

Article

Discovering Low Toxicity Ionic Liquids for *Saccharomyces cerevisiae* by Using the Agar Well Diffusion Test

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Abstract: Ionic liquids (ILs) are new solvents widely used in many technologies due to their unique and advantageous physicochemical properties. In biotechnological applications, ILs can be used along with microorganisms such as *Saccharomyces cerevisiae*. Due to the enormous number of ILs that can be synthesized through the combination of different anions and cations, it is necessary to have an easy and quick tool for the preliminary screening of their biocompatibility for being used in biotechnological applications. In this work, the agar well diffusion test was successfully applied as a rapid method to identify toxic/nontoxic ILs toward *S. cerevisiae*. Sixty-three ILs containing a diverse set of cations and anions were used. Through this methodology, nine fully biocompatible ILs toward *S. cerevisiae* were identified, including: [Bmim⁺] [NO₃[−]], [HOPmim⁺] [NO₃[−]], [Bmim⁺] [NTf₂[−]], [N_{8,8,1}⁺] [NTf₂[−]], [S_{2,2,2}⁺] [NTf₂[−]], [EMPyr⁺] [NTf₂[−]], [BMPi⁺] [NTf₂[−]], [Moxa⁺] [MeSO₄[−]] and [Chol⁺] [H₂PO₄[−]]. The analysis of the results also provides preliminary rules to enable the design of biocompatible ILs with *S. cerevisiae*. In this context, the toxicity was mainly determined by the cation nature although some anions can also display a strong influence on the IL biocompatibility as the bistriflimide anion. Besides, it was observed that an increase in the alkyl chain length of cations, such as imidazolium or pyridinium, involves an increase in the IL toxicity.

Keywords: ionic liquids; toxicity; *Saccharomyces cerevisiae*; agar well diffusion test; biocompatibility

1. Introduction

In recent years, there has been an increasing biotechnological interest in *Saccharomyces cerevisiae* [1], which has been used to produce specific fuels, chemicals, and pharmaceuticals, such as fatty acids and derivatives [2,3], terpenoids [4], ethanol [5], butanol and phenylethanol isomers [6] and pharmaceutical proteins [7].

At the same time, ionic liquids (ILs) are low-melting-point salts that have become increasingly attractive as green solvents for industrial applications. From an environmental point of view, their most important property is their practically zero vapor pressure. Furthermore, IL properties can be tailored for a specific application by accurately selecting the cation and the anion. Taking into account all these features, ILs are considered good candidates to be tested as extracting agents or solvents in

biotechnological applications [8,9]. ILs have also been employed along with *S. cerevisiae* in several processes such as the improvement of the enzymatic hydrolysis of cellulose before fermentation with *S. cerevisiae* [10] and in biphasic systems for the synthesis of enantiomeric compounds such as 2-phenylethanol [11] or ethyl 2-hydroxy-4-phenylbutyrate catalyzed by this microorganism [12]. One of the most important limitations of ILs is their potential toxicity to certain microorganisms, which could challenge their application in these types of industrial processes [13,14]. For this reason, the number of research works in literature focusing on the study of the biocompatibility of ILs with different microorganisms has significantly increased in recent years [15–22]. Furthermore, due to the enormous number of ILs that can be synthesized by the combination of different anions and cations, it is necessary to develop easy and quick tools for the preliminary selection of biocompatible ILs. The agar well diffusion test, used to assess the toxicity in solid media, is simple, inexpensive, requires little preparation, no specialized equipment and uses small quantities of sample. The antimicrobial activity is assessed through the diameter of the inhibition zones obtained by the addition of the ILs (in a pure state or high concentration solutions) in wells punched in the agar plate precultured with the fresh microorganism. In any case, this methodology could be used as a first stage to identify the most promising ILs for use in a wide range of processes [20,23–25].

In this context, this work aims at investigating the potential toxicity and biocompatibility of 63 ILs containing different cation and anion structures with *S. cerevisiae*, the most widely used microorganism for ethanol fermentation. Imidazolium, pyridinium, pyrrolidinium, piperidinium, morpholinium, oxazolinium, phosphonium, ammonium and sulfonium cations combined with different anions have been analyzed in solid media by using the well diffusion test. The results obtained are in-depth discussed and related to the structural characteristic of the ILs, which allows the identification of key factors for designing biocompatible IL toward *S. cerevisiae*.

2. Materials and Methods

2.1. Ionic Liquids and Chemicals

The 63 ILs investigated in the current work are grouped according to the type of cation present in their structure. Tables 1–6 include the name of each IL analyzed along with its structure and water solubility. The biocompatible water-insoluble ILs might be used as extraction agents of organic compounds from aqueous media while nontoxic water-soluble ILs could be employed as a reaction media in biocatalytic synthesis by using *S. cerevisiae*. The tables also include the nomenclature used for each IL (abbreviation name), as well as, the radius of inhibition (*RI*), caused on *S. cerevisiae*. All ILs were supplied by IoLiTec (Ionic Liquids Technologies, Germany) and were of the highest purity available.

