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Toxicity of Ionic Liquids Toward Microorganisms Interesting for Food Industry

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Considering the potential applicability of ionic liquids (ILs) as solvents in biotechnological process, such as the production of food supply via microbial synthesis, the work presented here aimed to evaluate the toxicity of these new solvents to microorganisms of interest in the food industry. Following the international standard method of CLSI (Clinical and Laboratory Standards Institute), the maximum non-toxic concentration (MNTC) was determined for nine ionic liquids (containing an imidazolium, cholinium or phosphonium cation) toward nine microorganisms, among them the bacteria *Bacillus subtilis, Lactobacillus delbrueckii* subs. *delbrueckii*, *Pseudomonas aeruginosa,* the actinobacteria *Streptomyces drozdowiczii*, the yeasts *Saccharomyces cerevisiae, Yarrowia lipolytica, Kluyveromyces marxianus,* and the filamentous fungi *Aspergillus brasiliensis* and *Rhizopus oryzae*. Among bacteria, *B. subtilis* and *P. aeruginosa* were more tolerant to hydrophilic imidazolium ILs with [C_2mim] cations, combined with the [$EtSO_4$], [$EtSO_3$] and [Cl] anions. When in the presence of hydrophilic choline and phosphonium based-ILs, the Gram-negative bacteria *P. aeruginosa* was more resistant than others. The same was observed for the [NTf_2]-based ILs, where just *P. aeruginosa* could growth. Regarding the fungi, it was observed that *A. brasiliensis* and *R. oryzae* tolerated high concentrations of ILs. Among the yeast, only *Y. lipolytica* was tolerant to all ILs tested. In general, ILs whose the cationic moiety was choline showed to be more biocompatible, since they allowed the growth of all microorganisms studied.

Introduction

The term ionic liquids (ILs) refer to a solvent that are composed only of ions, organic cations combined with organic or inorganic anions, whose melting point is below to 100ºC [1]. Due to this and other interesting features, these liquid salts have emerged as alternative solvents in bioprocess. The change of cations and anions which comprise the ILs, allows tuning properties of these salts, such as viscosity, density and hydrophobicity, in order to attend the requirements of a particular process, and it is the most attractive characteristic of ionic liquids. Since 1990, researches related to application of ionic liquids in biotechnology have been developed especially in enzymatic 2–4 and whole-cell processes. The latter application still remains quite limited in the literature, and restricted to processes [2–4] and whole-cell process.[2] The latter application still remains quite limited in the literature, and restricted to process involving biotransformation reactions, mainly asymmetric reduction ketones for production of chiral alcohols[5–10]. In whole-cell processes, water-immiscible ionic liquids can be used in a biphasic system, working as a reservoir of substrate and products that can act as inhibitors. Furthermore, other researches reported use of water-miscible ILs for increasing the availability of insoluble substrate.

An important requirement to obtain success in application of a solvent in a whole-cell process as an extractor or additive is present low toxicity toward microorganisms. Thus, the selection of an ionic liquid to be applied in a bioprocess requires a screening of different cations and anions in order to find a more biocompatible combination. Within the context of toxicity of ionic liquids, investigators have been searching for new compounds associated with efficient antimicrobial activity and their use as biocides.[11–16] In these studies, the toxic effect of ILs is evaluated toward a range of bacteria and fungi of importance for human health, such as *Micrococcus luteus, Staphylococcus aureus, Enterococcus faecalis, Escherichia coli, Klebsiela pneumoniae, Pseudomonas aeruginosa, Candida albicans, Rhodotorula rubra, Bacillus subtilis,* among other. There are some toxicological works in which the susceptibility of microorganisms considered ecotoxicological models are evaluated, such as the bacteria *Vibrio fischeri*.[17,18] In the context of biotechnology, there are still few studies and they are focused in microorganisms more commonly used, such as *E. coli* and *Saccharomyces cerevisiae,* and use mainly imidazolium-based ionic liquids, such as [C_4mim] and [C_6mim], with the anions [PF_6], [BF_4] and [NTf_2].[2]

In general, it was shown that antimicrobial activity is affected by the cation alkyl length, whereas longer is the alkyl chain, stronger is the biological activity for microorganisms[11–13,15,19]

and others organisms including the nematode *Caenorhabditis elegans*, [20] the freshwater snail *Physa acuta*,[21] and the green algae *Pseudokirchneriella subcapitata*.[22] This effect has been associated with increase in the molecular lipophilicity, through the QSAR concept (Quantitative Structure-Activity Relationship) that relates statistically the chemical structure, in this case lipophilicity parameters, to toxic effect,[11,23] and suggested that high lipophilicity of molecule imply increase of interaction with cell membrane as well as toxic effect. Thus the most biocompatible ionic liquids seem to be those with short alkyl chain.

