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Research Article

In silico Evaluation of Cross-linkers for Immobilizing Laccase in Scalable Polyethylene Terephthalate (PET) Biodegradation

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ABSTRACT

The rapid accumulation of polyethylene terephthalate (PET) in the environment has intensified the need for sustainable and efficient strategies for plastic degradation. Laccase, a flexible oxidoreductase able to attack and cleave the ester linkage for potential PET hydrolysis, is hindered in practical applications by limited stability, restricted reusability, and susceptibility to industrial conditions. This study employed in silico approaches, including molecular docking and molecular dynamics (MD) simulations, to evaluate the interactions and stability of laccase immobilized with a diverse set of cross-linkers: micromolecular (ethylene glycol, glutaraldehyde, and benzoquinone), macromolecular (chitosan, and dextran), and polysaccharide-based (amylopectin, dextran, and glucosamine). Among these, glucosamine exhibited the strong binding affinity, with ΔG values of –7.0 kcal/mol (AutoDock) and –7.98 kcal/mol (SwissDock), forming 4–7 persistent hydrogen bonds at a safe distance of 15–21 Å from the T1 copper catalytic centre. MD simulations over 10 ns confirmed enhanced structural stability of the laccase + glucosamine complex, evidenced by low RMSD values (~0.20–0.28 nm), reduced RMSF at catalytic loops (0.02–0.07 nm), consistent Rg (1.68–1.74 nm), and decreased SASA (175–185 nm²). According to these findings, glucosamine offers excellent structural stability, compactness, and preservation of active site integrity, highlighting its potential use as a biocompatible cross-linker. The study establishes an appropriate in silico framework for developing reliable immobilized laccase systems for industrial and environmental PET degradation applications.

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INTRODUCTION

The widespread utilization of plastics in modern society is largely attributed to their low production cost, versatility, and longevity. Despite these benefits, excessive plastic manufacturing and ineffective disposal techniques have led to a significant buildup of plastic debris in both terrestrial and marine environments (Evode et al., 2021) . Polyethylene terephthalate (PET) has emerged as one of the most widely used synthetic polymers worldwide, especially in the manufacture of beverage containers, textiles, fibres, and packaging materials. This is due to its superior mechanical strength, moisture resistance, and lightweight design, which makes it an ideal product for these applications (Soong et al., 2022). Regardless of these benefits, PET`s chemically persistent ester bonds severely restrict its biodegradation processes, resulting in increased environmental endurance that facilitates its gradual fragmentation into microplastics, which pose significant ecological toxicological concerns (Soong et al., 2022).

Microplastic accumulation has emerged as a growing environmental challenge in Malaysia. Recent reports have claimed that substantial microplastic waste is present in

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coastal and port environments, with 1.713 particles identified in Kuala Nerus and 621 particles found in Kuantan Port. In addition to the particle abundance, the alarming concentration further highlights the severity of the contamination, reaching 0.13-0.69 particles per litre in Kuala Nerus and 0.14–0.15 particles per litre in Kuantan Port, indicating significant microplastic loading in these aquatic systems (Khalik et al., 2018). Existing approaches for PET waste management, including landfilling, incineration, and mechanical recycling, remain insufficient to mitigate the growing pollution burden. For instance, landfilling promotes long-term environmental persistence, incineration poses risks through the release of toxic emissions, while mechanical recycling is constrained by deterioration of the polymer chain, declining material quality, and substantial energy requirements, all of which limit overall sustainability (Hasan et al., 2025).

Subsequently, to overcome these constraints, biological degradation using enzymes and microorganisms has gained attention as an environmentally friendly alternative for PET degradation. Horseradish peroxidase (HRP) and cutinase most commonly used in PET degradation, while lipase and esterases are frequently applied in the degradation of polyurethane (PUR) and short-chain polyesters (Jayasekara et al., 2023; Kibria et al., 2023; Raoufi et al., 2023). Notably, laccase, a multi-copper oxidase enzyme commonly derived from fungal (e.g., *Pleurotus ostreatus, Trametes versicolor*) and bacteria (e.g., *actinomycete Rhodococcus ruber*) are well-known for its capability of oxidative degradation of PE by reducing its molecular weight through cleavage of C-C backbone (Janusz et al., 2023). Recent reports have also highlighted its promising capabilities for targeting ester linkages in PET via oxidative chain scission mediated by reactive oxygen species (ROS). It will attack aromatic structures and backbone via hydrogen bonding and electrostatic interactions increasing the polymer`s susceptibility to hydrolysis (Ding et al., 2026; Zhang et al., 2023). Thus, this study has selected laccase as a promising enzyme for PET degradation.

