDAPFHSPKR  357

Sulfitobacter indolifex   GETVRITLANDTAFPHGIHLHGHHFYELAADGSLGDLRDTTLVAAGESRDVLCVFDNPGR  410
Lac 1326                  GETHRIQLYNDTGFPHGIHLHGHHFAADGADGSLGFLRDTTLVPAGEQRDLNCVFDNPGR  417

Sulfitobacter indolifex   WLIHCHMLSHAIGGMRTWV  429
Lac 1326                  WLVHCHMLGMALGGMDTWV  436
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Fig. 1: Amino acid sequence alignment of pLac$_{Si}$ and Lac1326 (that was reported by Yang et al. [34]). Conserved residues are highlighted in yellow. The four conserved copper binding regions are shown with a box and highlighted grey. The copper binding residues are coloured in pink (for those that bind Type 1 copper), red (for those that bind Type 2 copper) or blue (for those that bind Type 3 copper).

2.3 Production and purification of recombinant pLac$_{Si}$

The plasmid encoding pLac$_{Si}$ was transformed into *E. coli* BL21 (DE3) competent cells and plated on an LB agar plate containing 50 µg/mL kanamycin. A single colony was used to inoculate 100 mL of 2YT nutrient rich medium supplemented with 50 µg/mL kanamycin and grown shaking at 37 °C overnight. The starter culture was then diluted with fresh 2YT medium supplemented with 50 µg/mL kanamycin in a 1:100 ratio, which was then incubated at 37 °C with shaking until it reached an optical density (measured at 600 nm wavelength; OD_{600}) of 0.6, at which point protein expression was induced using 0.1 mM isopropyl 1-thio-β-D-galactopyranoside (IPTG). After induction, the culture was further incubated at 18 °C for 16 h. Cells were harvested by centrifugation. The harvested cells were resuspended in buffer A (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5% glycerol) and then lysed by sonication. The protein was purified using a His GraviTrap affinity chromatography column (GE Healthcare) charged with Ni^{2+} ions and the polyhistidine-tagged protein was eluted using buffer A supplemented with 500 mM imidazole. Protein purity was determined using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE), and protein concentration was measured using the "Protein A280" function on a NanoDrop 2000c spectrophotometer (Thermo Fisher Scientific). The purified pLac$_{Si}$ was buffer exchanged with buffer A to remove the imidazole and concentrated using an Amicon Ultra-15 30K centrifugal filter (Merck Millipore) and stored at -80 ºC until further use.

2.4 Tandem mass spectrometry for protein sequencing

An SDS-PAGE gel band corresponding to the molecular weight of pLac$_{Si}$ (~50 kDa) was dehydrated with acetonitrile. It was then subjected to reduction with dithiothreitol, alkylation with iodoacetamide and digestion with 12.5 ng/µL porcine trypsin (Promega) in a temperature-controlled

microwave (CEM) at 45 °C for 1 h. The digest was acidified and injected onto a 0.3 x 10 mm trap column packed with Reprosil C18 media (Dr Maisch) and desalted for 5 minutes at 10 μL/min. Following this, the peptide mixture was separated on a 0.075 x 200 mm picofrit column (New Objective) packed with Reprosil C18 media using a NanoLC 400 Ultra HPLC system (Eksigent).

The picofrit spray was directed into a TripleTOF 6600 (Sciex), a hybrid triple quadrupole-time-of-flight mass spectrometer, and scanned from 300-2000 m/z for 200 ms, followed by 40 ms MS/MS scans on the 35 most abundant multiply-charged peptides (m/z 80-1600). The mass spectrometer and HPLC system were under the control of the Analyst TF 1.7 software package (Sciex).

The resulting MS/MS data was searched against a database comprising Uniprot *E. coli* entries appended with a set of common contaminant sequences, plus the expected sequence for the expressed protein ($pLac_{Si}$), using ProteinPilot version 5.0 (Sciex). The peptide summary exported from ProteinPilot was further processed in Excel using a custom macro to eliminate inferior or redundant peptide spectral matches and to sum the intensities for all unique peptides from each protein.

2.5 Enzyme activity assay

Unless otherwise specified, laccase activity was measured at 30 ºC using 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) as substrate [22]. The assay mixture included 0.5 μM $pLac_{Si}$, 1 mM ABTS, 50 μM $CuCl_2$ in 0.04 M Britton-Robinson buffer (pH 6.0). The volume was 100 μL. The oxidation of ABTS to $ABTS^{\cdot+}$ was monitored spectrophotometrically by following the increase of absorbance at 420 nm ($\varepsilon_{420\ nm} = 36,000\ M^{-1}cm^{-1}$) using an EnSpire multimode plate reader (PerkinElmer). All assays were performed in triplicates in 96-well clear plates (Eppendorf).

Specific activity of the laccase was measured by the following equation:

$$\text{Specific activity} = [\Delta\text{Abs} \times V_T \times 1000]/[\varepsilon \times d \times V_E \times t \times C_E]$$

In which ΔAbs = changes of absorbance at 420 nm, V_T = volume of reaction mixture (in mL), ε = molar extinction coefficient of $ABTS^{\cdot+}$ at 420 nm (in $M^{-1}\ cm^{-1}$), d = path length of cuvette/well (in cm), V_E = volume of stock enzyme solution used (in mL), t = reaction time (in min), C_E = concentration

of stock enzyme solution (in mg/mL). The unit of specific activity was U/mg, where one unit (U) of enzyme activity is defined as the amount of enzyme that is needed to oxidize 1 μmol ABTS in 1 min.

2.6 *Enzyme stability assay*

For the studies of enzyme stability in buffers containing Cu^{2+}, residual enzyme concentration was used as the readout. 10 μM pLac$_{Si}$ was incubated with various concentrations of Cu^{2+} in Britton-Robinson buffer (pH 6.0) for 6 h at room temperature. The solution was centrifuged at 12,000 rpm for 10 mins to remove any protein debris. The residual protein concentration in the solution was measured using Bradford assay.

For the studies of enzyme stability in different buffer conditions (pH, temperature, and the presence of organic solvents), the residual catalytic activity of the enzyme was used as the readout. 10 μM pLac$_{Si}$ was incubated in the test condition for 6 h. The solution was then centrifuged at 12,000 rpm for 10 mins to remove protein debris. The supernatant was diluted 20 times in Britton-Robinson buffer (pH 6.0) and the residual activity was measured at 30 °C in presence of 1 mM ABTS and 50 μM Cu^{2+}. The activity of the enzyme without incubation measured at the optimal pH and temperature (pH 6.0 at 30 °C) was set at 100%.

