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DYE DECOLORISATION CAPACITY OF *Lignosus rhinocerotis* (Cooke) Ryvarden DIALYSED FRACTION AND ITS EXPRESSION OF NOVEL RECOMBINANT LACCASE IN *Pichia pastoris*

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Abstract

Azo dyes are widely used in the textile industry due to their bold colors and resistance toward degradation. This has made azo dyes the global burden in wastewater treatment as conventional dye removal methods have their limitations. Laccase has emerged as an alternative due to its ability to decolorise a wide range of dyes, producing water as by-product. In this study, we aim to assess the dye decolorisation capacity of the Basidiomycota, *L. rhinocerotis* dialysed fraction and produce recombinant laccase expressed in *Pichia pastoris*. Out of the 3 dyes tested, the dialysed fraction of *L. rhinocerotis* sclerotia was capable of decolorising around 90% of the Congo Red and Coomassie Brilliant Blue dye at the enzyme unit of 0.018 U/mL. The gDNA of *Lac1* [MG210944] was successfully updated in GenBank and the sequence was used to design the pPICZαA/Lac vector, which was successfully cloned and the recombinant *Lac1* was successfully expressed using the *Pichia pastoris* system. The recombinant *Lac1* expressed contain the 6x His-tag for purification but the binding towards the Ni-NTA resin was weak, therefore the purification can be optimised through reducing Imidazole concentration to compensate for the weak binding. In conclusion, *L. rhinocerotis* extract showed promising results in decolorising CR and CBB, while recombinant *Lac1* laccase successfully demonstrated its activity and can be further used for dye decolorisation in future studies.

INTRODUCTION

The textile and clothing industry are one of the global industries that are essential to our daily life. Globally the

textile industry is determined to be worth US1$ trillion and is contributing to 7% to the total world exports [1], with China being the main importer of textile products, followed by the European Union, India and USA [2]. Textile industry is also one of the biggest contributors in global wastewater pollution as the treatment process consumes high amounts of fuels and chemicals [3] and the dyes are difficult to degrade [1-3].

Annually, the world produces 7×10^5 tonnes of dyestuff [4-5], of which 40% are from the textile industry. Azo and reactive dyes are the most prominent classes of commercial synthetic dyes used because they are inexpensive and are available in a broad range of shades [5]. Azo dyes alone which are complex in structure and synthetic in nature contributed to 70% of the dyes used in textile manufacturing [6]. The aromatic and heterocyclic compounds make the dyes stable and resistant toward degradation by light, temperature and chemicals [7].

During the dyeing process, 15%-50% of the dyes are discharged without binding to any fabric [3], and the untreated dye effluent discharged contains high levels of biochemical oxygen demand (BOD) and chemical oxygen demand (COD) [3, 8]. The effluent contains between 10 and 200 mg/L of dyes, since most textile dyes can be detected by the human eye with a concentration lower than 1 mg/L, the effluent could prevent light from penetrating into the water body, which in turn will negatively impact the ecosystem of the marine life and the surrounding environment [8].

Azo dyes are readily soluble in water, making it hard to treat dye effluents using conventional separation methods [9]. Microbial biomass such as bacteria, fungi and micro-algae have been extensively studied because they are capable of dye absorption and their enzymes can decolorise dye effluents [9] without causing secondary pollution [10]. White-rot fungi are commonly applied for dye effluent treatment because it contains enzymes such as laccase, lignin peroxidase, manganese peroxidases and reductase that are capable of decolorising azo dyes [10]. However, fungal treatments are time-consuming [10] and require a controlled source of nitrogen [11], hence the best alternative would be using the enzyme itself.

Laccases (EC 1.10.3.2, oxidoreductase) are members of the large blue copper protein or blue copper oxidase family that are capable of catalysing oxidation of aromatic compounds with water and oxygen as by-products [11-14]. Such compounds include ortho and para-diphenols, aminophenols, polyphenols, polyamines and aryl diamines [13]. Laccase contains one type I copper (T1-Cu), one type II copper (T2-Cu) and two type III copper (T3-Cu) [13, 14]. The oxidation reaction occurs at the T1-Cu site while T2-Cu and T3-Cu form a trinuclear cluster, where the oxygen is reduced to water [14]. Laccase has a wide redox potential range and uses the molecular oxygen as a final electron acceptor. Additionally, the increase of laccase oxidation activity in the presence of a redox mediator makes laccase versatile and promising in application in biotechnological processes [11, 14].

