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## Molecular docking studies and in vitro degradation of four aflatoxins (AFB 1 , AFB 2 , AFG 1 , and AFG 2 ) by a recombinant laccase from *Saccharomyces cerevisiae*

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1     **Molecular docking studies and in vitro degradation of four**  
2     **aflatoxins(AFB1, AFB2, AFG1 and AFG2) by recombinant**  
3             ***laccase from Saccharomyces cerevisiae***

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24 **ABSTRACT:** Aflatoxins are widely distributed mycotoxins with high concentration in  
25 food and feed. They may have negative impacts on human health, animal productivity  
26 and the economy, these being accompanied by removal difficulties. Enzymatic  
27 degradation of aflatoxins is recently becoming an efficient strategy to ensure food and  
28 feed safety . Here, molecular docking was used to predict and compare interactions  
29 between laccase and four aflatoxins (AFB<sub>1</sub>, AFB<sub>2</sub>, AFG<sub>1</sub> and AFG<sub>2</sub>) as well as their  
30 degradation at a molecular level. Docking simulation studies indicated that aflatoxins  
31 may interact near the T1 copper center through H-bonds and hydrophobic interactions  
32 with amino acid residues His481 and Asn288, His481, Asn288 and Asp230, His481 and  
33 Asn288. Removal tests were performed in vitro in the presence of a recombinant fungal  
34 laccase. Degradation increased as incubation time increased from 12h to 60h and the  
35 maximum degradation obtained for AFB<sub>1</sub>, AFB<sub>2</sub>, AFG<sub>1</sub> and AFG<sub>2</sub> was 90.33%, 74.23%,  
36 85.24% and 87.58%, respectively. Maximum degradation of aflatoxins was obtained  
37 with a laccase total activity 4U at 30 °C in 0.1M phosphate buffer, pH 5.7 after 48h  
38 incubation. The experimental results are consistent with that of docking calculation on  
39 the removal of four aflatoxins by laccase.

40  
41 **Key Words: Laccases; degradability; aflatoxins.**

42

## 43 INTRODUCTION

44 Mycotoxins are low-molecular weight molecules produced as secondary metabolites  
45 by several species of fungi that contaminate various agricultural products, such as  
46 cereals, maize, oilseeds and nuts, either before or under post-harvest conditions[1, 2].  
47 Among various types of mycotoxins, aflatoxins produced by *Aspergillus flavus*, *A.*  
48 *parasiticus* and *A. nomius*, pose serious threat to public health and livestock  
49 productivity, even at relatively low concentration. They constitute a group of closely  
50 related compounds leading to highly toxic, mutagenic and carcinogenic[3, 4]. Therefore,  
51 it is necessary to remove them timely from foodstuffs.

52 Among the 20 well-known aflatoxins, aflatoxinB<sub>1</sub> (AFB<sub>1</sub>), aflatoxinB<sub>2</sub> (AFB<sub>2</sub>),  
53 aflatoxinG<sub>1</sub> (AFG<sub>1</sub>), aflatoxinG<sub>2</sub> (AFG<sub>2</sub>) are the most toxic ones (toxicity of AFB<sub>1</sub> >  
54 AFG<sub>1</sub> > AFB<sub>2</sub> > AFG<sub>2</sub>). They are both frequently found in many human dairy  
55 foodstuffs and animal feeds and difficult to remove. AFB<sub>1</sub> is the most potent naturally  
56 occurring hepatotoxic, immunotoxin and teratogenic properties reported to date  
57 (classified as Group 1 carcinogen by International Agency for Research on Cancer).  
58 Although it affects primarily the liver, AFB<sub>1</sub> is pluripotent acting on multiple organs  
59 and multiple systems [5], and is causing both acute and chronic toxicity [6]. The main  
60 toxic structures are the coumarin lactone ring, the double bond in the furan ring and the  
61 cyclopentenone ring.

62 Currently, many researchs are focused on aflatoxins degradation or removal, and  
63 various strategies have been reported to reduce aflatoxins levels. The most common  
64 way to remove aflatoxin is by physical treatment, such as heating at high temperatures,  
65 plasma degradation [7], extrusion or electromagnetic radiation treatment[8]. In addition,  
66 chemical treatments such as ammoniation, nixtamalization, ozonation [9], acids and  
67 bases treatments are also effective. However, these methods are so far not leading to  
68 practical applications because of consecutive nutritional quality losses and because they  
69 hardly comply with security, cost and productivity requirements for commercialization,  
70 as well as they may cause environmental damages. Biological processes, including  
71 degradation by plant extracts[10], microorganisms and enzymes, or the direct  
72 adsorption to the cell surface of microorganisms like *Saccharomyces cerevisiae* [11]  
73 and *Lactobacillus casei*[12], which are gentle, low cost and effective with less or no  
74 intermediates harmful to humans and animals, have been identified as promising routes.

