

1 **Heterologous expression of *Stlac2*, a laccase isozyme of *Setosphaeria turcica*, and the ability of**

2 **decolorization of malachite green**

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10

11 **Abstract**

12 Laccases can catalyze monoelectronic oxidation and have shown to have an increasing value in industrial  
13 application. In this study, as identified by Native-PAGE and ESI-MS/MS, ascomycetous fungus  
14 *Setosphaeria turcica* produced three laccase isozymes: Stlac1, Stlac2, and Stlac6. Stlac2 was  
15 heterologously expressed in both eukaryotic and prokaryotic expression systems. The eukaryotic  
16 recombinant Stlac2 expressed in *Pichia pastoris* was inactive, and also showed a higher molecular weight  
17 than predicted because of glycosylation. The depression of laccase activity was attributable to the  
18 incorrect glycosylation at Asn97. Stlac2 expressed in *Escherichia coli* and after being renatured from  
19 the inclusion body, the recombinant Stlac2 exhibited activity of 28.23 U/mg with 2,2-azino-bis(3-  
20 ethylbenzothiazoline-6-sulfonic acid) (ABTS) as the substrate. The highest activity was observed at pH  
21 of 4.5 and the temperature of 60 °C. The activity of recombinant Stlac2 was inhibited by 10 mM Na<sup>+</sup>,  
22 Mg<sup>2+</sup>, Ca<sup>2+</sup>, Mn<sup>2+</sup>, and increased by 10 mM of Fe<sup>3+</sup> with a relatively activity of 315% compared with no  
23 addition. Cu<sup>2+</sup> did not affect enzyme activity. Recombinant Stlac2 was capable of decolorizing 67.08%  
24 of 20 mg/L malachite green in 15 min without any mediators. It is suggested that Stlac2 has potential  
25 industrial applications.

26 **Importance**

27 *Setosphaeria turcica*, an ascomycetous fungus causes northern corn leaf blight, product three laccase  
28 isozymes identified by Native-PAGE and ESI-MS/MS. The major expression laccase gene *StLAC2* was  
29 expression in both eukaryotic and prokaryotic expression systems, which found incorrect glycosylation  
30 at Asn97 may result in the depression of laccase activity. The heterologous laccase Stlac2 decolorize  
31 organic dye malachite green, which had a potential industrial application.

32 **Keywords**

33 *Setosphaeria turcica*, Laccase isozyme, Expression, Enzyme characterization, Decolorization

34

35 **Introduction**

36 Laccases (EC1.10.3.2, benzenediol: oxygen oxidoreductases, p-diphenoloxidase) are multicopper  
37 oxidases (MCO) that can catalyze monoelectronic oxidation of more than 200 compounds, such as  
38 phenolic compounds and aromatic amines, as well as their derivatives. Using the redox mediators,  
39 laccases can expand the substrate range to catalyze polyphenol polymers like lignin(Giardina et al. 2010;  
40 Munk et al. 2015). Laccases are widely present in plant, insect, fungus, and bacterial species, though  
41 fungal laccase are the most studied and only used in biotechnological applications. In general, most  
42 fungal laccases are extracellular glycoproteins with molecular weight of 55-85 kDa and carbohydrate  
43 content of 10%–20% and even up to 25%(Maestre-Reyna et al. 2015). There are three cupredoxin-like  
44 domains in fungal laccase, and four copper ions of three types of metal binding sites (T1-T3). The  
45 substrates are bound in T1 and electrons transfer to the T2/T3 center, where O<sub>2</sub> is reduced to H<sub>2</sub>O. The  
46 sequence of amino acids and the glycosylation have influence in catalytic activity and redox potential of  
47 laccase, causing laccases to exhibit different characteristics even within the same strain. There are  
48 multiple isoforms of laccase that have been reported in a number of fungi. Laccase multigene familiy  
49 exhibit distinct expression patterns influenced by factors such as development, culturing condition, and  
50 inducers. *Pleurotus ostreatus* has 12 laccase isozymes that were investigated with different biochemical  
51 characteristics (Park et al. 2015). Homologously overexpressed 10 laccase-like MCO genes in  
52 *Aspergillus Niger* ATCC 1015 show different substrate specificities (Ramos et al. 2011). Owing to the  
53 similar molecular weight and pI, it is difficult to purify isozymes with conventional techniques. Native-  
54 PAGE is a gel-based electrophoretic system for analyzing active proteins. It is used to separate and  
55 identify laccase isozymes because of the property of color change on substrates like 2,2-azino-bis (3-  
56 ethylbenzothiazoline-6-sulfonic acid) (ABTS). In *Ganoderma* sp., multiple laccase isozymes with

57 molecular weight in the range of 40–66 kDa were separated and identified by Native-PAGE and MALDI-

58 TOF (Kumar et al. 2017).

59 Laccases have increasing value in industrial application, especially in dye and other xenobiotic

60 degradation, paper and pulp processing, organic synthesis of petrochemical industries, and food

61 processing(Upadhyay et al. 2016). Among these, the effective decolorization of dye in wastewater by

62 laccase has been widely studied. The typical triphenylmethane dye malachite green (MG) is used as dye

63 in textile, paper, and even fungicide in aquaculture, with toxicological and carcinogenic effects on

64 various fish species and certain mammals. Laccase can decolorize and reduce toxicity of MG

65 (Forootanfar et al. 2011; Mostafa and Abd El Aty 2018; Qu et al. 2018). For using laccase more

66 efficiently in biotechnology, many laccases have been purified and even expressed heterologous in

67 eukaryotic and prokaryotic expression systems (Antosova and Sychrova 2016; Forootanfar and

68 Faramarzi 2015). Although fungi laccase has a broad substrate range and high redox potential, its high

69 level of glycosylation, which plays a role in the structure and function of enzyme activity, causes

70 obstacles in heterologous expression(Ergun and Calik 2016).

71 *Setosphaeria turcica* (syn. *Exserohilum turcicum*), is an ascomycetous fungus that causes northern corn

72 leaf blight (NLB) in corn. Previously, the strain 01-23 was isolated from the disease sample of NLB from

73 Liaoning, China. There are 9 laccase-like MCOs sharing low identities of amino acid sequences, which

74 of genes are completely consistent with the published sequences from *S. turcica* Et28A

75 (<http://genome.jgi.doe.gov>)(Liu et al. 2018). Stlac2 (GenBank: XM\_008023812.1), which belongs to

76 ascomycetous MCO, is shown to be involved in cell wall integrity and DHN-melanin synthesis of

77 pathogens by gene-knockout. Further, the laccase activity of mutants of *StLAC2* was decreased, which

78 revealed its function as a laccase (Ma et al. 2017). In the present study, the laccase isozymes of *S. turcica*

79 were investigated and identified by Native-PAGE and ESI-MS/MS. The major expression laccase gene  
80 *StLAC2* was heterologous expression in both eukaryotic and prokaryotic expression systems to study the  
81 effect of glycosylation, as well as to characterize its physicochemical properties and decolorization to  
82 malachite green. This study proved ascomycete laccase in *S. turcica* to be a valuable resource for the  
83 application in the green industry for the future.

84 **Materials and methods**

85 **Fungi culture for laccase production and isolation of laccase isozymes**

86 *S. turcica* stain 01-23 as isolated from NLB samples of corn leaf from Liaoning province, China. The  
87 strain was deposited in the China General Microbiological Culture Collection Center (No.9857) and  
88 stored on PDA medium at 4 °C in the Key Laboratory of Hebei Province for Plant Physiology and  
89 Molecular Pathology. *S. turcica* were inoculated in 50 mL Erlenmeyer flasks containing 20 mL of  
90 Completed medium (CM)(Talbot et al. 1993) supplemented with 0.05 g/L CuSO<sub>4</sub> cultivated at 28 °C for  
91 7 days for laccase production. The filtered culture broth was concentrated using an Amicon Ultra-15  
92 membrane filter (Millipore, Germany) as extracellular crude protein samples for isolation. 0.2 g mycelia  
93 was ground in liquid nitrogen and dissolved in 10 mL phosphate buffered solution (PBS) for extracting  
94 intercellular crude protein. Laccase isozymes were isolated using Native-PAGE (10%) staining with 0.1  
95 M citrate-phosphate buffer (pH 4.0) containing 2 mM laccase substrates ABTS and 2,6-DMP (2,6-  
96 dimethoxyphenol), and incubated at room temperature.

