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Comparative *in Silico* analysis of the behavior of Laccases from marine and terrestrial origin against degradation of industrial dyes

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Abstract : Laccases are multicopper-oxidase enzymes that catalyze one-electron oxidation of phenolic compounds, and other electron-rich substrates with the concomitant reduction of O_2 to H_2O. One of the most important applications of these enzymes can be found in bioremediation processes, for which the search for enzymes derived from marine organisms can be considered strategic due to the physicochemical characteristics of contaminated effluents. In this study, a comparative molecular docking analysis was made considering the behavior of putative laccases of marine and terrestrial origin against six industrial dyes. Using sequences retrieved from NCBI and Uniprot databases, three-dimensional structures were obtained and validated by computational methods. These models were used for docking studies with the dyes Acid Orange 10, Amido Black, Reactive Blue 4, Reactive Yellow 14, Remazol Black B, and Trypan Blue. The results showed an outstanding behavior of the putative laccase from marine-derived bacteria *Pantoea agglomerans*, which showed the best affinity interaction with the dyes Amido Black (-8,2 kcal/mol), Reactive Blue 4 (-9,4 kcal/mol), and Reactive Yellow 14 (-8,6 kcal/mol). Likewise, the putative laccase from marine-derived bacteria *Bacillus stratosphericus* showed the best affinity interaction with the dyes Acid Orange 10 (-7.5 kcal/mol), and Remazol Black B (-8.3 kcal/mol). The findings obtained in the present study demonstratethe potential of microbial enzymes that can be found in marine ecosystems, and establish that specific sequences might be used for further construction of synthetic genes in experimental evaluations.

Keywords: Molecular docking, Multicopper oxidases, Homology modeling, Bioremediation, Textile effluent.

Introduction

Lignolytic enzymes are oxidoreductases of great importance due to the great variety of applications in which they can be used[1]. Within these enzymes, laccases represent an important group that has been employed for environmental management studies of pollutants, for which the treatment of industrial effluents in the dye industry, or the building of biosensors to detect phenolic pollutants,are important[2,3].

Laccases are enzymes widely distributed in nature, being present in fungi, higher plants, bacteria and insects [4-6]. However, they have been studied mainly in fungi because it isprimarily in these organisms where the enzymes have been found[7,8]. Despite this, bacterial laccases are of great interest because, although they have lower redox potential than fungal laccases, they are generally more stable at high temperatures and in a wider

pH range, and they are less susceptible to inhibitory agents[9]. These characteristics make bacterial laccases a commendable candidate for studying industrial applications.

Bioremediation of textile effluents is an important environmental application of laccases. The textile industry uses processes in which wastewater with a high content of phenols, sulfides, chromium and dyes is generated, with the generation of dyes being of high environmental concern due to their hard degradation and the negative impact generated in liquid effluents with values up to 400 mg/L[10]. Additionally, the removal of dyes through physicochemical processes can generate even more toxic substances, which requires the search for more efficient and environmentally friendly methods, such as biotechnology[11-13]. In that regard, marine bacteria represent a wide variety of microorganisms with scarcely studied enzymatic systems, which might have novel properties with respect to terrestrial microorganisms[14,15].

Molecular docking is a valuable tool for the study of these relatively unexplored systems, allowingfor the study of interactions between potential substrates and receptors [15-17]. In the present study, a comparative analysis was madeof the behavior of different putative laccases of bacteria from marine and terrestrial origin with respect to the industrial dyes Acid Orange 10, Amido Black, Reactive Blue 4, Reactive Yellow 14, Remazol Black B, and Trypan Blue.

Material and methods

Proteins and substrates

A set of putative laccase proteins of 12 marine-derived bacteria and 19 terrestrial bacteria was selected from the Uniprot and NCBI (National Center for Biotechnology Information) databases. Additionally, seven substrates for studying enzymatic interaction were taken from the PubChem database. The substrates include one positive control for activity, ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid), and six dyes, Acid Orange 10, Amido Black, Reactive Blue 4, Reactive Yellow 14, Remazol Black B, and Trypan Blue.

Phylogenetic Tree Construction

The putative laccase sequences were aligned using Muscle. Thephylogenetic model was selected with the Mega 6 software, which was also used for the phylogenetic construction using the maximum likelihood method, generating 1000 bootstrap replicates.

Physicochemical properties

Some physicochemical properties of the putative laccase sequences were calculated using the ProtParam tool on the ExPaSy server (http://web.expasy.org/protparam/) [18]. This method was used to obtain the theoretical pI, molecular weight, GRAVY index, and instability index. The presence of a signal peptide was evaluated in each sequence with the SignalP 4.1 server tool (http://www.cbs.dtu.dk/services/SignalP/) [19].