75 Laccases (EC1.10.3.2) belong to the family of the blue multicopper oxidases. They  
76 contain four copper ions, a type 1 copper acting as a primary electron acceptor from  
77 reductant species and a trinuclear cluster (TNC) involved in dioxygen reduction.  
78 Widely distributed in plants, fungi, bacteria, and insects, these enzymes have the  
79 catalytic ability to oxidize a wide range of aromatic substrates with a concomitant  
80 reduction of dioxygen to water[13]. Globally, fungal laccase have better pH/or  
81 temperature stability, higher metal tolerance and are able to oxidize a wider range of  
82 substrates as enzymes from other origin[14]. At present, they are broad application  
83 prospects of these enzymes in environment, food, medicine, cosmetics and other fields.  
84 Considering the structural characteristics of aflatoxins, laccases may have a great  
85 potential in AFs' biodegradation applications.

86 The use of laccases may result in rapid and significant recalcitrant substrate  
87 degradation[15]. With the maturity of computer simulation technology and the deep  
88 understanding of the three-dimensional structure of laccase, it becomes easier and faster  
89 to study the relationship between laccase and substrate through molecular docking.  
90 Laccase is taken as redox enzyme while molecular docking is used to conduct  
91 simulation studies focusing on substrates binding abilities and binding modes. Catalytic  
92 reactions then occur only if substrates get close enough to the T1 copper center of the  
93 enzyme. Molecular docking has been used in a good deal of researches to study the  
94 interaction of laccase with substrates. They agree that molecular docking is an effective  
95 analytical tool to assess the enzyme-substrate interaction. E.D. Morales-Alvarez[16] has  
96 used molecular docking to evaluate interactions between the recombinant laccases  
97 GILCC1 and POXA 1B enzymes with Crystal Violet (CV) or Malachite Green (MG)  
98 dyes. L. Dellafiora[17] have investigated the enzyme-substrate interaction for various  
99 enzyme isoforms through 3D molecular docking techniques. D. Mo[18] has employed  
100 molecular docking to study the interaction mechanism between nonylphenol and  
101 octylphenol isomers and a laccase from *Trametes versicolor*. Y. Zhang[13] has used  
102 molecular docking method to analyze the interactions between laccase produced by  
103 *Trametes versicolor* and Triton X-100. Comparing three-dimensional models of  
104 laccases A.K.S. Kameshwar[19] has performed molecular docking studies using lignin  
105 model compounds to understand the structural and functional properties of laccase. K.K.  
106 Sharma[20] has used molecular docking to study the interaction of different  
107 biomolecules with laccases from *C. neoformans* and different enteropathogenic bacteria  
108 to understand their probable role in the oxidation of cellular metabolites and the  
109 formation of the reactive intermediates.

110 In this work, we investigated the enzymatic degradation of aflatoxins by a laccase.  
111 An homology model of the enzyme was first obtained then molecular docking analysis  
112 was used to evaluate interactions between the laccase and four aflatoxins (AFB<sub>1</sub>, AFB<sub>2</sub>,  
113 AFG<sub>1</sub> and AFG<sub>2</sub>). This analysis was used to better understand the basis of the enzyme-  
114 substrates interaction at a molecular level, highlighting interaction areas around the  
115 catalytic site and specific differences among the aflatoxins in terms of enzyme surface-  
116 ligand recognition. Results were compared to degradation tests performed alongside.  
117 The comparison of theoretical and experimental data help to broaden our knowledge on  
118 the degradation of aflatoxins and provide a basis for later molecular modifications of  
119 laccase.

## 120 MATERIALS AND METHODS

### 121 Chemical and Reagents

122 LAC3 from *Trametes versicolor* produced heterologously in *Saccharomyces*  
123 *cerevisiae* was gift from Dr. Thierry Tron's lab. Aflatoxins (purity >99%) was obtained  
124 from J&K. ABTS was purchased from sigma. Methanol and formic acid were of high  
125 performance liquid chromatography grade. Britton-Robinson buffer consisting of  
126 0.04M phosphoric, 0.04M acetic acid, 0.04M boric acid was used during the  
127 experiments. Ultrapure water was used throughout all of the experiments.

128 **Preparation of Aflatoxins Working Solution**

129 Solid aflatoxins were dissolved into 10mL of methanol to obtain 0.1 mg/mL standard  
130 aflatoxins solutions and subsequently stored at -20°C.

131 **Preparation of Laccase**

132 Laccase secreting *Saccharomyces cerevisiae* cells were cultivated at 30°C for 4 days  
133 in S-Gal medium on a rotary shaker incubator at 160 rpm. Cells were pelleted by  
134 centrifugation (8000rpm for 30min). The recombinant laccase was found in the  
135 supernatant. Purification was carried out as previously described[21]

136 **Laccase Activity**

137 Laccase assay was based on 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonate)  
138 (ABTS) oxidation in 0.04M Britton-Robison buffer (pH 4.0) at 30°C. Oxidation of  
139 ABTS was monitored spectroscopically by absorbance measurements at 420nm ( $\epsilon=$   
140  $3.6 \times 10^4 \text{M}^{-1} \text{cm}^{-1}$ ). One unit of laccase oxidizes 1 $\mu$ mol of substrate per minute[22].

