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► **To cite this version:**

A. Arteaga-Castrejón, M. Trejo-Hernández, Yasmina Mekmouche, A. Amouric, P. Rousselot-Pailley, et al.. Relevance of Surface-Exposed Lysine Residues Designed for Functionalization of Laccase. *Molecular Biology*, 2022, 56 (4), pp.600-609. 10.1134/S0026893322040021 . hal-03842442

**HAL Id: hal-03842442**

**<https://hal.science/hal-03842442v1>**

Submitted on 26 Jan 2023

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1 **Relevance of surface exposed fonctionnalizable lysine in laccase for selective oxidation**  
2 **of substrates and future applications**

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17 **Keywords:** Laccase, UNIK site-directed mutagenesis. Oriented functionalization.

18

19 **Abstract:**

20 Fungal laccases are oxidoreductases with wide applications due to their low substrate  
21 recognition specificity. Laccase LAC3 from *Trametes* sp C30 is our laccase model and  
22 since few years, its functionalization by external modules is studied to add new properties.  
23 Surface-exposed Lys residues are usually chosen for functionalization by reductive  
24 amination. In this work, LAC3-K0, previously designed, was used as a platform to detect  
25 potential surface-exposed sites suitable to be replaced by a single lysine residue relevant for  
26 future functionalization studies. In a first approach, seven surface-exposed sites were  
27 selected on the LAC3-K0 3-D predicted model and the derivative single lysine mutants  
28 were obtained by site-directed mutagenesis (LAC3-UNIK<sub>238</sub>, etc). Moreover, additionally  
29 mutations were generated randomly from LAC3-K0 giving LAC3-UNIK<sub>N96D/V269K/A479T</sub>,  
30 that were all located on the laccase surface. All mutants were functionally expressed in  
31 *Saccharomyces cerevisiae* W303 as detected by oxidation of guaiacol or ABTS substrates.  
32 The catalytic parameters of the variants showed that UNIK<sub>238</sub>, UNIK<sub>269</sub> and  
33 UNIK<sub>N96D/V269K/A479T</sub> have a high affinity for ABTS with a Km (mM) of  $0.28 \pm 0.006$ ,  $0.27$   
34  $\pm 0.0007$  and  $0.30 \pm 0.004$  respectively. The maximum speed Vmax ( $\mu\text{M min}^{-1}$ ) for these  
35 variants was  $8.22 \pm 0.291$  for UNIK<sub>269</sub> and  $11.98 \pm 0.216$  for UNIK<sub>N96D/V269K/A479T</sub>,  
36 respectively. All variants were stable at acidic pH and lose stability at neutral pH just like  
37 LAC3 y K0. The temperature stability was steady between 15 and 55 °C, at 70 °C most of  
38 the variants were almost completely inactivated. Furthermore, in the oxidation tests of  
39 phenolic compounds it was observed that all mutants were able to oxidize them, however,  
40 their activity varied greatly only by changing one or two residues on the surface of the  
41 laccase K0 regardless to the distance to the T1 site. Despite the differences observed in the  
42 catalytic parameters and in their ability to oxidize phenolic compounds, all the unique  
43 variants studied in this work seem to be appropriate sites for new functionalization of the  
44 laccase.