2.7 *Measurement of enzyme kinetic parameters*

Enzyme kinetics was monitored using the activity assay described above with varying concentrations of ABTS. Absorbance was monitored 5 min after the start of the reaction to obtain the initial reaction rates according to the Lambert-Beer law. Initial rates were plotted against substrate concentrations and the data was fitted by non-linear regression to the Michaelis-Menten equation (shown below) with SigmaPlot 14.0 (Systat Software, USA) to obtain the kinetic parameters.

$$v = \frac{V_{max}[S]}{K_m + [S]}$$

In which v denotes the initial reaction rates, V_{max} denotes the maximum reaction rate, K_m as the Michaelis constant and [S] as the substrate concentration, which can be approximated as $[S]_{total}$ at the start of the reaction. All assays were conducted in triplicates.

2.8 Mass spectrometry analysis to measure the incorporation of metals in pLac$_{Si}$

The incorporation of Cu^{2+} (and the concentration of residual Ni^{2+}) to pLac$_{Si}$ was examined using inductively coupled plasma-mass spectrometry (ICP-MS) following a protocol reported by Li et al. [37]. Briefly, a 1 µM solution of the enzyme in Britton-Robinson buffer (pH 6.0) was buffer exchanged four times using the same buffer with an Amicon Ultra-15 30K centrifugal filter to wash off any unbound Cu^{2+} (and Ni^{2+}). The enzyme solution was then heated on a heat block at 100 °C to denature the protein and release the bound metal ions into the solution. This was followed by centrifugation at 12,000 rpm for 20 mins to remove the protein precipitates. The supernatant was diluted 10 times with 5% HNO_3 solution and the content of metal ions was analysed using an Agilent 770X ICP-MS instrument.

2.9 Dye decolourisation potential

Reaction mixture included 1 µM of purified pLac$_{Si}$, 1 mM ABTS, 50 µM $CuCl_2$, 0.05 mg/mL of dye in Britton-Robinson buffer (pH 6.0). The mixture was incubated overnight at 30 ℃. Dye decolourisation was determined by measuring the decrease of absorbance using the following wavelengths for the different dyes: Alizarin (496 nm), Acid Red 27 (520 nm), Congo Red (490 nm), Bromophenol Blue (592 nm), Coomassie Brilliant Blue R-250 (556 nm), Malachite Green (624 nm) and Indigo Carmine (610 nm). Dye decolourisation was expressed in terms of % decolourisation.

$$\% \text{ Decolourisation} = \frac{\text{Initial absorbance} - \text{Final absorbance}}{\text{Initial absorbance}} \times 100\,\%$$

2.10 Immobilisation of pLac$_{Si}$

Purified pLac$_{Si}$ was immobilised using three techniques: entrapment, support binding and cross-linked enzyme aggregates (CLEA).

For the enzyme entrapment method, the protocol by Varga et al. was followed [38]. In brief, sodium alginate was dissolved in 5 mL of enzyme solution containing 1 mg/mL purified pLac$_{Si}$ to produce a 2% (w/v) alginate solution with gentle stirring. This solution was added dropwise into a 25 mL solution of 2% (w/v) $CaCl_2$ using a 0.70 x 38 mm needle fitted to a 5 mL syringe from a height of 15 cm above the solution to produce equal sized beads. After allowing the beads to harden for 3 h, they were filtered using Whatman filter paper and washed two times with 10 mL of 2% (w/v) $CaCl_2$ solution and three times with deionised water. The beads were stored in Britton-Robinson buffer (pH 6.0) at 4 °C until further use.

For the support binding method, chitosan beads were used as the solid support. Purified pLac$_{Si}$ was covalently linked to chitosan beads through cross-linking with glutaraldehyde following the protocol by Zheng et al. [39]. In brief, 0.5 mg of chitosan was first dissolved in 20 mL of 1% (v/v) acetic acid solution. The solution was then added dropwise to 200 mL of a 2.0 M NaOH solution to generate the chitosan beads. After filtering and washing the beads with deionised water, they were then added to 5 mL glutaraldehyde solution (0.8% v/v) and stirred at 150 rpm for 3 h for cross-linking. The cross-linked beads were washed several times with deionised water to remove excess glutaraldehyde. 10 mL of purified laccase (1.85 mg/mL) was then immobilised onto the glutaraldehyde-cross-linked-chitosan beads by shaking at 150 rpm for 6 h, after which they were filtered with Whatman filter paper, washed several times with deionised water, dried at room temperature to constant weight and stored at 4 °C until further use.

For the CLEA method, enzyme aggregates were first generated by precipitation by mixing 1 mL pLac$_{Si}$ solution (5.54 mg/mL) with 2.5 mL of a saturated ammonium sulphate solution (4.1 M at 25 °C). The mixture was incubated with gentle shaking for 2 h [40]. The enzyme aggregates were collected by centrifugation at 12,000 rpm for 10 mins, which were then suspended in 3.5 mL of Britton-Robinson

buffer (pH 6.0). Next, cross-linking was carried out for 3 h by the addition of glutaraldehyde to a final concentration of 30 mM. The CLEAs were then collected by centrifugation at 12,000 rpm for 10 mins. These were then washed several times with Britton-Robinson buffer (pH 6.0) by centrifugation until no laccase activity was detected in the supernatant wash solution. They were stored in the same buffer at 4 °C until further use.

Loading efficiency, which is defined as the percentage of enzyme that has been successfully immobilised, was calculated using the equation as follows:

$$\text{Loading efficiency} = \frac{C_i V_i - C_f V_f}{C_i} \times 100\ \%$$

In which C_i denotes the initial enzyme concentration, V_i denotes the total volume of enzyme solution used, C_f denotes the concentration of free enzyme in the flow through after the final wash, and V_f is the total volume of the flow through after the final wash.

2.11 Determination of stability and reusability of CLEA-immobilised pLac$_{Si}$

The stability of CLEA-immobilised pLac$_{Si}$ was studied using the same method to the free enzyme as described above. A concentration of approximately 0.1 mg/mL of CLEA-immobilised pLac$_{Si}$ (representing the enzyme concentration in the CLEAs) was used for these studies. Residual activity was used as the readout for enzyme stability.

For the reusability experiments, Indigo Carmine was used as a model dye. The reaction mixture included CLEA-immobilised pLac$_{Si}$ (approximately 0.1 mg/mL), 1 mM ABTS, 50 µM $CuCl_2$, 0.05 mg/mL of dye in Britton-Robinson buffer (pH 6.0). The mixture was incubated overnight at 30 ºC. The solution was centrifuged at 12,000 rpm for 10 mins to recover the CLEA-immobilised pLac$_{Si}$. The absorbance of the supernatant was measured to calculate the decrease in absorbance of the dye solution. The CLEAs were then added to a fresh solution of dye supplemented with 1 mM ABTS and 50 µM $CuCl_2$ for a new decolourisation cycle. This process was repeated up to seven cycles. To test the

reusability of the free enzyme, fresh ABTS and dye were added to the existing solution at the end of each cycle without centrifugation as the enzyme cannot be extracted out of the solution.