Nevertheless, the application of laccase is hindered by intrinsic limitations, including low operational stability, susceptibility to denaturation under industrial conditions, reduced catalytic efficiency, and poor reusability. To overcome these limitations, enzyme immobilization has been introduced, which is successfully able to improve enzyme resilience and functionality (Gao et al., 2021). It works by carefully binding laccase to selected ligands or cross-linkers, improving structural stability, maintaining catalytic performance, and permitting repeated reusability (Khairul Anuar et al., 2022). Nevertheless, the relationship between cross-linkers and enzyme remains challenging; incorrect can obstruct active sites and reduce catalytic efficiency. Advanced computational approaches, particularly molecular docking and molecular dynamics (MD) simulations, have been indispensable for predicting and optimizing enzyme-cross-linker interactions by offering cost- and time-efficient insights into binding affinity and conformational alterations at the molecular level (Prabhakar et al., 2025).

In this study, an in silico immobilization framework is employed to systematically investigate the laccase interactions with a diverse spectrum of cross-linkers, encompassing micromolecular (ethylene, glutaraldehyde, and benzoquinone), macromolecular (chitosan and dextran), and polysaccharide-based (amylopectin, dextran, and glucosamine). The findings are anticipated to advance the rational design of efficient immobilization and support the development of durable, industrially applicable biocatalytic systems aimed at mitigating the persistent environmental burden posed by PET pollution.

MATERIALS AND METHOD

Materials

The chemicals and solvents used in this study were purchased from Sigma Aldrich (USA) and Thermo Fisher Scientific (USA) unless stated otherwise.

Enzyme Structure and Linkers Modelling

The 3D structure of laccase (PDB ID: 1GYC) was retrieved from the RCSB Protein Data Bank (PDB). (Liu et al., 2019). Prior to molecular docking, the protein structure was prepared by removing all non-essential components, including bound ligands, water molecules, inhibitors, and heteroatoms attached to the protein structure using PyMOL 3.1 to ensure an optimized receptor model before docking analysis (Rosignoli & Paiardini, 2022). Then, the PDB files of the cross-linkers (amylopectin, benzoquinone, chitosan, dextran, ethylene glycol, ethylene glycol bis (succinimidyl succinate) (EG-NHS), glucosamine, and glutaraldehyde) were extracted from PubChem (Kim et al., 2023).

Molecular Docking Analysis

Molecular docking analysis the ligands and protein structure for docking were prepared using AutoDock vina tools 4.2.6, by removing the water molecules and adding hydrogen atoms to the structures (Trott & Olson, 2010). Both receptor and ligand structures were converted from Protein Data Bank (PDB) to Partial Charge, and Torsions (PDBQT) format, following standard preparation protocols (Jailani et al., 2022). The protein docking of laccase and ligands were carried out using AutoDock Vina, with the grid box parameters (size: x = 126, y = 126, z = 124; centre: x = 16.74, y = 6.18, z = 37.33; and grid spacing = 1.0 Å). The other parameters of docking were set as default. Then, the binding energy (ΔG) of each laccase–ligand complexes were obtained from the docking process. The resulting protein-ligand interaction profile was visualized and interpreted using LigPlot+ to identify the key intermolecular contacts and binding surface residues (Laskowski & Swindells, 2011).

Molecular Dynamics (MD) Simulations

Molecular dynamics (MD) simulations were conducted using Groningen Machine for Chemical Simulations (GROMACS) (version 5.1.4) with the CHARMM27 united-atom force field. Each protein-ligand system was placed in a cubic box with a minimum distance of 1.0 nm between the solute and box edges, solvated with TIP3P water molecules and neutralized with Na^+/Cl^- ions, and energy-minimized to avoid steric clashes. Simulations were run under NVT and NPT conditions at 313 K (V-rescale thermostat) and 1 bar (Berendsen barostat) (Hess et al., 2008). Lennard-Jones (1 nm cut-off) and PME electrostatics handled non-bonded interactions. A 2-fs leap-frog integrator and LINCS constraints-maintained stability. VMD 1.9.3 was used to analyse RMSD, RMSF, and Rg to assess enzyme–cross-linker stability (Yip et al., 2024).