45

46

47 **1. Introduction**

48 Laccases (EC 1.10.3.2) are *p*-diphenol: oxidoreductases; they are multicopper blue enzymes  
49 characterized by the presence of four copper ions organized in 2 active centers. The type I  
50 copper site is involved in a cycle of the oxidation of phenolic substrates whereas the  
51 trinuclear copper center is involved in the concomitant O<sub>2</sub> reduction into water (Mate *et al*  
52 2016; Stanzione *et al* 2019). Their low specificity enable these enzymes to catalyze the  
53 oxidation of a wide variety of phenolic compounds, aromatic amines and organic  
54 compounds. Laccases are considered as “green catalysts” due to their ability to use  
55 molecular O<sub>2</sub> as an electron acceptor generating water as the only by-product (Bertrand *et*  
56 *al* 2017; Martínez *et al* 2017, Pardo, I *et al* 2018). Laccases are widely distributed in nature,  
57 however the most interesting, from a biotechnological point of view, are those from fungal  
58 origin. These enzymes perform a wide variety of biological functions including  
59 morphogenesis, stress defense , and lignin degradation (Su *et al* 2017). White-rot fungi are  
60 the most robust producers of these enzymes. Although the laccases produced by fungi have  
61 the greatest redox potential in nature, it is not yet enough to oxidize certain components of  
62 lignin and other substrates of higher redox potential, 730–790 mV (Pardo and Camarero,  
63 2015; Yin, Q *et al* 2019).

64 Since the past decades, laccases have received special attention thanks to their high electro-  
65 catalytic potential and environmentally friendly application. So their use has been extended  
66 in biotechnological processes including textile, cosmetic, food, pharmaceutical industry,  
67 biofuel industry, polymer synthesis and more recently in the nanobiotechnology industry  
68 (Su *et al* 2017; Pezzella *et al* 2015). This latter is emerging as a new frontier in the  
69 biotechnology field, where, by means of functionalization and immobilization techniques,  
70 enzymes can be grafted to diverse supports for the design of novel materials with enhanced  
71 characteristics and properties not yet discovered in nature or to mimetize nature’s abilities.  
72 Additionally, these techniques are powerful tools to improve the properties of the enzyme  
73 since its application often faces production issues such as stability long term storage,  
74 operating, conditions at certain pH and temperature intervals, recovery and recycling of the  
75 catalyst.

76 Several research groups are developing immobilization processes via especially covalent  
77 bonding (Mateo *et al*, 2007). As recently reviewed by Hoarau and colleagues that used  
78 chemically well-defined, self assembled monolayer (SAM) surfaces made of short ethylene  
79 glycol chains and cysteine-reactive maleimide groups that covalently attached the enzymes  
80 to the surface. This technique gives better control of the enzyme-support interaction, so  
81 which has been improved by the vectorial orientation of the enzyme leads also to an  
82 improvement in activity and selectivity (Hoarau *et al*, 2017; Shen *et al*, 2016; Liu *et al*  
83 2013).

84 To achieve this, surface-exposed amino acid residues are usually targeted as binding sites to  
85 module groups. As an example, covalent binding between laccase and the surface of  
86 nanoparticles were used for the synthesis of nanomaterials through of specific reactions of  
87 covalent functionalization (Lalaoui *et al* 2016). It involved only one reactive functional  
88 group, the primary amino groups of a lysine residue for which the reactivity can be adjusted  
89 by careful pH monitoring (Robert *et al* 2017; Barbosa *et al* 2013) followed by reductive  
90 amination with an aldehyde functionalized pyrene module.

91 The molecular orientation of the enzyme on the electrode surface allowed to observe a  
92 direct electron transfer (DET) from the electrode to the enzyme (Lalaoui *et al* 2017;  
93 Arrocha *et al* 2014). This DET was influenced by the controlled orientation of the protein  
94 increasing the number of "connected" enzymes (Lalaoui *et al*, 2016). As an other example,  
95 Martínez-Ortiz *et al* 2011 covalently coupled the laccase with the electrode surface through  
96 the T1 copper site and thus induced the direct transfer of electrons between the T1 and the  
97 graphite electrode surface. The DET of the laccase was successfully achieved, and the  
98 molecularly oriented laccase cathode showed 37% more power density and 43% more  
99 current density than the randomly bonded laccase cathode.