97 **Identification of laccase isozymes and glycosylation analysis by ESI-MS/MS**

98 Stained active bands of laccase isozyme were used for identifying by peptide finger printing (ESI-  
99 MS/MS analysis). The bands obtained by Native-PAGE were according to the batch procedures outlined  
100 by Beijing Protein Innovation Co., Ltd. The MS/MS spectra of the peptide fragments were searched in

101 the *Setosphaeria trucica* Et28A v1.0 protein database from the National Center for Biotechnology  
102 Information (NCBI). A truly positive peptide was defined to be located at rank one of the search results  
103 and reached a confidence level above 95% in the search against the substrate database. The spectra  
104 corresponding to the identified peptides were then manually examined.

105 **RNA extraction and cDNA synthesis**

106 Filtered mycelium of *S. turcica* was collected and ground using liquid nitrogen. Total RNA was extracted  
107 using RNA Simple Total RNA kit (Tiangen, China) and the quantity was estimated by Eppendorf Bio  
108 Photometer plus. Reverse-transcription was using Prime Script<sup>TM</sup> RT reagent Kit with gDNA Eraser  
109 (TaKaRa, Japan) to obtain cDNA for amplification of *StLAC2*.

110 **Expression of recombinant laccase *Stlac2* in *Pichia pastoris* KM71H**

111 cDNA of *StLAC2* for eukaryotic expression was amplified by PCR using a primer StLAC2-PP-F (5'-  
112 CATGGAATTCATGTCTTACAATGGTTC-3') incorporating a *Eco*RI restriction site and a primer  
113 StLAC2-PP-R(5'-CATGTCTAGATCAATGATGATGATGATGCAGGCCGAGTCGATCTG-3')  
114 incorporating a *Xba*I restriction site for cloning in pPICZ $\alpha$ A expression vector (Fig.S2b). PCR was  
115 carried out using Taq DNA polymerase and the amplified fragment was extracted for insertion into the  
116 pMD-19 vector for sequencing. The recombinant pMD-19-*StLAC2* was digested with *Eco*RI and *Xba*I,  
117 and then ligated into the pPICZ $\alpha$ A vector digested with the same restriction enzyme to construct the  
118 plasmid pPICZ $\alpha$ -*StLAC2*.

119 The pPICZ $\alpha$ -*StLAC2* plasmids with correct insertion confirmed by sequencing were transformed into the  
120 host strain *P. pastoris* KM71H by electro-poration. Transformants were selected and plated on YPDS  
121 plates containing 100  $\mu$ g/mL zeocin and incubated for 2 days at 28 °C. Resultant colonies were screened  
122 by PCR using  $\alpha$ -factor primer (5' -TACTATTGCCAGCATTGCTGC-3') and 3'AOX primer (5' -

123 GCAAATGGCATTCTGACATCC-3') (Fig.S2d).

124 The correct transformants were inoculated into 4 mL BMGY medium for 20 h at 28 °C and 220 rpm. The  
125 cells were then centrifuged and re-suspended in BMMY medium (OD600~1.0). Methanol was added to  
126 a final concentration of 1% (v/v) for 3 days. Expression cultures were subjected to 10% SDS-PAGE  
127 analysis. The empty vector pPICZ $\alpha$ A was used as the control. Western blot of supernatant of induced  
128 culture after 6 days was carried out according to the method as described previously(Ma et al. 2018).

129 **Expression and purification of recombinant laccase Stlac2 in *Escherichia coli* Rosetta (DE3)**

130 cDNA of *StLAC2* for prokaryotic expression was amplified by PCR using a primer StLAC2-EC-F (5'-  
131 CATGGAATTCATGTCTTACAATGGTCG-3') incorporating a *Eco*RI restriction site and a primer  
132 StLAC2-EC-R (5'-CATGAAGCTTCACAGGCCGAGTCGATCTGG-3') incorporating a *Hind*III  
133 restriction site for cloning in pET32 expression vector (Fig.S2c). The plasmid pET32-*StLAC2* was  
134 constructed as mentioned above, but by using *Eco*RI and *Hind*III, which was transformed into *E. coli*  
135 Rosetta (DE3). Transformants were selected on LB with 100  $\mu$ g/mL ampicillin and confirmed by PCR  
136 using T7 primer (5' -TAATACGACTCACTATAGGG-3') and T7 Terminator primer (5'-  
137 GCTAGTTATTGCTCAGCGG-3') (Fig.S2e).

138 The transformants with recombinant vector pET32-*StLAC2* were cultured at 37 °C and at 220 rpm with  
139 50  $\mu$ g/mL ampicillin. When the OD600 reached 0.7-0.8, 1 mM isopropyl  $\beta$ -D-thioacetamide (IPTG) was  
140 added to induce the expression of recombinant Stlac2. Cells were collected 3 h after adding IPTG and  
141 detected by 10% SDS-PAGE. The recombinant laccaseStlac2 was purified by 6  $\times$  His Ni-IDA Resin  
142 (Probe Gene, China).

143 The recombinant laccaseStlac2 was purified by denaturation and renaturation from inclusion body by the  
144 Inclusion Body Protein Extraction Kit (Sangon, China). The samples before and after renaturation were

145 tested by SDS-PAGE. BCA Protein Assay kits (Sangon, China) were used to analyze the protein  
146 concentration.

147 **Deglycosylation assay and identification of glycosylation sites**

148 For N-glycan removal, eukaryotic recombinant Stlac2 was denatured with Glycoprotein denaturing  
149 buffer at 100 °C for 10 min to be deglycosylated using PNGase F (NEB, USA) according to instructions.

150 The deglycosylated recombinant laccase was separated by SDS-PAGE. Stained band of recombinant  
151 laccase expressed in *P. pastoris* was used for identification of the sites of glycosylation and were  
152 conducted in accordance with the batch procedures outlined by Beijing Protein Innovation Co., Ltd.

153 N-glycosylation sites (Asn-X-Ser/Thr) were predicted with NetNGlyc 1.0  
154 (<http://www.cbs.dtu.dk/services/NetNGlyc/>). Glycosylation sites of Stlac2 were compared in the crystal  
155 structures of laccases – which have more similarities with Stlac2 including the laccase in *Melanocarpus*  
156 *albomyces*(PDB ID:1GW0), laccase from *Botrytis aclada* (PDB ID:3SQR), McOG from *Aspergillus*  
157 *niger*(PDB ID:5LM8), and Ascomycete fungal laccase from *Thielavia arenaria*(PDB ID: 3PPS). The  
158 alignment of amino acid sequence was performed by DNAMAN. For the analysis of the effect of  
159 glycosylation, the three-dimensional structure of Stlac2 was generated by YASARA (Land and Humble  
160 2018) and the result was revealed and analyzed by Pymol.

161 **Assay of laccase activity and biochemical characterization of recombinant Stlac2**

162 ABTS was used as a substrate for assaying recombinant Stlac2 activity at 420 nm ( $\epsilon_{420}=36 \text{ mM}^{-1}/\text{cm}^{-1}$ ).

163 One unit (U) of laccase activity was defined as the oxidation of 1  $\mu\text{mol}$  ABTS in one minute.

164 The enzyme activity was assayed in the pH range of 3.0-6.5 using 0.1 M of citrate buffer. The effect of  
165 temperature was determined by incubating the reaction mixture at different temperatures varying from  
166 30 °C to 80 °C under standard assay conditions at pH 4.5. The enzyme activity was expressed as percent

167 relative activity with respect to maximum activity, which was considered as 100%. The effects of various  
168 metal ions were determined by incubating the recombinant Stlac2 at a final concentration of 5 and 10  
169 mM for 5 min at 60 °C. The enzyme activity was expressed as percent relative activity with respect to no  
170 additional ions.

