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Ionic Liquid Toxicity in Biotechnological Yeasts: Experimental Assessment and Molecular Docking Insights

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ABSTRACT

Ionic liquids (ILs) are promising media for biotechnological applications, although their use in microbial systems is limited by their toxicity to living cells and biomacromolecules. In this study, the toxicity profiles of selected IL-derived ions were evaluated toward *Saccharomyces cerevisiae*, *Yarrowia lipolytica*, and *Kluyveromyces marxianus* by determining the maximum non-toxic concentration (MNTC), and the experimental trends were further investigated through molecular docking against the corresponding yeast hexokinases. The results revealed a species-dependent response, with *Y. lipolytica* displaying substantially higher tolerance than *K. marxianus* and *S. cerevisiae*. For the anionic series, toxicity increased consistently from Cl^- to $[NTf_2]^-$ in the three yeasts, with $[NTf_2]^-$ showing the lowest MNTC values in all cases. The cationic series also showed clear differences in toxicity, though in a more species-dependent manner. Docking analyses revealed a strong qualitative relationship between binding affinity and MNTC, particularly for the anions, indicating that ions with more favourable interactions with hexokinase were generally tolerated only at lower concentrations. Thus, the results indicate that IL toxicity in yeasts emerges from the interplay between ion-specific properties and species-dependent structural features of the target enzyme. By integrating microbiological and computational analyses, this study provides new insight into the molecular basis of ion-dependent and species-dependent IL toxicity in yeasts of biotechnological relevance.

1 | Introduction

Ionic liquids (ILs) have attracted considerable interest as alternative solvents in biotechnology and biorefinery processes due to their negligible vapor pressure, high thermal stability, low flammability, and wide structural tunability arising from the modular combination of cations and anions [1, 2]. This compositional versatility enables the rational design of media with tailored physicochemical properties, including polarity, hydrophobicity, viscosity, hydrogen-bonding ability, and solvation capacity, rendering ILs promising candidates for biomass processing, extraction, catalysis, and whole-cell biotransformations [3]. Nevertheless, despite these advantages, the successful integration of ILs into biological systems remains strongly constrained by their toxicity toward living cells and biomacromolecules, which has been widely reported in recent reviews addressing both their biological activity and their interactions with membrane systems [4, 5]. Therefore, in microbial platforms, the beneficial solvent-related properties of ILs must be balanced against their potential to impair cell viability, metabolism, and enzymatic function [6].

The biological effects of ILs are known to depend on their chemical identity and on the specific contribution of the constituent ions. A growing body of literature has demonstrated that IL toxic-

ity is highly ion-dependent, with both cation and anion structure governing the extent of toxicity as well as the dominant mode of biological perturbation [7–9]. At the cellular level, IL exposure may compromise membrane integrity, as demonstrated in studies on IL-induced membrane permeabilization, and may also alter ion homeostasis, disturb mitochondrial and metabolic functions, and ultimately reduce microbial growth and productivity in yeast systems [10]. Nevertheless, the same IL may produce significantly different effects depending on the microorganism, indicating that IL toxicity is determined by the interplay between ion-specific properties and the physiological resilience of the target organism [11, 12].

Among microorganisms of biotechnological relevance, yeasts occupy a central position due to their robustness, metabolic diversity, and long-standing industrial use. *Saccharomyces cerevisiae* remains the most established eukaryotic platform for fermentation and biocatalysis [13], whereas *Kluyveromyces marxianus* has attracted increasing attention for its rapid growth, thermotolerance, and ability to utilize diverse substrates [14]. Meanwhile, *Yarrowia lipolytica* is recognized as an unconventional yeast of major industrial interest, particularly for lipid metabolism, enzyme secretion, and tolerance to non-conventional media [15]. Therefore, these three yeasts represent valuable and complementary models for investigating how IL-derived ionic stress is translated into biological response. Importantly, their distinct physiological and metabolic characteristics also render them suitable for comparative studies aimed at identifying species-dependent patterns of tolerance [16].

Previous studies have shown that IL toxicity toward yeasts may vary substantially depending on both the chemical nature of the ions and the target microorganism. In comparative screenings, *Y. lipolytica* has frequently displayed broader tolerance windows than *S. cerevisiae* and *K. marxianus*, while certain anions, particularly bis(trifluoromethanesulfonyl)imide ($[NTf_2]^-$), have been associated with stronger toxic effects [16]. Likewise, imidazolium-based ILs have often been reported as biologically disruptive, especially in microorganisms with limited capacity to maintain membrane and metabolic homeostasis under solvent stress. Although these studies have provided important experimental evidence regarding ion- and species-dependent toxicity, the molecular mechanisms of this behavior remain only partially understood [16–19]. To our knowledge, there is still limited information on how the individual ionic components of ILs interact with metabolically relevant intracellular targets.

In this context, the analysis of central metabolic enzymes offers an attractive route for connecting whole-cell tolerance data with molecular-level behavior. Hexokinase is especially relevant in this regard as it catalyzes the first step committed to glycolysis, namely the ATP-dependent phosphorylation of glucose to glucose-6-phosphate, and therefore occupies a central position in carbon assimilation, energy generation, and glycolytic regulation. Any perturbation affecting its structural integrity or interaction environment may influence cellular metabolism in a biologically meaningful manner. Computational approaches such as molecular docking can provide useful insight into whether ion-dependent toxicity trends are accompanied by differences in the strength and spatial distribution of ion–protein interactions, particularly at catalytically and conformationally relevant regions of the enzyme.

Thus, the present study aimed to evaluate the toxicity profile of selected IL toward *S. cerevisiae*, *Y. lipolytica*, and *K. marxianus* through the determination of the maximum non-toxic concentration (MNTC), and to evaluate whether the differences observed experimentally among the three yeasts were consistent with distinct ion–hexokinase interaction patterns at the molecular level through molecular docking. Molecular docking is widely used to simulate binding affinity and the preferred interaction mode between receptor and ligand, thereby providing relevant insights into molecular interactions that may affect biocatalytic systems. By combining microbiological and computational analyses, this work aims to provide a more detailed understanding of ion-specific and species-dependent IL toxicity in yeasts of biotechnological relevance.