100 In conclusion, the modification of functional proteins by site-directed mutagenesis is an  
101 ideal approach to achieve module grafting to a protein (Ying *et al*, 2014). Due to its well-  
102 known topography some laccases are perfect candidates to gain insights for the modulation  
103 of surface-directed enzyme function. Laccase LAC3 from *Trametes sp.* C30 has been  
104 studied for the oxidation of unnatural compounds (Klonowska *et al* 2005; Mekmouche *et al*  
105 2015, Robert *et al* 2017) and their functionalization reactions have been shown to maximize  
106 DET so that in the present work we focus on obtaining variants of laccase LAC3 in

107 particular from the variant K0 (Robert, V *et al* 2017) by site-directed mutagenesis with a  
108 single lysine, as UNIK<sub>157</sub> and UNIK<sub>161</sub>, the goal of this work is to gain insights of the role  
109 of a single lysine residue on the laccase surface without affecting the structure and  
110 functionality of the Lac3 enzyme and these can be implemented in processes in future  
111 works of covalent functionalization.  
112

113

## 114 **2. Materials and methods**

### 115 *2.1 Strains, media and plasmids*

116 *Escherichia coli* strain DH5 $\alpha$  (F $\Delta$ 80*lacZ*  $\Delta$ M15  $\Delta$ (*lacZYAargF*)U169 *deoR recA1 endA1*  
117 *hsdR17(rk, mk +) phoA supE44 thi1gyrA96 relA1*  $\lambda$ ) was used for all DNA mutagenesis  
118 manipulations. Luria-Bertani (LB) broth was used for *E. coli* culture per litre (10 g  
119 tryptone, 5g yeast extract 10g NaCl). 100  $\mu$ g mL<sup>-1</sup> Ampicillin was added to LB medium  
120 when necessary. Plasmids and primers used in this work are listed in table 1. For the  
121 expression of recombinant laccases, the constitutive yeast expression plasmid pAKLAC3-  
122 KO (Klonowska *et al*, 2005) was used as the parent plasmid to obtain the mutant derivatives  
123 from laccase. *S. cerevisiae* W303-1A (MATa{*leu2-3,112 trp1-1 can1-100 ura3-1 ade2-1*  
124 *his3-11,15*}) was used as a host for laccase expression. *S. cerevisiae* transformants were  
125 routinely grown in selective medium (6.7 g L<sup>-1</sup> YNB without amino acids,; 5 g L<sup>-1</sup> casamino  
126 acids, 90 mg L<sup>-1</sup> adenine; 40 mg L<sup>-1</sup> tryptophan; 50 mM succinate buffer, pH 5.3 ; 0.5% v/v  
127 guaiacol; 100  $\mu$ M CuSO<sub>4</sub> and 20g L<sup>-1</sup> of glucose (SD) as a carbon source.

### 128 *2.2 Molecular model*

129 A replacement 3-D model was generated from the Swiss Model server  
130 (swissmodel.expasy.org) using the laccase 2 crystal structure 1GYC from *T. versicolor* as  
131 template downloaded from the Protein Data Bank database (<http://www.rcsb.org>). This  
132 model was used to predict the surface-exposed amino acids residues on LAC3 (GenBank  
133 AAR00925.1) with the GetArea and PyMOL programs from expasy.

### 134 *2.3 Mutants design and construction*

135 Plasmid pAK145-LAC3-KO (Robert *et al*, 2017), a derivative of LAC3, was used as DNA  
136 template to obtain single lysine mutants (UNIK). The quick-change mutagenesis kit  
137 (Thermo Scientific) was used to obtain UNIK derivatives named LAC3-UNIK Q238K;  
138 D254K; V269K; T270K; D283K; A291K and D293K. The sequence of the mutagenic  
139 primers designed in this work is shown in Table 1. All mutant sequences were confirmed  
140 by DNA sequencing. In two cases, due to DNA polymerase error, spontaneous mutants  
141 were detected as in the case of UNIK **N96D/V269K/A479T** and UNIK **D889/A291K**, were the  
142 unplanned mutations are indicated in bold (fig1). Because the spontaneous mutations

143 mapped in the surface of the 3-D model of LAC3-KO, and are enzymatically active, we  
144 decided to include them in this study.