Homology Modeling

The three-dimensional structure of the putative laccase proteins was predicted through the homology modeling approach using crystal structures of multicopper oxidases from the protein data bank as templates (PDB ID: 1GSK, 4HAK, 4E9Q, 1KV7, and 4HAL) and the Modeler 9.15 software. The program generates the three-dimensional structures of proteins and their assemblies by satisfaction of spatial restraints with optimization of molecular probability density function. The modeled structures are optimized with conjugate gradients and molecular dynamics with simulated annealing. The input–output HETAM function was set to true mode. The function was read in HETAM records from the PDB template, and the copper atoms were inserted in the query models. The quality of each laccase modeled structure was validated using the Procheck (https://www.ebi.ac.uk/thornton-srv/databases/pdbsum/Generate.html),Prosa (https://prosa.services.came.sbg. ac. at), Verify 3D and ERRAT (http://services.mbi.ucla.edu/SAVES/) tools.

Molecular Docking

Molecular docking simulations were performed with Autodock Vina in the Virtual Screening Tool PyRx 0.8.The substrate accessible pockets and active sites of putative laccases were determined using the Computed Atlas of Surface Topography of Proteins (CASTp) calculation [20]. Polar hydrogens were added to each protein, and charges were automatically included by Autodock Vinausing the Gasteiger method for each protein and substrate. According to the CASTp results, the grid was localized in the nearest place to the T1

copper active site pocket for each model. The grid box sizes were 25 x 25 x 25 Å, and an exhaustiveness of 15 was applied. Visualization and interaction analysis was achieved with Discovery Studio 4.5 and PyRx 0.8.

Results and Discussion

Phylogenetic Analysis

After the search for and selection of sequences, a total of 31 putative bacterial laccases were used for the phylogenetic analysis, with the idea of seeking possible biological differences that might otherwise not be apparent [21]. The phylogenetic tree (Figure 1) shows that the distribution of sequences is especially influenced by the taxonomic origin of the sequences and not by their marine or terrestrial provenance. Thus, it appears that most of the sequences are grouped according to their taxonomic clade, Proteobacteria, Firmicutes or Actinobacteria. Some exceptions are noted with the laccase of *Enterobacter* sp.,which is closely related to the sequences of *Streptomyces* sp. Also, this happens with laccases of *Sinorthizobium meliloti* and the marine actinobacterium evaluated. These results are similar to what was found by Hoegger et al.[22], whose phylogenetic analysis of laccases showed that the tree distribution is influenced mainly by the taxonomic origin, and secondarily by functional characteristics. The foregoing may be related to the lower and wide substrate specificity, which makes the functional differentiation from the sequence a difficult task [22].