RESULTS AND DISCUSSION

Molecular Docking Analysis

Cross-linking enhances enzyme stability and reusability by reinforcing the interactions with surface-exposed amino acid residues without altering the catalytic function (Yip et al., 2024). Molecular docking analysis was performed using both AutoDock and SwissDock as a rapid in silico screening tool to further validate the lowest binding free energy (ΔG) between laccase and selected crosslinker to justify the compatibility and stability of the enzyme-crosslinker interactions. Then LigPlot analysis was done to observe the number of hydrogen bonds, hydrophobic interactions and the distance of ligand binding to surface residues from the catalytic site as shown in **Table 1** and **Figure 1.**

Among the evaluated cross-linkers, binding free energy (ΔG) analysis revealed that chitosan (-7.1 kcal/mol^{-1}) showed the lowest binding free energy, followed by dextran, amylopectin, glucosamine, EG-NHS, ethylene glycol, benzoquinone, and glutaraldehyde (-6.6, -6.1, -6, -5.9, -5.16, -4.8, and -3.9 kcal/mol^{-1}, respectively). Consistently, SwissDock also predicted chitosan with lowest binding energy (-7.98 kcal/mol), followed by dextran, EG-NHS, amylopectin, glucosamine, glutaraldehyde, benzoquinone, and ethylene glycol (-6.96, -6.93, -6.2, -5.8, -5.76, -5.57, and -5.2 kcal/mol, respectively). The similar outcomes between both docking platforms highlight the consistency and reliability of the simulation outcomes. Similarly, studies on the in-silico approach on enzyme immobilization have reported that cross-validation using multiple docking engines improves the clarity of the predicted interactions hierarchies and better correlates with experimental immobilization outcomes (Agu et al., 2023).

Hydrogen bond analysis relatively supported these outcomes, as such chitosan (10 hydrogen bonds, 10 hydrophobic interactions), dextran (7 hydrogen bonds, 10 hydrophobic interactions), and glucosamine (6 hydrogen bonds, 3 hydrophobic interactions), displayed the highest number of hydrogen bond interactions. Alongside, corresponding hydrogen bond lengths with the interacting amino acid residues were ranged within an optimal range (2.7–3.2 Å) (Zhou et al., 2022). Collectively, these features indicate the formation of shorter, stronger, and more stable interactions with the enzyme. Both of these specialties highlight robust and energetically favourable non-covalent interactions at the enzyme interface, which greatly aid in the stability of the enzyme-crosslinker complex formation (Majewski et al., 2019).

In contrast, amylopectin (4 hydrogen bonds, 3 hydrophobic interactions), EG-NHS (3 hydrogen bonds, 10 hydrophobic interactions), ethylene glycol (2 hydrogen bonds, 4 hydrophobic interactions), benzoquinone (1 hydrogen bond, 4 hydrophobic interactions) and glutaraldehyde (1 hydrogen bonds, 2 hydrophobic interactions) were among the crosslinkers with the least hydrogen bonds and hydrophobic interactions with laccase. A lower number of hydrogen bonds implies less conformation rigidity and reduced enzyme stability. These crosslinkers rely primarily on hydrophobic or π–π stacking forces rather than strong polar interactions (Le et al., 2024). Although their corresponding hydrogen bond lengths generally fall (2.8–3.4 Å), within the acceptable range for hydrogen bonding, their weaker number of interactions causes weaker stabilization at the enzyme–cross-linker interface. Notably, ethylene glycol exhibited the longest average hydrogen bond distance (up to 3.5 Å), consistent with its weakest binding performance.

Additionally, analysis on the distance relative to the copper active-site centre (especially the T1 copper centre and its associated residues) revealed that glucosamine, EG-NHS, amylopectin, and glutaraldehyde bind at a safer distance and range (15–21.0 Å), minimizing steric overlap with the substrate binding positions at the T1 microenvironment (Ahmad et al., 2022). Although, chitosan has demonstrated the lowest binding energy (ΔG) and a greater number of hydrogen bonds, it binds at a closer proximity (< 15 Å) to the T1 copper centre, increases the risk of interfering with substrate binding by occluding the T1 copper access channel, reducing enzyme activity, lowering substrate affinity, and altering the catalytic efficiency (Biernat et al., 2023; Udoetok et al., 2024).

Therefore, glucosamine has consistently shown binding free energy (ΔG), number of hydrogen bonds, hydrophobic interactions, and distance of binding from active site, all within the optimal range, making it a compatible cross-linker for laccase. These findings contribute to the rational design of enzyme immobilization systems by emphasizing that optimal cross-linkers must balance strong binding with preservation of catalytic accessibility. The identification of glucosamine as a favourable candidate highlights its potential for developing robust, reusable laccase-based biocatalysts. Thisin silico framework is a reliable strategy for pre-screening immobilization agents, reducing experimental cost and accelerating the development of stable biocatalysts for industrial and environmental applications, including sustainable pollutant degradation and green bioprocessing.