141 **Extraction of residual Aflatoxins and analysis by HPLC-MS**

142 Aflatoxins were extracted three times from samples with chloroform (1 :1, v/v) as  
143 described by Teniola et al[23]. Then chloroform fractions were pooled and evaporated  
144 under nitrogen to dry.

145 The samples were dissolved in methanol, filtered (0.22 $\mu$ m) and separated on an  
146 Inertsil ODS-3 C18 column,(4.6 $\times$ 150mm, 3 $\mu$ m) coupled to a 1260 HPLC system  
147 equipped with 6240 Triple Quad LC/MS (Agilent, United States) detector. LC-MS  
148 conditions for Aflatoxins analysis were the followings: for regular analysis, 5 $\mu$ L of  
149 sample or standard solutions were injected; the mobile phase, pumped at a flow rate of  
150 0.2mL/min, consisted in an isocratic mixture of aqueous solution of 0.1% formic  
151 acid/methanol (3:7, v/v); column temperature was maintained at 30°C; the total  
152 operation time was 30 min; compounds were analyzed in positive mode. MS conditions  
153 were as follows: capillary temperature 300°C; sheath gas flow and auxiliary gas flow  
154 (11L/min and 3L/min, respectively) were adjusted to get stable spray; Fragmentor  
155 Voltage, Collision Energy, and Cell Accelerator Voltage were 135V, 30eV, 4KV,  
156 respectively. Data were collected in MRM Scan Type with the precursor ions of AFB<sub>1</sub>,  
157 AFB<sub>2</sub>, AFG<sub>1</sub>, AFG<sub>2</sub> measured at  $m/z$  313, 314, 328, 330 and product ions measured at  
158  $m/z$  285, 287, 243 and 245, respectively.

159 **Aflatoxins Degradation by Laccase**

160 Aflatoxins degradation experiments (methanol solution) were carried out in 10mL  
161 tubes. Laccase was incubated with toxins (0.1mg/mL) at intervals of enzyme activity,  
162 incubation periods and temperature. The control was prepared with addition of 0.1 M  
163 PBS pH 5.7 instead of laccase. Samples were placed in a shaker-incubator and  
164 incubated under constant agitation (200rpm/min) at 30°C. Afterward, the toxins content  
165 in the reaction mixture was extracted and determined as described above. Experiments  
166 were performed independently for each aflatoxin.

167 The effect of a variation of the enzyme concentration on degradation rates was

168 conducted with enzyme loads corresponding to 1-5U. The effect of the incubation time  
169 on degradation rates was investigated sampling the reactions every 12h for 60h. The  
170 effect of temperature on degradation rates was investigated in the range of 25-45°C.

171 All experiments were performed in triplicate and the percentage (%) of degradation  
172 was calculated with the following equation:

$$173 \quad C(\%) = [(A - B) / A] * 100$$

174 Where, A corresponds to the initial mass of the aflatoxins and B is the residual mass  
175 of the aflatoxins, C is the degradation rate of laccase for aflatoxins.

### 176 **Homology Modeling of the laccase LAC3**

177 The target sequence LAC3 was retrieved from Uniprot (Uniprot ID Q6TH77).  
178 Template crystal structures were screened through a BLAST search; the structure with  
179 the highest homology score was downloaded from the RCSB Protein Data Bank (PDB  
180 ID: 3KW7). Homology modeling was conducted in MOE v2014.0901 (Chemical  
181 Computing Group Inc, 1010 Sherbooke St. West, Suite#910, Montreal, QC, Canada,  
182 H3A 2R7. 2014). The protonation state of the protein and the orientation of the  
183 hydrogen atoms were optimized by LigX at a pH of 7 and a temperature of 300 K. First,  
184 the target sequence was aligned to the template sequence, and ten independent  
185 intermediate models were built. These different homology models were the result of the  
186 permutational selection of different loop candidates and side chain rotamers. Then, the  
187 intermediate model with the highest score according to the GB/VI scoring function was  
188 chosen as final model, and then subjected to further energy minimization using the  
189 AMBER12/EHT force field.

### 190 **Modeling of Aflatoxins**

191 AFB<sub>1</sub>, AFB<sub>2</sub>, AFG<sub>1</sub>, AFG<sub>2</sub> were used to perform docking analysis with the model  
192 constructed for LAC3 from *Trametes sp.* C30. 2D structures of the AFB<sub>1</sub>(CAS: 1162-  
193 65-8), AFB<sub>2</sub>(CAS: 7220-81-7), AFG<sub>1</sub>(CAS: 1165-39-5) and (AFG<sub>2</sub>, CAS: 7241-98-7)  
194 were drawn in ChemBio Draw 2014 and converted to 3D in MOE v2014.0901 through  
195 energy minimization. 2D structure of ligands are shown in Table 1.