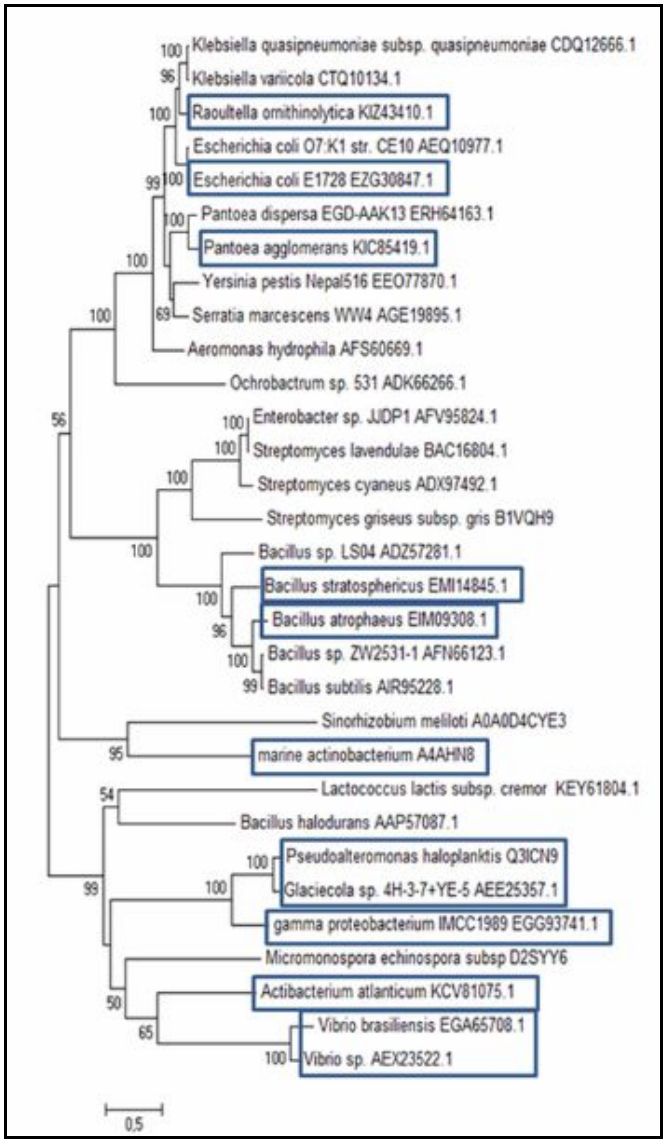

Fig. 1 Phylogenetic tree for the analysis of laccases from marine and terrestrial bacteria. In the blue boxes the marine microorganisms are highlighted

Physicochemical properties

Some of the physicochemical properties of the sequences were calculated by means of the ProtParam tool (Table 1), just as the presence of signal peptide was determined with the SignalP tool. This analysis highlights the negative hydrophobicity index (GRAVY),which indicates that all the selected proteins are hydrophilic. This behavior is common in many laccase proteins, although some studies show that protein sequences of *Pseudomonas* sp have a hydrophobic character [23]. The hydrophilic nature of the sequences studied is significant, since the pollutants that are considered in this study are present in aqueous effluents. Therefore, it is necessary for the enzymes studied to be soluble in water. With respect to the theoretical pI, a similar distribution of this characteristic was noted for both enzymatic origins, finding an acidic pI in all cases, except with the laccase of *L. lactis,*which had a slightly alkaline pI of 7.22. Additionally, the high values of the aliphatic index suggest a good thermal stability of proteins, which is an interesting property to be exploited in industrial applications. In general, thermal stability of laccases has been a property widely found in several studies by means of the aliphatic index[23, 24]. Finally, signal peptide prediction is important because it allows identifying protein segments that are not of the functional enzyme and can be discarded in the modeling step.

Homology Modeling

Analysis of the three-dimensional structure of proteins is a valuable tool for obtaining information about the function of proteins. In the absence of a three-dimensional structure, homology modeling can be a useful tool for obtaining structures to study enzymatic behavior. It is generally believed that to successfully perform a process of homology modeling, at least 30% of identity between the sequence to be modeled and the template sequence is required, provided that the length of the sequence is at least 100 amino acids [25]. Nonetheless, other authors indicate that the model quality is highly influenced by the percent identity, generally recognizing the following behavior: for values >90%, the accuracy of the models obtained is comparable to that achieved experimentally; in the 50-90% range, it is possible to obtain proper models with some local errors; and finally, in the 25-50% range it is common to obtain structures with regions with a high degree of error, in which homology modeling is not appropriate [26]. Due to this, it was decided to model only sequences that have enzyme templates with a percent identity greater than 50%. In order to identify these enzymes, a search was carried out using the Blast tool, available on the NCBI website (http://www.ncbi.nlm.nih.gov/), specifying the Protein Data Bank (PDB) as the database, which is composed of proteins with known three-dimensional structures. The results of this search yielded 14 multicopper oxidase sequences that were appropriate for homology modeling (Table 2). The modeling of putative bacterial laccases was carried out with the Modeller 9.15 software. Figure 2 shows the structure for two of the proteins studied, pointing out the copper centers T1 and T2/T3, which are of special importance in the catalytic activity of the enzyme [27].