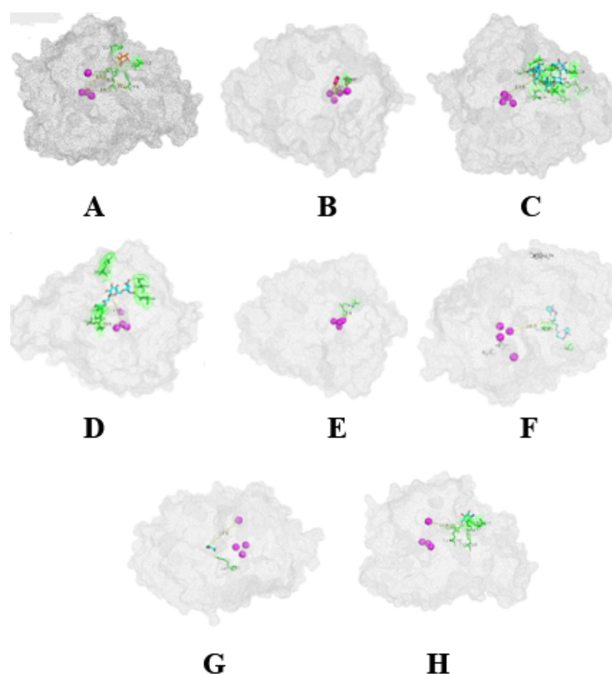

Figure 1 This figure shows the binding position of cross-linkers (green) consist of (A) amylopectin, (B) benzoquinone, (C) chitosan, (D) dextran, (E) ethylene glycol, (F) EG-NHS, (G) glutaraldehyde and (H) glucosamine on surface amino acid residues and distance from the T1 copper centre of laccase (pink).

Table 1 Binding position of cross-linker on Laccase`s surface

Crosslinkers	Binding Free Energy (ΔG) (kcal/mol)		Hydrogen Bond Interacting Residues	Hydrogen Bond Length (Å)	Distance between cross-linker from active site (Å)	Hydrophobic Interacting Residues	
	AutoDock Vina	SwissDock					
Amylopectin	-6.10	-5.80	Gln242 Tyr244 Arg423 Leu300 Ala410	3.11 2.85 2.97 3.02 3.13	18.0	Glu302 Phe239 Ile301	
Benzoquinone	-4.80	-5.57	His458	3.10	6.95	Phe265 Asp206 Gly392	
Chitosan	-7.10	-7.98	Arg423 Glu302 Glu237 Thr210 Ser296 Asp140 Asp138 Tyr137 Asn208 Leu300	3.05 2.79 2.84 3.01 3.18 3.09,2.90 2.71 3.24 3.18 2.95	11.50	Ser427 Gln242 Phe239 Ala410 Thr430	Gly429 Asn264 Pro394 Pro299 Ile301
Dextran	-6.60	-6.96	Asp101 Lys40 Gly225 Ala103 Asp444 Gln442 Arg310	2.89 3.11 3.13,2.78 3.05,2.93 2.89 2.89,312 3.13	12.20	Asp128 Gln102 Ile226 Ala403 Phe422 Pro314	Phe441 Met311 Asn227
Ethylene Glycol	-5.90	-6.93	Ser16 Phe31	3.04 3.20	2.20	Phe450 Pro79 Leu459	Leu112
EG-NHS	-5.9	-6.93	Ser16 Phe31	3.04 3.20	18.90	Pro17, Thr147 Leu174, Val145 Val30, Glu142 Val247	Pro32, Val15 Asn172 Pro123
Gluteraldehyde	-3.9	-5.76	Arg423	2.82	15.0	Gln237	
Glucosamine	-6.0	-5.78	Arg423 Tyr244 Gln237 Leu300 Glu302 Gln242	3.27 2.77 3.20 3.17, 3.06 3.05 2.96	18.6	Val211	

Validation of Docking Analysis Using Molecular Docking Simulation

RMSD analysis was performed as shown in **Figure 2**, below, to evaluate the global conformational stability of laccase upon immobilization with different crosslinkers. RMSD measures the time-dependent displacement of backbone atoms relative to the initial structure, thereby representing overall structural deviation during equilibration and production phases (Hollingsworth & Dror, 2018). For well-folded proteins like laccase, RMSD values stabilizing at approximately (0.15–0.30 nm) are generally indicative of conformational stability, whereas sustained increases or frequent fluctuations suggest destabilization or excessive structural rearrangement (Christensen & Kepp, 2013). In this study, the analysis revealed that free laccase exhibited an initial increase during the equilibration phase, reaching values approximately (~ 0.28–0.32 nm) within the first 10 ns, followed by moderate fluctuation, stabilizing at ~ 0.30 nm and throughout the trajectory.