2 | Materials and Methods

2.1 | Materials

1-ethyl-3-methylimidazolium chloride ($[C_2mim]Cl$), 1-ethyl-3-methylimidazolium acetate ($[C_2mim][C_1CO_2]$), 1-ethyl-3-methylimidazolium ethanesulfonate ($[C_2mim][EtSO_3]$), 1-ethyl-3-methylimidazolium ethyl sulfate ($[C_2mim][EtSO_4]$), 1-ethyl-3-methylimidazolium bis(trifluoromethanesulfonyl)imide ($[C_2mim][NTf_2]$), 1-butyl-3-methylimidazolium bis(trifluoromethanesulfonyl)imide ($[C_4mim][NTf_2]$), 2-hydroxyethyltrimethylammonium bis(trifluoromethanesulfonyl)imide ($[N_{111(2OH)}][NTf_2]$), and tributylmethylphosphonium bis(trifluoromethanesulfonyl)imide ($[P_{4441}][NTf_2]$) was purchased from Iolitec with purity > 99%, while dimethyl sulfoxide (DMSO), RPMI 1640, MOPS buffer, and resazurin supplied by Sigma-Aldrich. All ILs studied are liquids at room temperature and were used as received.

2.2 | Strains and Growth Media

The yeast strains used in this study were *S. cerevisiae* ATCC 2601, *Y. lipolytica* IMUFRJ 50682, and *K. marxianus* IMUFRJ 508152. Stock cultures were maintained on Sabouraud agar and periodically subcultures under appropriate storage conditions until use. For the toxicity assays, fresh inoculum were prepared from actively growing colonies and transferred to liquid medium before the experiments.

2.3 | Determination of MNTC

The MNTC of the investigated ionic-liquid ions was determined by broth microdilution in 96-well microplates, following the methodology previously described by Santos et al. [16]. Serial twofold dilutions were prepared from stock solutions of the investigated compounds in RPMI-MOPS medium (pH 7.2), yielding a concentration range suitable for evaluation of yeast growth inhibition. Each well contained 100 µL of culture medium with the corresponding concentration of the tested ion solution, followed by inoculation with the yeast suspension.

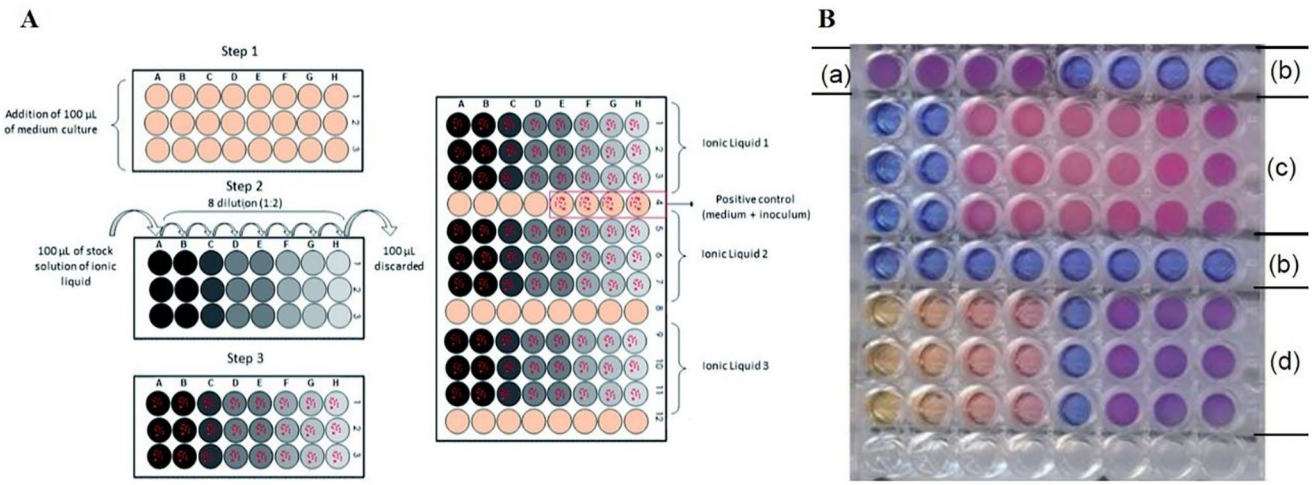

FIGURE 1 | Determination of the minimum non-toxic concentration (MNTC). (A) Schematic representation of the broth microdilution assay. (B) Representative experimental plate used for MNTC determination.

Microplates were incubated at room temperature for 48 h. Negative controls consisted of sterile, non-inoculated medium, whereas positive controls contained inoculated medium without the tested ions. After incubation, growth was initially assessed by visual inspection of turbidity and subsequently confirmed by adding resazurin solution under aseptic conditions. The plates were further incubated for 1 h. The MNTC was defined as the highest tested concentration at which yeast growth remained detectable under the assay conditions. An MNTC below 4.2 mM indicates that growth inhibition was already observed at the lowest concentration tested; therefore, the precise MNTC lies below the experimental range. Each assay comprised three replicates per concentration and was independently repeated on at least two occasions using separately prepared inocula. The MNTC assigned to each IL corresponded to the concentration consistently classified as non-toxic across the independent experiments (Figure 1). For the hydrophobic ILs, stock solutions were prepared in DMSO to ensure homogeneous solubilization and pipetting. Control assays containing the corresponding DMSO concentration were also performed to confirm that the solvent itself did not interfere with yeast growth under the experimental conditions employed.

2.4 | Molecular Docking

The three-dimensional structures of the hexokinases from *Y. lipolytica* (UniProt: F2Z672), *K. marxianus* (UniProt: V0FZV4), and *S. cerevisiae* (PDB: 3b8a) were used as receptor models for the molecular docking analyses. Protein structures were prepared for computational analysis by assigning the protonation states of titratable residues using the Protein Prepare tool available on the PlayMolecule web server (playmolecule.org). This preparation was carried out under physiological pH conditions, and all crystallographic water molecules, ions, and co-crystallized ligands were removed from the original protein files prior to docking calculations. The chemical structures of the investigated ions were built and optimized using Discovery Studio v21 (Accelrys, San Diego, CA, USA).

Three-dimensional coordinates were generated and subsequently energy-minimized using the Chem3D-MM2 protocol. The resulting ligand structures were then converted into .pdbqt format using AutoDockTools (ADT), with all rotatable bonds defined as active torsions whenever applicable. The prepared hexokinase structures were also converted into .pdbqt format using the same software. Docking simulations were performed using AutoDock Vina 1.1.2. For each receptor, the grid box was centered so as to cover the catalytically relevant region and surrounding areas of the enzyme, allowing the identification of interactions at both active and allosteric regions. The docking calculations were carried out using an exhaustiveness value of 100, num_modes = 10, and seed = 100. For each ligand, the binding pose with the lowest docking score was selected for further analysis.