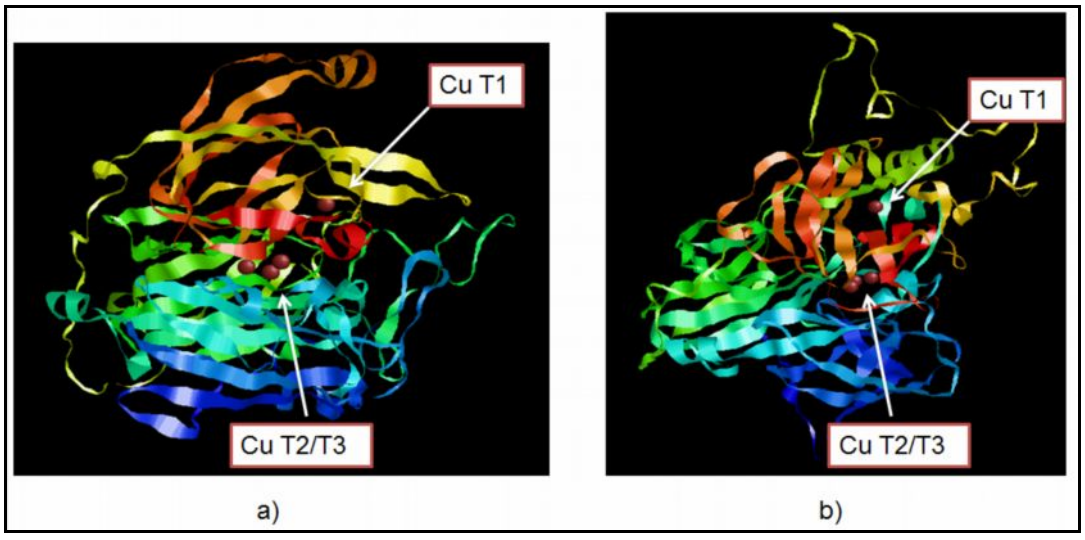

Fig. 2 Copper atoms in the modeled enzymes from a)*Bacillus stratosphericus*,b) *Klebsiella variicola*

Table 1 Physicochemical properties of putative laccases from marine and terrestrial origin

Origin	Organism	Thoretical pI	MW	Aliphatic index	GRAVY	Stability index	Signal peptide
Terrestrial	*Streptomyces griseus* subsp. griseus	6,02	70,98	83,3	-0,21	43,78	23-24
	*Micromonospora echinospora*subsp.challisensis	6,86	75,48	90,96	-0,063	43,12	No
	Sinorhizobium meliloti	5,65	70,47	84,14	-0,26	33,48	26-27
	Enterobacter sp. JJDP1	5,28	68,74	79,9	-0,346	43,17	No
	Bacillus sp. LS04	6,25	59,07	81,5	-0,527	40,03	No
	Bacillus sp. ZW2531-1	5,91	58,44	77,89	-0,566	45,03	No
	Streptomyces cyaneus	5,28	69,51	78,41	-0,479	43,92	No
	Bacillus halodurans	4,29	56,16	69,52	-0,45	33,47	No
	Bacillus subtilis	5,8	58,4	78,09	-0,562	45,58	No
	*Yersinia pestis*Nepal516	6,09	58,33	87,49	-0,133	36,14	28-29
	*Pantoea dispersa*EGD-AAK13	6,07	58,78	81,43	-0,252	36,8	28-29
	Aeromonas hydrophila	6,09	59,78	78,65	-0,324	42,44	No
	Ochrobactrum sp. 531	5,58	57,7	82,23	-0,223	32,93	No
	Streptomyces lavendulae	5,28	68,74	79,9	-0,346	43,17	No
	Escherichia coli O7:K1 str. CE10	6,28	56,67	84,11	-0,185	30,81	28-29
	*Serratia marcescens*WW4	6,77	60,6	75,33	-0,252	34,8	28-29
	Klebsiella quasipneumoniae subsp. quasipneumonia	6,23	57,8	81,67	-0,129	33,89	28-29
	Klebsiella variicola	6,11	58,21	82,48	-0,135	34,24	28-29
	Lactococcus lactis subsp. cremoris GE214	7,22	52,28	70,73	-0,613	27,12	29-30
Marine	*Pseudoalteromonas haloplanktis*TAC125	5,86	68,13	76,78	-0,283	34,4	No
	Vibrio brasiliensis LMG 20546	6,88	51,55	87,93	-0,176	39,01	No
	Vibrio sp.EJY3	6,25	50,81	85,19	-0,174	34,83	No
	Marine actinobacteriumPHSC20C1	4,89	52,29	88,91	-0,107	44,36	No
	*B. stratosphericus*LAMA585	5,82	58,69	80,08	-0,496	38,51	No
	*Bacillus atrophaeus*C89	5,96	58,26	76,57	-0,523	42,88	No
	*Escherichia coli*E1728	6,28	56,66	84,86	-0,166	31,47	28-29
	P. agglomerans	6,64	58,27	80,32	-0,265	42,72	28-29
	*Gamma proteobacterium*IMCC1989	5,83	63,71	68,14	-0,476	29,59	No
	Glaciecola sp.4H-3-7+YE-5	5,71	68,5	74,47	-0,304	39,76	No
	Actibacterium atlanticum	5,04	44,71	78,54	-0,282	35,06	No
	Raoultella ornithinolytica	6,23	58,9	78,11	-0,2	33,94	No

pI= Isoelectric point; MW= Molecular weight; GRAVY= Hydrophobicity index.