This behaviour reflects preservation of native structure. Upon binding with crosslinkers, such as laccase + amylopectin and laccase + glucosamine complexes, stable RMSD values ranging from (~ 0.20–0.28 nm), similar to baseline values. This indicates that the ligands effectively stabilized the enzyme without inducing excessive conformational strain. The branched architecture of amylopectin, as well as polar functional groups of glucosamine, significantly promotes surface anchoring while preserving native folding (Bertoft et al., 2024). In contrast, laccase + chitosan complexes exhibited elevated RMSD values (~ 0.26-0.32 nm), micromolecular crosslinkers like benzoquinone, ethylene glycol, EG-NHS, and glutaraldehyde also produced elevated RMSD values reaching up to approximately (~ 0.38 nm). This indicates a significant increase in conformational flexibility or structural instability of the enzyme (Wegrzynowska-Drzymalska et al., 2020). Therefore, glucosamine and amylopectin provide superior conformational stabilization compared to micromolecular crosslinkers, supporting previous studies' findings that larger weight and surface area of crosslinkers can form multipoint surface residue interactions that ensure stronger and more stable interactions that are essential for maintaining enzyme integrity during enzyme immobilization (Shen et al., 2025).

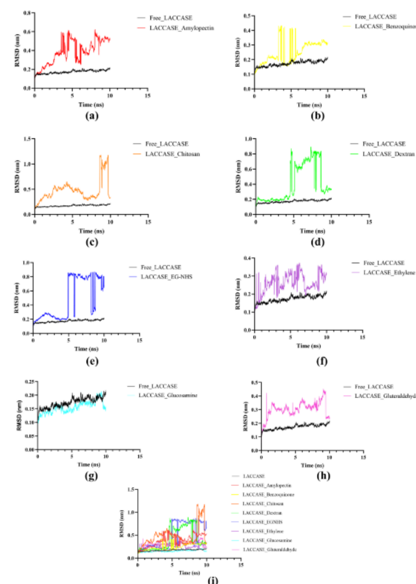

Figure 2 The RMSD plot consist of (a) Laccase + Amylopectin (red) (b) Laccase + Benzoquinone (yellow), (c) Laccase + Chitosan (orange), (d) Laccase + Dextran (green), (e) Laccase + EG-NHS (dark blue), (f) Laccase + ethylene glycol (purple), (g) Laccase + glutaraldehyde (pink), (h) Laccase + glucosamine (light blue), and (i) showing all RMSD plotted together over the course of 10 ns production simulations.

Next, as depicted in **Figure 3** below, the RMSF profiles revealed that free laccase RMSF value ranging (~ 0.02–0.08 nm) and displayed moderate fluctuations at loop regions surrounding the T1 copper-binding sites, consistent with the intrinsic flexibility required for catalysis (Brissos et al., 2024). The binding of amylopectin and glucosamine once again closely similar RMSF values (~ 0.02-0.07 nm) to free enzyme (~ 0.02-0.08 nm), particularly around T1-copper binding loops, indicates conformational stability (Zovo et al., 2022). While micromolecule cross-linkers like ethylene glycol, glutaraldehyde, and benzoquinone complex with laccase, once again showed moderated stabilization. Though the complexes retained RMSF values < 0.05 nm at catalytic residues, there were some significant fluctuations observed throughout the simulations. Reports have stated that micromolecules tend to introduce moderate covalent tethering while maintaining essential conformational flexibility (Konstantinidou et al., 2023). Additionally, laccase + chitosan and laccase + dextran, complexes which involve bulky cross-linkers, exhibited elevated RMSF peaks at residues associated with the T1 copper-binding loops, initiating destabilization due to steric crowding near substrate access channels (Delanoy et al., 2005).

This analysis revealed that immobilization induces enzyme-stabilizing effects in a crosslinker-dependent manner. Among the evaluated systems, glucosamine and amylopectin consistently provided the most favourable dynamic profiles, effectively enhancing structural rigidity while preserving the functional flexibility essential for enzymatic activity.