3 | Results and Discussion

3.1 | Toxicity Profile of IL Ions in Different Yeasts

The toxicity profile of the investigated IL-derived ions, measured as MNTC, revealed a notable species-dependent response in the three yeast strains presented in Figure 2, indicating that the biological impact of these ions is strongly modulated by the intrinsic physiological characteristics of each microorganism. *Y. lipolytica* exhibited substantially higher tolerance to most of the ions evaluated, particularly anions, whereas *K. marxianus* and *S. cerevisiae* were considerably more sensitive, with MNTC values generally restricted to the low millimolar range. This was especially evident for Cl^-, $[C_1CO_2]^-$, $[EtSO_3]^-$, and $[EtSO_4]^-$, whose MNTC values in *Y. lipolytica* ranged between 145 and 270 mM, in contrast to the corresponding values of 3.8–8.0 and 4.2–6.7 mM in *K. marxianus* and *S. cerevisiae*, respectively. The wide magnitude of the difference strongly suggests that *Y. lipolytica* is less susceptible to the disruptive effects of various IL-derived ions at the membrane, metabolic, or protein-interaction levels.

The species-dependent toxicity trend observed in the present study is in close agreement with the comparative screening

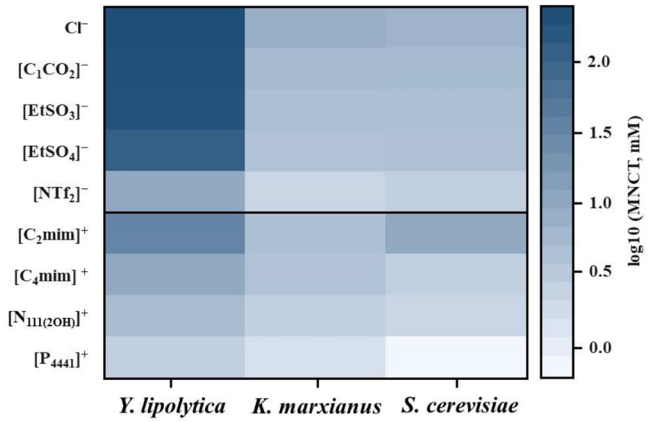

FIGURE 2 | Heatmap of maximum non-toxic concentration (MNTC) values for the ions investigated in Yarrowia lipolytica, Kluyveromyces marxianus, and Saccharomyces cerevisiae. Color intensity represents log10-transformed MNTC values. Anions were evaluated as ILs containing the common cation $[C_2mim]^+$, whereas cations are based on the common anion $[NTf_2]^-$.

reported by Santos et al. [16], who evaluated the MNTC of nine different ILs toward microorganisms of interest to the food industry, including *S. cerevisiae* ATCC 2601, *Y. lipolytica* IMUFRJ 506822, and *K. marxianus* IMUFRJ 508152. In this work, *Y. lipolytica* was the only yeast tolerant to all tested ILs, whereas *S. cerevisiae* and *K. marxianus* exhibited notably lower tolerance, particularly toward imidazole-based systems. For hydrophilic imidazolium ions, the authors reported that the MNTC values for *Y. lipolytica* ranged from 235 to 270 mM, while *S. cerevisiae* and *K. marxianus* did not grow within the concentration range used (< 4.2–< 6.7 mM). This trend demonstrates that the toxicity of ILs depends highly on the physiological context of the target microorganism. Walker et al. [17] demonstrated that *Y. lipolytica* (YlSR001) can grow in a medium containing up to 10% (v/v) 1-ethyl-3-methylimidazolium acetate ($[C_2mim][C_1CO_2]$, and that its tolerance was due to a restructuring of its membrane and sterol metabolism. Dickinson et al. [18] showed that imidazolium ILs disrupt key cellular functions in *S. cerevisiae*, with evidence pointing to mitochondrial impairment, altered membrane potential, and ion-homeostasis-related effects. More recently, Sokolov et al. [19] proposed that the primary toxic event of imidazolium ILs occurs at the plasma membrane, whose barrier properties are compromised upon exposure.

An evident ion-dependent trend was also observed within each yeast. For the anionic series, toxicity increased progressively from Cl^- to $[NTf_2]^-$ in all three microorganisms, as reflected by decreasing MNTC values along the sequence. This agreement among the three yeasts may be due to the fact that, despite clear species-specific differences in absolute tolerance, the relative toxic classification of the anions remains consistent. Thus, it can be concluded that the ion's systematic effect is more dominant than the intrinsic resistance of each microorganism. Within the anions, $[NTf_2]^-$ was consistently the most deleterious species, exhibiting the lowest MNTC values in all cases, which indicates that its toxic effect is associated with specific structural and intermolecular characteristics. This behavior is likely due to its bulky structure, highly delocalized charge, and high hydrophobicity, which reduce its hydration in aqueous media and promote stronger interactions with less polar regions of biological systems. As a result, $[NTf_2]^-$ can induce more severe cellular disruptions, even at lower concentrations, than smaller, more strongly hydrated anions.

A similarly strong convergence with the literature was observed for $[NTf_2]^-$. Santos et al. [16] identified this anion as particularly toxic to the microorganisms studied in their work, with hydrophobic $[NTf_2]$-based ILs exhibiting very low MNTC values for all yeasts. The present results are consistent with this trend, since $[NTf_2]^-$ also produced the lowest tolerance values among the investigated anions in all three yeasts. Missoun et al. [20] investigated the toxicity and biocompatibility of 63 ILs toward *S. cerevisiae* using the agar well diffusion test as a rapid screening method. The study included a broad structural diversity of cations, including imidazolium, pyridinium, pyrrolidinium, piperidinium, morpholinium, oxazolinium, phosphonium, ammonium, and sulfonium, combined with various anions. Based on the radius of the inhibition zone, the authors classified the ILs as very toxic, toxic, low toxic, or biocompatible, and concluded that toxicity was mainly determined by the cation nature, although some anions, particularly $[NTf_2]^-$, also exerted a strong influence on IL biocompatibility. In addition, they reported that increasing the alkyl chain length of cations such as imidazolium or pyridinium increased IL toxicity toward *S. cerevisiae*. This observation is consistent with pioneering studies showing that increasing the alkyl chain length of imidazolium-based ILs is associated with enhanced biological toxicity and membrane-disruptive effects [21].