The white-rot fungi *Lignosus rhinocerotis* (cooke) Ryvarden, formerly known as *Polyporus rhinoceros* is also known as the Tiger milk mushroom [15, 16], and it belongs to the Polyporaceae family. It is a medicinal mushroom used by the indigenous communities of Peninsular Malaysia [15] and is hailed as a 'natural treasure' in Malaysia [16]. *L. rhinocerotis* can also be found in countries such as Thailand, China, India, Japan, Indonesia, New Zealand and Australia [15, 16]. The mushroom is made of two parts: the fruiting body consisting of the pileus supported by the central stipe, and the sclerotia that is attached below the central stipe [16, 17]. The medicinal properties such as neuritogenesis [15], anti-coagulant [16], anti-inflammatory, anti-cancer, and antioxidant [15-17] are mostly attributed to the sclerotia. In recent years, industrial-scale cultivation for *L. rhinocerotis* had been achieved to meet commercial demand [17], but there are still limited reports on oxidases (particularly laccases) from *L. rhinocerotis* thus far. Following the genomic study by Yap [18] that found 4 putative laccase genes, *Lac1*, *Lac2*, *Lac3* and *Lac4* in *L. rhinocerotis* (Table 1), this study aimed to shed further light on *L. rhinocerotis'* sclerotia, specifically investigating its decolorisation capacity using the sclerotia's dialysed fractions, along with the heterologous expression of its recombinant laccase *Lac1* by *Pichia pastoris.*

Table 1. *Lignosus rhinocerotis* (Cooke) *Ryvarden* laccases found by Yap [18] and its abbreviation in this study

Abbreviation	Gene description	Accession number	e-value	Identity (%)
Lac1	laccase C [*Trametes* sp. 420]	AAW28938.1	0	82
Lac2	laccase C [*Trametes* sp. 420]	AAW28938.1	0	78
Lac3	laccase [*Coriolopsis gallica*]	AAF70119.2	0	69
Lac4	laccase [*Trametes* sp. C30]	AAR00925.1	0	83

MATERIALS AND METHODS

Total Protein Extraction and Ammonium Sulfate Precipitation

Fresh *L. rhinocerotis* Cultivar TM02 (TM02) was provided by Ligno Biotech Sdn. Bhd. TM02 was first cut into smaller pieces before it was homogenized using 0.1 M sodium acetate phosphate buffer (pH 6) at a ratio of 1:3 (w/v) and was centrifuged at 7,826 × g for 15 minutes. The supernatant was collected and precipitated with ammonium sulfate purchased from Merck, Germany at 70% saturation with continuous stirring for 6 hours. The solution was then centrifuged at 2,817 × g for 30 minutes and the pellet was dissolved with 0.1 M sodium phosphate buffer (pH 6) to be used in dialysis against the same buffer with a minimum of three buffer changes for a period of 2 days. The recovered dialysed *L. rhinocerotis* protein extract was stored at 4°C and laccase activity was determined.

Laccase Activity Determination

Laccase activity was measured spectrophotometrically using 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid (ABTS) (Amresco LLC, United States) as substrate according to Khammuang and Sarnthima's [19] method with slight modifications. The reaction mixture contained 930 µL of 0.1 M sodium acetate buffer (pH 4.5), 20 µL of 5 mM ABTS solution and 50 µL of sample. Mixture was incubated at 37°C for 10 minutes in the dark, followed by spectrophotometric absorbance reading against a blank at 420 nm. One unit of laccase enzyme activity was defined as the amount of laccase enzyme required to oxidize 1 µmol/min of ABTS substrate under assay conditions (e = 36 000 $M^{-1}cm^{-1}$) and the enzyme activity was calculated as units per mL of sample.

In Vitro Dye Decolorisation

The quantitative measurement of *in vitro* dye decolorisation of the respective Congo Red (CR) (Merck, Germany) at 20, 60 and 100 mg/L, Coomassie Brilliant Blue (CBB) (Merck, Germany) at 20, 60 and 100 mg/L and Bromophenol Blue (BB) (SYSTERM, Malaysia) at 10, 20 and 50 mg/L by the dialysed *L. rhinocerotis* protein extracts was carried out in sodium acetate buffer (pH 4.5). The dialysed *L. rhinocerotis* protein extracts were added at a ratio of 4:1 (dye solution: dialysed protein extract) in a 2 mL microcentrifuge tube and incubated at 37°C. A negative control was prepared for each concentration of the respective dyes by replacing the sample with 0.1 M sodium acetate buffer (pH 4.5). After 20 minutes, 200 µL of the treated dye solution was aliquoted and centrifuged at 6,528 × g for 1 min. The supernatant was transferred into the 96-well plate (SPL, South Korea) and absorbance was measured using a FLUOstar Omega microplate reader (BMG Labtech, Germany) at 508 nm for CR, 583 nm for CBB and 590 nm for BB. These were repeated after 1, 3 and 24 hours. The dye decolorisation was expressed as:

$$\text{Dye decolorisation (\%)} = [A_N - (A_S - A_B)]/A_N \times 100\%$$

where A_N represents the absorbance reading of negative control, A_S represents the absorbance reading of each CR, CBB and BB treated with sample and A_B represents absorbance reading of blank consisting of dialysed *L. rhinocerotis* protein extracts and 0.1 M sodium acetate buffer only.

Genomic DNA Extraction and Sequencing of *Lac1*

Lac1 was selected for genomic DNA extraction as it has the highest similarities with other laccase species as shown in Table 1. Genomic DNA of *Lac1* was extracted from TM02 using the NucleoSpin Plant II kit (MACHEREY-NAGEL GmbH & Co., US) according to the manufacturer's instructions. Amplification of *Lac1* genes was performed using the subsequent oligonucleotide couple: *Lac1* forward primer 5'-ATGTCGATGATCATGTCGAAGCTC-3'; *Lac1* reverse primer 5'-TTAGGCCTTCAATGAAGCCTCGTAC-3' which were designed based on the putative *Lac1* gene (*GME1037_g*) sequence respectively from Yap's study [18]. The PCR program was initiated at 95°C for 5 min, followed by 29 cycles of 95°C for 30 s, 51°C for 30 s followed by 72°C for 30 s and final elongation at 72°C for 5 min. The PCR product was purified using MEGAquick-spin Total Fragment DNA Purification kit (iNtRON Biotechnology, Korea), sequenced and aligned to obtain the full gDNA sequence.

RNA Extraction

Total RNA was extracted from TM02 following manufacturer's instructions from NucleoSpin RNA Plant kit (MACHEREY-NAGEL GmbH & Co., US). Reverse transcription reaction was performed using RevertAid Reverse Transcription (Thermo Scientific, US) with *Lac1* gene specific primer: 5'-TTAGGCCTTCAATGAAGCCTCGTAC-3'.
Amplifications of *Lac1* gene were performed with nested PCR using 10 µL of the synthesized cDNA and the subsequent oligonucleotide couple: forward primer 5'-ATGTCGATGATCATGTCGAAGCTC-3' and reverse primer 5'-TTAGGCCTTCAATGAAGCCTCGTAC-3' designed based on the putative *Lac1* gene (*GME1037_g*) sequence from Yap's study [18]. The PCR program was initiated at 95°C for 5 min, followed by 30 cycles of 95°C for 30 s, 51°C for 30 s, 72°C for 30 s and final elongation at 72°C for 5 min. The PCR product obtained was purified using MEGAquick-spin Total Fragment DNA Purification kit (iNtRON Biotechnology, Korea) and cloned into TOP10F' *Escherichia coli* using pGEM-T Easy vector

system (Promega, USA) following the manufacturer's instructions. The positive transformants were selected for DNA extraction and sequencing, and the results obtained were analysed using BLASTn and aligned against the *GME1037_g* sequence.

Recombinant *Lac1* Laccase Expression

The EasySelect ™ Pichia Expression Kit (Invitrogen, US) was selected for the expression of recombinant laccase. The recombinant laccase was designed based on the *Lac1* cDNA and was synthesized into pPICZαA vector by Genescript named pPICZαA/Lac (Figure 1). The growth of the *Pichia pastoris* (*P. pastoris*) X-33, linearization of pPICZαA/Lac vector, preparation of electro-competent *P. pastoris* X-33, electroporation at 1500V for 5 ms using Eppendorf Multiporator (Eppendorf, Germany) and expression of recombinant *Lac1* laccase using Buffered Methanol-complex Medium (BMMY) for secreted protein were done following the manufacturer's instructions.

