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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-UNIK₂₃₈, etc). Moreover, additionally
29 mutations were generated randomly from LAC3-K0 giving LAC3-UNIK_{N96D/V269K/A479T},
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 UNIK₂₃₈, UNIK₂₆₉ and
33 UNIK_{N96D/V269K/A479T} have a high affinity for ABTS with a Km (mM) of 0.28 ± 0.006 , 0.27
34 ± 0.0007 and 0.30 ± 0.004 respectively. The maximum speed Vmax ($\mu\text{M min}^{-1}$) for these
35 variants was 8.22 ± 0.291 for UNIK₂₆₉ and 11.98 ± 0.216 for UNIK_{N96D/V269K/A479T},
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 O₂ 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 O₂ 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 UNIK₁₅₇ and UNIK₁₆₁, 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 α (F Δ 80*lacZ* Δ M15 Δ (*lacZYAargF*)U169 *deoR recA1 endA1*
117 *hsdR17(rk, mk +) phoA supE44 thi1gyrA96 relA1* λ) 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 μ g mL⁻¹ 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⁻¹ YNB without amino acids,; 5 g L⁻¹ casamino
126 acids, 90 mg L⁻¹ adenine; 40 mg L⁻¹ tryptophan; 50 mM succinate buffer, pH 5.3 ; 0.5% v/v
127 guaiacol; 100 μ M CuSO₄ and 20g L⁻¹ 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 **DS89/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 CuSO₄ and 0.5% v/v guaiacol. For laccase
151 activity quantification, each clone was grow in microtiter plate cultures at 28° C on a rotary
152 shaker at 120 rpm. Production of wild type LAC3 and LAC3-K0, 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 OD₆₀₀ 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 μ L of laccase concentrate extract, 180 μ L of 100 mM acetate buffer, pH 5.0
207 and 20 μ 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 Δ Abs per μ 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_n where n indicate the position of the new lysine insertion on the LAC3 sequence.
228 The furthest K substitution from T1 was UNIK₂₈₃ (LAC3-K0-D283K) at 46.5 Å of distance
229 and the closest was UNIK₂₆₉ (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 (UNIK₂₃₈, UNIK₂₅₄, UNIK₂₇₀, UNIK₂₈₃,
242 UNIK₂₉₁ and UNIK_{S89A/D291K} 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 UNIK₂₉₃ does not present significant differences in activity, with 0.025 U / mL. Two
245 mutants, UNIK_{D96N/V269K/A479T} and UNIK₂₆₉, 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 UNIK₂₃₈, UNIK₂₅₄, UNIK₂₇₀ are two to three times below the values of the controls. In the
249 case of mutants UNIK₂₆₉, UNIK₂₈₃, UNIK₂₉₁ no differences were observed compared to the
250 controls. Notably, the values of specific activity of UNIK₂₉₃, UNIK_{D96N/V269K/A479T} and
251 UNIK₂₆₉ 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 UNIK₂₃₈, UNIK₂₅₄ and UNIK_{S89A/D291K} 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 UNIK₂₇₀, UNIK₂₈₃, UNIK₂₉₃
260 and UNIK₂₉₁ showed values ranging from 1.98 to 2.6 U / mL; on the other hand, UNIK₂₆₉
261 and UNIK_{D96N/V269K/A479T} 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 UNIK₂₉₃, UNIK_{D96N/V269K/A479T} and UNIK_{S89A/D291K}
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 UNIK₂₃₈, UNIK₂₆₉ and UNIK_{D96N/V269K/A479T}
271 variants showed a high affinity for the ABTS substrate with a Km of 0.28 ± 0.006 mM,
272 0.27 ± 0.0007 mM and 0.30 ± 0.004 mM respectively, the maximal velocity (Vmax) for
273 these variants was 8.22 ± 0.291 ($\mu\text{M min}^{-1}$) for the UNIK₂₆₉ variant and 11.98 ± 0.216 (μM
274 min^{-1}) for UNIK_{D96N/V269K/A479T}. 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 UNIK₂₃₈,
283 UNIK₂₇₀ (0.07 U/mL) and UNIK_{D96N/V269K/A479T} (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_{D96N/V269K/A479T} 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_{S89A/D291} 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*

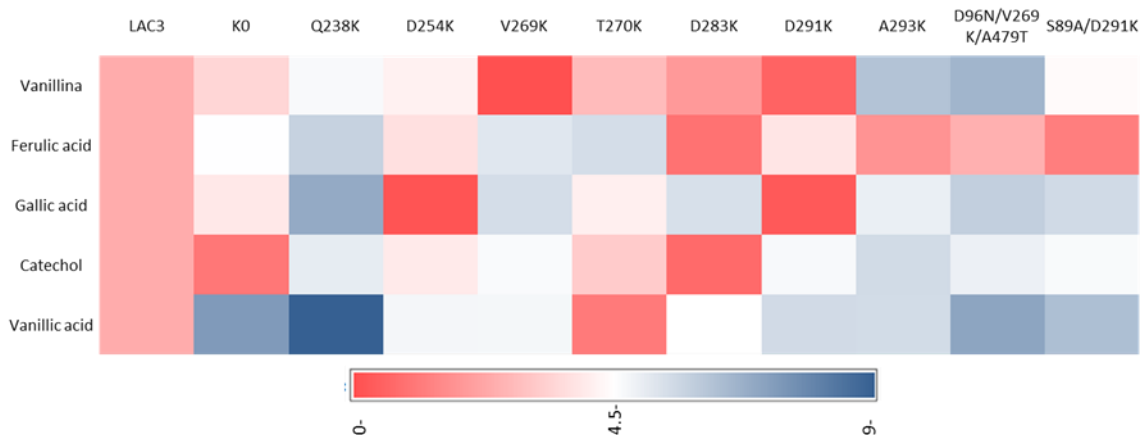
294 Fungi laccases are known for their ability to oxidize compounds derived from lignin (Wang
295 *et al* 2018). Five phenolic compounds with different chemical structures (Table 2) were
296 selected as substrates. In order to study the oxidation of phenolic compounds we used the
297 enzyme extract concentrated 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 UNIK₂₃₈, UNIK₂₉₃, UNIK_{D96N/V269K/A479T} and UNIK_{S89A/D291K} 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 (UNIK₂₃₈ at 17 Å, and UNIK_{D96N/V269K/A479T} 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 (UNIK₂₆₉ 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 (UNIK₂₅₄ and UNIK₂₉₁) presentaron actividades relativas por debajo de LAC3 y
 313 para el catechol solo la mutante UNIK₂₈₃ y K0 la actividad fue la más baja. Para Vanillic
 314 acid solo una mutante presentó dificultades para oxidar UNIK₂₇₀, el resto de las mutantes
 315 fueron presentaron actividades relativas por encima de LAC3 siendo UNIK₂₃₈ la de mejor
 316 actividad. En el cuadro 5 además se puede observar como UNIK₂₃₈, UNIK₂₆₉ y su triple
 317 mutante UNIK_{D96N/V269K/A479T} así como UNIK₂₉₃ y UNIK_{S89A/D291K} 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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