The cation series also revealed significant differences, although the trend was slightly more species-dependent than that observed for the anions. In *Y. lipolytica*, MNTC values decreased from 37 mM for $[C_2mim]^+$ to 2.5 mM for $[P_{4441}]^+$, showing a substantial increase in toxicity across the cationic series. In *S. cerevisiae*, the same general trend of higher toxicity for the more deleterious cations was also evident, with $[P_{4441}]^+$ displaying the lowest MNTC value of the entire cation series (0.6 mM), followed by $[N_{111(2OH)}]^+$ (1.9 mM) and $[C_4mim]^+$ (2.5 mM). *K. marxianus* yeast exhibited a narrower MNTC range between cations, with values of 1.3–4.5 mM for $[P_{4441}]^+$ and $[C_2mim]^+$. Thus, the cation also plays an important role in determining toxicity, although the magnitude of its effect appears to depend more strongly on the microorganism than on the anionic IL-derived ions.

It is important to note that the relative contribution of anions and cations to toxicity is not equivalent between the three yeasts. In *Y. lipolytica*, the less-toxic anions were tolerated at concentrations considerably higher than those observed for most cations, which may lead to cationic effects to be a dominant limiting factor once anionic toxicity is sufficiently reduced. Meanwhile, in *K. marxianus* and *S. cerevisiae*, both classes of ions performed similarly within low concentration ranges, which could result in these yeasts having a higher susceptibility to ionic stress, regardless of the corresponding ionic charge.

Lai et al. [22] investigated the effects of ILs on enzyme activity and stability using two model enzymes, *Penicillium expansum* lipase and mushroom tyrosinase, in aqueous solution supplemented with 14 different ILs. The authors showed that ILs can markedly alter enzyme performance and that these

effects are strongly dependent on ion identity, particularly through influences on active-site conformation, surface pH, catalytic mechanism, and protein structural organization. In addition, they concluded that IL cations may play a predominant role in affecting enzyme behavior, especially in the lipase system. Mehmood et al. [14] investigated the response of *K. marxianus* to two imidazolium ILs, 1-ethyl-3-methylimidazolium acetate ($[C_2mim][C_1CO_2]$), and 1-ethyl-3-methylimidazolium methylphosphonate ($[C_2mim][MeO(H)PO_2]$), for second-generation ethanol production. It was reported that *K. marxianus* tolerated up to 2% $[C_2mim][C_1CO_2]$ and 6% $[C_2mim][MeO(H)PO_2]$, demonstrating a clear anion-dependent effect on toxicity. At subinhibitory concentrations, some IL levels even increased ethanol production, which the authors associated with a metabolic shift from respiratory to fermentative metabolism.

3.2 | Molecular Docking Analysis

Hexokinase was selected as a representative intracellular target as it catalyzes the first committed step of glucose utilization, thereby controlling the entry of glucose-derived carbon into glycolysis and its subsequent distribution toward central metabolic pathways. In addition to its catalytic function, Hxk2 from *S. cerevisiae* participates in glucose sensing and the transcriptional regulation of glucose-repressible genes. Previous biochemical studies have also demonstrated that yeast hexokinase can be inhibited or undergo conformational changes in the presence of exogenous compounds, supporting its relevance as a model enzyme for investigating possible effects on central carbon metabolism. However, MNTC is a whole-cell endpoint that results from multiple processes, including membrane perturbation, cellular uptake, efflux, intracellular accumulation, ionic homeostasis, and interactions with multiple molecular targets. Therefore, this docking analysis does not establish hexoquinase as the primary determinant of IL toxicity, although its interactions are predicted to be biologically relevant and require direct biochemical validation [23–25].

3.2.1 | Relationship Between Binding Affinity and MNTC

The MNTC patterns showed ion- and strain-dependent trends, confirming the hypothesis that specific interactions between the components of ion-ILs and the yeasts may contribute to the observed biological effects. In this sense, computational molecular docking analyses provide important insights into whether the differences in tolerance observed among the three yeast strains are associated with distinct interaction profiles between the IL-derived ions and the target protein at the molecular level. Interactions between ligands and amino acids can involve hydrogen bonds, hydrophobic interactions, electrostatic forces, and van der Waals interactions. The absolute affinity values (kcal mol^{-1}), the conformations of each ligand for the yeast hexokinases studied, the best binding pose, and the molecular modelling affinities, as well as the type of interaction and the geometric distance (Å), are presented in Tables S1–S3 in the Supporting Information. The docking affinity values, the corresponding MNTC data, and the numbers of predicted interactions with the active and allosteric regions of the hexokinases from *Y. lipolytica*, *K. marxianus*, and *S. cerevisiae* are summarized in Tables 1–3. As the Vina scoring function does not explicitly account for the electrostatic and desolvation contributions that dominate the behavior of monatomic ions, the score calculated for Cl^- was not considered sufficiently reliable for quantitative interpretation. Consequently, Cl^- was excluded from the docking affinity analysis, especially since no specific interactions with the investigated hexokinases were predicted.

The comparative analysis revealed a strong qualitative relationship between binding affinity and MNTC, showing that ions with stronger interactions with the target enzyme tend to be associated with lower non-toxic concentration limits. This trend was especially evident for the anionic series. Among the three yeasts, $[NTf_2]^-$ consistently exhibited the most negative docking affinities among the anions, followed by the lowest MNTC values in all strains. Intermediate anions, such as $[C_1CO_2]^-$, $[EtSO_3]^-$, and $[EtSO_4]^-$, followed the same trend, with increasingly negative affinity values being paralleled by decreasing tolerance. These results indicate that ions with more favorable interactions with hexokinase were generally tolerated only at lower concentrations [16, 20, 26, 27].

A similar, although somewhat less regular, tendency was observed for the cationic series. In *Y. lipolytica*, the value decrease in MNTC from $[C_2mim]^+$ to $[P_{4441}]^+$ was followed by increasingly negative affinity values, supporting the existence of a coherent relationship between stronger predicted binding and higher toxicity [28]. In *K. marxianus* and *S. cerevisiae*, the same general trend was observed, although variations in affinity among some cations were not consistently reflected in the corresponding MNTC values. Even so, the most deleterious cations, particularly $[P_{4441}]^+$ and $[N_{111(2OH)}]^+$, were also among those exhibiting the most favorable docking energies, whereas less toxic cations such as $[C_2mim]^+$ generally showed weaker interactions [29].