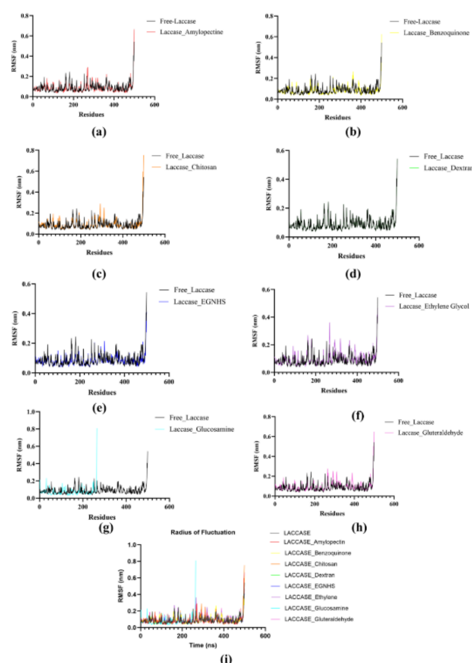

Figure 3 Average RMSF plots of Cα atoms for free_Laccase (black). The RMSF of cross-linker-Laccase complexes at (a) Laccase + Amylopectin (red) (b) Laccase + Benzoquinone (yellow), (c) Laccase + Chitosan (orange), (d) Laccase + Dextran (green), (e) Laccase + EG-NHS (dark blue), (f) Laccase + ethylene glycol (purple), (g) Laccase + glutaraldehyde (pink), (h) Laccase + glucosamine (light blue), and (i) showing all RMSF plotted together over the course of 10 ns production simulations.

The radius of gyration (Rg) as depicted in **Figure 4** was evaluated to assess global structural compactness and folding stability of laccase complexes throughout the simulation trajectories (Jailani et al., 2022). Rg provides a quantitative measure of mass distribution around the enzyme`s centre of geometry, where lower Rg values reflect increased compactness and tighter molecular packing, while higher values indicate structural expansion and conformational loosening (Kumar et al., 2025; Lobanov et al., 2008).Notably, maintaining an Rg profile close to the free enzyme`s equilibrium state is considered a hallmark of a stable immobilized conformation. In this study, the analysis of Rg on the enzyme complexes was depicted in **Figure 4**, below.

The free enzyme structures analyzed and depicts stable Rg baselines ranging (~ 1.70-1.74 nm) were demonstrated, validating the accurate folding state used as a reference for comparison (Sinha et al., 2022). In this analysis, micromolecule cross-linkers (e.g., ethylene glycol, glutaraldehyde) yielded almost identical and slightly reduced Rg values (1.68-1.74 nm), almost similar to the baseline value, demonstrating preserved compactness. Though micromolecular linkers induce significant compactness, based on molecular docking analysis, it binds closer to the active site region of enzymes, which might alter the catalytic function of the enzyme; hence, it's not a reliable complex for immobilization studies.

Besides, chitosan showed an elevation in Rg values ranging (~ 1.78-1.83 nm). These findings were evident in a previous report in which chitosan tends to introduce stabilizing or destabilizing effects towards most of the enzymes (Krainer & Glieder, 2015). Additionally, glucosamine in Rg analysis showed weak stability with reduced compactness towards laccase with an elevated Rg value (~ 2.21–2.28 nm) along with frequent fluctuation as compared to the free laccase system (~ 2.21–2.24 nm), which maintained a more compact structure with minimal fluctuation. This trend may be attributed to weaker and non-specific interactions between glucosamine and the residues on the enzyme`s surface, leading to insufficient effectiveness to stabilize protein structure, thus causing an increase in structural flexibility and conformational mobility, which was observed in a previous study on enzyme immobilization of laccase (Patel et al., 2014).

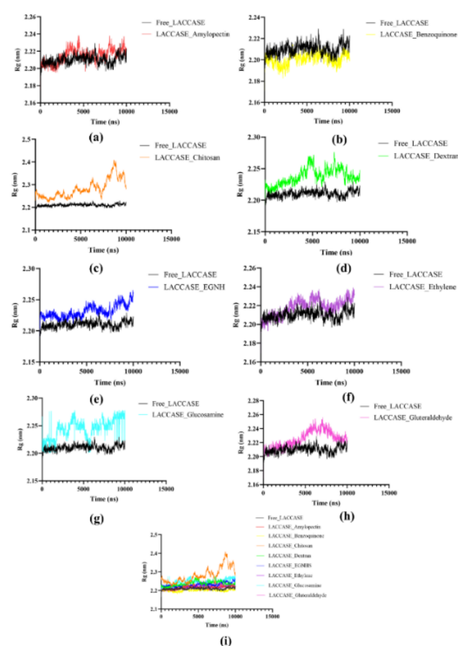

Figure 4 Average radius of gyration (Rg) is shown as a function of 100 ns simulation time at 313 K, free_ Laccase (black). The Rg of cross-linker- Laccase complexes at (a) Laccase + Amylopectin (red) (b) Laccase + Benzoquinone (yellow), (c) Laccase + Chitosan (orange), (d) Laccase + Dextran (green), (e) Laccase + EG-NHS (dark blue), (f) Laccase + ethylene glycol (purple), (g) Laccase + glutaraldehyde (pink), (h) Laccase + glucosamine (light blue), and (i) showing all Rg plotted together over the course of 10 ns production simulations.