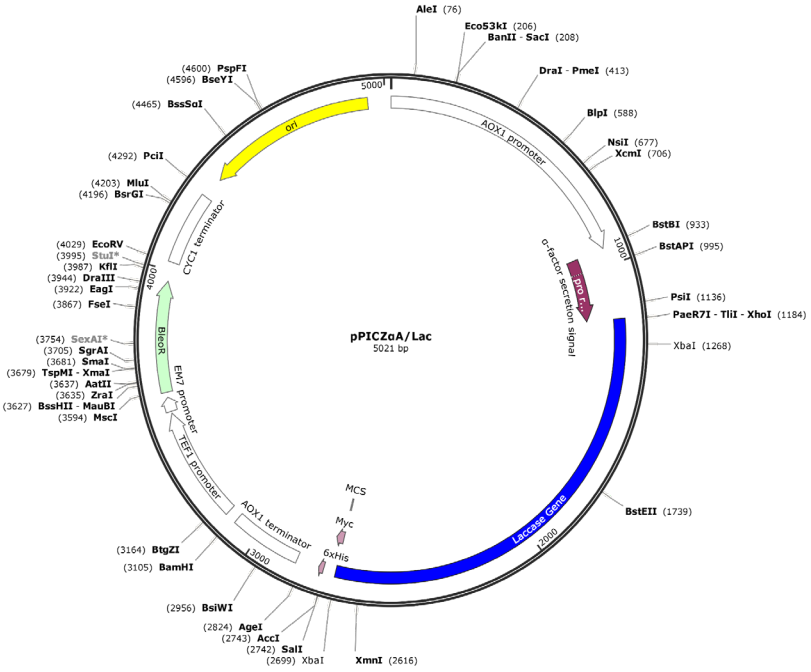

Figure 1. pPICZαA/Lac construct and its recombinant laccase DNA sequence (On a separate file)

Ni-NTA Affinity Chromatography

The Ni-NTA Spin Kit 2008 (Qiagen, US) was used to collect the desired His-tagged laccase with slight modification to the method. Briefly, 4 mL of secreted recombinant laccase supernatant was mixed with 20 mL of cold NPI buffer. Then, 1 mL of the mixture was placed into the column containing 1 mL of Ni-NTA resin and resuspended with the resin. The total mixture was transferred into a 50 mL falcon tube. The resin-mixture was shook on ice at 200 rpm for 1 hour in the KS 4000 I Shaking Incubator (IKA, US) to allow the binding of the recombinant laccase to the resin. After 1 hour, 2 mL of the resin-mixture was then placed into the column. After the resin had settled, the plug was removed and the flow-through was collected. 1 mL of the resin-mixture was added to the column continuously until all the mixture was used up.

The resin was then washed with 1 mL of NPI buffer for 3 times and the flow-through was collected in 3 different microcentrifuge tubes, each holding 1 mL of the flow-through. Then, 1 mL of cold NPI-50 buffer was added into the resin to wash away the contaminants for a total of 5 times. Lastly, NPI-500 buffer was added 3 times to elute the His-tagged laccase. All the collected flow-through and elutions were subjected to laccase activity determination. Flow-through that showed laccase activity were concentrated together using Amicon Ultra – 2 mL Centrifugal Filters (Merck, Germany) with NMWL of 10,000 and were centrifuged using an Allegra X-22 centrifuge (Beckman Coulter, US) with setting 7,000 × *g* for 30 min at 4°C. Lastly, 500 µL of NPI-10 buffer were added into the amicon and was centrifuged again with setting 5,000 × *g* for 5 min at 4°C before the resulting concentrated flow-through (24-fold) were collected.

SDS-PAGE and Western Blot

Two SDS-PAGE gel consisting of 12% resolving and 4% stacking gel was prepared in parallel as described by

Sambrook [20]. The SDS-PAGE gels were run at 80 V until the marker dye passed the stacking gel, followed by 100 V for 1 hour and 30 min. The first SDS-PAGE gel was viewed using the GS-800 calibrated densitometer (Bio-Rad, US) with the Quantity One software version 4.6.9 (Bio-Rad, US).

Western blot was done on the second SDS-PAGE gel as described by Bio-Rad (Bulletin 6376) at 100 V for 60 min to transfer the proteins from the SDS-PAGE gel into the nitrocellulose membrane. After blotting, 6x His-tagged protein detection was done using the HisDetectorTM Western Blot Kit, AP Colorimetric (Seracare, US) following the manufacturer's instructions. The picture of the developed membrane was captured using the GS-800 calibrated densitometer (Bio-Rad, USA) with the Quantity One software version 4.6.9 (Bio-Rad, USA).