Although a qualitative relationship between docking affinity and MNTC was observed for the overall ion series, this trend was not strictly linear for all cases. In particular, some intermediate anions exhibited relatively similar docking affinities despite showing markedly different MNTC values, indicating that toxicity cannot be explained solely on the basis of predicted binding affinity. This limitation is consistent with previous studies showing that membrane binding or permeabilization-related parameters alone are not always sufficient to account for the magnitude of the biological response [10]. In microbial systems, toxicity is inherently multifactorial and may also depend on hydration effects, membrane partitioning, ion transport, intracellular accumulation, interactions with multiple molecular targets, and broader metabolic disturbance. In addition, molecular docking relies on simplified scoring functions and static receptor conformations, which may not fully capture the dynamic and multicomponent nature of ion-induced toxicity.

The apparent ordering between docking scores and MNTC should be interpreted cautiously because both parameters may be influenced by shared physicochemical properties of the investigated ions. Larger, more hydrophobic, weakly hydrated, and charge-delocalized ions may receive more favorable empirical docking scores while also displaying greater membrane partitioning and

TABLE 1 | Docking affinity values, maximum non-toxic concentration (MNTC), and number of predicted interactions between the investigated ions and the active and allosteric sites of the Hexokinase from *Yarrowia lipolytica*.

Ions	Affinity (kcal mol^{-1})	MNTC (mM)	Active site	Allosteric site
$[C_2mim]^+$				
Cl^-	n.a.	270	0	0
$[C_1CO_2]^-$	−3.1	250	1	2
$[EtSO_3]^-$	−3.6	235	2	3
$[EtSO_4]^-$	−3.9	145	4	8
$[NTf_2]^-$	−6.1	37	5	14
$[NTf_2]^-$				
$[C_2mim]^+$	−3.5	37	0	3
$[C_4mim]^+$	−3.8	10	0	4
$[N_{111(2OH)}]^+$	−4.3	5	4	9
$[P_{4441}]^+$	−4.6	2.5	2	12

TABLE 2 | Docking affinity values, maximum non-toxic concentration (MNTC), and number of predicted interactions between the investigated ions and the active and allosteric sites of the Hexokinase from *K. marxianus*.

Ions	Affinity (kcal mol^{-1})	MNTC (mM)	Active site	Allosteric site
$[C_2mim]^+$				
Cl^-	n.a.	8.0	0	1
$[C_1CO_2]^-$	−3.4	6.2	1	1
$[EtSO_3]^-$	−3.6	5.5	3	1
$[EtSO_4]^-$	−3.8	4.8	4	1
$[NTf_2]^-$	−5.3	4.5	5	1
$[NTf_2]^-$				
$[C_2mim]^+$	−3.5	4.5	0	7
$[C_4mim]^+$	−3.6	3.8	2	1
$[N_{111(2OH)}]^+$	−3.8	2.5	4	6
$[P_{4441}]^+$	−4.1	1.3	5	13

TABLE 3 | Docking affinity values, maximum non-toxic concentration (MNTC), and number of predicted interactions between the investigated ions and the active and allosteric sites of the hexokinase from *S. cerevisiae*.

Ions	Affinity (kcal mol^{-1})	MNTC (mM)	Active site	Allosteric site
$[C_2mim]^+$				
Cl^-	n.a.	6.7	0	0
$[C_1CO_2]^-$	−2.9	5.2	1	0
$[EtSO_3]^-$	−3.1	4.5	2	8
$[EtSO_4]^-$	−3.4	4.2	3	9
$[NTf_2]^-$	−5.2	3.7	5	12
$[NTf_2]^-$				
$[C_2mim]^+$	−3.0	3.7	0	0
$[C_4mim]^+$	−3.5	2.5	0	9
$[N_{111(2OH)}]^+$	−3.6	1.9	4	8
$[P_{4441}]^+$	−3.7	0.6	2	4

whole-cell toxicity. Therefore, the observed trend cannot be attributed exclusively to specific recognition by hexokinase and is considered here only as a covariation requiring biochemical validation [9, 30, 31]. Toxicity in yeast is necessarily multifactorial and can also involve membrane perturbation, ion transport, osmotic imbalance, and broader metabolic effects. Therefore, the present docking results do not imply that hexokinase is the sole determinant of the observed toxicity profiles. Instead, they support the interpretation that interaction with this key glycolytic enzyme may represent one relevant molecular component within a more complex cellular response [9, 22]. In this context, the fact that ions to bind more favorably to hexokinase were, in general, tolerated only at lower concentrations reinforces the hypothesis that enzyme-level interference may contribute to the reduced physiological compatibility of the most deleterious ions [15].

Another important aspect is the affinity–MNTC relationship, which was preserved despite the significant differences in absolute tolerance among the three yeast strains. Thus, although *Y. lipolytica* consistently exhibited substantially higher MNTC values than *K. marxianus* and *S. cerevisiae*, the order of the ions based on docking affinity remained broadly consistent with the experimentally observed tolerance profile within each species. This could be due to the distinct toxicities observed for the three microorganisms, which likely result from completely different mechanisms of interaction related to each yeast's ability to resist or compensate for similar molecular disturbances induced by ions [32].

A more detailed inspection of Tables S1–S3 further suggests that lower MNTC values were generally associated with broader interaction networks involving catalytically and allosterically relevant polar or ionizable residues, whereas higher MNTC values tended to occur for ions exhibiting more limited or predominantly hydrophobic contacts. This observation reinforces that the inhibitory behavior is more likely governed by the combined strength, extent, and spatial distribution of the interaction network than by association with any single amino acid residue.

3.2.2 | Anion-Specific Interaction Patterns

Beyond the general relationship between docking affinity and MNTC, the anionic set revealed a particularly ordered interaction profile at the level of hexokinase. This profile was determined by the magnitude of the binding affinities and by the distribution of anions between the enzyme's active and allosteric sites. In contrast to the cation series, which exhibited behavior that was more species-dependent, the anions followed a more conserved interaction pattern across the three yeast species, due to their molecular association with hexokinase being governed by a more consistent structural response to anion identity [33, 34]. Docking poses of the IL-anions in the hexokinases of the three yeast species are shown in Figure 3. The anion, Cl^-, was not included since no relevant interactions were predicted under the adopted docking conditions.

A notable feature of the anionic series is that the increase in binding affinity was followed by enhanced interactions with the amino acids that compose the enzyme's catalytic and allosteric sites. However, the amount of these interactions varied among the three hexokinases. In *Y. lipolytica*, the anions displayed increased interactions in both the catalytic and allosteric sites due to their broad ability to bind to different amino acids in the enzyme. In this context, the less hydrophobic anions, such as Cl^- (log$P$ = −4.51), $[C_1CO_2]^-$ (log$P$ = −1.38), $[EtSO_3]^-$ (log$P$ = −1.36), and $[EtSO_4]^-$ (log$P$ = −1.32), exhibited weaker interaction profiles than $[NTf_2]^-$ (log$p$ = 1.49), which showed the most favorable docking affinity and the lowest tolerance values. This behavior suggests that the higher hydrophobicity of $[NTf_2]^-$ may enhance its association with less polar regions of the enzyme, favoring stronger binding and potentially more pronounced conformational perturbations, which may ultimately contribute to the lower tolerance observed experimentally [16].