Table 2 Bacterial sequences suitable for homology modelling

Origin	Target Sequence	Target Organism	Template Sequence	Template Organism	% Identity
Terrestrial	ADZ57281.1	*Bacillus* sp. LS04	1GSK	*B. subtilis*	66
	AFN66123.1	*Bacillus* sp. ZW2531-1	1GSK	*B subtilis*	99
	AIR95228.1	*B. subtilis*	1GSK	*B.subtilis*	99
	AEQ10977.1	*E. coli* O7:K1 str. CE10	4HAK	*E. Coli*	99
	CTQ10134.1	*K. variicola*	4E9Q	*E. Coli*	78
	CDQ12666.1	*K. quasipneumoniae* subsp. *quasipneumoniae*	4HAK	*E. Coli*	78
	ERH64163.1	*P. dispersa* EGD-AAK13	1KV7	*E. Coli*	66
	AGE19895.1	*S. marcescens* WW4	4HAK	*E. Coli*	66
	EEO77870.1	*Y. pestis* Nepal516	4HAK	*E. Coli*	65
Marine	EIM09308.1	*B. atrophaeus* C89	1GSK	*B. subtilis*	81
	EMI14845.1	*B. stratosphericus* LAMA 585	1GSK	*B. subtilis*	69
	EZG30847.1	*E. Coli* 1728	4HAK	*E. Coli*	99
	KIC85419.1	*P. agglomerans*	1KV7	*E. Coli*	65
	KIZ43410.1	*R. ornithinolytica*	4HAL	*E. Coli*	77

After modeling, all the structures obtained for the selected sequences (Figure 3) were validated by the Procheck, Verify 3D, ERRAT and ProSa tools (Table 3). The validation results clearly show the attainment of good enzymatic models that can be used for further study of molecular docking. For the Procheck parameter, structures with about 90% of residues in the most favorable region can be noted, which is evidence of the good stereochemical quality of the models. Similarly, Verify 3D and ERRAT show values corresponding to high quality models. For Verify 3D, all the models achieved more than 80% of the residues above the 0.2 score, which implies a good correspondence between the aminoacid sequence and the three-dimensional structure obtained [28]. Regarding the ERRAT parameter, this tool evaluates the arrangement of different types of atoms with respect to each other; given that the value normally accepted as high quality is greater than 50 [29], the results obtained show the achievement of good models. Finally, the ProSa quality factor (Z score) must be within the range reported for native proteins, since such result is consistent with a good quality model [30]. In this case, all the values obtained were within those reported for other structures, ensuring the attainment of good quality models.

Table 3 Validation parameters for the proteins modeled.

Origin	Protein source	Procheck	Verify 3D	ERRAT	PROSA (Z score)
Terrestrial	*Bacillus* sp.LS04	89,3	92,59	62,178	-7,73
	Bacillus sp.ZW2531-1	90,6	91,23	69,843	-8,29
	B. subtilis	90,3	87,91	74,851	-8,27
	E. coli O7:K1 str. CE10	92,8	95,87	84,6	-9,03
	K. variicola	91,7	95,87	84,6	-8,05
	K. quasipneumoniae subsp. *quasipneumoniae*	91,3	93,68	74,364	-8,43
	P. dispersa EGD-AAK13	88,3	90,2	59,281	-7,28
	S. marcescens WW4	89	90,53	61,895	-7,64
	Y. pestis Nepal516	89,9	90,91	67,404	-8,18
Marine	*B. atrophaeus* C89	90,6	87,13	70,495	-7,62
	B. stratosphericus LAMA 585	89	86,27	63,347	-7,59
	E. Coli 1728	93,6	95,49	91,295	-9,36
	P. agglomerans	89,5	92,05	65,226	-7,42
	R. ornithinolytica	92,2	89,65	82,4	-7,78