RESULTS AND DISCUSSIONS

Laccase Activity

ABTS assay showed that dialyzed *L. rhinocerotis* protein extract was capable of oxidizing ABTS, yielding enzyme activity of 0.018 U/mL at 37°C. The specific laccase activity from dialyzed *L. rhinocerotis* was comparably lower (0.0024 U/mg) to specific laccase activity from other white-rot fungus species (Table 2). Despite the low enzyme activity, this shows the presence of oxidizing factors not limited to laccase found in *L. rhinocerotis* as reported in Yap's study [18]. Heterologous expression of recombinant laccase was carried out in this study with the aim to increase the production of *Lac1* laccase.

Table 2. Purification table of laccase purification from *L.rhinocerotis* compared to reported white-rot fungus

Organism	Purification step	Volume (mL)	Enzyme activity (U/ml)	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Purification fold	Yield (%)	Reference
L. rhinocerotis	Crude	50	0.0006	0.03	101	0.0003	1	1	
	NH_4SO_4 precipitation	4	0.018	0.072	29.8	0.0024	8	240	
Pleurotus sp.	Crude	800	36.25	29000	2300	36	1	100	[21]
	NH_4SO_4 precipitation	60	300	18000	1300	107	2.97	62	
Perenniporia tehropora	Crude	3450	4.12	14214	328	43.34	1	100	[22]
	NH_4SO_4 precipitation	25	456	11400	255	44.71	1.03	80	
Pleurotus ostreatus	Crude	500	125	62500	2360	134.95	100	1	[23]
	NH_4SO_4 precipitation	30	1602	48060	138	348.26	76	2.5	

Note: NH_4SO_4 precipitation = ammonium sulphate precipitation

Dye Decolorisation Capacity

Table 3 shows that the dialysed *L. rhinocerotis* protein extract could decolorise all concentrations of CR and CBB dyes. The decolorisation of azo dye (CR) and triphenylmethane dyes (CBB and BB) in this study was in line with Laura [24], who stated that fungal laccases from *Galerina sp.* are known for their ability to decolorise azo dyes and a few other classes of dyes such as triphenylmethane and anthraquinonic dyes. The average percentage for CR and CBB decolorisation was > 90% after 20 minutes for all three different concentrations (20, 60 and 100 ppm), with slight decrease in dye decolorisation (%) observed overtime for 60 and 100 ppm of CR. It was hypothesised that the decrease in CR decolorisation (%) may be due to reformation of azo bonds from some of the intermediate products, thus the dye regains its colour [25]. Li and Guthrie [26] also suggested that the intermediate product of stage 1 showed that complete degradation of azo dyes involves a two stages mechanism, as shown in below:

Stage 1: $2e^- + 2H^+ + (R\text{-}N{=}N\text{-}R') \rightarrow (R\text{-}NH\text{-}NH\text{-}R')$

Stage 2: $2e^- + 2H^+ (R\text{-}NH\text{-}NH\text{-}R') \rightarrow (R\text{-}NH_2) + (R'\text{-}NH_2)$

Hence, the intermediate product of stage 1 (R-NH-NH-R') would be unstable, yet colourless [26]. Further degradation of the intermediate product of azo dyes is therefore necessary for a complete degradation (stage 2).

The decolorisation of BB showed decolorisation of < 50% (Table 3) at concentration of 10, 20 and 50 ppm after 24 hours of treatment. The BB dye decolorization (%) was found to be inversely proportional to the concentration of BB (ppm). A maximum of 14.6% decolorisation of 10 ppm BB was obtained after 20 minutes of treatment. Similar results were reported in decolorisation of triphenylmethane dyes by

Table 3. *In vitro* dye decolorisation by dialysed *L. rhinocerotis* protein extract compared to the negative control at treatment time of 20 min, 1, 3 and 24 hrs

Dye	λ_{max}	Concentration (ppm)	Dye decolorisation (%)			
			20 min	60 min	180 min	1440 min
Congo red	508	20	91.2	92.3	97.9	96.0
		60	96.7	95.9	93.1	93.0
		100	96.7	95.5	91.3	92.3
Coomassie brilliant blue G-250	583	20	94.2	95.9	97.7	96.9
		60	93.3	94.2	95.8	97.0
		100	88.8	95.0	95.8	97.1
Bromophenol blue	590	10	14.6	10.1	10.5	10.1
		20	11.2	7.1	6.4	9.1
		50	7.0	5.7	0.0	3.2

Results were representative of mean ± S.D. (n = 3), where S.D. <0.5

laccase from another white-rot fungi *T. versicolor* [27] where four triphenylmethane dyes possessing significant structural variability expressing different dye decolorisation abilities. The highest decolorisation (%) was observed for bromocresol purple (97.8%), bromophenol blue (19.9%), phenol red (182%) and bromochlorophenol blue (15.1%) after 7 days of treatment. Chmelová and Ondrejovič [27] previously noted the presence of halogen groups in BB that decrease laccase-catalyzed decolorisation. This was also observed in our results with only 10% decolorisation of BB compared to decolorisation of CBB and BB after 24 hours of treatment.