171 **MG decolorization by recombinant Stlac2**

172 To evaluate the application, triarylmethane dye MG was used to analyze the degradation by Stlac2. The  
173 reaction mixture contained 100 mM Tris buffer with 20, 50, 100, and 200 mg/L MG were conducted and  
174 50 µg purified recombinant Stlac2 in 0.5 mL reaction volume, and incubated at room temperature. The  
175 decolorization of MG was followed by measuring absorbance at 618 nm, where the degradation  
176 efficiency was calculated as a percentage(Yang et al. 2017).

$$177 \text{Decolorization}(\%) = \frac{A_0 - A_f}{A_0} \times 100$$

178 where  $A_0$  = initial absorbance and  $A_f$ = final absorbance.

179 All experiments were performed in triplicate; controls were not treated with recombinant Stlac2.

180 **RESULTS**

181 **Isolation and identification of laccase isozymes**

182 Intracellular and extracellular crude protein were collected from *S. turcica* in liquid CM culture 7d using  
183 for isolation of laccase isozymes by Native-PAGE staining with ABTS and 2,6-DMP. It is more sensitive  
184 than staining with ABTS than with 2,6-DMP (Fig. S1). There are two bands in the intracellular crude  
185 protein and one in concentrated extracellular crude protein staining with ABTS (Fig.1a). The two bands  
186 were tested by ESI-MS/MS to identify laccase isozymes, and the peptide fingerprints were compared  
187 with the protein database of *S. turcica* downloaded from JGI (Fig.1b). The peptide sequence of proteins

188 matched with Stlac2 (Gi: 482813379) and Stlac6 (Gi: 482808389) of band I; Stlac1 (Gi: 482805212),

189 and Stlac6 (Gi: 482808389) of band II, all of which are classified to be laccase *sensu stricto* in *S. turcica*.

190 The molecular weight of the laccase isozymes did not match the specific bands of protein markers shown.

191 This is attributable to the fact that protein separation in Native-PAGE depended not only on molecular

192 size, but also the charge the protein carried.

193 **Recombinant laccase Stlac2 expression in *Pichiapastoris*KM71H**

194 To characterize laccase Stlac2, the coding sequence was cloned and inserted into the plasmid pPICZ $\alpha$ A

195 including  $\alpha$ -factor signal and 6 histidine tag (Fig. S2b). The constructed plasmid pPICZ $\alpha$ -*StLAC2* was

196 transformed into *P. pastoris*KM71H, and the transformants grew well on the YPD medium containing

197 zeocin(100  $\mu$ g/mL) was verified by PCR (Fig. S1d) using primers of  $\alpha$ -factor and AOX, indicating that

198 pPICZ $\alpha$ -*StLAC2* was integrated into the AOX sites of *P. pastoris*.

199 Recombinant laccase Stlac2 with  $\alpha$ -factor signal was detected in the cell culture supernatant after

200 methanol induction by SDS-PAGE with a single apparent band of different positive transformant samples

201 of pPICZ $\alpha$ -*StLAC2*, though no bands appeared in the samples of induced control with empty vector

202 pPICZ $\alpha$ -*StLAC2* or non-induced positive transformants (Fig. 2a). The recombinant laccase was

203 confirmed by Western blot (Fig. 2b). The molecular weight of recombinant laccaseStlac2 was about 100

204 kDa compared with the protein marker, but Stlac2 (554 amino acids) had the predicted molecular weight

205 of 61.64 kDa. In order to exclude the possibility of protein dimer, the reducing agents of 20%  $\beta$ -mercapto

206 ethanol and 1.0 mol/L DTT was added to depolymerize. The results showed that the single apparent band

207 did not change (data not shown), meaning that recombinant laccase Stlac2 was monomer with a much

208 larger molecular weight than predicted.

209 **Recombinant laccase Stlac2 expression in *E. coli* Rossetta (DE3) and purification**

210 To verify the confused molecular weight of recombinant Stlac2, the coding sequence was inserted into  
211 the plasmid pET32a without signal peptide (Fig. S2c). The constructed plasmid pET32-*StLAC2* was  
212 transformed into *E. coli* Rosetta (DE3), and the transformants growing on the LB medium containing  
213 ampicillin (50 µg/mL) was verified by PCR (Fig. S1e) using T7 primers.  
214 Recombinant laccase Stlac2 with an apparent molecular weight about 80 kDa with histidines tag but not  
215 signal peptide was detected in the whole-cell extract of cultural cells after IPTG induction compared with  
216 non-induction (Fig. 3a). The recombinant laccase was purified by Ni-IDA (Fig. 3b), and the specific band  
217 of Stlac2 was present in the precipitate, indicating that the expressed protein was in the inclusion bodies  
218 of about 70% of purity. The target protein was purified by denaturation and renaturation from inclusion  
219 bodies with the purity of 87% and the concentration of 1.03 mg/mL. The activity of purified recombinant  
220 Stlac2 using ABTS as substrate at pH4.5 citrate buffer and room temperature was 28.23 U/mg.

221 **Glycosylation of Stlac2 recombinant protein in *P. pastoris***

222 When the activity of Stlac2 recombinant protein in *P. pastoris* was detected using ABTS, the color of  
223 reaction mixture did not change, compared with the same concentration of protein of Stlac2 recombinant  
224 protein in *E. coli* and crude protein from *S. turcica*, which changed the color of ABTS to dark green and  
225 increased over time (Fig. 4a). The glycosylation of Stlac2 recombinant protein in *P. pastoris* was detected  
226 (Fig. 4b). After the deglycosylation using PNGase F, there were two bands with molecular weight of  
227 approximately 80 kDa and 90 kDa, respectively, compared with 100 kDa before deglycosylation,  
228 suggesting that recombinant Stlac2 in *P. pastoris* was glycosylated at two sites of Asn-X-Ser/Thr.  
229 7 potential N-glycosylation (Asn-X-Ser/Thr) sites were detected by NetNGlyc 1.0 server, including Asn4,  
230 Asn97, Asn206, Asn373, Asn382, Asn383, and Asn475. From these, Asn382 was indicated as non-  
231 glycosylated with the 'potential' score is less than the default threshold of 0.5. In order to identify the

232 glycosylation sites affecting laccase activity, the stained band of recombinant laccase Stlac2 expressed  
233 in *P. pastoris* was identified by peptide mass fingerprinting (ESI-MS/MS analysis) and positive peptide  
234 was located in the sequence of Stlac2, as shown in Fig.5. The peptides containing the predicted  
235 glycosylation sites Asn206, Asn373, and Asn475 was detected with no glycosylation, but the peptides  
236 containing Asn4, Asn97, Asn382, and Asn383 were not covered. Stlac2 and the laccases of which N-  
237 glycosylation sites were known were aligned to analyze potential glycosylation sites of fungal laccase.  
238 It was found that N-glycosylation of asparagine residue in positions 197 and 382 are highly conserved  
239 among laccase proteins, but only the position of Asn382 in Stlac2 had the possibility of N-glycosylation.  
240 Three-dimensional structural simulation of Stlac2 showed that, when the carbohydrate chain of N-acetyl-  
241 D-glucosamine attaches to Asn97, it may block the egress for the water molecules, resulting from the  
242 reduction of the dioxygen molecule, potentially affecting the activity of laccase.