#### 145 *2.4 Yeast transformation and laccase activity assay*

146 Plasmid transformation of *S. cerevisiae* W303-1A was performed with the lithium acetate/  
147 ssDNA carrier/PEG protocol with a change in the time of incubation for 90 min (Gietz and  
148 Woods, 2002). After transformation, cells were spread on selective SD medium plates and  
149 were incubated at 28° C for 4 days. Qualitative laccase activity in the transformants was  
150 first detected on SD plates containing 100µM CuSO<sub>4</sub> and 0.5% v/v guaiacol. For laccase  
151 activity quantification, each clone was grown in microtiter plate cultures at 28° C on a rotary  
152 shaker at 120 rpm. Production of wild type LAC3 and LAC3-KO, used as controls in this  
153 study, was carried out as previously described in Zhou *et al* 2018. All the assays were  
154 performed in triplicate as follows: A 96-well microtiter plate was filled with 300µL of SD  
155 medium and each yeast colony was inoculated, the cells were grown for 3 days at 30°C. A  
156 10µL aliquot of this cell suspension was taken and inoculated into 300µL of fresh SD  
157 medium, the cultures were grown for 24h at 30°C at 160 rpm (Zhou *et al* 2018). The cells  
158 were pelleted at 3000 rpm for 2 min, and the supernatant (SN) was transferred to a clean  
159 96-well microtiter plate for measuring protein concentration by Bradford (Bradford, 1976)  
160 and laccase activity. The supernatant was centrifuged at 3000 rpm for 2 min to remove any  
161 solid particles. Laccase activity from 5 µL of supernatant was determined at 25 °C by  
162 oxidation of 1 mM 2, 2'-azino-bis (3-ethylbenzothiazoline-6 sulphonic acid) (ABTS) in  
163 100 mM sodium acetate buffer (pH 4) in a total volume of 1 ml. The oxidation of ABTS  
164 was determined at 436 nm ( $\epsilon = 29,300 \text{ M}^{-1} \text{ cm}^{-1}$ ) using an UV/Vis spectrophotometer (DU  
165 640 Beckman). Enzyme activities were expressed in international units (U). One unit of  
166 enzyme activity was defined as the amount of enzyme required to oxidize 1 mol of ABTS  
167 per min. The enzyme concentration was determined by the method of Bradford colorimetric  
168 assay on microtiter plates.

#### 169 *2.5 Production of laccase mutants*

170 Yeast cultures transformed with the different mutants were cultivated for 60 hours with an  
171 initial OD<sub>600</sub> of 0.1 in 50 mL of SD medium in 250 mL flasks at 28 °C on a rotary shaker at  
172 160 rpm. After centrifugation (4200 rpm, 10 min), the culture supernatants were filtered  
173 through filter paper (Whatman Nom. 1) then through glass-fibre filters (porosity 1.6 µM)



174 and then concentrated in an ultrafiltration cell equipped (Amicon) with a membrane 10 kD  
175 (Millipore). The enzyme concentrate was stored at -80 °C in 30% (w/v) glycerol. Protein  
176 concentration was determined according to the method of Bradford, using BSA for the  
177 calibration curve.

## 178 *2.6 Characterization of crude laccase kinetic parameters and stability conditions.*

### 179 *2.6.1 Kinetics parameters (Vmax and Km).*

180 The kinetics parameters of the Michaelis-Menten equation were calculated according to  
181 Lineweaver-Burk plots (Lineweaver and Burk., 1934). The concentration used for ABTS  
182 was 0.25 mM, 0.5 mM, 1 mM, 2.5 mM, 3 mM and 5 mM. All the assays were conducted in  
183 triplicate in conditions previously described.