Although ionic liquids have been denoted as green solvent because they have negligible vapor pressure and non-flammability in contrast to molecular solvents, theses solvents can be very toxic for organisms in the environment and poorly biodegradable.[24] Beside on this, the number of works with ionic liquids based on biocompatible ions such as cholinium cation,[16,25] the anions saccharinate, acesulfamate, [26–28] and based on organic acids[29] have been raised. Aiming the possibility of applying ionic liquids in biocatalytic process of food industry chemicals, such as organic acids, enzymes, vitamins, polysaccharides, among other; the present work evaluated the tolerance of a range of microorganisms used in the food industry or with a potential of use in this field. In this way, short alkyl chain ionic liquids with more common cations, such as imidazolium and phosphonium and sustainable cations, such as choline, associated with different anions was applied to test hydrophilic and hydrophobic ILs. The objective was to identify some differences in tolerance between groups of microorganisms and also combinations of cations and anions more biocompatible with potential to be used as solvent in biotechnology.

Results and discussion

Hydrophilic ILs

Growth inhibition effect of nine ionic liquids was evaluated toward nine microorganisms, among them: bacteria, actinobacteria, yeast and filamentous fungi. MNTC value was considered as the maximum concentration which was possible to observe microbial growth and viable cells, in other words, the higher MNTC values, the lower toxicity of ionic liquid.

As the relationship between the increase of alkyl chain length of the cationic portion and the increase of toxic effect to a variety of organisms is previously described in literature, this work applied cations with short alkyl chain, between 2-4 carbons.

For hydrophilic imidazolium-based ionic liquids, showed in Table 1, it can be observed higher toxicity to yeasts, mainly *S. cerevisiae* and *K. marxianus*, which did not grow at the studied range concentration. Among yeasts, only *Y. lipolytica* showed some resistance to hydrophilic imidazolium-based ILs. The filamentous fungi *A. brasiliensis* and *R. oryzae* were quite tolerant to ILs mentioned, presenting growth at higher tested concentration. *R. oryzae* growth was not inhibited at 825.5 mM of [C_2mim][Cl] (Table 1). Among bacteria, *B. subtilis* and *P. aeruginosa* were comparatively more resistant to hydrophilic imidazolium-based ionic liquids. Apparently, this effect is not related to membrane and cell wall composition, since these two bacteria differ in this regard.

It was not possible to observe a clear pattern between results obtained for [Cl], [$EtSO_4$] and [$EtSO_3$] anions, in general, demonstrating low toxicity toward tested microorganisms. In others works with *Clostridium butyricum*[30] and *E. coli*,[31] alkylsulfates anions were also considered non-toxic and promising for producing of biocompatible and biodegradable ionic liquids.

Table 1 MNTC values relative to water miscible imidazolium-based ionic liquids

Microorganisms	MNTC (mM)		
	[C_2mim][Cl]	[C_2mim][$EtSO_4$]	[C_2mim][$EtSO_3$]
Gram-positivebacteria			
Bacillus subtilis	426	270	581
Lactobacillus delbrueckii subsp. *delbrueckii*	53	67,5	72
Actinobacteria			
Streptomyces drozdowickii	27	34	36
Gram-negativebacteria			
Pseudomonas aeruginosa	852,5	135	145
Yeast			
Saccharomyces cerevisiae	< 6,7	< 4,2	< 4,5
Yarrowia lipolytica	235	270	145
Kluyveromyces marxianus	< 6,7	< 4,2	< 4,5
Filamentousfungi			
Aspergillus brasiliensis	426	>540	>581
Rhizopus oryzae	> 852,5	>540	290