2.2. Culture of *S. cerevisiae*

The yeast *S. cerevisiae* was selected as a microorganism to evaluate the potential toxicity of different ILs. A suspension of 1 g L^{-1} of the yeast was prepared in physiological water (9 g L^{-1}) and then, $200 \mu\text{L}$ of this solution was transferred to yeast extract-peptone-dextrose (YPD) agar plates with the following composition in g L^{-1} $20_{\text{D-glucose}}/20_{\text{peptone}}/10_{\text{yeast extract}}/20_{\text{agar}}$ and incubated for 48 h. The inoculum was prepared by adding a single colony of the yeast to a flask containing 100 mL of YPD liquid medium prepared with $20_{\text{D-glucose}}/20_{\text{peptone}}/10_{\text{yeast extract}}$ (g L^{-1}). The flask was incubated in an orbital shaker at 170 rpm and 30°C for 24 h. Both solid and liquid YPD media were autoclaved at 121°C for 20 min. All the reagents were purchased from Sigma Aldrich and were of the highest purity available.

2.3. Toxicity Analysis: Agar Well Diffusion Test

The toxicity of the different ILs to *S. cerevisiae* was studied by using the well agar diffusion test [26,27]. This method consists of making wells of 6 mm of diameter by punching an agar plate with a glass tube under sterilized conditions. Aliquots of $50 \mu\text{L}$ of each IL were placed on the wells, using physiological water for the control wells without IL [25,28,29]. The agar plates were incubated

at 30 °C for 48 h and then, the radius of the inhibition zone around the wells was determined (see Figure 1). Each IL was tested in triplicate and the size of the inhibition zone was measured by using a Vernier scale. The results showed are the average of the triplicates and the standard deviations.

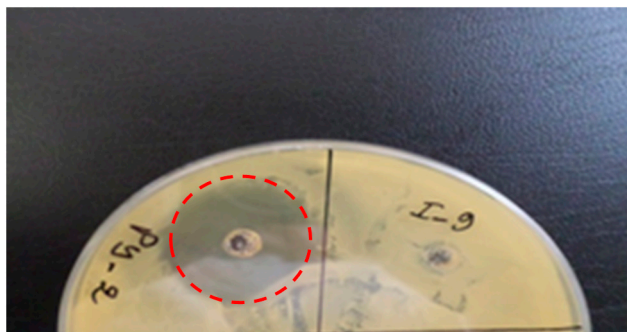


Figure 1. Inhibition zone in well agar diffusion test for the 1-butyl-3-methylpyridinium tetrafluoroborate [Bm⁺py⁺] [BF₄⁻] (very toxic) on the left side of the agar plate, and 1-butyl-3-methylimidazolium bis (trifluoromethylsulphonyl) imide [Bmim⁺] [NTf₂⁻] (biocompatible) on the right side of the agar plate.

3. Results and Discussion

The biocompatibility of 63 ILs toward *S. cerevisiae* was assessed by using the agar well diffusion test previously described. The names of these compounds and the results obtained are shown in Tables 1–6. The importance of evaluating the compatibility of ILs with *S. cerevisiae* lies in the development of new biotechnological processes that combine ILs and *S. cerevisiae* as it has been recently reported by de los Ríos et al. [19]. In this previous work, they evaluated the toxicity of nine water-insoluble ILs toward *S. cerevisiae* using the agar diffusion test (in pure form and 3% v/v IL solution), specific growth rates (μ , h⁻¹) in liquid media at 3% (v/v) IL and the final dry-weight concentration of yeast at 48 h [19]. The toxicity results found by the authors with the agar diffusion test were similar to those achieved in the rest of the toxicity assays evaluated (growth in liquid media and final dry weight concentration), which confirms the suitability of the well diffusion test as a simple method to estimate the toxicity of ILs toward *S. cerevisiae* [19]. In this context, the growth rate *S. cerevisiae* in the presence of ILs which exhibited zero inhibition radius (agar diffusion test, pure ILs) was similar to the growth rate of the control without IL. These preliminary results allow us to classify the toxicity of ILs as ‘very toxic’ when the inhibition radius is higher than 1 cm, as ‘toxic’ when the inhibition radius is between 1 and 0.5 cm, as ‘low toxic’ when it is between 0.5 and 0.0 cm and as ‘biocompatible’ when the inhibition radius is equal to zero. The IL [Omim⁺] [dca⁻] is very toxic and, on the contrary, [HOPmim⁺] [NO₃⁻] and [Bmim⁺] [NTf₂⁻] can be considered biocompatible with *S. cerevisiae* (see Table 1). On the other hand, it was found that water solubility was not directly related to the toxicity of the ILs toward *S. cerevisiae* as can be observed in Table 1. For instance, some ILs that are water-insoluble are very toxic (e.g., [P_{6,6,6,14}⁺] [C₉COO⁻], see Table 2) while other water-soluble ILs are low toxic or even biocompatible with the yeast (e.g., [Omim⁺] [dca⁻], see Table 1). This implies that it is necessary to analyze the relationship between toxicity and IL structure. In the following sections, we systematically analyze the qualitative relationship between IL structure and toxicity.

Table 1. Biocompatibility data of imidazolium-based ionic liquids toward *S. cerevisiae* using agar well diffusion test.