3 Results and discussion

3.1 Identification of a putative laccase from Sulfitobacter indolifex through sequence alignment search

As we are interested to identify novel laccases that function at cold to ambient temperatures, we first conducted protein sequence alignment searches by using the protein sequence of a recently reported cold-adapted laccase, Lac1326, which was isolated from an uncultured bacterium extracted from the South China Sea, as the query sequence [34]. As all laccases contain four conserved copper binding regions **(Fig. 1)** that consist of a total of ten histidines and one cysteine at the active site [41], this criterion was used as a selection filter for putative laccases. A NCBI BLAST search for sequence homologs of the Lac1326 protein resulted in 67 putative laccase sequences in species of bacterial origin with similarity greater than 50%. Out of these, an enzyme of the multicopper oxidase family from the HEL-45^{T} strain of *Sulfitobacter indolifex* (accession code: WP_040700410) showed the maximum sequence similarity (64%) with the query sequence and contained the four conserved regions **(Fig. 1)**. *S. indolifex* is a Gram-negative, non-motile rod and obligate aerobe isolated from the North Sea, 2 km offshore of the archipelago of Heligoland where temperature typically ranges between 0 and 15 °C [35,36]. Hence, this putative laccase (termed $pLac_{Si}$ for this study) was chosen for its potential cold adaptability. The amino acid sequence of this putative laccase has also reasonable similarity with bacterial laccases from *K. pneumoniae* (WP_064183572)*, E. coli* (P36649), *S. ipomoeae* (ABH10611) *and B. pumilus* (AGO57931) (31.23%, 29.47%, 28.64% and 22.75% respectively), all of which have previously shown to possess dye decolourisation ability [28–31].

3.2 Overexpression and purification of the recombinant $pLac_{Si}$

Recombinant pLac$_{Si}$ was expressed in *E. coli* BL21 (DE3) cells with an N-terminal polyhistidine tag. SDS-PAGE analysis confirmed that the recombinant pLac$_{Si}$ was produced as a soluble intracellular enzyme, as the gel showed a thick intense band at around 50 kDa in the cell lysate fraction **(Fig. 2a)**. This is in accordance with its predicted molecular weight of 46.60 kDa plus the molecular weight of the N-terminal polyhistidine tag with a thrombin cleavage site (2.18 kDa). Purification of the recombinant pLac$_{Si}$ was achieved by using immobilised metal affinity chromatography with Ni^{2+}, which yielded proteins with high degree of purity (>95%) as judged by SDS-PAGE **(Fig. 2a)**. The sequence of the purified protein was confirmed using tandem mass spectrometry. Overall, the typical yield of purified enzyme was ~12 mg/L of culture media. Furthermore, native PAGE analysis illustrated that pLac$_{Si}$ migrated as a single band in the absence and in the presence of reducing agents, suggesting the purified pLac$_{Si}$ exists as a single species in solution **(Fig. 2b)**.

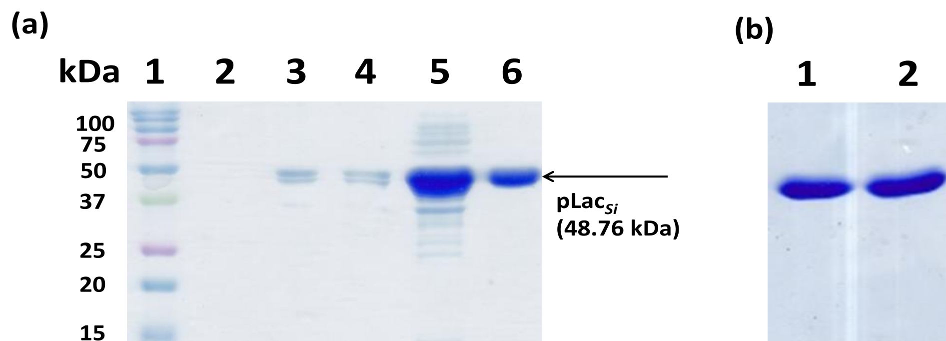


Fig. 2: a) Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis of pLac$_{Si}$ expression and purification. Lanes 1-6 represent the following: protein marker (lane 1), centrifugal supernatant of fermentation broth (lane 2), whole cell proteins (lane 3), centrifugal sedimentation after cell lysis (lane 4), cell lysate (lane 5) and protein purified with affinity column chromatography (lane 6). b) Native-PAGE analysis of purified pLac$_{Si}$. Lane 1 represents the protein in absence of any reducing agents and lane 2 represents the protein in presence of 2 mM β-mercaptoethanol.

3.3 Characterisation of the recombinant pLac$_{Si}$

Laccases typically have broad substrate specificity and they could catalyse the oxidation of a wide range of substrates including mono-, di-, poly-, and methoxy-phenols, aromatic amines and

thiols, hydroxyindoles and benzothiazoles [16,18,19]. The substrate spectra of laccases often overlap with other polyphenol oxidases like catechol oxidase and tyrosinase, particularly catechols and their derivatives [42,43]. Hence, the selection of a suitable substrate is key for its characterisation. However, this is not a trivial task. For example, the oxidised products of laccase-catalysed reactions often react with each other to form polymerised aggregates that precipitate out of solution. This may lead to inaccurate kinetics measurements if the precipitation is not taken into proper account. In addition, some substrates such as catechols may get oxidised by free copper ions alone [44]. Therefore, a careful selection of the substrate and its reaction conditions is important. In this study, we chose ABTS as a model substrate to characterise our recombinant pLac$_{Si}$ because it showed high specificity for laccases and, upon oxidation, forms a highly stable cation radical, ABTS$^{\cdot+}$, which is highly water soluble and absorbs strongly at 420 nm ($\varepsilon_{420\,nm}$ = 36,000 $M^{-1}cm^{-1}$) [45]. A concentration of 1 mM was chosen. This was based on previous laccase studies employing ABTS as a redox mediator [34,46].

A spectrophotometer-based activity assay was used to follow the oxidation reaction catalysed by pLac$_{Si}$. We found that the reaction mixture changes from colourless to green upon the addition of pLac$_{Si}$, indicating that ABTS is oxidised to ABTS$^{\cdot+}$. The same change was also observed with cell lysates overexpressed with pLac$_{Si}$. In contrast, negligible colour change was observed when cell lysates that did not contain pLac$_{Si}$ was added to ABTS. These experiments showed that pLac$_{Si}$ possesses laccase activities. The specific laccase activity for the cell lysate and for the purified pLac$_{Si}$ were 22.64 mU/mg and 625 mU/mg respectively at 30 °C and pH 6.0.