Ions based on natural products have been shown as a good choice to form biocompatible and biodegradable ionic liquids,[32] for example the choline cation, a essential micronutrient of complex B vitamins.[16] [Ch][Cl] (or B4 vitamin) is an organic salt of low cost, used, for example, as feed additive for chickens. It has a high melting point (298-304ºC), therefore this salt is not considered as an ionic liquid, however is used in choline-based ionic liquids by anion substitution,[27] and is also applied in deep eutectic solvents (DES) synthesis. DES are produced from the mixture of two substances in specific proportion, whose melting point is lower than when are separated, as a example, the mixture of choline chloride and urea.[33] Thus it was interesting to study choline chloride, since presents a potential use as component of deep eutectic solvents.

Water-miscible ionic liquids with choline cation (Table 2) were, in general, more biocompatible than the previous studied with imidazoliumcation, since allowed the growth of all microbial strains studied.

Some studies involving other ionic liquids containing tetralkylammoniumnon-cyclic cations evidenced that these solvents are less toxic than those with cyclic quaternarium ammonium, such as alkylimidazolium.[30,34]Furthermore, previous results showed that the introduction of hidroxyl in the alkyl chain substituent, as is the case of choline (2-hidroxyethyltrimethylammonium), tends to produce non-toxic salts both for microbial [31]and for mammalian cells strains.[35]

The gram-positives bacteria, including *S. drozdowikzii*, showed low MNTC values to [Ch][CH_3COO] and [Ch][Cl] in relation to imidazolium-based ionic liquids studied in this work. Choline-based cation is structurally similar to quaternary ammonium compounds (quats), which are cationic surfactants with biocide activity. These substances have capacity to attract negatively charged compounds, such as proteins, as well as change the superficial tension, solubilize and denature proteins and disintegrate the cell membrane, being more effective against gram-positives bacteria than gram-negative ones.[36] Since, it was expected that *P. aeruginosa* exhibited high tolerance to choline-based ILs compared to gram-positive bacteria, suggesting a similar mechanism of action of these compounds. In a previous work,[23] the authors also observed the same trend

between *P. aeruginosa* and gram positive bacteria, when evaluated antimicrobial activity of quaternary ammonium choline-based compounds

Table 2 MNTC values relative to water miscible choline and phosphonium-based ionic liquids.

Microorganisms	MNTC (mM)		
	[Ch][CH_3COO]	[Ch][Cl]	[P_{4441}][$MetSO_4$]
Gram-positive bacteria			
Bacillus subtilis	24,5	28	< 3,75
Lactobacillus delbrueckii subsp. delbrueckii	24,5	112	8
Actinobacteria			
Streptomyces drozdowikzii	24	112	< 3,75
Gram-negative bacteria			
Pseudomonas aeruginosa	98	448	15
Yeast			
Saccharomyces cerevisiae	98	224	1.95
Yarrowia lipolytica	196	448	7.8
Kluyveromyces marxianus	98	224	< 3,75
Filamentous fungi			
Aspergillus brasiliensis	196	448	8
Rhizopus oryzae	392	>896	8

associated with chloride anion.

For filamentous fungi and yeasts, [Ch][CH_3COO] and [Ch][Cl] determined high MNTC's, being less inhibitory to *Y. lipolytica, A. brasiliensis* and *R oryzae.* The cell wall of many fungi contains chitin, a polymer of N-acetylglicosamine that form microfibrils with a crystalline structure. Due to crystallinity, chitin is one of the most insoluble natural substances.[37] The content of chitin in the cell wall of fungi used in this study are estimated in 1% to 3 % for yeasts *K. marxianus* and *S. cerevisiae;*[38] about 15 % for *Y. lipolytica*;[39] and has been reported in the literature a content ranging from 7 % to 26 % for *Aspergillus* species and 3% to 8 % for *Rhizopus* species.[40] It may be suggested that highest content of this crystalline substance in the cell wall of *Y. lipolytica, A. brasiliensis* and *R. oryzae* made them more resistant to ILs dissolution. In addition to this, some results about the ability of ILs to dissolve chitin have been reported for imidazolium cation associated with chloride, acetate[41] and bromide anions,[42,43] submitted at extreme conditions of temperature (100-110 ºC) and high ILs concentration. In general, ILs with chlorides and bromide anions are not efficient in chitin dissolution,[41,42] unlike acetates, which seems to be more effective in form clear liquids.[41,44] It have been suggested that weak acids, as acetate, characterized by basicity, in other words stronger hydrogen bonding acceptability, interact with the H-bond of the chitin destroying their compact crystal structure.[41] This was confirmed with choline-based ILs, wherewith was possible to note a decreased of MNTC values relative to [Ch][CH_3COO] when compared to [Ch][Cl] in all cases, confirming the possible association with the role of chitin in resistance to ILs effect.