#### 243 **The activity of the recombinant laccase under different temperatures, pH values, and ions**

244 The oxidation of ABTS by the purified laccase in 0.1 M citrate buffer was evaluated at different levels  
245 of pH, temperature, and metal ions. The purified recombinant laccase showed the highest activity at pH  
246 4.5 (Fig. 6a) and 60 °C (Fig. 6b). The effects of different concentrations of metal ions on purified laccase  
247 activity were tested by evaluating oxidation of ABTS at the optimum pH and temperature for 5 min in  
248 the presence of different ions (Fig. 6c). The results showed that the activity of Stlac2 was relatively stable  
249 in the presence of Cu<sup>2+</sup>, which is a structural component of the catalytic center. The relative activity of  
250 Stlac2 increased to 315% when the concentration of Fe<sup>3+</sup> increased up to 10 mM. 5 mM Mn<sup>2+</sup> increased  
251 the laccase activity, while the activity decreased when the concentration of Na<sup>+</sup>, Mg<sup>2+</sup>, Ca<sup>2+</sup>, and Mn<sup>2+</sup>  
252 was 10 mM.

#### 253 **Decolorization ability of Stlac2 on MG**

254 Malachite green is an organic compound that is used as a dyestuff, potentially causing carcinogenic  
255 symptoms. The decolorization activity of recombinant Stlac2 on malachite green was investigated to  
256 demonstrate its industrial applicability. The decolorization of recombinant Stlac2 were analyzed after  
257 being treated at 20, 50, 100, and 200 mg/L malachite green for 15 min, 30 min, 45 min, 1 h, 1.5 h, and 3  
258 h (Fig.7). Stlac2 displays an excellent decolorization activity without any redox mediators under 20 mg/L  
259 malachite green, as Stlac2 decolorized 67.08% MG in 15 min and more than 70% of 50 mg/L malachite  
260 green after 3 h of incubation. With the increase of MG concentration, the decolorization rates showed the  
261 trend of gradual reduction. The decolorization efficiency was not significantly different when the  
262 concentration of MG was more than 100 mg/L.

263 **Discussion**

264 In this work, using Native-PAGE and ESI-MS/MS identification, there are 3 laccase isozymes produced  
265 by *S. turcica*, while 9 laccase-like multicopper oxidases were found in the genome using a Hidden  
266 Markov Model for three Pfam copper oxidase families (Liu et al. 2018). Laccases multigene families are  
267 diverse in fungi with different expression patterns. There are 17 laccase genes in *Coprinopsis cinerea*  
268 and 4 laccase-type multicopper oxidases in *Leptosphaerulina* sp. ( Kilaru et al. 2006; Copete et al. 2015).  
269 However, only several isozymes of the multifamily can be detected and separated. For example, 12  
270 laccase genes are identified in the whole genome sequence of *P. ostreatus*, but only six laccases have  
271 been isolated and characterized(Jiao et al. 2018). *Ganoderma lucidum* contains 16 laccase genes in its  
272 genome, and three to five isozymes are secreted under different cultural conditions (Dongbo et al. 2012;  
273 Fang et al. 2015). Further, isozymes show different substrate specificity even for the common substrates  
274 ABTS and DMP. All 17 laccase isozymes of *C. cinerea* showed laccase activities with DMP but only  
275 eight of them with ABTS(Kilaru et al. 2006). In *S. turcica*, 9 laccase-like multicopper oxidases were

276 identified with low sequence similarity (19.79–48.70%) and they were found with different expression  
277 levels during the growth and infection process(Liu et al. 2018). *StLAC2*, *StLAC6*, and *StLAC8* were  
278 highly expressed with different degrees when detected by q-PCR. They have the similarly predicted  
279 molecular weight of 60.83–68.10 kDa and pI, signifying that it may be difficult to separate. Native-PAGE  
280 was used to separate isozymes here to find out the active laccase in *S. turcica*. The results showed that in  
281 *S. turcica*, the bands are the same when stained with ABTS and DMP. One band was identified as *Stlac2*  
282 and *Stlac6*. *Stlac2* has a lower predicted molecular weight of 61.64 kDa and low predicted pI of 5.00,  
283 and it was mostly secreted intracellularly. The other band was identified as *Stlac1* and *Slac6*, which were  
284 detected both intracellularly and extracellularly. *Stlac1* and *Stlac6* shared the highest identity of 48.70%  
285 in 9 laccase-like multicopper oxidases of *S. turcica*; had a similar predicted molecular weight of 65.73  
286 kDa and 65.85 kDa, respectively. Since predicted pI of *Stlac6* is 5.07, lower than 5.65 of *Stlac1*, and  
287 predicted molecular weight larger than *Stlac2*, the band of *Stlac6* should be detected between *Stlac1* and  
288 *Stlac2*(Liu et al. 2018). But *Stlac6* was detected in both of two bands, which means there may be different  
289 glycosylation states(Rai et al. 2010). The other laccases with high gene expression levels, such as *Stlac8*,  
290 were not detected. Different cultural conditions and additions of aromatic compounds may also lead to  
291 differential production of laccase isozymes(Kumar et al. 2017). Thus, further understanding of laccase  
292 isozymes in *S. turcica* for basic and applied purposes will be investigated in future studies.  
293 *Stlac2* was heterologously expressed in eukaryotic expression system *P. pastoris* KM71H, and the results  
294 showed that recombinant *Stlac2* is incapable of oxidizing the substrates ABTS and DMP (data of DMP  
295 was not showed). Most fungal laccases are reported as glycoprotein with a carbohydrate content of 10%–  
296 20% and even up to 25% (Maestre-Reyna et al. 2015; Morozova et al. 2007). Owing to the fact that the  
297 *P. pastoris* expression system has the ability of post-translational modifications, it is often used to

298 heterologously express laccase(Fonseca et al. 2018; Kumar et al. 2018). Glycosylation in general  
299 increased the thermostability of fungi enzymes, but depending on the glycosylation position it might lead  
300 to increased or reduced catalytic activity(Ergün and Çalik 2015). In our case, recombinant Stlac2 can be  
301 expressed in *P. pastoris*, detected extracellularly, but it is inactive. The higher molecular weight of  
302 recombinant Stlac2 than predicted is attributable to the glycosylation status. There are a predicted 7  
303 potential N-glycosylation, and the glycosylation of 3 potential glycosylation sites were excluded by ESI-  
304 MS/MS analysis of recombinant Stlac2. The glycosylation site of Asn382 is conserved compared with  
305 the glycosylation sites of the known laccase proteins, revealing that its glycosylation has no obvious  
306 influence on enzyme activity. When a three-dimensional structural simulation of Stlac2 was used for the  
307 analysis, it was shown that the glycosylation of Asn97 the undetected glycosylation site may result in the  
308 depression of laccase activity by blocking the release route of water molecules in the channel of dioxygen  
309 reduction(Bento et al. 2005). When the prokaryotic expression system *E. coli* was used, recombinant  
310 protein without glycosylation achieved the activation of ABTS and even the degradation of MG, which  
311 seems like glycosylation of Stlac2 inessential for function.  
312 Here, the recombinant Stlac2 expressed in *E. coli* precipitated in inclusion bodies may affect enzyme  
313 activity when renaturated, though it still has a relatively high activity on ATBS. Compared with bacterial  
314 laccase, less fungal laccases have been expressed in *E. coli* (Brissos et al. 2010; Claudio et al. 2013; Ece  
315 et al. 2017; Suzuki et al. 2003). The heterologous expression of the gene LACP83 (encoding laccase)  
316 from *P. ostreatus* in *E. coli* was obtained with activity of 3740 U/L, which was similar to that reported  
317 for the native strain of *P. ostreatus* at 144 h of culture(Grandes-Blanco et al. 2017). A recombinant laccase  
318 of *Rigidoporus lignosus* expressed in *E. coli* can be used as a new enzymatic biosensor for medical  
319 purposes. We have heterologously expressed Stlac4 of *S. turcica* in *E. coli* and the activity of up to 127.78