### 196 **Molecular Docking Experiments**

197 MOE Dock in MOE v2014.0901 was used for molecular docking simulations of the  
198 aflatoxins and prediction of their “binding affinity” to the homology model of LAC3.  
199 The 3D structure of the LAC3 was predicted through homology modeling. And then,  
200 the protonation state of the enzyme and the orientation of the hydrogens were optimized  
201 by LigX, at the PH of 7 and temperature of 300 K. Prior to docking, the force field of  
202 AMBER12: EHT and the implicit solvation model of Reaction Field (R-field) were  
203 selected. The docking workflow followed the “induced fit” protocol, in which the side  
204 chains of the receptor pocket were allowed to move according to ligand conformations,  
205 with a constraint on their positions. The weight used for tethering side chain atoms to  
206 their original positions was 10. All docked poses of aflatoxins molecules were ranked  
207 by London dG scoring first, then a force field refinement was carried out on the top 30  
208 poses followed by a rescoring of GBVI/WSA dG.

## 209 **Statistical Analysis**

210 All the experiments were performed in triplicate. All the data are graphically presented  
211 as means  $\pm$  standard deviation of triplicates( $n=3$ ), and the data were analyzed using  
212 single factor analysis of variance(ANOVA). ANOVA was performed using SPSS  
213 software(Version 8.5). Difference among the means were considered for significance at  
214  $P < 0.05$  using Duncan's multiple range test.

## 215 **RESULTS**

### 216 **Interaction of Aflatoxins with the laccase**

217 In the absence of an available 3D structure of LAC3, the investigation of the binding  
218 mode of aflatoxins with this enzyme required first the construction of an homology  
219 model. Results are displayed in Figure 1 and Table 1. Analysis of the Ramachandran  
220 plot revealed that ninety-nine percent of LAC3 residues map in allowed regions which  
221 underlines that our homology model of LAC3 conforms to stereo chemical rules (Figure  
222 2). Therefore, we docked aflatoxins AFB<sub>1</sub>, AFB<sub>2</sub>, AFG<sub>1</sub> and AFG<sub>2</sub> on the LAC3  
223 homology model.

224 Docking scores of interactions between aflatoxins and LAC3 are reported in Table 3.  
225 The minor differences observed among the values obtained for each aflatoxins may be  
226 related to their chemical similarities. The different binding models between ligands and  
227 laccase may result from different binding ability. The computational result indicates  
228 that each of the aflatoxins tested can interact with laccase with a binding ability of  
229 AFB<sub>1</sub>>AFG<sub>2</sub>>AFG<sub>1</sub>>AFB<sub>2</sub>.

230 The lowest energy configurations of aflatoxins and their binding mode at the surface  
231 of LAC3 are illustrated in Figure 3. We assume that these predictions correspond to the  
232 most likely configurations. In our models, the oxygen atom of the methoxy group of  
233 AFB<sub>1</sub>, regarded as hydrogen bond acceptor, forms one hydrogen bond with the side  
234 chain of His481 which is coordinated to the nearby copper ion (i.e. T1 Cu<sup>II</sup>) [24]  
235 whereas the oxygen atom of the terminal furan ring, regarded as hydrogen bond  
236 acceptor, forms one hydrogen bond with the side chain of Asn288. Similarly, a carbon  
237 atom of the benzene ring in AFB<sub>2</sub>, regarded as hydrogen bond acceptor, forms one  
238 hydrogen bond with the sidechain of His481. Two carbon atoms of the terminal furan  
239 ring of AFG<sub>1</sub>, regarded as hydrogen bond acceptors, form two hydrogen bonds with the  
240 sidechain of Asn288 whereas the oxygen atom of 5-membered heterocyclic ring of  
241 AFG<sub>1</sub>, regarded as hydrogen bond acceptors, form one hydrogen bond with the  
242 sidechain of Asp230. The carbon and oxygen atoms of the terminal furan ring of AFG<sub>2</sub>,  
243 regarded as hydrogen bond acceptors, forms two hydrogen bonds with the side chain  
244 of Asn288 in laccase. Eventually, the carbon atom of methoxy group of AFG<sub>2</sub>, regarded  
245 as hydrogen bond acceptor, forms one hydrogen bond with the side chain of His481.

### 246 **Degradation of Aflatoxins using the Recombinant Laccase LAC3 Produced in** 247 ***Saccharomyces cerevisiae***

248 The aflatoxins(B<sub>1</sub>, B<sub>2</sub>, G<sub>1</sub> and G<sub>2</sub>) detoxifying efficacy of LAC3 was tested at



249 different incubation times, enzyme activities and temperatures. Generally speaking, the  
250 degradation of aflatoxins by the laccase was a rapid process.