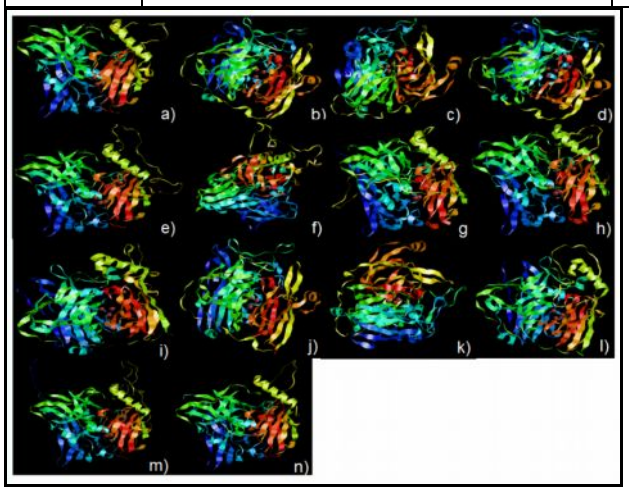

Fig. 3 Modelled putatuve laccases from marine and terrestrial origina) *E. coli O7:K1 str CE10,* b) *Bacillus sp. ZW2531-1,* c) *Bacillus sp. LS04,* d) *B. subtilis,* e) *K. quaipneumoniae,* f) *K. variicola,* g) *P. dispersa,* h) *S. marcescens,* i) *Y. pestis,* j) *B. atrophaeus,* k) *B. stratosphericus,* l) *P. agglomerans,* m) *R. ornithinolytica,* n) *E. coli E1728.*

Molecular Docking Simulation

The active site of the enzyme was searched using the CASTp tool, which can find accessible cavities in the enzyme that can give access and connect substrates to its active site. Between 80 and 100 pockets were estimated for each enzyme. For molecular docking studies, the pocket nearest to the T1 copper binding site was chosen (Figure 4). The affinity of each enzyme with the industrial dyes was evaluated along with the ABTS (2,2-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid), which serves as a positive control for laccase activity. The results showed that for many enzymes the affinity with the industrial dyes was greater than that with the positive control (Table 4). Additionally, the high affinity values were remarkable for marine bacteria *P. agglomerans* and *B. stratosphericus*, which scored the best affinity for 5 of 6 the dyes evaluated, thereby highlighting the potential of microorganisms from marine origin, whose laccases have been scarcely studied so far. The foregoing is even more interesting, if it is considered the potential for the enzymes of the marine environment to be tolerant to saline environments [31], for which the benefit could be even greater.

Table 4 Affinity interaction between enzymes of marine and terrestrial origin with industrial dyes.

Origin	Organism	ABTS*	Acid Orange 10*	Amido Black*	Reactive Blue 4*	Reactive yellow 14*	Remazol Black B*	Trypan Blue*
Terrestrial	*Bacillus sp.* LS04	-7,7	-7,4	-7,7	-8,1	-8,3	-8	-9,7
	Bacillus sp. ZW2531-1	-8,3	-7,4	-8,1	-9,1	-8,5	-8,2	-9,3
	B. subtilis	-7,9	-7,4	-7,9	-9,1	-7,8	-7,9	-9,7
	E. coli O7:K1 str. CE10	-5,6	-6,1	-7,1	-8,1	-6,7	-7	-7,2
	K. variicola	-6,8	-6,2	-6,7	-7	-6,6	-7,5	-8,1
	K. quasipneumoniae subsp. *quasipneumoniae*	-6,4	-6,3	-7,1	-7,9	-6,3	-5,5	-3,2
	P. dispersa EGD-AAK13	-5,7	-4,8	-6,4	-6	-6,4	-5,1	-4,7
	S. marcescens WW4	-5,8	-5,4	-6,3	-7	-6,1	-6,2	-4,8
	*Y._pestis_*Nepal516	-6	-6	-5,9	-6,8	-6,6	-7,4	-7,2
Marine	*B._atrophaeus_C89*	-7,6	-7	-8	-8,9	-7,4	-7,7	-9,3
	B._stratosphericus LAMA585	-8	-7,5	-7,9	-8,7	-8,2	-8,3	-8,7
	E. Coli 1728	-6,9	-6,2	-6,9	-7,8	-7,2	-7,1	-7,5
	P. agglomerans	-7,7	-6,9	-8,2	-9,4	-8,6	-7,2	-8,6
	R. ornithinolytica	-6,5	-6,2	-6,6	-7,9	-6,4	-6,2	-7,1

*Each value represents the affinity energy in kcal/mol. The more negative this value is, the more interaction occurs between the enzyme and the dye.