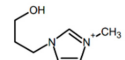
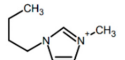
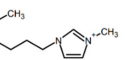
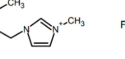
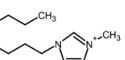
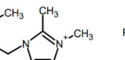
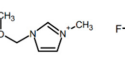
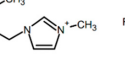
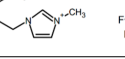
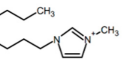
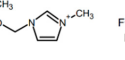
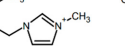
Full Name	Abbreviation	Structure	Water Miscibility	State 25 °C	Ref.	Radius of Inhibition (RI) (cm)
1-(2-hydroxypropyl)-methylimidazolium chloride	[HOPmim ⁺] [Cl [−]]		Soluble	Liquid	[19]	0.1 ± 0.0
1-butyl-3-methylimidazolium chloride	[Bmim ⁺] [Cl [−]]		Soluble	Solid	[29]	0.6 ± 0.1
1-hexyl-3-methylimidazolium chloride	[Hmim ⁺] [Cl [−]]		Soluble	Liquid	[19]	1.3 ± 0.5
1-butyl-3-methylimidazolium tetrafluoroborate	[Bmim ⁺] [BF ₄ [−]]		Soluble	Liquid	[30]	0.7 ± 0.2
1-methyl-3-octylimidazolium tetrafluoroborate	[Omim ⁺] [BF ₄ [−]]		Insoluble (decomposes in water)	Liquid	[31,32]	1.3 ± 0.4
1-butyl-2,3-dimethylimidazolium tetrafluoroborate	[BDImim ⁺] [BF ₄ [−]]		Soluble	Liquid	[19]	0.30 ± 0.15
1-methoxymethyl-3-methylimidazolium tetrafluoroborate	[MOMmim ⁺] [BF ₄ [−]]		Soluble	Liquid	[19]	0.2 ± 0.0
1-methoxyethyl-3-methylimidazolium tetrafluoroborate	[MOEmim ⁺] [BF ₄ [−]]		Insoluble	Liquid	[19]	0.7 ± 0.1
1-butyl-3-methylimidazolium hexafluorophosphate	[Bmim ⁺] [PF ₆ [−]]		Insoluble	Liquid	[33]	0.4 ± 0.1
1-methyl-3-octylimidazolium hexafluorophosphate	[Omim ⁺] [PF ₆ [−]]		Insoluble(decomposition in water)	Liquid	[31,32,34]	0.5 ± 0.1
1-methoxymethyl-3-methylimidazolium hexafluorophosphate	[MOMmim ⁺] [PF ₆ [−]]		Soluble	Solid	[31]	2.0 ± 0.5
1-butyl-3-methylimidazolium nitrate	[Bmim ⁺] [NO ₃ [−]]		Soluble	Liquid	[19]	0.0

Table 1. Cont.

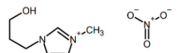
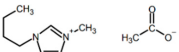
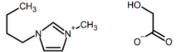
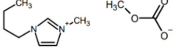
Full Name	Abbreviation	Structure	Water Miscibility	State 25 °C	Ref.	Radius of Inhibition (RI) (cm)
1-(2-hydroxypropyl)-3-methylimidazolium nitrate	[HOPmim ⁺] [NO ₃ [−]]		Soluble	Liquid	[19]	0.0
1-butyl-3-methylimidazolium acetate	[Bmim ⁺] [CH ₃ COO [−]]		Soluble	Liquid	[19]	1.0 ± 0.2
1-butyl-3-methylimidazolium glycolate	[Bmim ⁺] [CH ₂ OHCOO [−]]		Soluble	Liquid	[19]	0.3 ± 0.1
1-butyl-3-methylimidazolium methylcarbonate	[Bmim ⁺] [MeCOO ₂ [−]]		Soluble	Liquid	[31]	1.0 ± 0.3

Table 2. Biocompatibility data of phosphonium-based ionic liquids toward *S. cerevisiae* using agar well diffusion test.

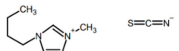
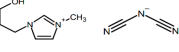
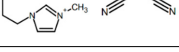
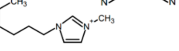
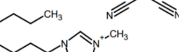
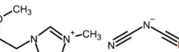
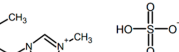
Full Name	Abbreviation	Structure	Water Miscibility	State at 25 °C	Ref.	Radius of Inhibition (RI) (cm)
1-butyl-3-methylimidazolium thiocyanate	[Bmim ⁺] [SCN [−]]		Insoluble	Liquid	[35]	0.8 ± 0.3
1-(2-hydroxypropyl)-3-methylimidazolium dicyanamide	[HOPmim ⁺] [dca [−]]		Soluble	Liquid	[19]	0.05 ± 0.00
1-butyl-3-methylimidazolium dicyanamide	[Bmim ⁺] [dca [−]]		Soluble	Liquid	[36]	0.6 ± 0.2
1-hexyl-3-methylimidazolium dicyanamide	[Hmim ⁺] [dca [−]]		Soluble	Liquid	[19]	0.5 ± 0.2
1-methyl-3-octylimidazolium dicyanamide	[Omim ⁺] [dca [−]]		Soluble	Liquid	[36]	2.0 ± 0.2
1-methoxyethyl-3-methylimidazolium dicyanamide	[MOEmim ⁺] [dca [−]]		Soluble	Liquid	[31]	1.0 ± 0.2
1-butyl-3-methylimidazolium hydrogen sulfate	[Bmim ⁺] [HSO ₄ [−]]		Soluble	Liquid	[31]	0.5 ± 0.1

Table 2. Cont.