251 The kinetic of degradation of aflatoxins was investigated at an enzyme activity of 3U  
252 and at 30°C. As it can be seen from Figure 4(A), degradation rate increased first from  
253 12 to 48h and then no significant difference was observed from 48 to 60h. In these  
254 conditions, the maximum degradation of AFB<sub>1</sub>, AFG<sub>1</sub> and AFG<sub>2</sub>, respectively 90.33%,  
255 85.24%, 87.58%, was observed after 48h of incubation time, while for AFB<sub>2</sub> the  
256 maximum of degradation was reached after 60h of incubation. This may be explained  
257 that the activity sites of laccase were more and the concentration of aflatoxins were  
258 higher during the beginning of the reaction, the declining activity sites limited the  
259 reaction.

260 Degradation experiments with laccase were tested at different enzyme activity (i. e.  
261 different enzyme loads). Experiments were performed at 30°C for 12h and results are  
262 presented in Figure 4(B). Expectedly, degradation increased with an increase in the  
263 enzyme load. Starting to plateau at 4U of laccase, the maximum degradation of AFB<sub>1</sub>,  
264 AFB<sub>2</sub>, AFG<sub>1</sub>, AFG<sub>2</sub>, respectively 74.87%, 62.36%, 66.94%, 70.88%, was obtained at  
265 5U of laccase. It is more likely to predict that the substrate-binding sites may have  
266 reached to the saturation point as the activity of laccase was above 4U.

267 The effect of temperature on degradation rate of aflatoxins was conducted at an  
268 enzyme activity of 3U for 12h and the results are shown in Figure 4(C). The percentage  
269 of degradation of aflatoxins by laccase increased slightly from 25 to 40°C and then  
270 decreased with an increase of temperature from 40 to 45°C. The maximum degradation  
271 of AFB<sub>1</sub>, AFB<sub>2</sub>, AFG<sub>1</sub>, AFG<sub>2</sub> respectively, i.e., 77.45%, 51.84%, 56.06%, 68.33%, was  
272 observed at 30°C. LAC3, like most of fungal laccase, has been described as a fairly  
273 temperature tolerant enzyme. However, temperature tolerance is function of the  
274 exposure time. Therefore, the decrease in activity observed when the reaction is  
275 performed at a temperature over 40°C is probably a consequence of the long exposure  
276 (12h) of the enzyme at this temperature. Hence, the optimal temperature was set at 30°C  
277 in future studies.

278 All together the experimental are in agreement with the theoretical study that  
279 predict aflatoxins “binding affinity” for LAC3, in particular that of AFB<sub>2</sub> that was found  
280 minimum among the aflatoxins tested.

## 281 **DISCUSSION AND CONCLUSION**

282 It is important to reduce the mycotoxin contents in food and feed for public health  
283 and globe trade. A wide number of effective strategies are already used to control toxin  
284 accumulation levels at pre- or post-harvest. Comparing with cultivation techniques,  
285 conventional breeding, genetic engineering and other physical or chemical methods,  
286 biological treatments have good application potential due to their ability to transform  
287 toxins to non - or less harmful compounds during food processing [25]. Among these,  
288 the degradation by means of enzymes is considered as the strategy with the most highest  
289 potential for the mitigation of toxins content. So far, J.F. Alberts et al. have tried to  
290 degrade AFB<sub>1</sub> by laccase from white rot fungi *Peniophora*, *Pleurotus ostreatus* and  
291 *Trametes versicolor* achieving degradation of 40.45%, 35.90% and 87.34%,

292 respectively[26]. Zeinvand-Lorestani H et al. also applied a commercial laccase  
293 preparation from *Trametes versicolor* to AFB<sub>1</sub> with 67% of the toxic substrate  
294 removal[27]. Loi M et al. have shown that AFB<sub>1</sub> and AFM<sub>1</sub> degradation by a pure  
295 enzyme form *Pleurotus pulmonarius* is greatly enhanced from 23% to more than 90%  
296 in the presence of redox mediators [28]. However, screening diversity to find out proper  
297 active enzymes is challenging and time-consuming, and more importantly does not  
298 allow to study an interaction mechanism between ligands and the target enzyme.  
299 Investigation of potential enzyme-substrate interactions by in silico methods could be  
300 used as screening tool as it provides increasingly reliable and informative insights [17].  
301 While taken alone there may be still some deviations due to computer simulation,  
302 performing alongside molecular docking analysis and in vitro experiment allows a  
303 benchmarking of methodologies. The consistency between in vitro experimental results  
304 and that of docking calculation will contribute to find out a faster, more precise and  
305 effective way in aflatoxins removal.