320 U/mg(Ma et al. 2018), which is higher than 28.23 U/mg of Stlac2, which is likely attributable to the  
321 formation of inclusion body in Stlac2. Compared with recombinant Stlac4 of *S. turcica* and other purified  
322 laccase, Stlac2 has similar activation with temperature and pH. Stlac2 has more tolerance to metal ions  
323 like  $\text{Na}^+$ ,  $\text{Mg}^{2+}$ , and  $\text{Ca}^{2+}$  with the concentration of 5 mM, inhibiting the activity of Stlac4. However,  
324 when the concentration is increased to 10 mM, the activity of Stlac2 was decreased, similar to Stlac4.  
325 The effect of  $\text{Cu}^{2+}$  on Stlac2 was not as obvious as Stlac4. When 10 mM $\text{Cu}^{2+}$  was added, the relative  
326 activity of Stlac2 was 108.0%, while that of Stlac4 was 217.4%. The activity of Stlac2 is increased by  
327 adding up to 10 mM of  $\text{Fe}^{3+}$ mM, which is consistent with Stlac4, but contrary to other laccases where  
328 they were inhibited by  $\text{Fe}^{3+}$ (Pawlik et al. 2016). It seems like laccases of *S. turcica* were more sensitive  
329 to  $\text{Fe}^{3+}$ . Laccases have shown the ability to decolorize industrial dyes in the presence of redox mediators  
330 like ABTS and HBT(Wang et al. 2016; Wang and Zhao 2017), but some laccases are more eco-friendly  
331 and can decolorize dyes without mediators(Campos et al. 2016; Yang et al. 2015). Recombinant Stlac2  
332 can rapidly decolorize 67.08% MG of 20 mg/L in 15 min without any mediators. It is suggested that  
333 recombinant Stlac2 have potential application and benefits in both the textile and environmental  
334 industries.

335 To conclude, there are three laccase isozymes expressed in *S. turcica*, in which Stlac2 analyzed the  
336 characteristic by heterologous expression. Eukaryotic recombinant Stlac2 in *P. pastoris* was inactive  
337 caused by the incorrect glycosylation, as the glycosylation of Asn97 may result in the depression of  
338 laccase activity. Stlac2 expressed in *E. coli* was found to be a potential catalyst with industrial application  
339 as it can decolorize organic dye MG. However, the possible application is limited by the approach for  
340 production of recombinant laccase from *E. coli*. For our future studies, optimization of the production  
341 parameters for inducible expression system and even for large-scale treatment will be investigated.

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345 **Compliance with ethical standards**

346 **Ethical approval**

347 This article does not contain any studies with human participants or animals performed by any of the  
348 authors.

349 **Competing interests**

350 The authors declare that they have no competing interests.

351 **References**

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465

466 **Fig. 1 Separation and identify of laccase isozymes in *S. turcica*.**

467       **a** Separation of intercellular and extracellular crude protein. 1: zymogram of laccase in  
468           extracellular crude protein. 2: zymogram of laccase in intracellular crude protein. Band I and  
469           band II were collected to confirm the isozymes by ESI-MS/MS.

470       **b** The results of laccase isozymes confirmed by ESI-MS/MS.

471 **Fig. 2 Expression and identify of Stlac2 recombinant protein in *P. pastoris*.**

472       **a** SDS-PAGE gel result of cell culture supernatant. EV: the control *P. pastoris*KM71H with empty  
473           vector pPICZ $\alpha$ -*StLAC2* after methanol induction. CK: samples of non-induced positive  
474           transformant. 1-2: supernatant of the induced culture samples of two different positive  
475           transformants. M: the Standard Protein Marker (14-116kDa)

476       **b** Identify of 6  $\times$  His-tagged recombinant protein by western blot. 1-2: supernatant of the induced  
477           culture samples.

478 **Fig. 3 Expression and purification of Stlac2 recombinant protein in *E. coli*.**

479       **a** SDS-PAGE gel result of cell culture samples. CK: whole-cell extract from non-induced cells. 1  
480           and 2: whole-cell extract from induced cells. M: the Standard Protein Marker (14.4-100 kDa).

481       **b** SDS-PAGE gel result of purification. 1: the precipitate; 2: clear lysate; 3: flow thru; 4-6: elutes.  
482           M: the Standard Protein Marker (14-116kDa).

483       **c** Renaturation result of the inclusion body. 1: sample before renaturation; 2: sample after  
484           renaturation. M: the Standard Protein Marker (14.4-100 kDa).

485 **Fig. 4 Deglycosylation of Stlac2 recombinant protein in *P. pastoris*.**

486       **a** The color of ABTS changed when treated with recombinant proteins and crude protein from *S.*  
487           *turcica*. CK: 5mM ABTS in 0.1 M Tris buffer (pH 6.8). 1: ABTS treated with Stlac2

488 recombinant protein in *P. pastoris*; 2: ABTS treated with Stlac2 recombinant protein in *E. coli*.

489 3: ABTS treated with crude protein from *S. turcica*.

490 **b** SDS-PAGE gel result of deglycosylation. 1: Stlac2 recombinant protein in *P. pastoris*; 2:

491 deglycosylated Stlac2 recombinant protein using PNGase F.

492 **Fig. 5 The analysis of glycosylation sites in Stlac2 recombinant protein in *P. pastoris*.**

493 **a** Amino acid alignment of Stlac2 and other laccases. Asparagine residues of potential glycosylation

494 sites of Stlac2 are marked in blue and the positive peptide by ESI-MS/MS analysis of recombinant

495 laccase Stlac2 expressed in *P. pastoris* are marked in yellow. Four copper-binding conserved

496 domains of typical laccase are indicated by solid lines. Asparagine residues of known glycosylation

497 sites in fungal laccases found are marked in red including laccases in *M. albomyces* (PDB ID:

498 1GW0), *B. aclada* (PDB ID: 3SQR), *A. niger* (PDB ID: 5LM8), and *T. arenaria* (PDB ID: 3PPS).

499 **b** The channels giving access to the trinuclear center for dioxygen (at the top) and egress for the water

500 molecules resulting from the reduction of the dioxygen molecule (at the bottom). Left: laccase from

501 *Bacillus subtilis* (Bento et al. 2005); Right: Stlac2.

502 **Fig. 6 Effects of pH, temperature, and metal ions to recombinant Stlac2.**

503 **a** The optimal pH was determined at pH from 3.0 to 6.5 at room temperature. Enzyme activity is

504 plotted as percentage (% relative activity) relative to the maximum value.

505 **b** The optimal temperature was measured with temperatures from 30 to 80 °C in 0.1 M citrate-

506 phosphate buffer of pH 4.5. Enzyme activity is plotted as percentage (% relative activity)

507 relative to the maximum value.

508 **c** Concentration (1, 5, and 10 mM) of metal ions (Na<sup>+</sup>, Mg<sup>2+</sup>, Ca<sup>2+</sup>, Mn<sup>2+</sup>, Fe<sup>3+</sup>, and Cu<sup>2+</sup>) on the

509 activity of laccase were assayed in 0.1 M citrate-phosphate buffer of pH 4.5, at 60 °C. Enzyme

510 activity is plotted as percentage (% relative activity) relative to the value of samples without

511 metal ions.

512 Error bars correspond to standard error of mean.

513 **Fig. 7 Decolorization of MG by recombinant Stlac2.**

514 Error bars correspond to standard error of mean for triplicates.

515 **Fig. S1 Zymogram of laccase by Native-PAGE stained with ABTS and 2,6-DMP.**

516 **Fig. S2 Construction and verification for expression of StLAC2 in *P. pastoris* and *E. coli*.**

517 **a** cDNA amplification of StLAC2 for eukaryotic expression and prokaryotic expression .

518 **b** The construction of pPICZ $\alpha$ -StLAC2 expression vector .

519 **c** The construction of pET32-StLAC2 expression vector.

520 **d** The confirmation of transformants with  $\alpha$ -factor primer and 3'AOX primer.

521 **e** The confirmation of transformants with T7 primers.