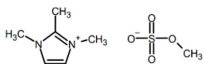
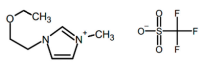
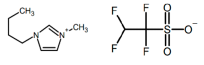
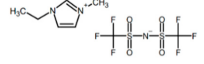
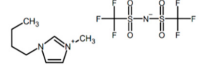
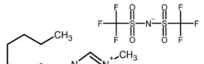
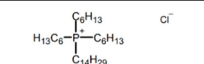
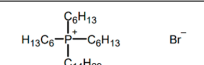
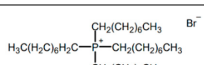
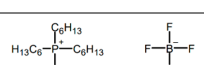
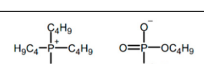
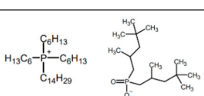
Full Name	Abbreviation	Structure	Water Miscibility	State at 25 °C	Ref.	Radius of Inhibition (RI) (cm)
1,2,3-trimethylimidazolium methylsulfate	$[(\text{CH}_3)_3\text{IM}^+][\text{CH}_3\text{SO}_4^-]$		Partly soluble	Solid	[37]	0.3 ± 0.1
1-(ethoxyethyl)-3-methylimidazolium trifluoromethanesulfonate	$[\text{EOEmim}^+][\text{CF}_3\text{SO}_3^-]$		Soluble	Liquid	[38]	0.5 ± 0.1
1-butyl-3-methylimidazolium 1,1,2,2-tetrafluoroethanesulfonate	$[\text{Bmim}^+][\text{TFES}^-]$		Soluble	Liquid	[39]	0.9 ± 0.2
1-ethyl-3-methylimidazolium bis (trifluoromethylsulfonyl) imide	$[\text{Emim}^+][\text{NTf}_2^-]$		Insoluble	Liquid	[40]	0.10 ± 0.09
1-butyl-3-methylimidazolium bis (trifluoromethylsulfonyl) imide	$[\text{Bmim}^+][\text{NTf}_2^-]$		Insoluble	Liquid	[36]	0.0
1-methyl-3-octylimidazolium bis (trifluoromethylsulfonyl) imide	$[\text{Omim}^+][\text{NTf}_2^-]$		Insoluble	Liquid	[36]	0.3 ± 0.1
Tetradecyl (trihexyl) phosphonium chloride	$[\text{P}_{6,6,6,14}^+][\text{Cl}^-]$		Insoluble	Liquid	[31,41]	0.20 ± 0.05
Tetradecyl (trihexyl) phosphonium bromide	$[\text{P}_{6,6,6,14}^+][\text{Br}^-]$		Insoluble	Liquid	[31,42]	0.90 ± 0.25
Tetraoctylphosphonium bromide	$[\text{P}_{8,8,8,8}^+][\text{Br}^-]$		Insoluble	Solid	[31,43]	0.5 ± 0.1
Tetradecyl (trihexyl) phosphonium tetrafluoroborate	$[\text{P}_{6,6,6,14}^+][\text{BF}_4^-]$		Insoluble	Liquid	[31,44]	0.60 ± 0.15
Tetrabutylphosphonium dibutyl phosphate	$[\text{P}_{4,4,4,4}^+][\text{Bu}_2\text{Phos}^-]$		Soluble	Solid	[31,45]	0.5 ± 0.1
Trihexyl (tetradecyl) phosphonium bis 2,4,4-(trimethylpentyl) phosphinate	$[\text{P}_{6,6,6,14}^+][\text{TMPPhos}^-]$		Insoluble	Liquid	[31,46]	0.5 ± 0.1

Table 2. Cont.

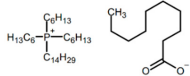
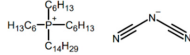
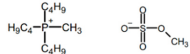
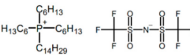
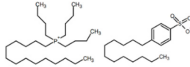
Full Name	Abbreviation	Structure	Water Miscibility	State at 25 °C	Ref.	Radius of Inhibition (RI) (cm)
Trihexyl (tetradecyl) phosphonium decanoate	$[P_{6,6,6,14}^+][C_9COO^-]$		Insoluble	Liquid	[31,47]	1.5 ± 0.5
Tetradecyl (trihexyl) phosphonium dicyanamide	$[P_{6,6,6,14}^+][dca^-]$		Insoluble	Liquid	[31,48]	0.5 ± 0.1
Tributylmethylphosphonium methylsulphate	$[P_{4,4,4,1}^+][CH_3SO_4^-]$		Soluble	Solid	[31,49]	0.9 ± 0.2
Trihexyl (tetradecyl) phosphonium bis (trifluoromethylsulfonyl) imide	$[P_{6,6,6,14}^+][NTf_2^-]$		Insoluble	Liquid	[31,50]	0.2 ± 0.1
Tributyltetradecylphosphonium dodecylbenzenesulfonate	$[(C_4H_9)_3(C_{14}H_{29})P^+][C_{12}H_{25}C_6H_4SO_3^-]$		Insoluble	Liquid	[31,51]	0.50 ± 0.15

Table 3. Biocompatibility data of pyrrolidinium-based ionic liquids toward *S. cerevisiae* using agar well diffusion test.

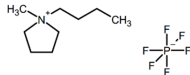
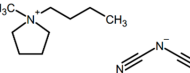
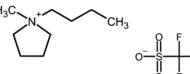
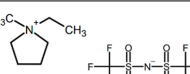
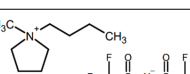
Full Name	Abbreviation Name	Structure	Water Miscibility	State 25 °C	Ref.	Radius of Inhibition (RI) (cm)
1-butyl-1-methyl pyrrolidinium hexafluorophosphate	$[BMPyr^+][PF_6^-]$		Insoluble	Solid	[52,53]	0.7 ± 0.1
1-butyl-1-methyl pyrrolidinium dicyanamide	$[BMPyr^+][dca^-]$		Soluble	Liquid	[54,55]	0.4 ± 0.1
1-butyl-1-methyl pyrrolidinium triflate	$[BMPyr^+][TfO^-]$		Soluble	Liquid	[56,57]	0.20 ± 0.05
1-ethyl-1-methyl pyrrolidinium bis[(trifluoromethyl) sulfonyl]imide	$[EMPyr^+][NTf_2^-]$		Insoluble	Solid	[31,58]	0.0
1-butyl-1-methyl pyrrolidinium bis (trifluoromethylsulfonyl) imide	$[BMPyr^+][NTf_2^-]$		Insoluble	Liquid	[57,59]	1.10 ± 0.35

Table 4. Biocompatibility data of pyridinium-based ionic liquids toward *S. cerevisiae* using agar well diffusion test.