306 Here, we employed molecular docking to study the interaction of four aflatoxins with  
307 the surface of a typical fungal laccase aiming to highlight possible differences in ligand-  
308 enzyme interactions. According to our models, relevant differences exist among  
309 aflatoxins in terms of interaction with the enzyme surface. AFB<sub>1</sub> is likely interacting  
310 the most favorably with laccase among all the aflatoxins. Docking simulation studies  
311 indicate that the residue His481 present in the coordination sphere of the T1 copper  
312 may interact with aflatoxins and therefore could mediate the electron transfer during  
313 oxidation[29]. Different hydrogen bonding patterns arise from calculation: in addition  
314 to His481, AFB<sub>1</sub> may interact with Asn288, AFB<sub>2</sub> may interact with His481, AFG<sub>1</sub>  
315 may interact with both Asn288 and Asp230, AFG<sub>2</sub> may interact with both His481 and  
316 Asn288. Amino acid residues involved in H-bonds are considered as key residues for  
317 the interaction of laccase with a given ligand[18]. H-bonds details as shown in Table 4  
318 suggest different interactions between the laccase and the ligands which may  
319 correspond to differences in binding ability with AFB<sub>1</sub>>AFG<sub>2</sub>>AFG<sub>1</sub>>AFB<sub>2</sub>, an order  
320 consistent with that drawn from experimental results on the biodegradation of  
321 aflatoxins by LAC3 by , and the of aflatoxins at the same time is  
322 AFB<sub>1</sub>>AFG<sub>2</sub>>AFG<sub>1</sub>>AFB<sub>2</sub>.

323 The binding affinity is related to the efficiency of enzyme-ligand interactions which  
324 is affected by structural characteristics and distorting extent of ligands and the shape  
325 complementation between the ligand and the enzyme surface[30, 31]. With the higher  
326 binding affinity, the ligands have stronger capability to reach the pocket-like structure  
327 of enzyme. Differences in toxin degradation appears to be related primarily to group  
328 contacts established with the laccase surface. The stronger the aflatoxin-laccase  
329 interaction is the more efficient is the oxidation of the toxin.

330 In silico analysis provides insight into the interaction of aflatoxins with laccase and  
331 allows to point structures interactions relevant for the degradation. An in-depth  
332 mapping of enzyme-ligand potential interactions will provide a theoretical basis for  
333 molecular modification strategies of laccase set to improve degradation or for screening  
334 for natural laccase with high toxins degrading capabilities from a variety of  
335 microorganisms. In both cases, molecular docking will be advantageous to improve the

336 biodegradation techniques.

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444

445 Table Legends

446 Table 1: Homology modeling of LAC3 (*Trametes* sp. C30)

447 Table 2: 2D structure of ligands

448 Table 3: Docking score of molecules binding to Laccase

449 Table 4: H-bonding distances between laccase and aflatoxins



450 Figure Legends

451 Fig. 1: Homology models of laccase protein sequences (*Trametes* sp. C30)

452

453 Fig. 2: Ramachandran Plot: dark green dots represent the residues in most favored  
454 regions; yellow dots represent the residues in additional allowed regions; Small red  
455 cross represents the residues in not-allowed regions.

456

457 Fig. 3: Models of AFB<sub>1</sub>, AFB<sub>2</sub>, AFG<sub>1</sub> and AFG<sub>2</sub> interacting with the laccase LAC3. A.  
458 The binding model of AFB<sub>1</sub> on molecular surface of the laccase. B. The interaction  
459 model of AFB<sub>2</sub> with the laccase. C. The binding model of AFB<sub>2</sub> on molecular surface  
460 of the laccase. D. The interaction model of AFB<sub>2</sub> with the laccase. E. The binding model  
461 of AFG<sub>1</sub> on molecular surface of the laccase. F. The interaction model of AFG<sub>1</sub> with  
462 the laccase. G. The binding model of AFG<sub>2</sub> on molecular surface of the laccase. H. The  
463 interaction model of AFG<sub>2</sub> with the laccase. The ligands are colored in cyan, the T1  
464 copper ion is colored in pink and the surrounding residues in the binding pockets are  
465 colored in orange. The backbone of the receptor is depicted as lightblue ribbon.

466

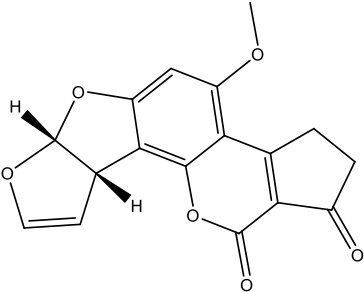
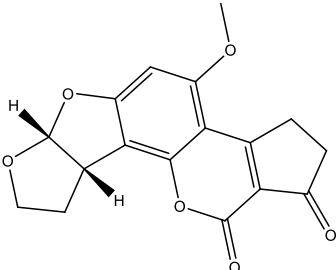
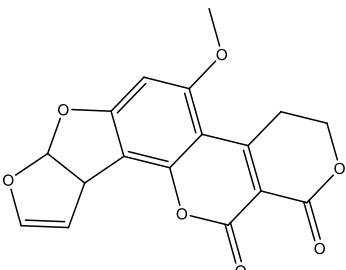
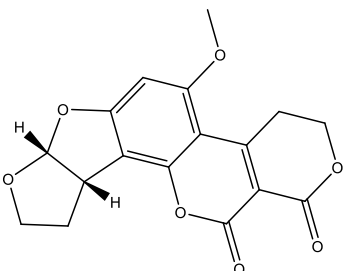
467 Fig. 4: Effect of incubation time (A), enzyme activity (B) and temperature (C) on the  
468 degradation of aflatoxins by the laccase LAC3. Values are means of three replicates and  
469 their standard errors. Means with different letters representing differences within the  
470 group are significantly different according to Duncan's test( $P \leq 0.05$ ).