Isolation and Sequencing of *Lac1*

The gDNA of *Lac1* [GenBank: MG210944] is 2,262 bp long, while the cDNA of *Lac1* is 2,210 bp suggesting that the cDNA could be amplified from pre-mRNA. While the sequence alignment of *Lac1* gDNA and cDNA with *GME1037_g* showed 99.9% similarity, there were still gaps between the ATG (Starting codon) and UUG (Stop codon) on the gDNA and cDNA sequence, suggesting the gaps were intron regions which is in line with characteristics of fungal introns with 5'-GT and AG-3' dinucleotides of the consensus splicing sites and lengths of 49-85 bases [28]. The 1,578 bp *Lac1* gene was obtained through alignment with *GME1037_g* sequence. The sequence alignment of *Lac1* gene with *GME1037_g* showed 99.9% similarity and homology comparison was done with the other laccase genes available in the NCBI database, with BLASTn results indicating 68% identity with laccase (TvLac3) from *T. versicolor*, showing that amplicons were laccase-specific.

***Lac1* Laccase Expression**

The pPICZαA/Lac vector received from Genescript showed an additional 2,018 bp compared to pPICZαA vector (Figure 2) and the sequencing result shows the recombinant *Lac1* gene was present. After cloning, a total of 3 surviving colonies named SA1, SA2 and SA3 all contain the 2,018 bp gene (Figure 2) indicating the cloning was successful. Besides that, the 3 surviving colonies were also capable of oxidizing ABTS as seen in Figure 3. This suggests that *Lac1* laccase is able to oxidize ABTS, and likely the dyes tested as well. However, the laccase of SA1 stopped expressing from day 2 onwards, which could be caused by ectopic recombination, where the plasmid was transformed into a heterologous region in the chromosome, causing genetic material to exchange when the yeast goes through meiosis. This could in turn cause the loss of various important genes leading to harmful effects on the yeast [29]. SA2 and SA3 that were still expressing its laccase on the 5th day. SA2 was thus selected for purification. The binding of laccase toward the resin was successful as there were no traces of oxidizing activities observed in the washing step without imidazole, but were only observed in the presence of imidazole as shown in Figure 4, suggesting further optimizing is required with lower imidazole concentration.