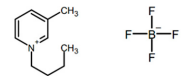
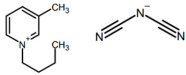
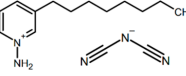
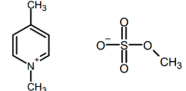
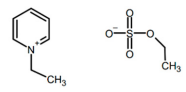
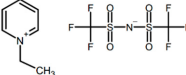
Full Name	Abbreviation Name	Structure	Water Miscibility	State 25 °C	Ref.	Radius of Inhibition (RI) (cm)
1-butyl-3-methylpyridinium tetrafluoroborate	[BMPy ⁺] [BF ₄ [−]]		Soluble	Liquid	[31,60]	1.4 ± 0.2
1-butyl-3-methylpyridinium dicyanamide	[BMPy ⁺] [dca [−]]		Soluble	Liquid	[31,61,62]	1.1 ± 0.3
1-methyl-3-octylpyridinium dicyanamide	[MOPy ⁺] [dca [−]]		Insoluble	Liquid	[31]	0.5 ± 0.2
1,4-dimethyl pyridinium Methylsulfate	[MMPy ⁺] [Me SO ₄ [−]]		Soluble	Solid	[31]	0.4 ± 0.1
1-ethylpyridinium ethyl sulfate	[EPy ⁺] [EtSO ₄ [−]]		Soluble	Liquid	[31,63]	0.5 ± 0.2
1-ethylpyridinium bis(trifluoromethylsulfonyl)imide	[EPy ⁺] [NTf ₂ [−]]		Insoluble	Liquid	[31,63,64]	0.5 ± 0.15

Table 5. Biocompatibility data of ammonium-based ionic liquids toward *S. cerevisiae* using agar well diffusion test.

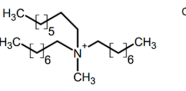
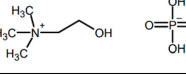
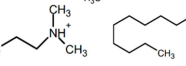
Full Name	Abbreviation Name	Structure	Water Miscibility	State at 25 °C	Ref.	Radius of Inhibition (RI) (cm)
Methyltriocylammonium chloride	[N _{8,8,8,1} ⁺] [Cl [−]]		Insoluble	Liquid	[31,65]	0.6 ± 0.2
Choline dihydrogen phosphate	[Chol ⁺] [H ₂ PO ₄ [−]]		Soluble	Solid	[31,66]	0.0
N,N-dimethylbutylammonium 2-hexyldecanoate	[N _{4,1,1,0} ⁺] [C ₈ C ₆ CHCOO [−]]		Soluble	Liquid	[67]	0.5 ± 0.1

Table 5. Cont.

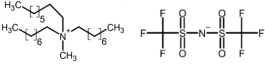
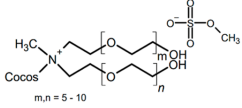
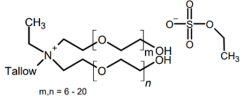
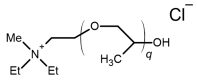
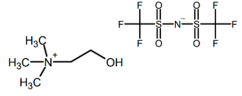
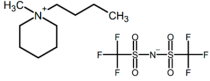
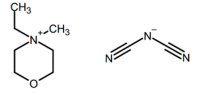
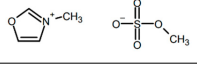
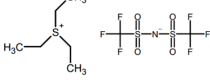
Full Name	Abbreviation Name	Structure	Water Miscibility	State at 25 °C	Ref.	Radius of Inhibition (RI) (cm)
Methyltrioctylammoniumbis (trifluoromethylsulfonyl) imide	[N _{8,8,8,1} ⁺] [NTf ₂ ⁻]		Insoluble	liquid	[68]	0.0
Cocosalkylpentaethoximethylammonium methylsulfate	[C1EG ⁺] [MeSO ₄ ⁻]		Soluble	Liquid	[31,69]	1.0 ± 0.3
Tallowsalkylpentaethoximethylammonium methylsulfate	[T2EG ⁺] [MeSO ₄ ⁻]		Insoluble	Liquid	[31,70]	0.7 ± 0.2
TEGO®-IL-P9	[221PG ⁺] [Cl ⁻]		Soluble	Liquid	[31,71]	0.20 ± 0.05
Choline bis (trifluoromethylsulfonyl) imide	[Chol ⁺] [NTf ₂ ⁻]		Insoluble	Liquid	[31,72,73]	0.1 ± 0.0

Table 6. Biocompatibility data of piperidinium-, morpholinium-, oxazolinium- and sulfonium-based ionic liquids toward *S.cerevisiae* using agar well diffusion test.