## Table Legends

**Table 1:**

<b>Protein</b>	<b>Template</b>	<b>Query Cover</b>	<b>Identity</b>
<b>Laccase</b>	3KW7 , A	93%	77%

**Table 2 :**

Name	Structure
AFB <sub>1</sub>	 <p>The chemical structure of AFB<sub>1</sub> (Aflatoxin B<sub>1</sub>) consists of a difuran ring system fused to a coumarin ring system, which is further fused to a cyclopentenone ring. A methoxy group (-OCH<sub>3</sub>) is attached to the coumarin ring at the 7-position. The stereochemistry at the two chiral centers in the difuran ring is (1R,2S).</p>
AFB <sub>2</sub>	 <p>The chemical structure of AFB<sub>2</sub> (Aflatoxin B<sub>2</sub>) is similar to AFB<sub>1</sub>, but the difuran ring system is fused to a cyclopentane ring instead of a cyclopentenone ring. The stereochemistry at the two chiral centers in the difuran ring is (1R,2S).</p>
AFG <sub>1</sub>	 <p>The chemical structure of AFG<sub>1</sub> (Aflatoxin G<sub>1</sub>) is similar to AFB<sub>1</sub>, but the difuran ring system is fused to a cyclohexane ring instead of a cyclopentenone ring. The stereochemistry at the two chiral centers in the difuran ring is (1R,2S).</p>
AFG <sub>2</sub>	 <p>The chemical structure of AFG<sub>2</sub> (Aflatoxin G<sub>2</sub>) is similar to AFB<sub>1</sub>, but the difuran ring system is fused to a cyclohexane ring instead of a cyclopentenone ring. The stereochemistry at the two chiral centers in the difuran ring is (1S,2R).</p>

**Table 3:**

<b>Ligands</b>	<b>Receptor</b>	<b>Docking score (kcal/mol)</b>
<b>AFB<sub>1</sub></b>	laccase	-6.4532
<b>AFB<sub>2</sub></b>	laccase	-5.5306
<b>AFG<sub>1</sub></b>	laccase	-5.9708
<b>AFG<sub>2</sub></b>	laccase	-6.3328

**Table 4 :**

<b>Enzyme-ligand</b>	<b>H-bonds</b>	<b>Enzyme residues</b>	<b>Ligand atom</b>	<b>H-bond length(Å)</b>
<b>LAC-AFB<sub>1</sub></b>	2	His481	O	3.16
		Asn288	O	3.2
<b>LAC-AFB<sub>2</sub></b>	1	His481	C	3.14
<b>LAC-AFG<sub>1</sub></b>	3	Asn288	C	3.17
		Asn288	C	3.16
		Asp230	O	2.76
<b>LAC-AFG<sub>2</sub></b>	3	His481	C	2.98
		Asn288	C	3.19
		Asn288	O	2.93