Next, the kinetics of the recombinant pLac$_{Si}$-catalysed reaction was studied by determining the initial reaction rate of ABTS oxidation at different substrate concentrations (0-5 mM). The Michaelis constant (K_m) for ABTS at 30 °C was calculated to be 3.21 ± 0.27 mM, and the corresponding values for maximum velocity (V_{max}), catalytic constant (k_{cat}) and catalytic efficiency (k_{cat}/K_m) were 30.97 ± 0.51 μM min^{-1}, 1.03 ± 0.01 s^{-1} and 0.32 ± 0.03 mM^{-1} s^{-1} respectively. Comparing to other dye-degrading/decolourising bacterial laccases (that all used ABTS as substrate), pLac$_{Si}$ has similar kinetic

properties to a laccase from *Thermus thermophiles* that was reported by Kumari et al. [46] **(Table 1)**. However, the numbers vary from Lac1326 that was reported by Yang et al., and a *Bacillus subtilis* endospore laccase that was reported by Martins et al [34,47] **(Table 1)**. These differences are common across laccases from different species, presumably due to their sequential diversity [48].

Table 1: Comparison of the kinetic parameters of pLac$_{Si}$ with three other reported bacterial laccases using ABTS as substrate.

Kinetic parameters	**pLac$_{Si}$ (current study)**	**Laccase from *T. thermophilus*** [46]	**Lac1326** [34]	**Laccase from *B. subtilis*** [47]
Assay temperature	30 ºC	50 ºC	60 ºC	37 ºC
K_m (mM)	3.21 ± 0.27	4.40	0.21	0.11
V_{max} (μM min^{-1})	30.97 ± 0.51	14.0	-----	-----
k_{cat} (s^{-1})	1.03 ± 0.01	1.58	34.39	16.80
k_{cat}/K_m (mM^{-1} s^{-1})	0.32 ± 0.03	0.36	163.76	152.73

3.4 Optimisation of Cu^{2+} concentration for laccase activity assays

Laccase is a copper-containing enzyme. However, as the recombinant pLac$_{Si}$ was purified by immobilised metal affinity chromatography that was charged with Ni^{2+}, there is a possibility that the enzyme may be contaminated with Ni^{2+}. Hence, ICP-MS was used to examine the presence of Ni^{2+} and Cu^{2+} in the enzyme. We found that the purified enzyme does not contain any significant amount of Ni^{2+} ions (<0.001 μg/mL in a 1 μM pLac$_{Si}$ solution, i.e., a nickel-to-enzyme ratio of <0.1). Interestingly, it was also found that the copper-to-protein ratio was only around 2.6. Since laccases have a four-copper active site, the ICP-MS results showed that our purified recombinant pLac$_{Si}$ are not saturated with copper ions. We then investigated the effect of supplementing extra Cu^{2+} to the enzyme. Our results showed that, at a protein concentration of 0.5 μM, pLac$_{Si}$ exhibits maximum activity when

the mixture was supplemented with 50 μM Cu^{2+} (**Fig. 3a**). However, any concentration greater than 50 μM may lead to protein precipitation (**Fig. 3b**). This is perhaps not surprising as cupric ion is a strong Lewis acid and heavy metal ions are known to cause protein aggregation at high concentrations [49]. Thus, all activity assays of $pLac_{Si}$ were conducted by supplementing the reaction mixture with 50 μM Cu^{2+}.

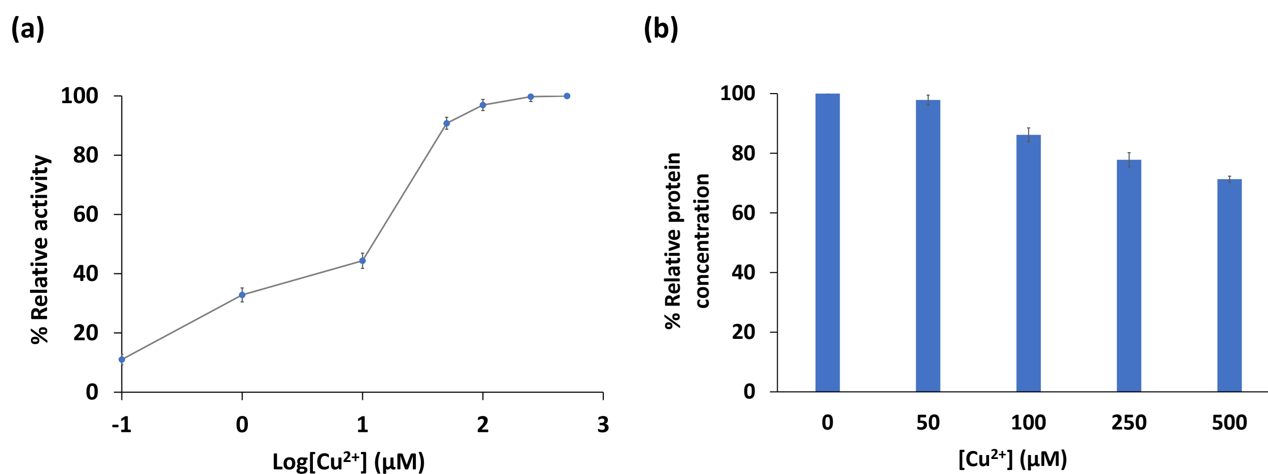


Fig. 3: The effect of Cu^{2+} supplementation on the activity and stability of purified recombinant $pLac_{Si}$. (a) The addition of at least 50 μM Cu^{2+} is required to achieve maximum $pLac_{Si}$ activity towards the oxidation of ABTS. (b) The presence of additional Cu^{2+} may lead to the precipitation of $pLac_{Si}$, which is reflected by the residual protein concentration in solution after 6 h of incubation. $pLac_{Si}$ concentration in absence of Cu^{2+} was set at 100%. All experiments were conducted in triplicate and error bars represent standard deviation.

3.5 Effect of pH on activity and stability of $pLac_{Si}$

As dye-containing wastewater may possess different pH values, it is important to understand the effect of pH on the activity and stability of our enzyme. We first tested the activity of $pLac_{Si}$ at different pHs by using ABTS as substrate (**Fig. 4a**). The results indicated that $pLac_{Si}$ is most active at pH 5.0. The activity of the enzyme only dropped to about 60% as the pH value was increased to 8.0. However, its activity dropped sharply at highly acidic or basic conditions, with the enzyme retaining only ~20% of maximum activity at pH 3.0 and it became completely inactive beyond pH 9.0. The results are in partial agreement with previous studies involving ABTS oxidation with bacterial laccases, which typically showed that these laccases do not work in basic pH ranges. However, different bacterial

laccases may have different optimal acidic pH ranges. For instance, Lac1326 from uncultured bacterium showed high activity between pH 5.0-8.0, laccases from *K. pneumoniae* and *E. coli* have narrow pH working ranges of 3.5-4.5 and 2.5-4.5 respectively, and the *B. pumilu*s laccase was most active between pH 3.0 and 6.5 [28–30,34]. Having an arsenal of different laccases that work at different pHs is advantageous for dye bioremediation as the dye-containing wastewater may have different acidity or basicity.