Full Name	Abbreviation Name	Structure	Water Miscibility	State at 25 °C	Ref.	Radius of Inhibition (RI) (cm)
1-butyl-1-methylpiperidiniumbis (trifluoromethylsulfonyl) imide	[BMPi ⁺] [NTf ₂ ⁻]		Insoluble	Liquid	[31,74]	0.0
Ethyl methyl morpholinium dicyanamide	[EMMOR ⁺] [dca ⁻]		Soluble	Liquid	[19,75]	0.6 ± 0.3
Methyloxazolinium methylsulfate	[Moxa ⁺] [MeSO ₄ ⁻]		Soluble	Liquid	[19,31]	0.0
Triethylsulfonium bis (trifluoromethylsulfonyl) imide	[S _{2,2,2} ⁺] [NTf ₂ ⁻]		Insoluble	Liquid	[76]	0.0

3.1. Influence of the Alkyl Substituent of the Cation of Ionic Liquid on the Toxicity toward *S. cerevisiae*

According to the results obtained, an increase of the alkyl chain length in the IL cation (considering the same anion) involves an increase in the toxicity of the ILs to *S. cerevisiae*. This behaviour is found for the imidazolium cation (see Table 1) from [Bmim⁺] [Cl[−]] to [Hmim⁺] [Cl[−]] (in which the ILs range from toxic to very toxic), from [Bmim⁺] [BF₄[−]] to [Hmim⁺] [BF₄[−]], from [MOMmim⁺] [BF₄[−]] to [MOEmim⁺] [BF₄[−]], from [Bmim⁺] [dca[−]], [Hmim⁺] [dca[−]] to [Omim⁺] [dca[−]] and from [Bmim⁺] [PF₆[−]] to [Omim⁺] [PF₆[−]]. This behavior was also observed for the pyrrolidinium cation (see Table 3) from [Empyr⁺] [NTf₂[−]] to [Bmpyr⁺] [NTf₂[−]] (0 and 1.1 cm of inhibition radius, respectively). Sendovski et al. [11] studied the toxicity of nine immiscible ILs toward *S. cerevisiae*, used in biphasic systems in the synthesis of 2-phenylethanol catalyzed by this yeast. To this aim, *S. cerevisiae* was grown in the presence of the selected ILs. The results found are in line with those obtained in the present work since the longer the alkyl side chain on the imidazolium ring, the lower biocompatibility of the ILs. In other microorganisms, it was also found that the toxicity was directly related to the chain length of the alkyl substituent on the cation [19,77–80]. The influence of an increasing chain length of the imidazolium cation moiety on the cytotoxicity in marine bacteria and two types of mammalian cell cultures were also evident in HeLa cells [81,82]. This effect is currently known as the ‘side-chain effect’ [83]. For highly lipophilic cations, cytotoxicity does not significantly increase with lipophilicity anymore. It is well known that lipophilicity relationships with biological activity are only linear over a restricted range [84]. The ‘side-chain effect’, in which carbon atoms are added, involves a high hydrophobic character in ILs. It would increase the possibility of their interaction with phospholipid bilayers of the cell membranes and the hydrophobic domains of the membrane proteins, leading to the disruption of the membrane physiological functions and, consequently, to cell death [81,82,85,86]. On the contrary, as can be observed in Table 1, the inclusion of an oxygen atom in the alkyl substituent of the imidazolium ring can significantly decrease the toxicity of imidazolium-based ILs. It has been observed that [HOPmim⁺] [Cl[−]] (*RI* = 0.1 cm) displayed much lower toxic effects than [Bmim⁺] [Cl[−]] (*RI* = 0.6 cm), so the substitution of the methyl by a hydroxy group converts a toxic IL into a slightly toxic IL. This behavior is in agreement with the work of Álvarez-Guerra and Irabien [87], who reported that the presence of oxygenated groups in the structure of cations can lead to a decrease in the ecotoxicity of the IL. In line with these results, Tether et al. [88] reported the decrease in the ionic liquid toxicity toward *Escherichia coli* and *Staphylococcus epidermidis* through the side chain oxygenation by using the agar diffusion test. In this context, the presence of the oxygen makes the IL more hydrophilic, and therefore, less toxic. It is also important to remark the high toxicity observed for [Bmim⁺] [BF₄[−]] compared to [BDImim⁺] [BF₄[−]]. The inclusion of the methyl substituent in the R2 position of the imidazolium ring could reduce the acidic proton in 2, so lowering the toxicity of the IL. This effect was also recently observed for *Shewanella* [89].

3.2. Effect of the Ionic Liquid Cation on Toxicity toward *S. cerevisiae*

In order to study the effect of the cation structure, ILs with the same anion and different cation were compared. In general, the cation toxicity for other microorganisms has been higher for ILs containing aromatic cations, such as imidazolium and pyridinium cations, in comparison to nonaromatic cations, e.g., pyrrolidinium. A higher hydrophobic character of aromatic cations could increase the possibility of interaction with the cell membrane [81,82,85]. Furthermore, the planarity of the cation ring in imidazolium and pyridinium appeared to be also a relevant parameter for increasing IL toxicity, as reported in [90]. This fact could be due to the lower steric hindrance of the aromatic cation, which might favor the interactions with the lipidic membrane to a greater extent. For *S. cerevisiae*, a high toxicity was found for pyridinium and imidazolium cations compared with pyrrolidinium cations for the series [Bmpyr⁺] [dca[−]] (*RI* = 1.1 cm), [Bmim⁺] [dca[−]] (*RI* = 0.6 cm) and [BMPyr⁺] [dca] (*RI* = 0.4 cm) and also for the series [Epyr⁺] [NTf₂[−]] (*RI* = 0.6 cm), [Emim⁺] [NTf₂[−]] (*RI* = 0.1 cm) and [Empyr⁺] [NTf₂[−]] (*RI* = 0.0 cm). The IL [Bmpyr⁺] [BF₄[−]] (*RI* = 1.4 cm) was also found to be more toxic

than [Bmim⁺] [BF₄[−]] (*RI* = 0.7) to *S. cerevisiae*. On the contrary, the toxicity of [Bmim⁺] [NTf₂[−]] was lower than the toxicity of [BMPyr⁺] [NTf₂[−]]. Similar results were reported for *Shewanella* [89].