Next, the solvent accessible surface area (SASA) in **Figure 5**, is a fundamental indicator of protein stability, compactness, and conformational integrity during molecular dynamics simulations. It signifies the portion of the protein accessible to the solvent molecules (Kamaraj & Purohit, 2013). Lower and more uniform SASA values typically correlate with tighter protein packing, reduced solvent exposure, and enhanced structural stability qualities that are desirable in enzyme immobilization strategies aimed at improving catalytic performance, thermostability, and operational lifespan (Bagewadi et al., 2023). In this present study, SASA behaviour was visualized across the enzyme complexes as depicted in **Figure 5** by comparing to the baseline value laccase (175-190 nm^2), cutinase (100-108 nm^2).

The comparative SASA evaluation on enzyme laccase demonstrated that glucosamine binding consistently produces substantial reductions in solvent exposure, indicating increased conformational compactness and

structural stability. Laccase + amylopectin (~ 185-205 nm²) remained close to their baseline values, indicating beneficial compaction. Glucosamine consistently reduced or stabilized SASA, consistent with its ability to form dense hydrogen-bonded networks that restrict surface fluctuations, align with trends reported by (Li et al., 2010; Srivastava et al., 2022). While chitosan showed moderate compactness when interacting with laccase, with SASA values ranging (~ 182-205 nm²), slightly narrower than their respective baselines, yet still maintaining moderate surface solvent exposure; however, there are variable fluctuations (~12-15 Å²) seen throughout the trajectory.

Chitosan is a biopolymer with an abundance of hydroxyl and amino groups that tend to undergo rapid exchange, causing localized fluctuations and molecular motion within this seemingly rigid matrix (Szymańska & Winnicka, 2015). Moreover, complexes immobilized with glutaraldehyde, benzoquinone, ethylene glycol, and EG-NHS caused structural instability due to significant structural expansion or linker-induced conformation strain, with SASA values ranging from (~ 200-215 Å²), greatly surpassing the baselines of the free enzymes. These degrees of structural expansion could be attributed to steric rigidity and strong cross-linking chemistry that induces localized strain, thereby increasing surface exposure (Chen et al., 2025).

In SASA analysis reveals that laccase + glucosamine produces the most stable immobilized enzyme complexes, characterized by the lowest SASA values and minimal fluctuations. These linkers consistently enhanced protein compactness by reducing solvent exposure of surface residues, suggesting strong compatibility and stabilizing intermolecular interactions. While chitosan offered partial improvements in structural compactness but lacked in maintaining a uniform trend throughout the trajectory. In contrast, micromolecular cross-linkers such as benzoquinone, glutaraldehyde, and EG-NHS induced significant SASA expansion, marking them as the least stable complexes.

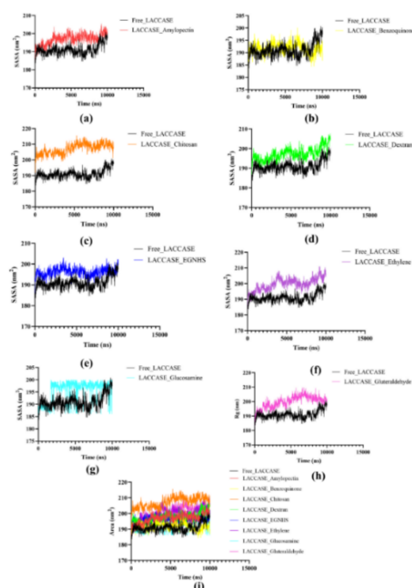

Figure 5 Average SASA is shown as a function of 10 ns simulation, free_ Laccase (black). The Rg of cross-linker-Laccase complexes at (a) Laccase + Amylopectin (red) (b) Laccase + Benzoquinone (yellow), (c) Laccase + Chitosan (orange), (d) Laccase + Dextran (green), (e) Laccase + EG-NHS (dark blue), (f) Laccase + ethylene glycol (purple), (g) Laccase + glutaraldehyde (pink), (h) Laccase + glucosamine (light blue), and (i) showing all SASA plotted together over the course of 10 ns production simulations.