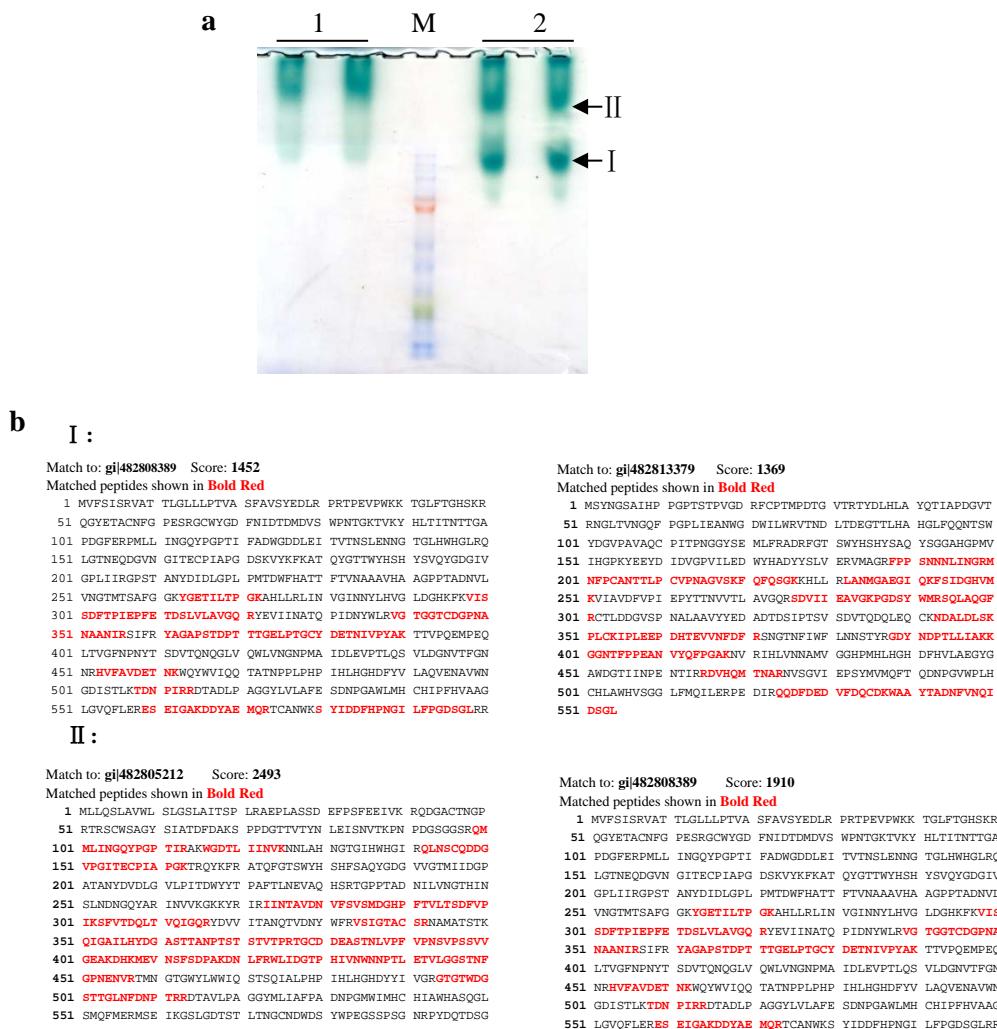
522 5'AOX1, TT and 3'AOX1: promoter region, transcription terminator and 3' region of alcohol  
523 oxidase 1 gene, respectively.

524 6 $\times$ HIS : histidinol dehydrogenase gene.

525 Arrows were the primers used in PCR verification of transformants.

526 EV indicating the control *P. pastoris* KM71H with empty vector pPICZ $\alpha$ -*StLAC2*.

527



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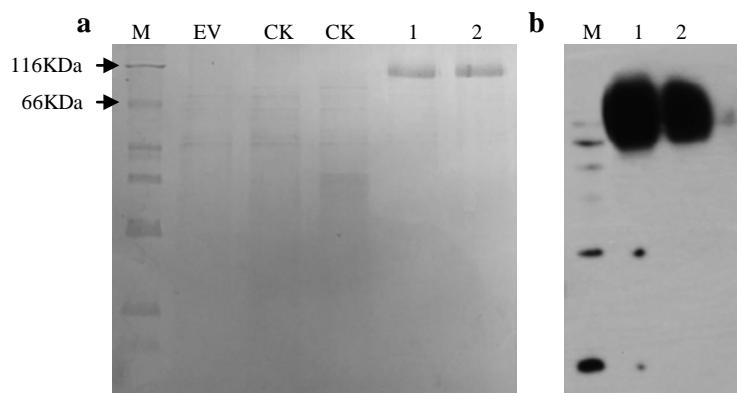
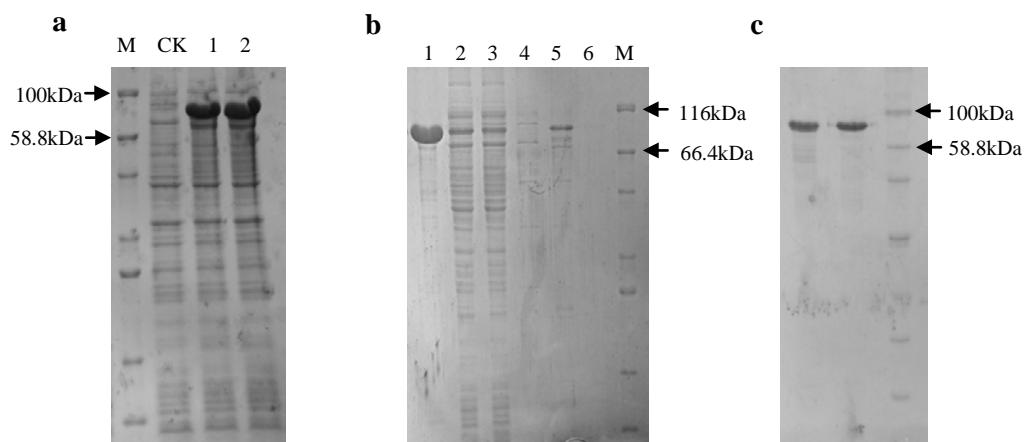


Fig.2

530

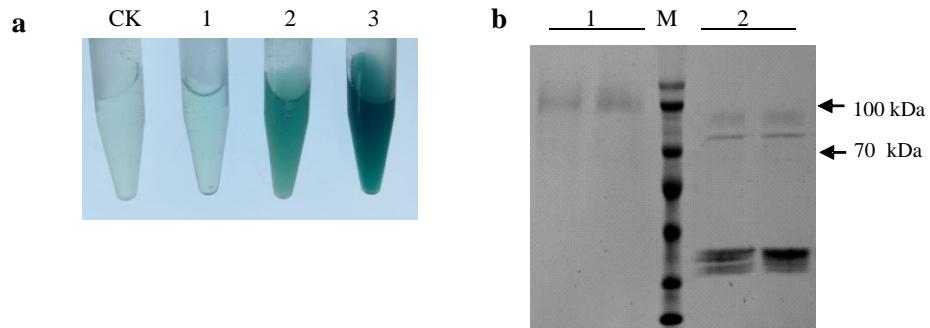
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532