### 184 *2.6.2 Temperature and pH stability of the laccase.*

185 The thermostability of selected variants was investigated by incubating the crude  
186 supernatant from the growth culture of the variants at various temperatures (15, 25, 37, 55,  
187 and 70 °C) for 120 minutes and then cooling at 4°C. The laccase activity was then assayed  
188 at 25 °C in the conditions previously described, with 1 mM ABTS as substrate, 100 mM  
189 sodium acetate buffer (pH 4.4). The effect of pH on the laccase stability was studied by  
190 incubating the supernatants at distinct pH values in 100 mM acetate buffer (pH 3.5 - 4.5) or  
191 100 mM phosphate buffer (pH 5 - 7.5) for 180 min at 25 °C, then, the supernatants were  
192 transferred to standard reaction conditions for laccase activity with 1 Mm of ABTS as the  
193 substrate.

### 194 *2.6.3 Effect of temperature and pH on laccase activity*

195 To study the effect of the temperature, supernatants were incubated at 15, 25, 37, 55, and  
196 70 °C for 30 min, and laccase activity was then assayed at the corresponding temperature in  
197 standard conditions (Zhou et al., 2018). To study the influence of pH, the laccase activity  
198 was assayed in the concentrated supernatant in 100 mM acetate buffer/100 mM phosphate  
199 buffer (pH 3.5 - 7.5) at 25 °C with 1 mM of ABTS as the substrate.

## 200 *2.7 Screening for the oxidation of phenolic compounds*

201 For the oxidation assays with different phenolic compounds, the protocol described by  
202 Pardo *et al* 2015 was followed with some modifications, using catechol, vanillic acid, gallic  
203 acid, ferulic acid and vanillin were purchased from Sigma-Aldrich (Germany). The stock  
204 solutions for the different phenols were prepared at 10 mM in 20% ethanol except catechol

205 which was dissolved in water, the reaction was performed for each mutant in 96-well plates  
206 by adding 10  $\mu$ L of laccase concentrate extract, 180  $\mu$ L of 100 mM acetate buffer, pH 5.0  
207 and 20  $\mu$ L of stock solution in each well and the control reaction without enzyme was  
208 included. The reactions were run in duplicate for each mutant in a Bio-Rad plate reader  
209 (Model 680 microplate reader) for each substrate at the appropriate wavelength (400-450  
210 nm) and lectures were taken respectively at reaction times 0, 30 min, 1 h, 2 h, 4 h, 6 h, 8 h,  
211 and 24 h. As described in Pardo *et al* 2015 (with a change in protein concentration units)  
212 average  $\Delta$ Abs per  $\mu$ g of protein was calculated for each mutant and normalized against  
213 parent type (LAC3) and the results were represented in a heat map.

### 214 **3. Results**

#### 215 3.1.1 *Design and expression of laccase UNIK variants*

216 To make some LAC3 fonctionnalisable tools by grafting modules which can be placed at  
217 different distance from the T1 or T2/3 copper sites on the LAC3 surface. In this goal, the  
218 eighty-five amino acid residues exposed on the surface of LAC3-K0 were identified and  
219 residue identity was analyzed. Surface exposed glycine and proline residues were not  
220 considered to avoid potential major changes in the structure-function relationship. Each of  
221 the other exposed residues could be replaced by a lysine (AAA or AAG codon) however  
222 seven of them were selected according to the distances with the T1 copper binding site.  
223 These distances were calculated from the laccase model of LAC3-K0 (see figure 1). Each  
224 of the mutants were constructed by site directed mutagenesis using pAKLAC3-UniK0 as  
225 template, a variant of LAC3 deprived of any lysine (by substitution of K40 (K40M) and  
226 K71 (K71H) (Lalaoui *et al*, 2017). Each of the K variants obtained where also called  
227 UNIK<sub>n</sub> where n indicate the position of the new lysine insertion on the LAC3 sequence.  
228 The furthest K substitution from T1 was UNIK<sub>283</sub> (LAC3-K0-D283K) at 46.5 Å of distance  
229 and the closest was UNIK<sub>269</sub> (LAC3-K0-V269K) at 23 Å of distance. Table 3 shows the  
230 amino acids that were changed by lysine on the surface of K0, and a comparison of its  
231 characteristics was made, also shows the distance of such residue to the T1 copper junction  
232 site and the the theoric pI calculated for each variant, where no differences are found.