Next, we tested the stability pLac$_{Si}$ by incubating the enzyme at different pHs for 6 h. Here, the stability of the laccase was measured by residual activities (i.e., after incubating the enzyme in the test condition for 6 h, the debris were removed by centrifugation and the remaining enzyme solution was diluted back to pH 6.0 for measurements). We found that pLac$_{Si}$ has a broad pH stability profile. For example, between pH 6.0-11.0, pLac$_{Si}$ retains over 80% residual activity (i.e. little loss of the protein). Even at very basic condition (e.g. pH 12.0), pLac$_{Si}$ retains 60% activity after 6 h of incubation. However, we found that pLac$_{Si}$ was not stable at or below pH 5.0 **(Fig. 4a)**. Our results are in agreement with other bacterial laccases, which are usually stable in weakly acidic to weakly basic conditions. For example, Lac1326 was stable between pH 5.0-8.0 whilst the *K. pneumoniae* laccase showed stability in the pH range of 5.0-9.0 [30,34]. The significant stability of pLac$_{Si}$ at high pH further showcase its suitability as a biocatalyst for the decolourisation of dyes in wastewater, especially industrial wastewater, particularly those from the textile industry, is usually highly basic [50].

Combining the results from the optimal activity and stability, we reasoned that pLac$_{Si}$ works best at around pH 6.0 as it possesses high stability and high activity at this pH. Hence, all further characterisations of this enzyme were conducted at pH 6.0.

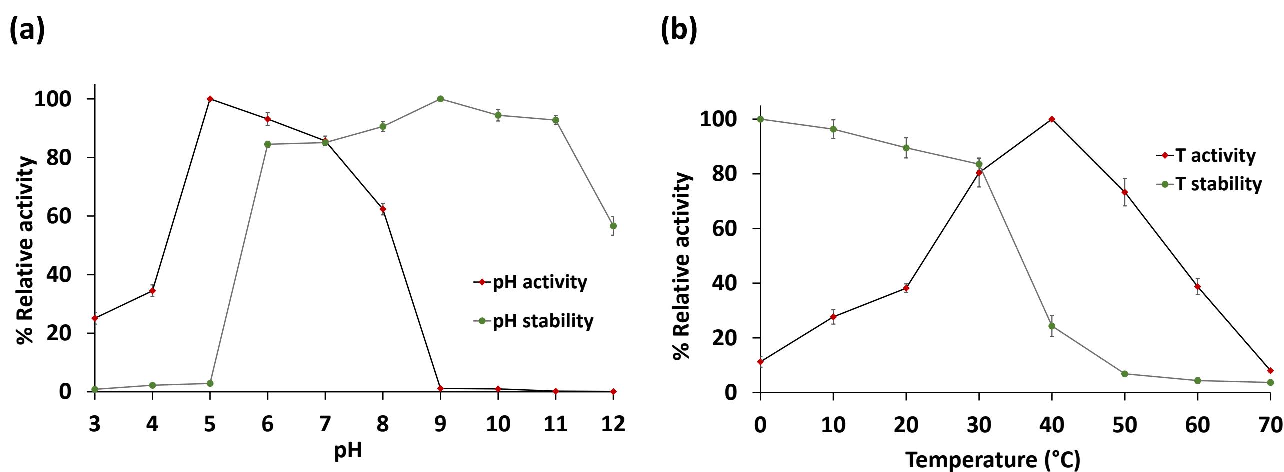


Fig. 4: The dependence of the activity and stability of purified recombinant pLac$_{Si}$ with varying (a) pH and (b) temperature. Protein stabilities were measured in terms of residual activity after pre-incubation in the test conditions. Conditions with maximum activity was set at 100%. All experiments were conducted in triplicate and error bars represent standard deviation.

3.6 Effect of temperature on activity and stability of pLac$_{\mathrm{Si}}$

The effect of temperature on the catalytic ability of pLac$_{Si}$ was then studied by measuring its activity between 0 to 70 °C using ABTS as the substrate at pH 6.0. Our results showed that pLac$_{Si}$ was most active at 40 °C. It also showed significant activity at lower temperatures, retaining ~80%, ~40% and ~30% activities at 30, 20 and 10 °C respectively (**Fig. 4b**). It also retained ~75% of maximum activity at 50 °C and ~40% at 60 °C. We then proceeded to study the thermal stability of pLac$_{Si}$ by incubating the enzyme at different temperature for 6 h. Our results showed that pLac$_{Si}$ is most stable at low temperature between 0 and 30 °C. The enzyme was found to be unstable above 40 °C (**Fig. 4b**). To further confirm this observation, we performed differential scanning fluorimetry experiments, which showed that pLac$_{Si}$ has a denaturation temperature (T_m) of 42 °C. Combining the results from the activity and stability assays, pLac$_{Si}$ appears to work best at ambient temperatures (~30 °C) as this temperature gives the enzyme a good balance of activity and stability. The high activity and stability of pLac$_{Si}$ at lower temperatures is potentially advantageous in an industrial setting as it is able to catalyse chemical transformations without need of extra heating.

3.7 *Tolerance of pLac*$_{Si}$ *towards organic solvents and salts*

Industrial wastewater is usually accompanied by a range of impurities such as salts as well as organic solvents [3,50]. As such, tolerance towards these contaminants is a prerequisite for a reliable biocatalyst for the treatments of wastewater. The effect of these contaminants on the stability of pLac$_{Si}$ was therefore screened (by using residual activity as described above) by pre-incubating the enzyme in different concentrations of organic solvents or NaCl for 6 h.

We first tested the tolerance of pLac$_{Si}$ towards high salt concentrations (0.1 M, 0.5 M and 1.0 M NaCl concentration). Our results also showed that pLac$_{Si}$ is stable even at 1.0 M NaCl concentration. Salt may help pLac$_{Si}$ to increase its solubility by increasing hydration of the protein surface. However, care must be taken at very high salt concentrations, as competition between salt ions and protein molecules for hydration may eventually lead to the deactivation of the protein [51].

We then studied the stability of pLac$_{Si}$ in aqueous-organic solvent mixtures. Five different organic solvents, methanol, ethanol, acetone, acetonitrile and dimethyl sulfoxide (DMSO), were tested at concentrations of 10%, 30% and 50% (v/v). The results indicated that methanol, acetone and acetonitrile had adverse effect on the stability of the protein, but the laccase retained some activity in aqueous solutions containing up to 30% DMSO or 10% ethanol (**Table 2**). Although some laccases including Lac1326 or CueO from *E. coli* have shown to be relatively unaffected by the presence of solvents [29,34], not all laccases are tolerant to water-miscible solvents, as were seen with laccases from *Trametes versicolor* and *Pleurotus ostreatus* [52]. The reduction in protein stability caused by solvents may be attributed to conformation changes due to direct interaction with solvent molecules or inactivation of the enzyme caused by exchanging water molecules from the active site [53]. Solvents such as alcohols and acetone can disrupt intra-protein hydrogen bonds. Acetonitrile could weaken hydrophobic interactions, while DMSO may reduce protein solubility by sequestering water molecules and disrupt hydrogen bond interactions [54,55]. These effects are reflected by the low stability of pLac$_{Si}$ in the presence of these solvents. The lack of stability of pLac$_{Si}$ in water/organic solvent mixtures

is an obvious disadvantage to its potential application as a biocatalyst to remove dyes in wastewater. We therefore attempted to mitigate this effect by engineering the biocatalyst though enzyme immobilisation, which is discussed below.