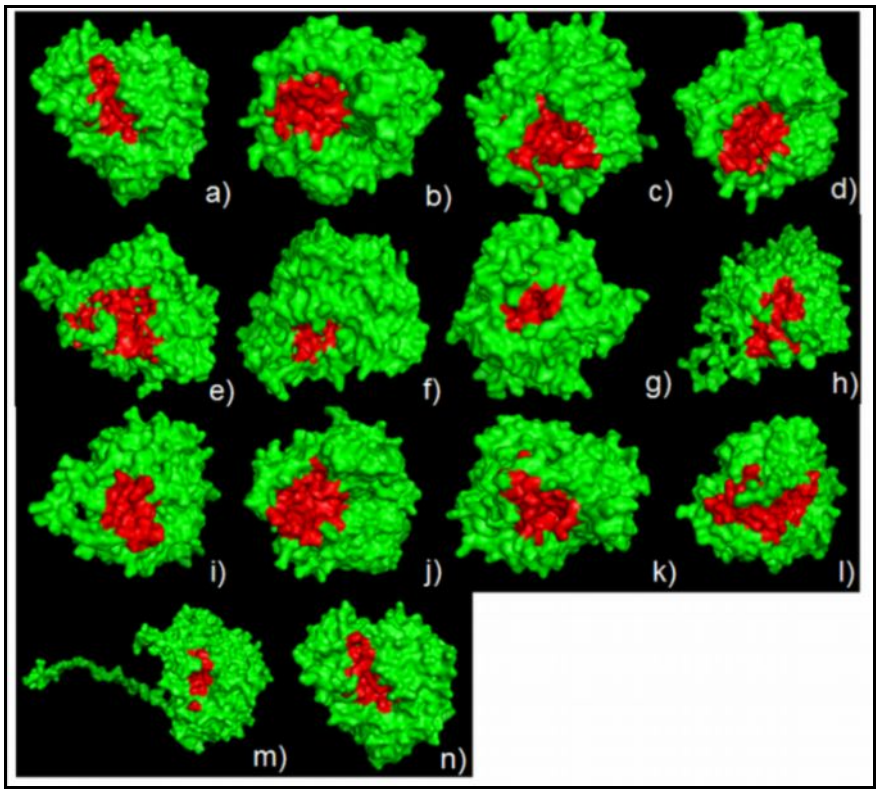

Fig. 4 Active site pockets of putative laccases from marine and terrestrial origin a) *E. coli O7:K1 str CE10,* b) *Bacillus sp. ZW2531-1,* c) *Bacillus sp. LS04,* d) *B. subtilis,* e) *K. quaipneumoniae,* f) *K. variicola,* g) *P. dispersa,* h) *S. marcescens,* i) *Y. pestis,* j) *B. atrophaeus,* k) *B. stratosphericus,* l) *P. agglomerans,* m) *R. ornithinolytica,* n) *E. coli E1728*

In studying the interactions of enzymes with higher affinity with each dye (Figure 5), it is noted that in each case there are specific residues involved in the interaction, which depends on the enzyme and substrate. However, despite the different poses that the substrates can take at the active site and that may influence the interaction with different residues in the pocket of the enzyme, it is possible to see some common residues in the binding sites. Thus, in the case of the interaction of *P. agglomerans* with the dyes Amido Black, Reactive Yellow 14, and Reactive Blue 4, the important role of Lys 274, Gly 353, and Ala 426 is evident, which may have different types of favorable interactions with the substrate, either by hydrogen bonding, pi-alkyl interaction, electrostatic attraction, or van der Waals forces. For the interaction of *B. stratosphericus* with the dyes Acid Orange 10 and Remazol Black B, it is evident that His 497 and Thr 262 play an important role, owing to the interactions by hydrogen bonding and electrostatic attraction. Additionally, it is interesting to note that, for Remazol Black B, His 497 also has unfavorable interactions due to the repulsion of positive charges with the nitrogen double bond in the dye. Nevertheless, the interaction with this dye is more favorable than with Acid Orange 10. In general, due to the favorable interactions noted in this work, it is possible to pose the putative laccase of *P. agglomerans* and *B. stratosphericus* for the design of synthetic genes that allow the expression and experimental analysis of these enzymes. Similar approaches have been successfully adopted in the study of fungal laccases in *Pleorutus ostreatus* and *Ganoderma lucidum*[32]. However, to date similar reports involving bacterial laccases have not been found, and even less so for laccases of marine origin.

Finally, although some factors, such as the concentration of enzymes, dyes, and bioavailability, can limit the experimental application of enzymes, it is worth mentioning that docking is a valuable tool that can help in the identification of potential substrates and the comparison and selection of candidate enzymes for bioremediation processes.