On the other hand, the toxicity of imidazolium cations ranges from very high for [Omin⁺] [dca[−]] (*RI* = 2 cm), which is an IL with a long alkyl chain, to zero (*RI* = 0.0 cm) as in the case of the IL [OHPmim⁺] [NO₃[−]], whose structure contains a hydrophilic substituent. It is worth noting that [Bmim⁺] [NO₃[−]] was also biocompatible toward *S. cerevisiae*, maybe due to the presence of the nitrate anion in its structure. Existing studies on the toxicity of imidazolium-based ILs to *S. cerevisiae* were performed taking into account the cell growth in the presence of [Bmim⁺] [PF₆[−]], [Bmim⁺] [BF₄[−]] and hexane. A slight reduction in the growth was observed with [Bmim⁺] [PF₆[−]] compared to the control without IL. The growth in the presence of [Bmim⁺] [BF₄[−]] was lower than in [Bmim⁺] [PF₆[−]], and the growth in hexane was much lower than in [Bmim⁺] [BF₄[−]] [91]. Furthermore, it was demonstrated that the presence of [Bmim⁺] [Cl[−]] during an ethanol fermentation process inhibits yeast growth [22]. These results are in good agreement with those found in the agar well diffusion test since the *RI* was 0.4, 0.7 and 0.6 cm for [Bmim⁺] [PF₆[−]], [Bmim⁺] [BF₄[−]] and [Bmim⁺] [Cl[−]], respectively.

For pyridinium-based ILs, the toxicity varies from an *RI* equal to 1.4 cm ([bmppy⁺] [BF₄[−]]) to 0.4 cm ([mmpy⁺] [MeSO₄[−]]). All ILs based on the pyridinium cation were fully biocompatible with *S. Cerevisiae*. Regarding the pyrrolidinium-based ILs assayed, lower toxicity values were obtained, [Empyr⁺] [NTf₂[−]] being fully biocompatible with *S. cerevisiae* (*RI* = 0.0 cm). The only piperidinium IL tested ([Bmpi⁺] [NTf₂[−]]) was also biocompatible with the yeast (*RI* = 0.0 cm), which might be due to the nonaromatic character and the presence of bis (trifluoromethylsulfonyl) imide as counteranion.

For ILs containing the ammonium cation, different toxicity values were found depending on the alkyl chain length of the cation and the type of anion. The toxicity values ranged from the highest toxicity for [C1EG⁺] [MeSO₄[−]], [T2EG⁺] [MeSO₄[−]], (*RI* = 1 cm) to the lowest toxicity for [N₈₈₈₁⁺] [NTf₂[−]] and [Chol⁺] [H₂PO₄[−]] (*RI* = 0.0 cm). The anion [NTf₂[−]] is present again in ILs with low toxicity, maybe due to its structure or its low water solubility. The IL [Chol⁺] [H₂PO₄[−]] contains a cation with heteroatoms and short alkyl chains. Other research works report that this IL is biocompatible with *Shewanella* sp. and even with the enzyme laccase, with overactivity reached in the presence of this IL [31,89]. Santos et al. [92] determined the maximum nontoxic concentration of choline-based ILs to *S. cerevisiae* finding significant biocompatibility when the ILs were water-soluble. The biocompatibility with choline-based hydrophilic IL was lower. All these results are in good agreement with those reported in the present study.

In the case of the phosphonium family, it was found that long alkyl chains promote higher toxic effects toward the bacterium *Vibrio fischeri* [83], following the ‘side-chain effect’ mentioned above. The toxicity values for phosphonium-based IL toward *S. cerevisiae* ranged from *RI* = 1.5 cm ([P_{6,6,6,14}⁺] [C₉COO[−]]) to *RI* = 0.2 cm ([P_{6,6,6,14}⁺] [Cl[−]]). In the existing literature, the toxicity of [P_{4,4,4,1}⁺] [CH₃SO₄[−]] was obtained by determining the maximum nontoxic concentration of the IL for *S. cerevisiae*, resulting in toxicity toward *S. cerevisiae* [92]. The same result was obtained in this work by using the agar well diffusion test.

On the other hand, the morpholinum-based IL was found to be biocompatible with *S. cerevisiae*, which might be due to the inclusion of a heteroatom in the imidazolium ring and also to the low alkyl chain constituent of the aromatic ring. Finally, only a sulfonium-based IL was assessed, [S_{2,2,2}⁺] [NTf₂[−]], which proved to be biocompatible with *S. cerevisiae*. These results might be related to the nature of its counteranion.

3.3. Effect of the Ionic Liquid Anion on Toxicity toward *S. cerevisiae*

The effect of the anion composition on the IL toxicity was analyzed by comparing ILs with different anions and the same cation. Several authors have reported that the toxicity of ILs on several microorganisms is directly related to the cation nature, while the anion seems to modulate the toxicity to some extent and in specific cases [77–80,93]. This behavior was observed in several ILs in which the toxicity values were similar when sharing the same cation but contain different anions. For example,

Table 7. Cont.