A distinct profile was observed for the hexokinase of *K. marxianus*. In this case, the interaction with the anion was mostly focused on the active site, while interactions with the allosteric sites remained minimal throughout the study. Even the anion with the strongest interaction capacity, $[NTf_2]^-$, exhibited only a single allosteric interaction, in contrast to the higher allosteric involvement of this ion with the other yeasts. Thus, the anionic component can exert its molecular effect in a more localized way, with interactions near the catalytically relevant regions of the enzyme [28]. A trend of binding to anions was observed for the intermediate anions $[C_1CO_2]^-$, $[EtSO_3]^-$, and $[EtSO_4]^-$, whose docking profiles occupied a well-defined region between the weakly interacting chloride and the extensively associated $[NTf_2]^-$. Although differences in anion basicity may contribute to the interaction behavior of the intermediate species, particularly by modulating hydrogen-bonding ability, the order of the interactions with the catalytic and allosteric sites found here is more likely governed by a broader set of structural factors, including charge delocalization, hydration, and conformational adaptability [35, 36]. Zhao et al. [9] highlighted that weakly hydrated, more chaotropic anions may disrupt enzyme function by mechanisms often involving destabilization of protein structure and activity.

3.2.3 | Cation-Specific Interaction Patterns

Regarding the cations studied, a more heterogeneous interaction profile was observed, in which cation–enzyme binding is more strongly influenced by specific structural features of the protein [37]. Although the cations generally followed the same trend as the anions, an increase in binding energy led to lower MNTC values; an increase in the relative interactions between the enzyme's catalytic and allosteric regions was observed. In *Y. lipolytica*, the cations displayed a gradual increase in both affinity and interaction number, with $[C_2mim]^+$ and $[C_4mim]^+$ showing fewer interactions with the amino acid located at the catalytic and allosteric sites, whereas $[N_{111(2OH)}]^+$ and $[P_{4441}]^+$ performed several interactions in both regions. This increase in binding may be due to a higher affinity for different regions of the enzyme [3]. The docking poses corresponding to the cations-IL are described in Figure 4.

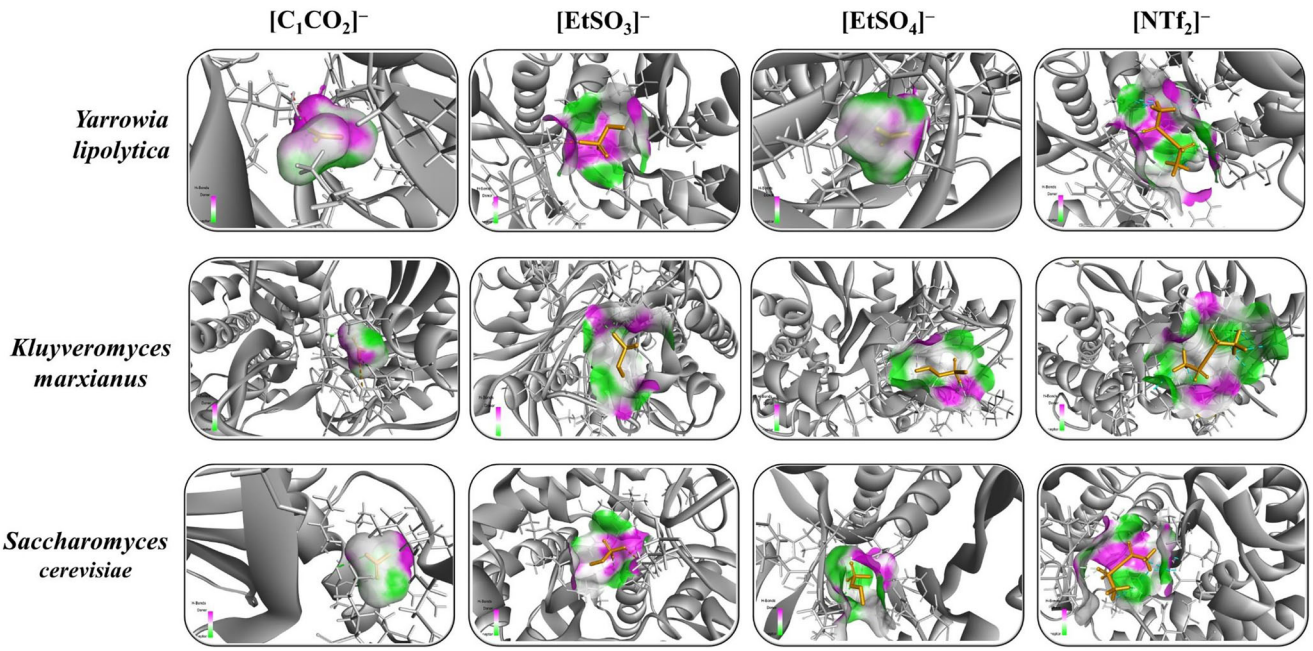

FIGURE 3 | Docking poses of the studied anions in the hexokinases of *Yarrowia lipolytica*, *Kluyveromyces marxianus*, and *Saccharomyces cerevisiae*. Lines correspond to the yeast species, and columns correspond to the anions $[C_1CO_2]^-$, $[EtSO_3]^-$, $[EtSO_4]^-$, and $[NTf_2]^-$. Protein structures are shown as grey, whereas ligands are displayed as orange. Highlighted surface regions indicate the predicted interaction environment of each anion.