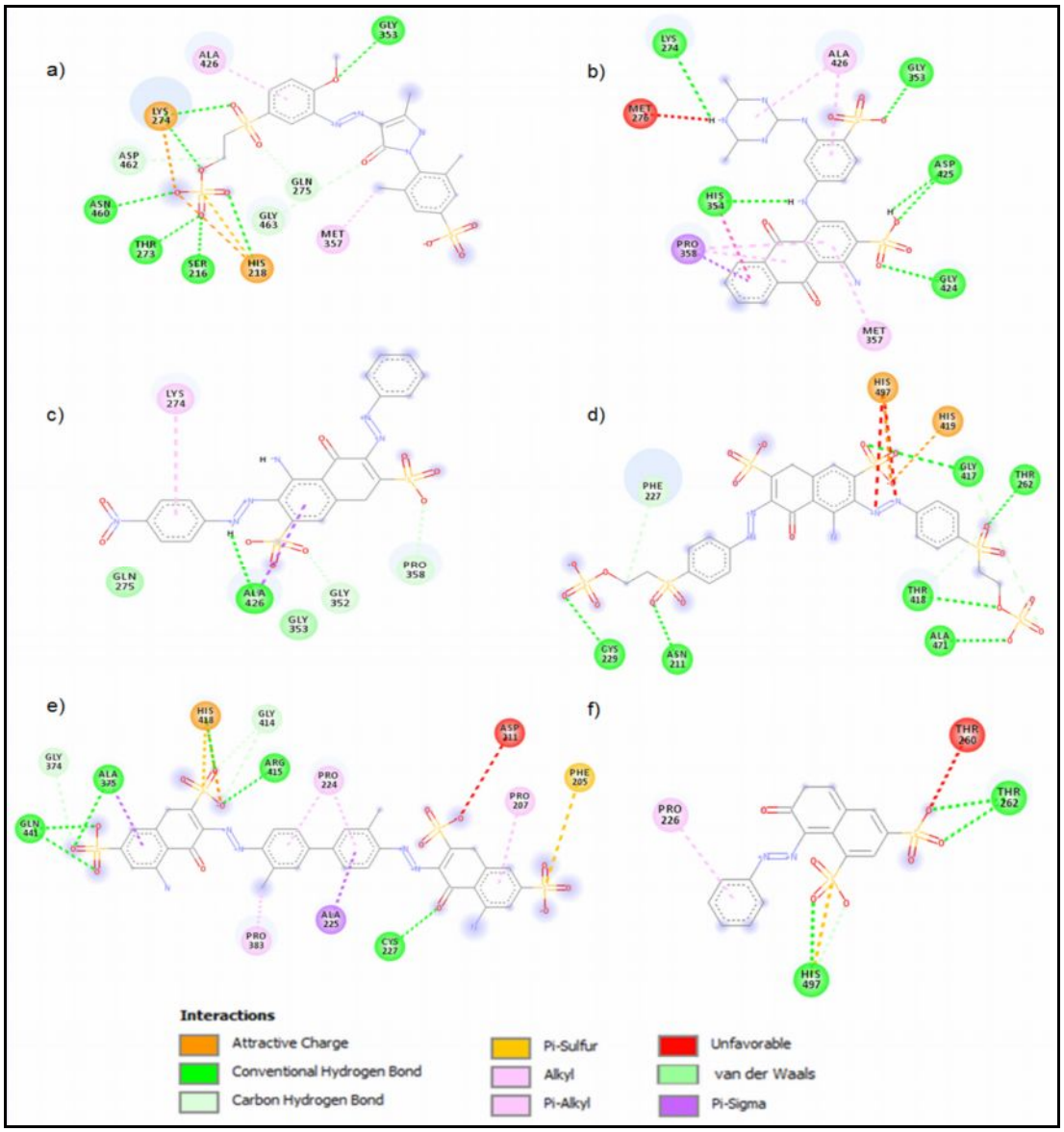

Fig. 5 2D representation of the best molecular interaction for every industrial dye with the corresponding enzyme a) Reactive yellow 14, b) Reactive Blue 4, c) Amido Black, d) Remazol Black B, e) Trypan Blue, f) Acid Orange 10

Conclusion

To conclude, the results showed an outstanding behavior of the putative laccase from the marine-derived bacteria *P. agglomerans,* which presented the best affinity interaction with the dyes Amido Black, Reactive Yellow 14, and Reactive Blue 4. Similarly, putative laccase of the marine-derived bacteria *B. stratosphericus* showed the best affinity with the dyes Acid Orange 10 and Remazol Black B. The remarkable behavior of the enzymes of marine origin in this study demonstrates the potential of marine microorganisms and the importance of exploring biotechnological products in this ecosystem, particularly enzymes. Also, this work can be regarded as an important step in considering the production of synthetic genes of the putative laccases studied that presented the best interaction results.

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Ethical Statement/Conflict of Interest

The authors declare that they have no conflict of interest.

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