Moreover, hydrogen bonds and their relative strength in a water environment are essential to facilitate protein-ligand binding. According to (Wan & Thompson, 2024), a hydrogen bond forms when an electronegative atom in an H-bond acceptor approaches a hydrogen atom bonded to another electronegative atom in an H-bond donor. In this study, intermolecular hydrogen bonds formed between cross-linkers and laccase are determined and depicted in **Figure 6**, below. It is also worth emphasizing that the MD simulations revealed the changes in the number of hydrogen bonds, which differed from the hydrogen bonds formed during molecular docking due to the rigid sampling score of the latter (Al-Karmalawy et al., 2021).

The hydrogen bond formation, as shown in **Figure 6** represent clear crosslinker-dependent variability, highlighting the importance of linker chemistry in stabilizing enzyme–crosslinker interactions. Laccase + glucosamine exhibited the strongest and most persistent hydrogen bonding profile, consistently maintaining approximately 4–7 hydrogen bonds throughout the simulation, indicating stable polar interactions with the enzyme surface. Moderate hydrogen bond formation was observed for laccase + amylopectin, laccase + dextran, and laccase + chitosan, where the number of hydrogen bonds generally fluctuated between 1 and 3, particularly during the early to mid-stages of the simulation, before showing minor fluctuations likely due to conformational rearrangements of the polysaccharide chains (Ma et al., 2025). In contrast, micromolecular crosslinkers such as ethylene glycol, glutaraldehyde, and benzoquinone exhibited weak and transient hydrogen bonding behaviour, typically maintaining 0–1 hydrogen bonds, reflecting their limited capacity to establish multipoint interactions. This part of the analysis demonstrates that polysaccharide-based crosslinkers, especially glucosamine and amylopectin, provide superior stabilization of laccase by enabling sustained and multipoint hydrogen bonding, whereas smaller crosslinkers contribute only marginally to interaction stability.

Overall, the H-bonding analysis demonstrates that glucosamine is the strongest stabiliser for laccase. These interaction profiles are consistent with RMSD, Rg, and SASA trends, indicating that glucosamine is an effective crosslinker for maintaining the stability and enhancement of catalytic activity of laccase.

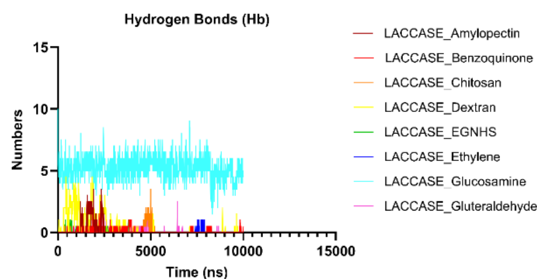

Figure 6 Intermolecular hydrogen bonds along 10 ns simulation time in free_Laccase (black). The hydrogen bonds of cross-linker- Laccase complexes at (a) Laccase + amylopectin (red) (b) Laccase + benzoquinone (yellow), (c) Laccase + chitosan (orange), (d) Laccase + dextran (green), (e) Laccase + EG-NHS (dark blue), (f) Laccase + ethylene

glycol (purple), (g) Laccase + glutaraldehyde (pink), (h) Laccase + glucosamine (light blue), and (i) showing all hydrogen bonds plotted together over the course of 10 ns production simulations.

CONCLUSION

This study demonstrated that the chemical nature of cross-linkers plays a critical role in determining the structural stability and functional preservation of immobilized laccase, with glucosamine emerging as the most effective candidate. Docking analysis showed that glucosamine exhibited the most favourable binding free energy and extensive hydrogen-bond interactions while binding distantly from the T1 copper catalytic centre, thereby preserving substrate accessibility. These outcomes were validated by molecular dynamics simulations, which revealed enhanced structural stability, reduced conformational fluctuations, sustained hydrogen bonding, and maintained compactness of the laccase-glucosamine complex. These findings rational framework for selecting biocompatible cross-linkers in enzyme immobilization approaches that ensure enhanced catalytic activity, reusability, and functional longevity. Future research should focus on experimentally validating these in silico predictions by creating cross-linked enzyme aggregates. Additionally, mutant laccases could be developed by analyzing the binding pattern of glucosamine on surface amino acid residues. Careful selection of cross-linker binding sites, far from the active site, will help avoid disruption of the enzyme–substrate complex. In silico analyses should continue to guide cross-linking efficiency and site-directed mutagenesis to optimize enzyme–cross-linker interactions, reducing experimental cost and time while improving biocatalyst design.

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Conflict of Interest

The author(s) declare(s) that there is no conflict of interest regarding the publication of this paper.

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