#### 233 3.1.2 *Detection of laccase activity in 96-well plate*

234 Laccase UniKn variants were transformed into *S. cerevisiae* W303 and laccase activity was  
235 detected directly on a SD plate containing 0.5 v/v Guaiacol after 72 h incubation at 28 ° C.

236 All LAC3-UniK variants tested positive for laccase activity. For quantification of laccase  
237 activity of yeast transformants 10 ul of culture supernatant of each variant was taken after  
238 24 hours of growth in liquid medium and was performed in 96-well microtiter plate, the  
239 activity was measured under standard conditions in the presence of 1 mM ABTS, all assays  
240 were performed in triplicate compared to wild type LAC3 and LAC3-KO, Fig. 2A shows  
241 the laccase activity data; six out of nine variants (UNIK<sub>238</sub>, UNIK<sub>254</sub>, UNIK<sub>270</sub>, UNIK<sub>283</sub>,  
242 UNIK<sub>291</sub> and UNIK<sub>S89A/D291K</sub> showed a lower volumetric activity (0.01 to 0.017 U / mL)  
243 values compared to the K0 and LAC controls, 0.023 and 0.026 U / mL respectively; mutant  
244 UNIK<sub>293</sub> does not present significant differences in activity, with 0.025 U / mL. Two  
245 mutants, UNIK<sub>D96N/V269K/A479T</sub> and UNIK<sub>269</sub>, showed a higher activity compared to the  
246 controls. These results indicate that all these mutations on the surface do not affect the  
247 oxidation of ABTS and Guaiacol. Figure 2B shows that the specific activity of mutants  
248 UNIK<sub>238</sub>, UNIK<sub>254</sub>, UNIK<sub>270</sub> are two to three times below the values of the controls. In the  
249 case of mutants UNIK<sub>269</sub>, UNIK<sub>283</sub>, UNIK<sub>291</sub> no differences were observed compared to the  
250 controls. Notably, the values of specific activity of UNIK<sub>293</sub>, UNIK<sub>D96N/V269K/A479T</sub> and  
251 UNIK<sub>269</sub> were improved from 3 to 5 fold higher compared to the controls.

### 252 3.1.3 Laccase Activity from scaled-up cultures

253 Laccase activity of LAC3-Unik mutants was assayed in 200mL cultures for analysis of the  
254 biochemical characteristics, temperature and pH, stability experiments, range of substrates  
255 and kinetic parameters. Table 4 summarizes the values of the volumetric specific activity of  
256 the concentrated supernatants for each variant, where it can be seen that the variants  
257 UNIK<sub>238</sub>, UNIK<sub>254</sub> and UNIK<sub>S89A/D291K</sub> show a volumetric activity of 2.6 to 3.5 U / mL  
258 respectively, close to the K0 control with 3 U / mL, but even higher than the activity  
259 observed in LAC3 with 2.6 U / mL were obtained. Variants UNIK<sub>270</sub>, UNIK<sub>283</sub>, UNIK<sub>293</sub>  
260 and UNIK<sub>291</sub> showed values ranging from 1.98 to 2.6 U / mL; on the other hand, UNIK<sub>269</sub>  
261 and UNIK<sub>D96N/V269K/A479T</sub> attained values of two to three times than the controls, with 6.19  
262 and 9.76 U / mL, respectively. However, in the results of specific activity it is observed that  
263 the majority of the mutants and even the controls had values between 2.42 to 4.44 U / mg.  
264 It is interesting to observe that mutants UNIK<sub>293</sub>, UNIK<sub>D96N/V269K/A479T</sub> and UNIK<sub>S89A/D291K</sub>  
265 6.07, 18.58 and 21.02 U / mg presented values that are 2.4 to 8.3 times above that of K0  
266 and 2.1 to 7.5 times with respect to LAC3.