**Fig.3**

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**Fig.4**

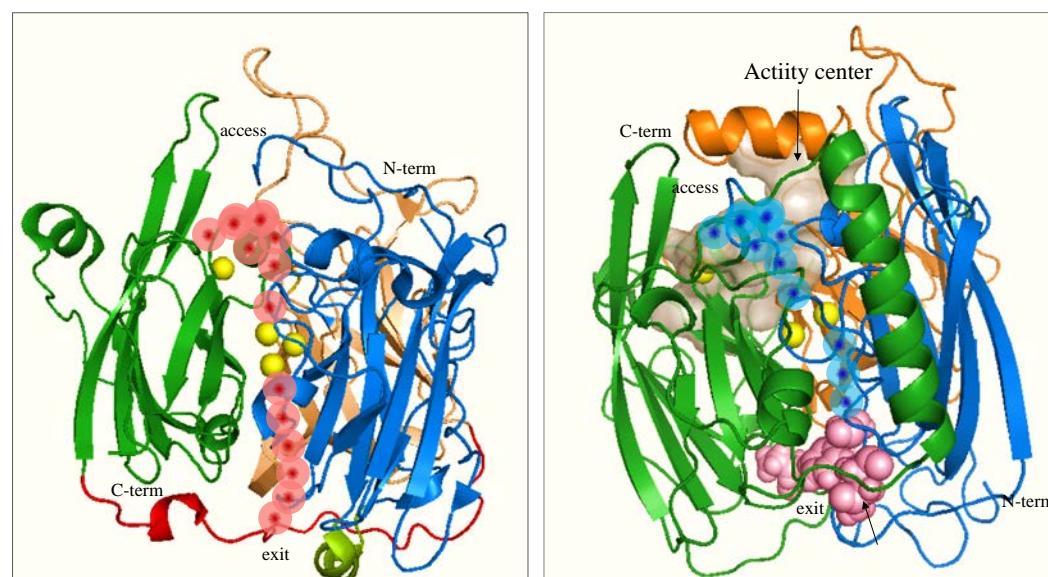
536

1GW0_A	.....	EPCTCNTPSNRACWSGDFDINIDYEVSTPDIGVTSQSYF	110
3PPS_A	MKSWAALMVGILSPHAAAAPPANPVRDMLQVLEARQSGPTCNTPSNRACWINGFDINIDYEVSTPDIGVTSQSYF	110	
3SQR_A	.....MKYPTIVPTALTAFLAQASASAIPAVRSTLTPRQNTTASCANSATSPSOWGEYSIDINWVDVTP.TGVIREWLSE	100	
5LM8_A	.....NTPWQGZDINININYETIPQINWVREYWFIDV	56	
StLAC2	.....MSYCSAIIHPGPCTPVGDRFCPTIMPDTGVRITYDHLAY.QTIAPIGVRN.GLTNGQGPGLI	65	
1GW0_A	VANWGDTEVIVINNLLVINGTISIHWHGLHQKDINLHDGANGVTECIP	179	
3PPS_A	RANWGDNIEVIVINNLLKINGTISIHWHGLRQLGNVFNIDGANGVTECIP	220	
3SQR_A	.....TADWGDNLLIHVINNLLKINGTISIHWHGLRQLGNVFNIDGANGVTECIP	209	
5LM8_A	EANNGDWTWKVHVINRMENNGTAAHPIHPIRQLYNNQMDGVAALTQCPVPP.NSSVYVWRAEEYGSWVHSHFSLQAWBGFQGILLHGPS	165	
StLAC2	.....TAEYDHDLGWVFLNDWHSQH	175	
<b>Cu I</b>			
1GW0_A	ADDLVHPTQNN.APPFSNDVNLING.....TAVNPNTIGEYQYANVILTPGKRRHRLRLLNTSTENHFQVSLVNHMTIVIAADMVPVNAMIVDSLFLAVGQRYDVVIDAS	280	
3PPS_A	ADELVHPTQSN.GAPPSNDVNLNG.....TARHPEITGAGQWYAVILTPGKRRHRLRLLNTSTENHFQVSLVNHMTIVIAADMVPVNATFIVSSLFLAVGQRYDVVIDAN	321	
3SQR_A	VFEIWDITARLG.APPAELNTMNGINTIDCSASTDPNCVGGKKFELITFVEGTYKRLRLLNIGDISHPEFAIDNHLLIVIANDLPVIPVYTTDILLIGIGQRYDVIVEAN	318	
5LM8_A	.....VANWQSVLESQNPFHQFQGLLINGSNIW..VITADNQIVG..RRFQTEFVGQYRVAQNAHFRPSNIDHLLTVAESDIPVTFITNNVPIGMQRYDIIVITAN	270	
StLAC2	.....YSSLVERVMAG.RFPPSNNNLNLGRMNP.CATILPCVPNAGVSKFQFQSKKHLRLANNGAGBQKPSIDGHVMKVIAVDWVPEPYTINWVTLAVGQKSDVIIIEAV	283	
1GW0_A	.....RAPDNNWPEVTFGG..QACACGSINLHPHAAIFHYAGAPGGLPTDGGTPVHDQCLDILDLVPRVWRSVPVNSFVKRPDNILPVALDITGTP..LFVWVKVNSDINVDNG	385	
3PPS_A	.....SPVGNYWPEVTFGG..DGCGSSNNKFPAAIFRYQGAPATITDQGLPVNPNNMCLDNLNLTPTVVTRSAPVNPNSVKRPSNITLGVDLGGTP..LFVWVKVNSDINVDNG	425	
3SQR_A	.....AAADNYWIRGNWGI..TCSINNEAATGILRYD..SSS1ANPTSVGJTPRG..TCEDEPVASLVPHLALD..VGGYSLVDBQVS.SAFINY..FTWITNSSSLIDWS	416	
5LM8_A	.....CAPDNYWIRAIPOS..FCSDNANSNDNKGVLHWHGADNSDPTSTIKWDYDDICQCLDFSLDELWVPLADIGAQMAESDWDFTPFGDVPFLYLWIMGNALNLSWK	376	
StLAC2	.....KPGDGSYWMRSQI LAQGFRCITLDDGVSPNLAALAVYYEDADTIDSPISTSVDTQDQLEQCKNDALDISKPLCKIPLLEPDHTEVNFEDRSQGIN..FTWPLNNSTYRQDYN	391	
<b>Cu II</b>			
1GW0_A	KPIIYDYLITGNISYPVSDNIVQVDADWQTYWLIENDPEGP..FSLPHPMHLHGHDFLVLRGRSPDVPAASQQRFVFDPAVLARLNGDNPQRRDTIMLPAGGWLLIAFRID	494	
3PPS_A	.....NGPQWLFHCHIAWHVSGGLSVDPLERPADLQRQIS..QEDEDDFNRVCDERWRAYWPINPYPKI.....DSGL	535	
3SQR_A	.....NGAWLFHCHIAWHVSGGLSVDPLERPADLRTQTSNKAQRADRDIFNRVCREWNAWYWPINPPPKI.....DSGL	580	
5LM8_A	.....NGSWLILHCHIWAHASEGLAMQFVESQSSIAVKMD..TAIFEDTCANWNAYTPTQLEFAED..DSGI	549	
StLAC2	.....DPTLQQTIFEDPDKMDWKAQSQGVIEAIKNWVTLVQDILP..VPHPIHLHGDFYLLAQGFCQFNQN..VTLKIHNPQRRDTALMATAOPEN..GGGGVWIGFPAD	492	
<b>Cu III</b>			
1GW0_A	.....NPGAWLFHCHIAWHVSGGLSVDPLERPADLQRQIS.....QEDEDDFNRVCDERWRAYWPINPYPKI.....DSGL	559	
3PPS_A	.....NGAWLFHCHIAWHVSGGLSVDPLERPADLRTQTSNKAQRADRDIFNRVCREWNAWYWPINPPPKI.....DSGL	604	
3SQR_A	.....NGSWLILHCHIWAHASEGLAMQFVESQSSIAVKMD..TAIFEDTCANWNAYTPTQLEFAED..DSGI	580	
5LM8_A	.....NGGWLILHCHIWFHATEQFAQOIVVERQSEFNIFFS..EDLLENICDAWDEYAKVNPVGHQYRALACPVESGI	549	
StLAC2	.....NGVWPLHCHLAWHVSGGLFWQILERPEDIRQQDFD.....EDVFD..QCDKWAAYTADNFVNQI.....DSGL	554	
<b>Cu IV</b>			
1GW0_A	.....NPGAWLFHCHIAWHVSGGLSVDPLERPADLQRQIS.....QEDEDDFNRVCDERWRAYWPINPYPKI.....DSGL	559	
3PPS_A	.....NGAWLFHCHIAWHVSGGLSVDPLERPADLRTQTSNKAQRADRDIFNRVCREWNAWYWPINPPPKI.....DSGL	604	
3SQR_A	.....NGSWLILHCHIWAHASEGLAMQFVESQSSIAVKMD..TAIFEDTCANWNAYTPTQLEFAED..DSGI	580	
5LM8_A	.....NGGWLILHCHIWFHATEQFAQOIVVERQSEFNIFFS..EDLLENICDAWDEYAKVNPVGHQYRALACPVESGI	549	
StLAC2	.....NGVWPLHCHLAWHVSGGLFWQILERPEDIRQQDFD.....EDVFD..QCDKWAAYTADNFVNQI.....DSGL	554	