Table 2: Effect of organic solvents and salt on the stability of free and CLEA-immobilised pLac$_{Si}$. The residual activity of free and CLEA-immobilised laccase was measured after incubation in aqueous-organic solvent mixtures or aqueous solution at various salt concentrations for 6 h. Activity in buffer alone was set at 100%. All experiments were conducted in triplicate and the uncertainties represent standard deviation.

Buffer/Organic solvent	Concentration (% v/v)	Relative activity (%) (Free pLac$_{Si}$)	Relative activity (%) (CLEA pLac$_{Si}$)
Britton-Robinson buffer (pH 6.0)	---------	100.0 ± 0.0	100 ± 0.0
Deionised water	---------	82.1 ± 4.7	114.0 ± 2.6
Methanol	10	27.2 ± 3.8	96.2 ± 7.3
	30	7.2 ± 3.6	71.7 ± 6.8
	50	4.6 ± 2.7	31.8 ± 4.7
Ethanol	10	96.2 ± 4.4	101.7 ± 8.7
	30	7.0 ± 1.5	66.4 ± 6.2
	50	3.5 ± 1.3	26.7 ± 1.5
Acetone	10	36.3 ± 3.6	105.4 ± 4.5
	30	7.6 ± 2.4	56.4 ± 6.3
	50	0.2 ± 0.2	39.2 ± 2.6
Acetonitrile	10	32.3 ± 2.1	106.9 ± 8.9
	30	3.3 ± 0.1	9.6 ± 2.3
	50	5.5 ± 1.3	5.2 ± 1.0
DMSO	10	93.3 ± 4.8	138.8 ± 7.9
	30	45.1 ± 3.7	119.4 ± 6.3
	50	12.2 ± 1.5	27.0 ± 0.7

Salt	Concentration (mM)	Relative activity (%) (Free pLac$_{Si}$)	Relative activity (%) (CLEA pLac$_{Si}$)
NaCl	100	127.3 ± 0.8	143.3 ± 5.2
	500	102.6 ± 5.5	141.4 ± 3.2
	1000	77.0 ± 9.6	137.7 ± 2.8

3.8 Dye decolourisation potential

Laccases catalyse the oxidation of substrates that contain phenolic or aromatic amine groups. Organic dye molecules usually bear auxochromic groups (e.g., hydroxy, methoxy or amino groups) attached to a highly conjugated aromatic ring system. As such, dye molecules are potential substrates for laccases. It is also possible to increase the spectrum of dyes that could be decolourised and degraded by the use of redox mediators such as ABTS [56]. In the presence of a redox mediator, laccases could catalyse its oxidation, with the resulting oxidised product (e.g. in the case of ABTS, a stable radical) then acts as an electron shuttle between the laccase and the dye by accepting electrons from the electron-rich portion of the dye [57]. However, it is important to note that, for the purpose of bioremediation, the use of a redox mediator may significantly increase the cost. Detailed studies of the fate of the redox mediator should also be conducted.

To determine the ability of pLac$_{Si}$ to decolour dyes, seven common industrial dyes encompassing different dye classes were tested. These were Alizarin (anthraquinones), Acid Red 27 and Congo Red (azo dyes), Bromophenol Blue, Coomassie Brilliant Blue R-250 and Malachite Green (triphenylmethane dyes) and Indigo Carmine (indigoid dyes). These dyes were chosen because they represent a diverse range of substrate classes for laccases. For example, Alizarin, Acid Red 27 and Bromophenol Blue contain phenolic groups; Coomassie Brilliant Blue R-250, Malachite Green and Congo Red possess aniline sub-structures; and Indigo Carmine contains an indole scaffold (**Supplementary Fig. S1**).

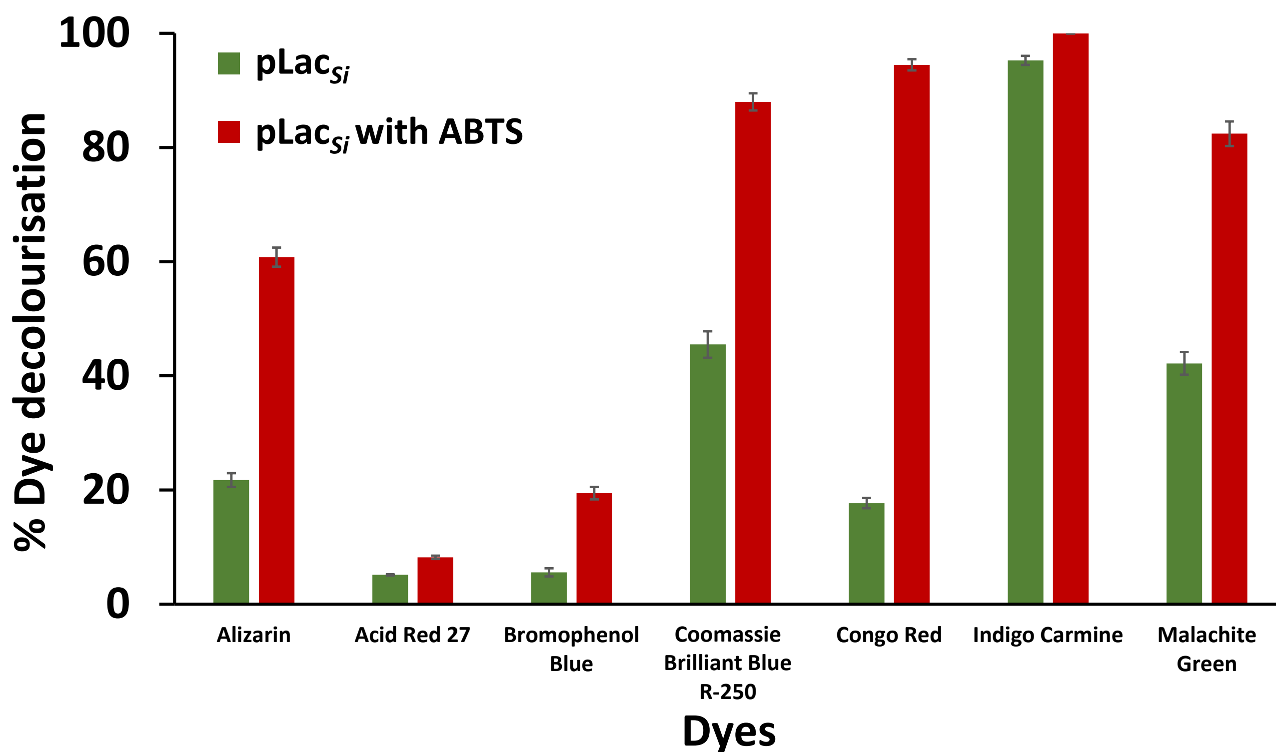


Fig. 5: Decolourisation rate of seven dyes by pLac$_{Si}$ in the absence and presence of ABTS as a redox mediator. The decrease in absorbance of the dyes at their wavelengths of maximum absorbance was used to calculate the percentage decolourisation. All experiments were conducted in triplicate and error bars represent standard deviation.