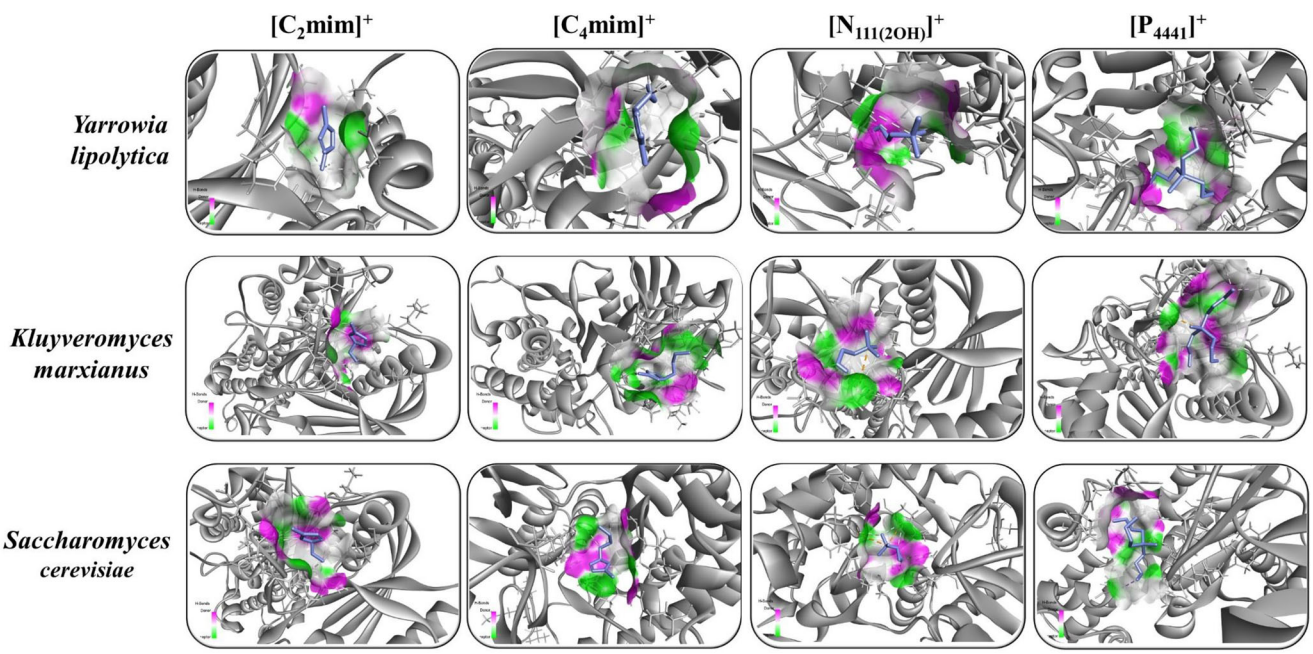

FIGURE 4 | Docking poses of the studied cations in the hexokinases of *Yarrowia lipolytica*, *Kluyveromyces marxianus*, and *Saccharomyces cerevisiae*. Lines correspond to the yeast species, and columns correspond to the cations $[C_2mim]^+$, $[C_4mim]^+$, $[N_{111(2OH)}]^+$, and $[P_{4441}]^+$. Protein structures are shown as grey, whereas ligands are displayed as blue. Highlighted surface regions indicate the predicted interaction environment of each cation.

The hexokinase from *K. marxianus* showed the greatest number of interactions with the enzyme's active regions among the yeasts studied in this cationic series. Although $[C_2mim]^+$ did not interact with amino acid residues at the catalytic site, it established seven contacts in the allosteric region, and $[P_{4441}]^+$ combined extensive active-site interaction with the highest allosteric contact number among the three yeasts. This behavior is due to cation binding, which is not restricted to the catalytic environment and may be strongly influenced by peripheral or conformationally responsive regions of the enzyme [38]. A different trend for *S. cerevisiae* was observed, in which the cationic series was marked by a strong contribution of the allosteric region for $[C_4mim]^+$ and

$[N_{111(2OH)}]^+$, whereas $[P_{4441}]^+$, despite being the most deleterious cation according to the experimental data, displayed a more limited number of bindings with amino acid at the catalytic and allosteric sites than in the other two yeast. This result shows that the toxicity of the cation may depend on the absolute number of interactions formed with the enzyme, as well as on the specific nature and structural location of these interactions [18, 19].

The imidazolium-based cations, $[C_2mim]^+$ and $[C_4mim]^+$, occupied an intermediate position in this series. In particular, $[C_4mim]^+$ consistently displayed broader interaction patterns than $[C_2mim]^+$, which is in agreement with its lower MNTC values. This behavior shows that structural changes, such as increased carbon chain length, are sufficient to alter the interaction pattern with hexokinase [3, 39].

Buarque et al. [15] investigated the inhibitory effects of DESs on *Y. lipolytica* by combining experimental microbial growth with molecular docking against the yeast hexokinase (YlHxk). The authors evaluated 18 DES and identified the catalytic and allosteric regions of YlHxk, as well as the most relevant transport tunnels for substrates, products, and solvent components. According to their results, molecular docking revealed that ligands transported through the same tunnel as the natural substrate exhibited similar binding patterns, particularly involving the ASN273 residue in the active site, suggesting that computational analysis can provide structural insight into microorganism inhibition at the enzyme level. Buarque et al. [40] studied the effects of DESs on the activity of the lipase YlLip2 from *Y. lipolytica*. The docking results showed that none of the HBA/HBD combinations directly interacted with the catalytic triad residues, whereas the most relevant interactions occurred at the allosteric sites. DES components with higher affinity energies also exhibited a higher number of allosteric interactions, indicating that modulation of enzyme activity may occur through interactions outside the catalytic center. Barbosa et al. [41] evaluated the impacts of several phosphonium-based ILs on the activity of *Burkholderia cepacia* lipase (BCL) by combining experimental assays with molecular docking. The authors investigated ILs composed of different phosphonium cations, namely $[P_{4444}]^+$, $[P_{444(14)}]^+$, and $[P_{666(14)}]^+$, combined with the anions Cl^-, Br^-, $[Deca]^-$, $[Phosp]^-$, and $[NTf_2]^-$, in order to assess the individual roles of the ionic species on enzyme performance. According to the experimental data, increasing the alkyl chain length of the cation decreased enzymatic activity, whereas the anions $[Phosp]^-$ and $[NTf_2]^-$ promoted higher activity. The molecular docking analysis further showed that the cations preferentially interacted with Leu17, a residue located in the oxyanion hole, while the anion [Deca]– mainly interacted by hydrogen bonding with Ser87, a residue of the catalytic triad. In contrast, [Phosp]– and $[NTf_2]^-$ exhibited high binding energies and preferentially interacted with side-chain amino acids outside the catalytic center, which the authors associated with enhanced lipase activity.