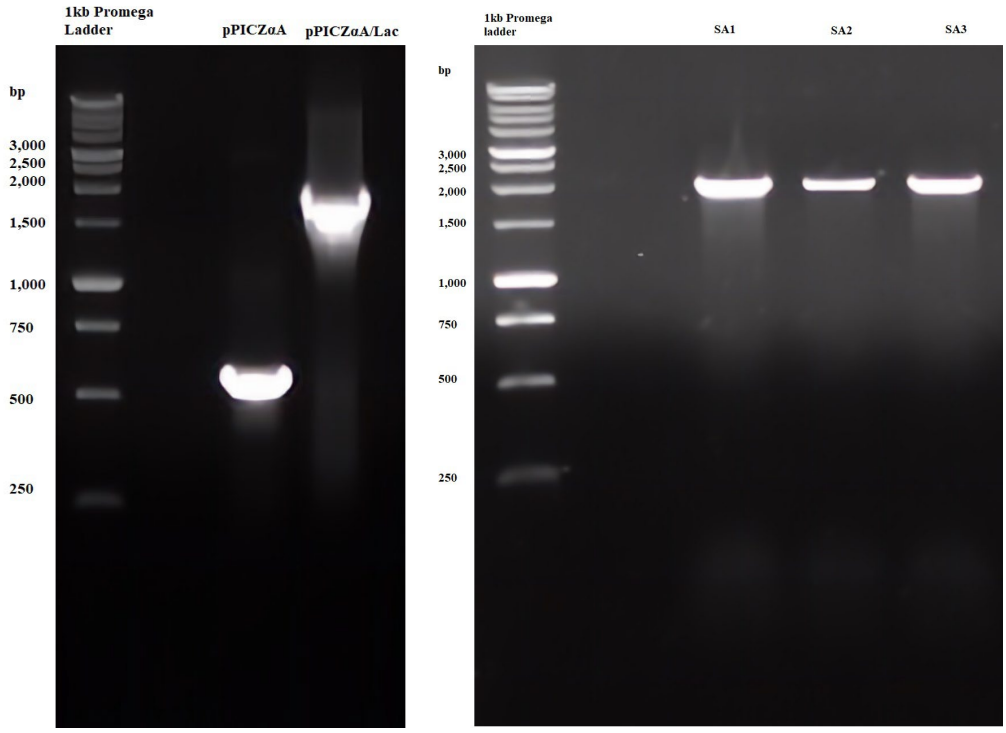

Figure 2. Comparison between pPICZαA vector and pPICZαA/Lac vector (left); PCR result of SA1, SA2 and SA3 (right), using *AOX1* sequencing primer set

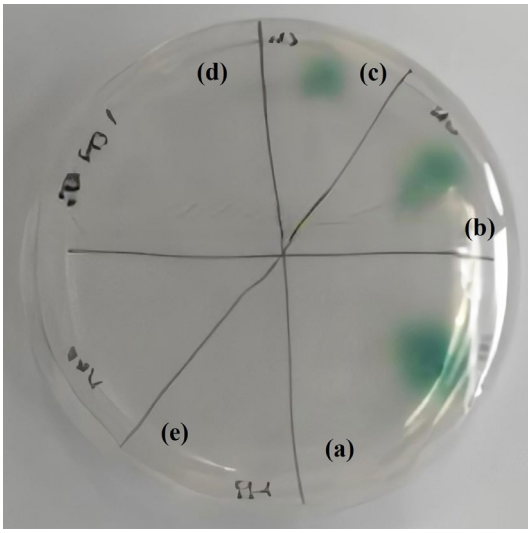

Figure 3. Minimal Methanol Agar with ABTS result of all the surviving colonies. Three positive colonies named (a) SA1, (b) SA2, and (c) SA3, along with (d) pPICZαA control and (e) Negative control *P. pastoris* X-33

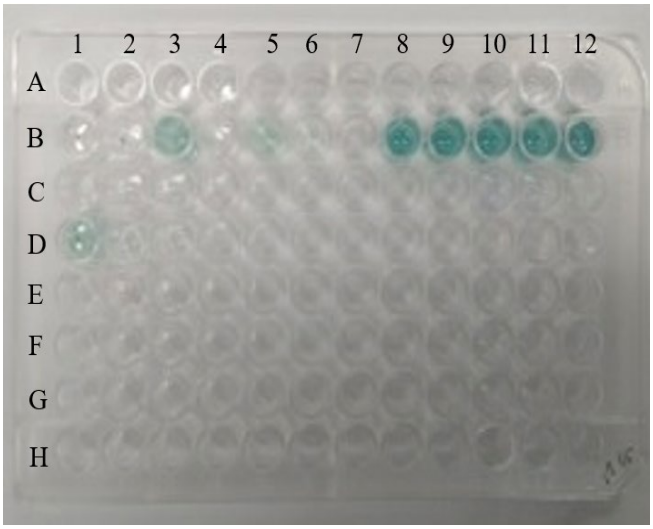

Figure 4. ABTS assay of Ni-NTA chromatography purified SA2 supernatant. Negative control (B1), flow- through (B3), first wash with NPI buffer (B5), washing with NPI-50 buffer (B8 - B12), and first elution with NPI-500 buffer (D1)

SDS-PAGE and Western Blot

The SDS-PAGE result in Figure 5 showed 3 visible bands near 46, 58 and 75 kDa for both SA2 and SA3 respectively. The gel showed the expected recombinant *Lac1* laccase, 55 kDa, present along with two other unknowns. Upon carrying out Western blot (Figure 5), a single band near the 58 kDa mark was visible indicating presence of *Lac1* in SA2. Two other contaminants were not present due to the lack of 6x His-tagged protein and thus they were not of interest in this study. There is no visible band for SA3, this data suggests that SA3 6x His-tagged *Lac1* expression was lower than SA2.