Dye solutions were incubated overnight with the laccase in Britton-Robinson buffer (pH 6.0) in the absence and presence of ABTS (redox mediator). Our results showed that in absence of ABTS, only Indigo Carmine was efficiently being decolourised (95% decolourisation) (**Fig. 5** and **Supplementary Fig. S2**). This is followed by Coomassie Brilliant Blue R-250 and Malachite Green, which were found to be decolourised to ~40%. The decolourisation of the other four dyes was negligible. These results showed that Indigo Carmine, Coomassie Brilliant Blue R-250 and Malachite Green are able to act as direct substrates of pLac$_{Si}$. The addition of ABTS made a stark difference, which enabled the decolourisation of Coomassie Brilliant Blue R-250, Congo Red, Indigo Carmine and Malachite Green up to or above 80% and Alizarin up to 60%. However, there was still no

significant decolourisation of Acid Red 27 and Bromophenol Blue. Liu et al. observed that the decolourisation of Bromophenol Blue by *K. pneumoniae* laccase may be significantly enhanced at low pH [30]. We therefore attempted the decolourisation of Acid Red 27 and Bromophenol Blue in both acidic (pH 4.5) and basic (pH 8.0) conditions. However, these conditions made little differences in the ability for pLac$_{Si}$ to decolour these two dyes either directly or indirectly via ABTS.

Laccases can only directly catalyse the oxidation of substrates if the substrate binds at the active site at the correct orientation and that the molecule possesses a redox potential that is lower than the enzyme. Hence, the inability of pLac$_{Si}$ to catalyse the decolourisation of Alizarin, Acid Red 27, Bromophenol Blue and Congo Red in the absence of ABTS is therefore likely due to steric effect and/or oxidation potential. Although the addition of ABTS helped increased the range of dyes that could be decolourised, Acid Red 27 and Bromophenol Blue remained unaffected by pLac$_{Si}$. In the case of Acid Red 27, the highly electron withdrawing sulphonate group located at the ortho position to the hydroxy group may reduce the electron density of the phenolic moiety, thus increasing its redox potential (when compared to other azo dyes such as Congo Red). This proposal is supported by the observation that Acid Red 27 could be degraded or decolourised by some fungal laccases [58,59], which typically have higher redox potential than bacterial laccases. In the case of Bromophenol Blue, its inertness against laccase-catalysed oxidation may be due to the presence of bromo groups on its phenolic rings, which are both bulky and electronegative.

3.9 Immobilisation of pLac$_{Si}$

In order for our laccase to be industrially facile, it is vital that we could recover and reuse the enzyme so that they can be applied in multiple catalytic cycles. However, the recovery and reuse of soluble enzymes are not trivial. Enzyme immobilisation may help achieve better recovery and reusability. Besides, enzyme immobilisation may also improve operational stabilities such as temperature and pH stability as well as tolerance towards contaminants [60].

In our study, the immobilisation of our purified laccase was examined by using three different immobilisation techniques. These include entrapment, covalent linkage to a solid support, and self-immobilisation (**Supplementary Fig. S3**) [61]. For the entrapment method, pLac$_{Si}$ was immobilised into a solid porous matrix of alginate beads. For the covalent linkage method, the enzyme was covalently linked to a support of chitosan beads via cross-linking with glutaraldehyde. In the final method, the laccase was self-immobilised by the formation of CLEAs. The loading efficiency, specific activity and storage stability of the three immobilised enzymes were then tested. When compared between the three immobilisation methods, we found that the CLEA-immobilised pLac$_{Si}$ offered maximum loading efficiency and activity (**Table 3**). This is likely due the presence of large areas of non-catalytic surface in support-based immobilisation methods like entrapment and support binding, which may dilute enzyme activity and/or affect diffusion of substrates/products through the carriers [62].

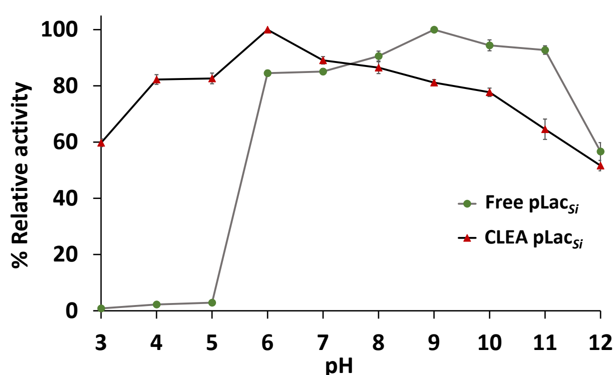
We then tested the long-term storage stability of the immobilised pLac$_{Si}$ at 4 ºC. We found that CLEA-immobilised pLac$_{Si}$ exhibited superior long-term storage stability (stable over 6 months when compared to 2 weeks for the other two methods) (**Table 3**). Their lack of long-term storage stability from the support-based immobilisation methods may be caused by the deformation of the beads. Such effects have been observed before. For example, Varga et al. observed a loss of 33% activity of a laccase immobilised into alginate beads over 5 days of storage [38]. In contrast, Vršanská et al. reported an activity retention of around 80% over a period of 70 days for a biocatalyst that has been subjected CLEA immobilisation [63]. These observations prompted the selection of CLEA as the system of choice for further examination.

Table 3: A table summarising the effect of pLac$_{Si}$ immobilisation.