267        *3.1.4 Determination of kinetic parameters Vmax and Km*

268        The kinetic parameters, Vmax and Km, from the different variants, are shown in Table 3.  
269        The kinetic constant was obtained using the Michaelis-Menten equation to evaluate the  
270        effect of the substrate concentration. The UNIK<sub>238</sub>, UNIK<sub>269</sub> and UNIK<sub>D96N/V269K/A479T</sub>  
271        variants showed a high affinity for the ABTS substrate with a Km of  $0.28 \pm 0.006$  mM,  
272         $0.27 \pm 0.0007$  mM and  $0.30 \pm 0.004$  mM respectively, the maximal velocity (Vmax) for  
273        these variants was  $8.22 \pm 0.291$  ( $\mu\text{M min}^{-1}$ ) for the UNIK<sub>269</sub> variant and  $11.98 \pm 0.216$  ( $\mu\text{M}$   
274         $\text{min}^{-1}$ ) for UNIK<sub>D96N/V269K/A479T</sub>. The other mutants did not show significant differences  
275        between them nor with LAC3.

276        *3.1.5 pH and Temperature stability*

277        To determine the effect of pH and temperature stability, the laccase activities were  
278        measured after several pretreatments respectively. As shown in figure 3A, as for LAC3 and  
279        LAC3-K0, all variants were stable at acidic pH with a top at pH 4.5. However, at neutral  
280        pH the stability was decreased until near zero at pH 7.5. The laccase stability at different  
281        temperatures was steady between 15 and 55 ° C, while at 70 ° C and 120 minutes of  
282        incubation most of the variants were almost completely inactivated except for UNIK<sub>238</sub>,  
283        UNIK<sub>270</sub> (0.07 U/mL) and UNIK<sub>D96N/V269K/A479T</sub> (0.018 U/mL), figure 3B.

284        *3.1.6 Effect of temperature and pH on laccase activity in the variants*

285        The laccase activity of the variants is modified without major changes from pH 3.5 to pH  
286        7.5 in ABTS as a substrate showing a slight increase to pH 6.5 for some variants among  
287        which the variant UNIK<sub>D96N/V269K/A479T</sub> and K0 variant, figure 4A. For the temperature, an  
288        optimal activity is shown between 15 and 55 ° C, giving a remarkable decrease for all  
289        variants at 70 ° C, although, contrary to the stability tests in this test, all variants remained  
290        active, however, not all showed the same behavior, the UNIK<sub>S89A/D291</sub> variant remains with  
291        it in the same ranges of activity up to 70 ° C, as shown in figure 4B.