3.2.4 | Species-Dependent Differences in Hexokinase–Ion Interactions

A comparative analysis of molecular docking results revealed that the molecular contribution of the ions under study should be evaluated in relation to binding affinity and the spatial distribution of interactions between the active and allosteric sites. This insight is important because interactions at the catalytic site directly affect substrate recognition and metabolism, whereas interactions in allosteric regions can alter the enzyme's conformation and, by extension, indirectly modulate its catalytic performance. In this context, ion-dependent effects on hexokinase may derive from a combination of direct binding to the catalytic site and structurally mediated allosteric disruption. These observations are consistent with previous studies showing that the ionic medium's effects on enzymatic performance may depend heavily on amino acid residues directly involved in catalysis or on more distant regions involved in structural regulation [42].

Although a qualitative relationship was observed between docking affinity and MNTC across the three yeasts, the interaction trends were not identical among the corresponding hexokinases. These differences are due to the absolute tolerance of each microorganism, which is influenced by the general strength of ion–enzyme association, as well as by species-dependent structural features that govern how each ion is accommodated at the protein surface. In this sense, the three hexokinases responded to the studied ions according to a shared overall trend, whose distinct spatial interaction profiles suggest that the same ionic species may perturb homologous enzymes through partially different molecular routes [43, 44].

Among the three microorganisms, *Y. lipolytica* consistently exhibited the highest MNTC values, indicating higher tolerance to both anions and cations. However, this higher tolerance did not correspond to an absence of interaction with hexokinase. On the contrary, several ions displayed extensive binding to amino acids forming the catalytic and allosteric sites, suggesting that the biological impact of ion binding may be more effectively buffered at the cellular level, even when the target enzyme shows substantial interaction with the ionic species [17]. Therefore, the higher tolerance of this yeast is more likely associated with an enhanced capacity to withstand or compensate for ion-induced molecular perturbations than with a fundamentally reduced susceptibility of its hexokinase to interaction [45]. On the other hand, *K. marxianus* and *S. cerevisiae* showed markedly narrower MNTC ranges, indicating greater physiological sensitivity to the investigated ions. Nevertheless, the two yeasts did not respond identically to the docking results. In *K. marxianus*, anion interactions were predominantly concentrated at the active site, whereas the cationic series displayed the strongest allosteric involvement among the three enzymes [14]. This combination suggests that the molecular response of *K. marxianus* hexokinase is highly dependent on ionic class, with anions acting more directly at the catalytic site and cations showing a stronger tendency to interact with peripheral or conformationally responsive regions. In *S. cerevisiae*, both anions and cations frequently showed extensive allosteric contributions, indicating that conformationally mediated effects may be particularly relevant in this enzyme [45].

These differences in interaction patterns are likely due to structural differences among the hexokinases of the different yeast species. Variations in accessibility, polarity, and conformational flexibility of regions around the active and allosteric sites can alter the way ions are accommodated, leading to distinct interaction patterns. This finding is consistent with the observation that some

ions maintained a similar order of affinity across species, even though these ions differed substantially in the number of bonds they formed with the enzyme's active sites. Thus, the species-dependent variation in tolerance can be attributed to differences between the cations and anions formed by the ILs studied and the structural context of the target enzyme in each microorganism [46, 47]. Imam et al. [3], who reviewed ionic-liquid applications in whole-cell and isolated enzyme biocatalysis and reported that the biological response to ILs varies substantially across microorganisms. According to the studies in that review, microbial resistance is not uniform, and *S. cerevisiae* is often a relatively sensitive organism in whole-cell biocatalytic systems, whereas other microorganisms may tolerate a wider range of ionic environments. Furthermore, it was also emphasized that ion structure, alkyl chain length, and the balance between solvent properties and toxic effects on metabolism are central to determining biocompatibility.

4 | Conclusion

This study demonstrated that the toxicity of ILs in yeasts is strongly influenced by both the chemical identity of their constituent ions and the physiological characteristics of the target microorganism. Among the three yeasts investigated, *Y. lipolytica* displayed the highest tolerance overall, with MNTC values ranging from 145 to 270 mM for the less toxic anions (Cl^-, $[C_1CO_2]^-$, $[EtSO_3]^-$, and $[EtSO_4]^-$), whereas the corresponding values in *K. marxianus* and *S. cerevisiae* were much lower, ranging from 4.8 to 8.0 mM and 4.2 to 6.7 mM, respectively. For the anionic series, toxicity increased progressively from Cl^- to $[NTf_2]^-$ in the three yeasts, with $[NTf_2]^-$ showing the lowest MNTC values, namely 37 mM in *Y. lipolytica*, 4.5 mM in *K. marxianus*, and 3.7 mM in *S. cerevisiae*. The cationic series also showed clear differences in toxicity, with MNTC values decreasing from 37 to 2.5 mM in *Y. lipolytica*, from 4.5 to 1.3 mM in *K. marxianus*, and from 3.7 to 0.6 mM in *S. cerevisiae*, confirming that cations contribute substantially to the observed toxic response.

The molecular docking results were consistent with the experimental toxicity profiles and revealed a clear qualitative relationship between binding affinity and MNTC, particularly for the anionic series. $[NTf_2]^-$ showed the most favorable affinities, with values of −6.1, −5.3, and −5.2 kcal mol^{-1}, respectively. For the cationic series, the most favorable docking affinities were observed for $[P_{4441}]^+$, reaching −4.6 kcal mol^{-1} in *Y. lipolytica*, −4.1 kcal mol^{-1} in *K. marxianus*, and −3.7 kcal mol^{-1} in *S. cerevisiae*, in agreement with its lower tolerance values. In addition to binding strength, the spatial distribution of interactions also differed among the three enzymes, revealing distinct contributions of active and allosteric regions to ligand association. Thus, these results indicate that IL toxicity in yeasts can be explained by physicochemical properties and by molecular interaction patterns with metabolically relevant targets, such as hexokinase. By integrating microbiological and computational approaches, this work advances the understanding of IL toxicity in yeasts and provides a useful framework for the rational selection of more biocompatible ionic-liquid systems for biotechnological applications.

Author Contributions

Filipe S. Buarque: conceptualization, methodology, investigation, formal analysis, writing – original draft, writing – review and editing. **Ariane G. Santos**: methodology, investigation, data curation. **Bernardo D. Ribeiro**: conceptualization, supervision, writing – review and editing. **Matheus M. Pereira**: molecular docking analysis, formal analysis, writing – review and editing. **Mara G. Freire**: conceptualization, supervision, writing – review and editing. **Maria Alice Z. Coelho**: conceptualization, supervision, project administration, funding acquisition, writing – review and editing. All authors have read and approved the final version of the manuscript.

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Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The data supporting the findings of this study are available within the article and its supporting Information. Additional data are available from the corresponding author upon reasonable request.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section.

Supporting Material: bab70204-sup-0001-SuppMat.docx