## Figure Legends

**Fig. 1**



Fig. 2

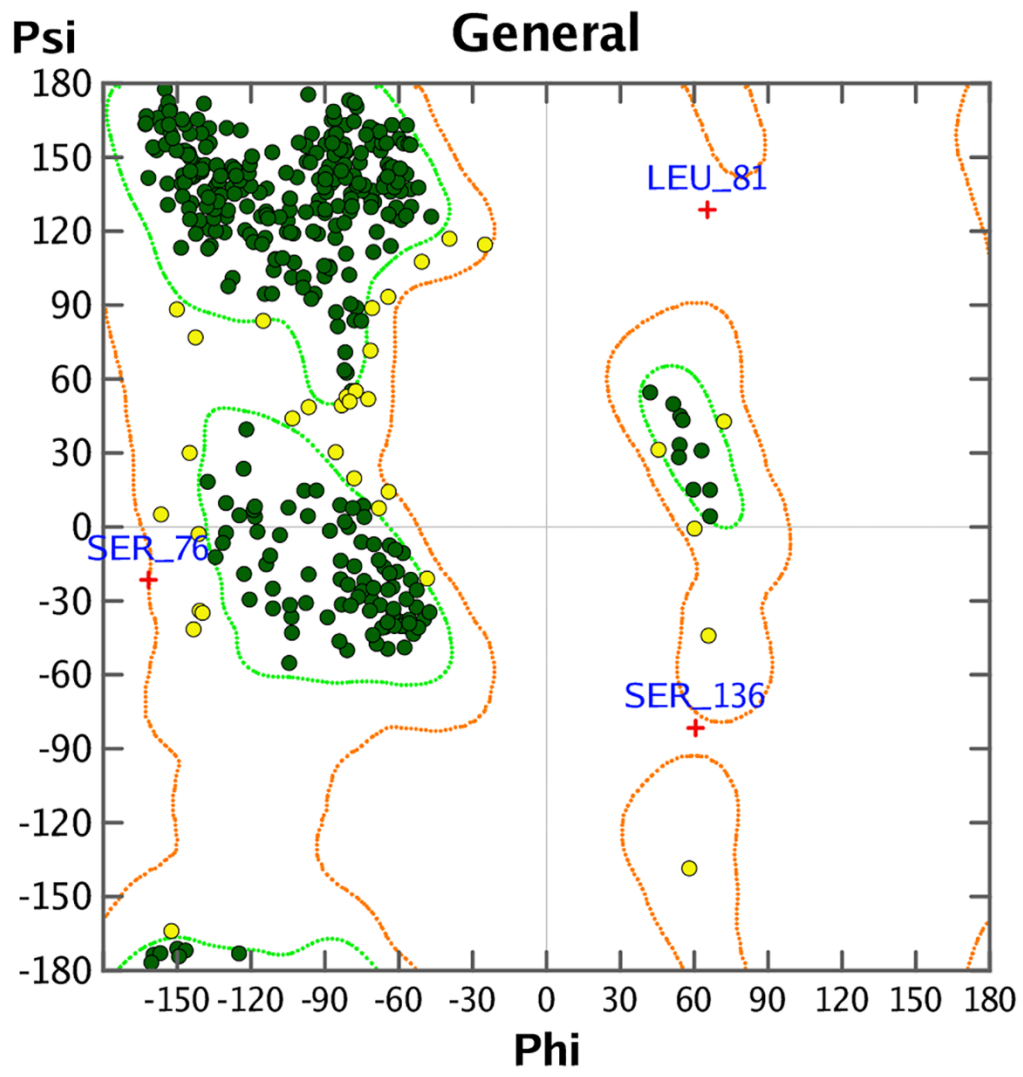
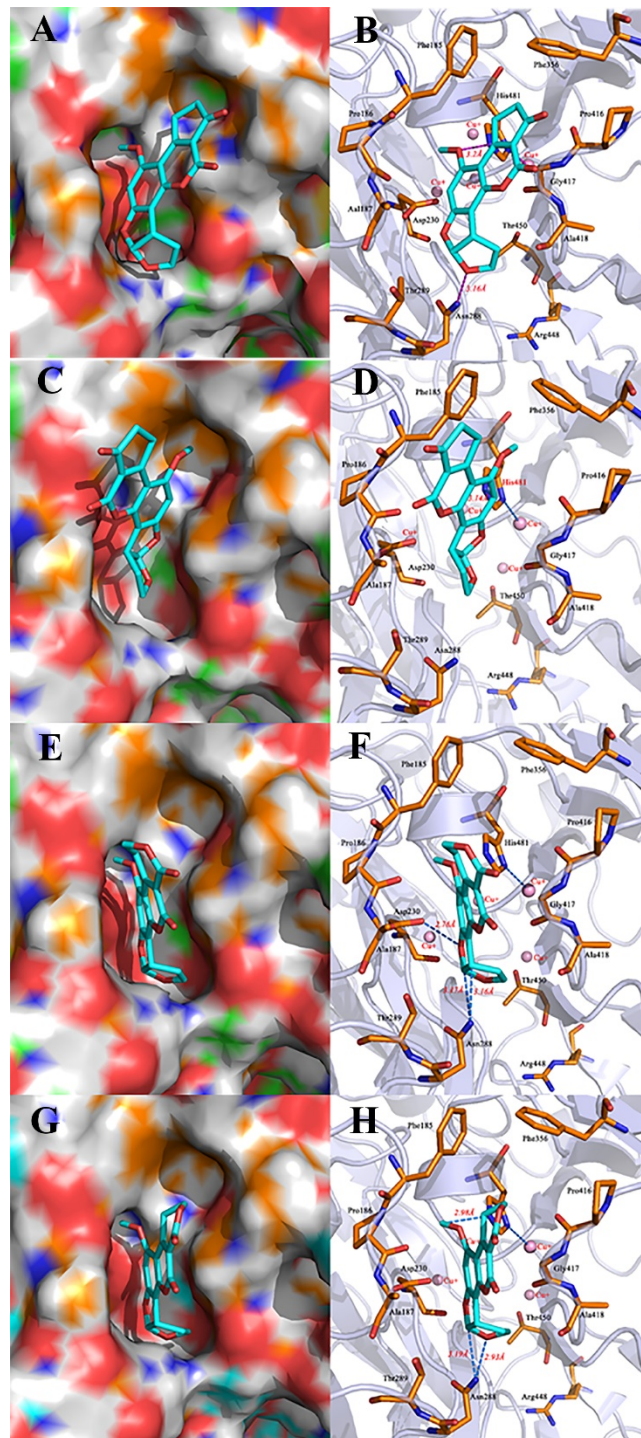


Fig. 3



**Fig. 4**