292

293        *3.1.7 Oxidation of phenolic compounds*

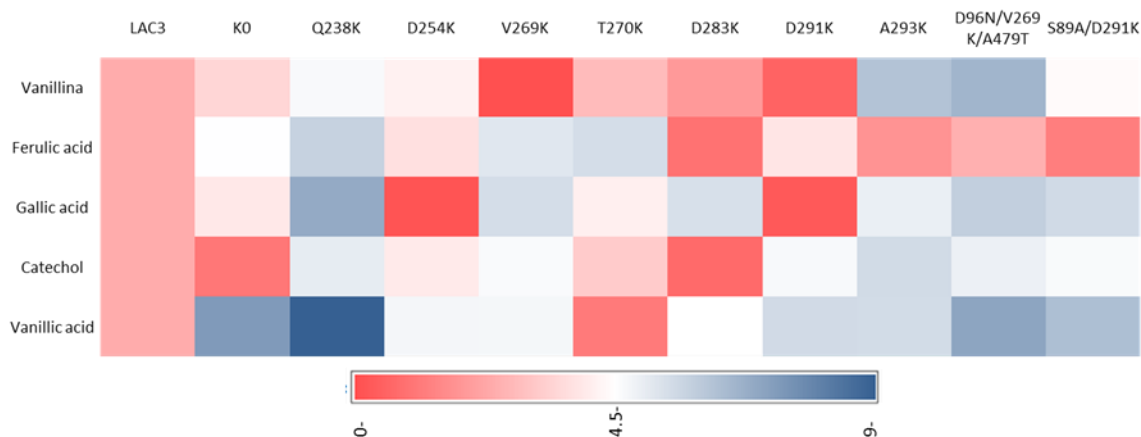
294        Fungi laccases are known for their ability to oxidize compounds derived from lignin (Wang  
295        *et al* 2018). Five phenolic compounds with different chemical structures (Table 2) were  
296        selected as substrates. In order to study the oxidation of phenolic compounds we used the  
297        enzyme extract concentrated obtained from each laccase variant (Table 4). All the variants,

298 K0 and the control LAC3 have the ability to oxidize all five compounds, The relative  
 299 activity for each clone with respect to the control LAC3 was calculated according to the  
 300 formula (Pardo, I *et al* 2015):

$$301 \quad \frac{((\Delta \text{Abs}_X - \Delta \text{Abs}_{\text{blank}})/[\text{protein}]_X)/((\Delta \text{Abs}_{\text{control}} - \Delta \text{Abs}_{\text{blank}})/[\text{protein}]_{\text{control}})}$$

302 La vainillina fue uno de los compuestos más difíciles de oxidar ya que solo 4 clonas  
 303 UNIK<sub>238</sub>, UNIK<sub>293</sub>, UNIK<sub>D96N/V269K/A479T</sub> and UNIK<sub>S89A/D291K</sub> presentan una actividad  
 304 relativa muy por arriba de LAC3, dos de estas cuatro mutantes presentan la mayor cercanía  
 305 de la K al T1 (UNIK<sub>238</sub> at 17 Å, and UNIK<sub>D96N/V269K/A479T</sub> at 20Å). Para el ácido ferulico se  
 306 observa que solo las mutantes UNIK 238, UNIK269, UNIK 270 y K0 fueron capaces de  
 307 oxidarlo mejor que LAC3 y el resto de las mutantes, tres de las cuatro enzimas ya  
 308 mencionadas presentan la mayor cercanía de K a T1 (UNIK<sub>269</sub> at 20Å y UNIK 270 at 27Å).  
 309 For all laccases variants, several common tendencies regarding their ability to oxidize  
 310 different substrates can be observed. El ácido galico y el catechol fueron de los compuestos  
 311 oxidados con mayor facilidad para la mayoría de las enzimas, para el ácido galico solo dos  
 312 mutantes (UNIK<sub>254</sub> and UNIK<sub>291</sub>) presentaron actividades relativas por debajo de LAC3 y  
 313 para el catechol solo la mutante UNIK<sub>283</sub> y K0 la actividad fue la más baja. Para Vanillico  
 314 ácido solo una mutante presentó dificultades para oxidar UNIK<sub>270</sub>, el resto de las mutantes  
 315 fueron presentaron actividades relativas por encima de LAC3 siendo UNIK<sub>238</sub> la de mejor  
 316 actividad. En el cuadro 5 además se puede observar como UNIK<sub>238</sub>, UNIK<sub>269</sub> y su triple  
 317 mutante UNIK<sub>D96N/V269K/A479T</sub> así como UNIK<sub>293</sub> y UNIK<sub>S89A/D291K</sub> fueron capaces de  
 318 oxidar la mayoría de los compuestos con una actividad relativa superior a la de las otras  
 319 mutantes.

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