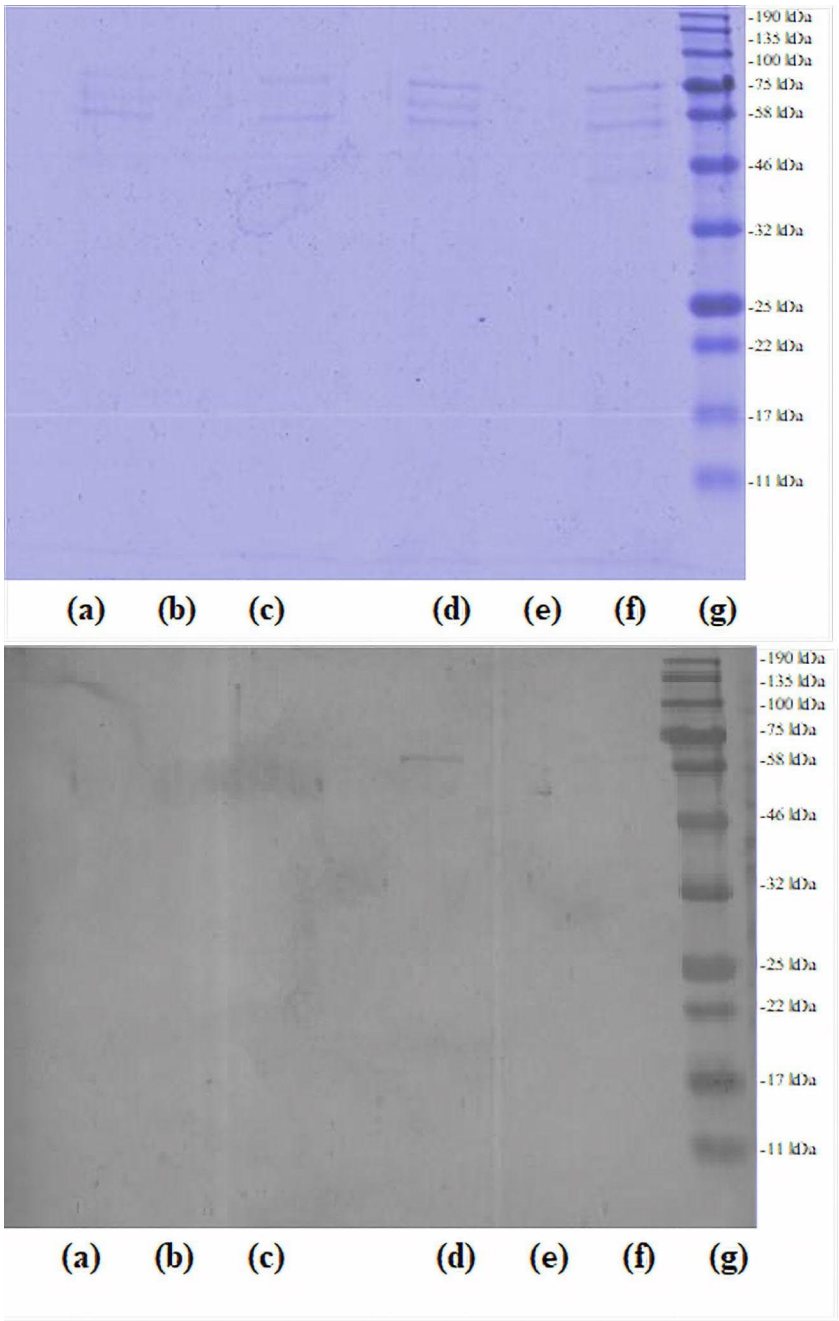

Figure 5. SDS-PAGE (top) and western blot (bottom) of SA2 and SA3. (a) concentrated SA3 using Amicon centrifugal filter, (b) SA3 first elution (c) SA3 first flow through, (d) concentrated SA2 using Amicon centrifugal filter, (e) SA2 first elution, (f) SA2 first flow-through, and (g) Blue protein standard marker (NEB, UK)

CONCLUSION

The present study unravels the potential of *Lignosus rhinocerotis* sclerotia in decolorising the textile dyes Congo red and Coomassie brilliant blue dye with decolorising capacity of ~90%. The gDNA of *Lac1* [MG210944] was successfully updated in GenBank. The *Lac1* laccase gene which was previously identified by Yap was successfully cloned into the *P. pastoris* X-33 while also demonstrating its activity. This opens the door for future application of this recombinant laccase in the remediation of dye effluent from textile industry and industrial run-off treatment.

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CONFLICT OF INTERESTS

The authors declare that there is no conflict of interests regarding the publication of this manuscript.

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