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**b**



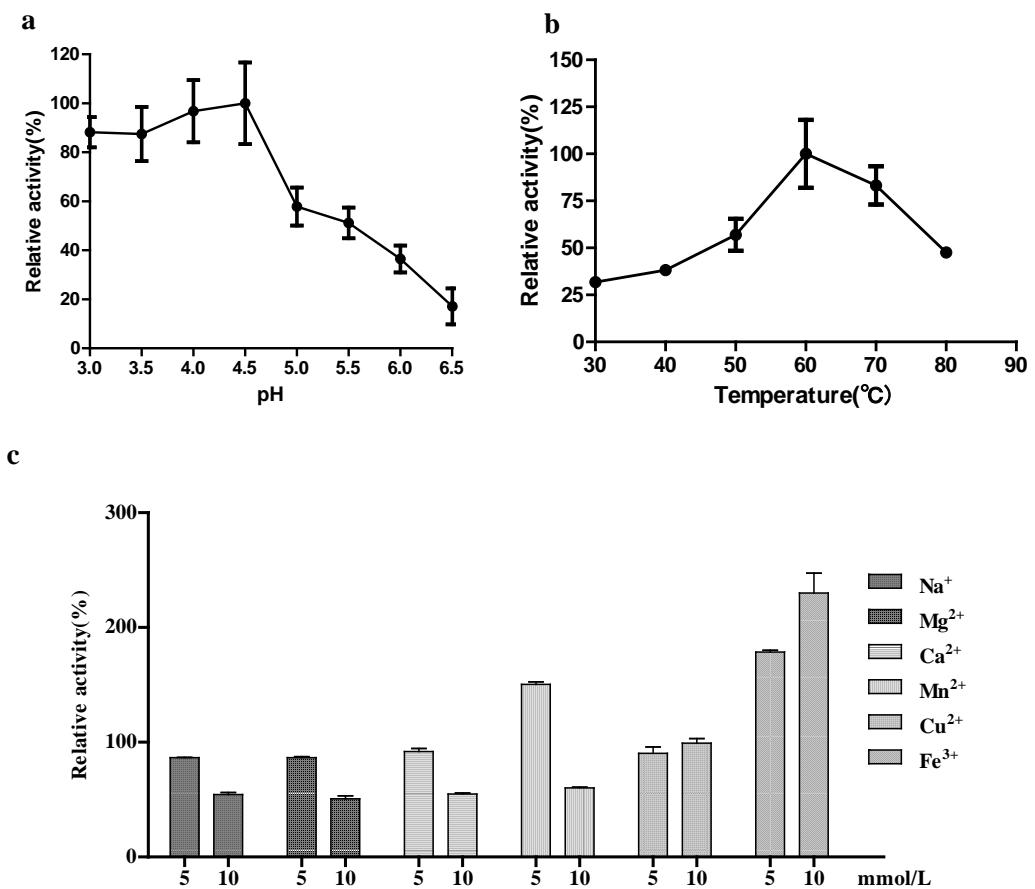
● Oxygen transport channels in *Bacillus subtilis*      ● Oxygen transport channels in *S. turcica*

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**Fig.5**

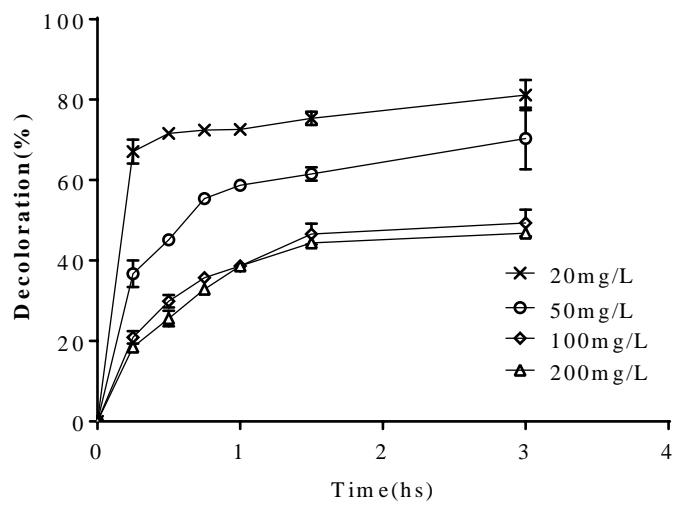
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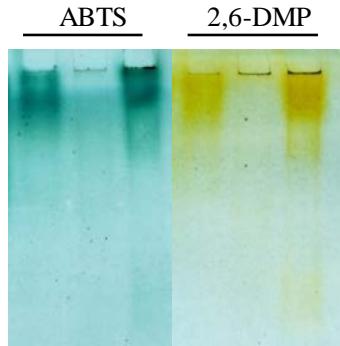
**Fig.6**



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**Fig.7**

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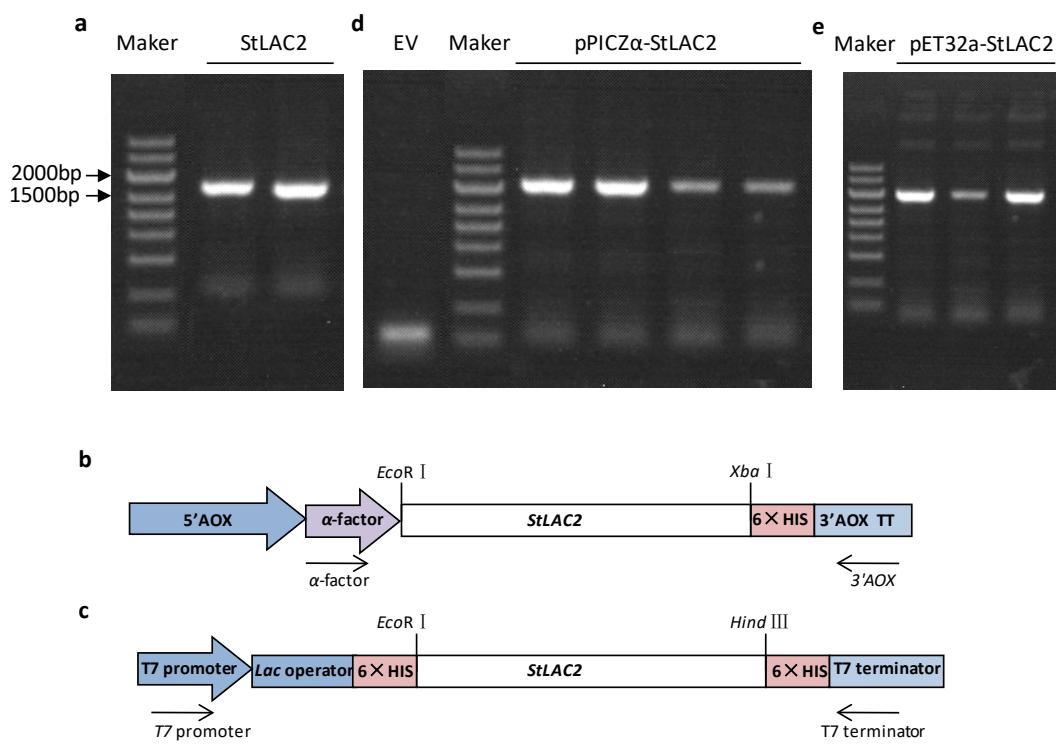


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**Fig. S1**

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**Fig. S2**