Cation Family	Anion Sequence from Lower to Higher Toxicity and Radius of Inhibition (RI)	
[N _{8,8,8,1} ⁺]	[NTf ₂ ⁻] (0)	[Cl ⁻] 0.6
[Chol ⁺]	[H ₂ PO ₄ ⁻] (0)	[NTf ₂ ⁻] (0.1)

3.4. Mechanisms of IL toxicity

Relatively few mechanisms have been suggested to explain the toxicity of ILs towards microorganisms, but membrane disruption is considered the most common [98,99]. The ability of the ILs to disrupt the cell wall of different microorganisms seems to be due to their hydrophobicity, caused by the length of the alkyl side chain of the cation or the presence of aromatic cations, which is in line with the results obtained in the present work.

Furthermore, the inclusion of a heteroatom such as oxygen leads to reduce the hydrophobicity of the IL and thus toxicity. Weuster-Botz [100] studied the membrane integrity of *S. cerevisiae* cells after 5 h of exposure to biphasic systems comparing pure buffer and [Bmim⁺] [PF₆⁻], [Bmim⁺] [NTf₂⁻] and [Omim⁺] [NTf₂⁻]. The membrane integrity of *S. cerevisiae* with the ILs was similar to that in aqueous media (90%). Slightly higher membrane integrity was found for [Omim⁺] [NTf₂⁻]. Those results are in concordance with the results showed in the present work, in which [Bmim⁺] [NTf₂⁻] and [Omim⁺] [NTf₂⁻] have been classified as biocompatible, and [Bmim⁺] [PF₆⁻] as low toxic. Other molecular mechanisms cannot be excluded when explaining the toxicity of ILs. Dickinson and Piotrowski et al. [101] studied the toxicity mechanism of [Emim⁺] [Cl⁻], [Bmim⁺] [Cl⁻] and [Emim⁺] [CH₃COO⁻]. They found that these ILs likely target mitochondria. High-throughput chemical proteomics showed the effects of ILs on mitochondrial protein levels. ILs induced abnormal mitochondrial morphology, as well as, altered the polarization of mitochondrial membrane potential, similar to valinomycin.

3.5. Ionic Liquids Biocompatible with *S. cerevisiae*

The agar well diffusion test can serve as an easy and quick decision-making tool when it comes to choosing sustainable ILs for biotechnological applications involving *S. cerevisiae*. In this case, ILs are deemed as biocompatible with *S. cerevisiae* when the radius of inhibition is equal to zero. In this sense, those classified as biocompatible are [Bmim⁺] [NO₃⁻] in which the low toxicity could be explained by the anion; [HOPmim⁺] [NO₃⁻] due to the anion and the hydroxyl imidazolium substituent; [Bmim⁺] [NTf₂⁻], [N_{8,8,8,1}⁺] [NTf₂⁻] and [S_{2,2,2}⁺] [NTf₂⁻] in which the bistriflimide anion strongly contributes to the biocompatibility; [EMPyr⁺] [NTf₂⁻] and [BMPi⁺] [NTf₂⁻] due to the anion and the nonaromatic structure of the cation; [Moxa⁺] [MeSO₄⁻] due to the inclusion of the oxygen heteroatom in the imidazolium ring, which consequently increases its polarity, and [Chol⁺] [H₂PO₄⁻] due to the hydroxyl short alkyl chain length and the nature of the anion. It is important to highlight that the studies on the toxicity of ILs in yeast are still low, but the few that exist corroborate the results reported in the current work. Sendovski et al. [11] also found that [Bmim⁺] [NTf₂⁻] and [N_{8,8,8,1}⁺] [NTf₂⁻] were biocompatible with *S. cerevisiae*. It is also important to note that the agar well diffusion method for the assessment of IL toxicity toward *S. cerevisiae* has been validated not only by comparison with other methods in previous works of our research group ([19]) but also with the results reported by other authors in this field, as commented throughout this work.

4. Conclusions

This work assesses the toxicity of a high number of ionic liquids toward *S. cerevisiae* using the agar well diffusion test. This method enables the easy and quick analysis of their biocompatibility toward *S. cerevisiae* with the aim to open up new synergy technologies between *S. cerevisiae* and ILs. With this method, nine fully biocompatible ILs toward *S. cerevisiae* have been identified, including [Bmim⁺]

[NO₃[−]], [HOPmim⁺] [NO₃[−]], [Bmim⁺] [NTf₂[−]], [N_{8,8,8,1}⁺] [NTf₂[−]], [S_{2,2,2}⁺] [NTf₂[−]], [EMPy⁺] [NTf₂[−]], [BMPI⁺] [NTf₂[−]], [Moxa⁺] [MeSO₄[−]] and [Chol⁺] [H₂PO₄[−]]. The results obtained also allowed us to establish several toxicity-structure relationships which could even help to make important predictions about IL toxicity, without further experimentation. Thus, it was observed that an increase in the alkyl chain length in cations such as imidazolium or pyridinium involves an increase in the hydrophobicity of the ILs and therefore, an increase of their toxicity. Furthermore, the toxicity is mainly determined by the cation but, certain anions have a strong influence on IL toxicity as in the case of the [NTf₂[−]] anion, which dramatically reduces the toxicity of the IL towards *S. cerevisiae*.

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