Immobilisation method	Loading efficiency	Specific activity (mU/mg)	Storage stability at 4 °C
Entrapment (Alginate beads)	85.7%	7.05 ± 0.08	20% residual activity after 2 weeks
Support binding (Chitosan-glutaraldehyde beads)	93.5%	3.01 ± 0.03	15% residual activity after 2 weeks
Self-Immobilisation (CLEA)	96.1%	28.46 ± 1.59	90% residual activity after 6 months

3.10 Stability and reusability of CLEA-immobilised pLac$_{Si}$

We first tested the stability of the CLEA-immobilised pLac$_{Si}$ at different conditions including pH, temperature and the presence of salt and organic solvents. When compared to the free enzyme, which was unstable in highly acidic conditions, we found that the CLEA-immobilised enzyme has a broader pH stability range between 3.0-12.0 (**Fig. 6a**). However, CLEA immobilisation did not significantly improve the thermal stability of pLac$_{Si}$ (**Fig. 6b**). In addition to a broader pH stability, we found that the CLEA-immobilised enzyme showed significantly improved stability towards organic solvents and salt (**Table 2**). The CLEA-immobilised pLac$_{Si}$ can tolerate aqueous solutions containing methanol and acetone of up to 30% (v/v) and acetonitrile up to 10% (v/v). It has also increased stability in aqueous solutions containing ethanol, DMSO and NaCl when compared to the free enzyme. The reason of the increased stability towards pH and solvent denaturation is likely due to CLEA immobilisation reducing the contact between the enzymes and the solvent. These results are significant as we showed that enzyme immobilisation may enhance the usability of this enzyme for industrial operations.



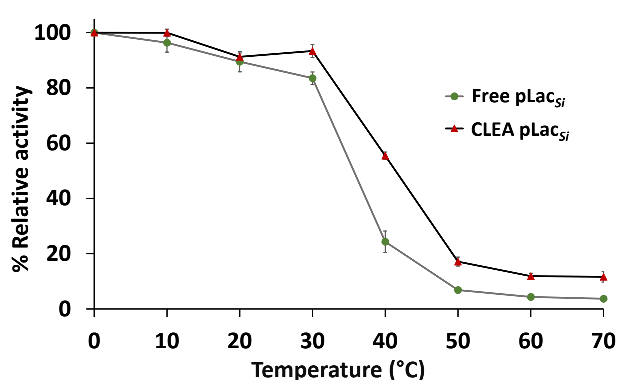


Fig. 6: The effect of (a) pH and (b) temperature on the stability of free and CLEA-immobilised pLac$_{Si}$. Relative activity of the enzyme after pre-incubation in the test conditions was used as a readout for protein stability. Conditions with maximum activity were set as 100%. (625 mU/mg for the free enzyme and 28.5 mU/mg for the CLEA-immobilised enzyme). All experiments were conducted in triplicate and error bars represent standard deviation.

The recovery and reusability of CLEA-immobilised pLac$_{Si}$ was then studied. We found that the free enzyme could not be reused for more than one cycle (<50% activity left after 1 cycle). In addition, the free enzyme could not be efficiently recovered without extensive buffer exchange and purification processes. In contrast, we found that the CLEA-immobilised enzyme could be efficiently recovered through centrifugation and filtration, and they appeared to be relatively stable over several catalysis/recovery cycles (~50% activity left after four cycles) (**Fig. 7a**). This slight improvement in reusability is promising although the observation that the CLEA-immobilised pLac$_{Si}$ lost almost 50% of its activity after four cycles is still not ideal. This is likely due to wear-and-tear of the immobilised enzymes during the recovery process (especially the enzymes were not bound to a solid support). Further optimisation to the mechanical stability of the immobilised enzyme is therefore required. Finally, the operational stability of the CLEA-immobilised laccase at incubation condition of pH 6.0 at 30 °C was also studied. We found that free pLac$_{Si}$ lost more than half of its initial activity over 24 h while the CLEAs retained over 90% activity over the same period (**Fig. 7b**), which is in line with our observation with the high storage stability of the CLEA-immobilised enzyme.

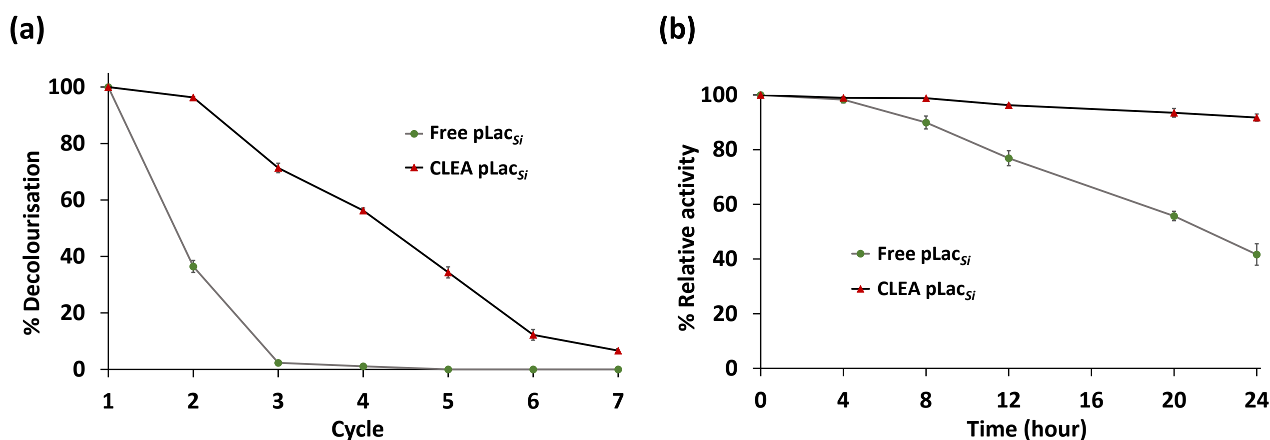


Fig. 7: (a) Comparison of the reusability of free and CLEA-immobilised pLac$_{Si}$ in the decolourisation of Indigo Carmine. Experiments were monitored up to 7 cycles. (b) Comparison of the stability of free and CLEA-immobilised pLac$_{Si}$ over a period of 24 h in the same incubation condition that is used for dye decolourisation (pH 6.0 at 30 ºC). Relative residual activities were measured at 4 h intervals in presence of 1 mM ABTS and 50 µM Cu^{2+}. All experiments were conducted in triplicate and error bars represent standard deviation.

4 Conclusions

In this study, we have recombinantly produced and characterised a putative laccase from *Sulfitobacter indolifex*. The enzyme was found to be stable and functional in ambient to cold temperature. It exhibits a broad pH stability profile, and it is stable in aqueous solutions containing up to 30% DMSO or 10% ethanol. Our results showed that the enzyme could effectively decolour five out of the seven dyes that were tested by using ABTS as a mediator. Furthermore, we found that the immobilisation of the laccase by forming CLEAs significantly improves its operational stability, including pH tolerance as well as stability in aqueous solutions containing organic solvent impurities. The use of bacterial laccases for dye degradation and decolourisation have been explored by many groups in the recent years. As dye-containing wastewater may have different physical characteristics (such as temperature, pH, and the presence of different impurities), for successful dye bioremediation, it is imperative that we build up our arsenal of laccases that could work under different operational conditions. Hence, our work adds to the existing literature about the use of bacterial laccases that can serve as an industrial catalyst for bioremediation.

Conflicts of interest

There are no conflicts to declare.

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Supplementary material

Electronic supplementary data has been provided for this article.

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