In another case, also was observed the effect more toxic of acetate anion, [C_2mim][CH_3COO],when compared with the same cation associated with chloride anion, [C_2mim][Cl], on *S. cerevisiae* growth.[45] Other authors also observed that between seven anions, among them acetate and chloride compounding pyridinium-based ionic liquids, acetate was more inhibitory than chloride for the growth of diverse microorganisms.[46]

The phosphonium cation, even though was less toxic than imidazolium one for yeasts, showed low MNTC values in all cases. After resazurin addition in the medium, it was observed immediate change of color in the first four wells, even without growth. Since resazurin is a redox indicator, this change of color can be attributed to oxidative stress, which may have contributed to growth inhibition.

Hydrophobic ILs

Hydrophobic ionic liquids with [NTf_2] anion were, in general, more toxic to microorganisms, noting the important role of anion on toxicity of ILs (Table 3). Both cations [C_2mim] and [Ch] presented discrepant results when associated with another anions, e.g. gram-positive bacteria which tolerated concentration up to 200mM when [C_2mim] was associated with others hidrophilic anions, on the other hand [C_2mim][NTf_2] inhibited the growth of it. Different results were observed with the gram-negative bacteria *P. aeruginosa*, which was able to tolerate high concentrations of [NTf_2] based ionic liquids used in this work. The mechanism of action related to this result is unknown, however it have been known that gram-negative bacteria, particularly species of gender *Pseudomonas*, dispose of diverse tolerance mechanism to organic solvents,[47] which could also act in this situation.Gram-negative bacteria has an outer membrane, in contrast with gram-positive bacteria, which constitutes a semipermeable barrier to the uptake of antibiotics and substrate molecules, and this is especially true to *P. aeruginosa.*[48]Furthermore, this bacteria also appears to have a low permeability toward hydrophobic solutes, as reported at literature.[49]

Table 3 MNTC values relative to immiscible water ionic liquids.

Microorganisms	MNTC (mM)		
	[C_2mim][NTf_2]	[C_4mim][NTf_2]	[Ch][NTf_2]
Gram-positive bacteria			
Bacillus subtilis	< 5	< 4,7	10
Lactobacillus delbrueckii subsp. delbrueckii	< 5	< 4,7	< 5
Actinobacteria			
Streptomyces drozdowikzii	< 5	< 4,7	< 5
Gram-negativebacteria			
Psedomonas aeruginosa	10	300	74
Yeast			
Saccharomyces cerevisiae	< 2,5	< 2,5	10
Yarrowia lipolytica	10	5	37
Kluyveromyces marxianus	< 2,5	< 2,5	< 2,5
Filamentousfungi			
Aspergillus brasiliensis	160	150	74
Rhizopus oryzae	20	> 300	74

In agreement with data to hydrophilic ILs, the yeasts *S. cerevisiae* and *K. marxianus* did not grow in the presence of hydrophobic imidazolium-based ionic liquids, suggesting that the imidazolium cation is quite toxic to these microorganisms. To support this hypothesis, *S. cerevisiae* had growth, though in low concentration (10 mM), in contact with [Ch][NTf_2]. Some works have been asserted that the anion portion does not play a significant role in the ionic liquid toxicity.[17,50] However, other works,[30,45,51] in addiction to results obtained in this work, show that the ionic liquid toxic effect also depends of anion structure. In previous studies performed with *S. cerevisiae*,[8] the IL [C_4mim][NTf_2], in contrast with results showed in the present work, was considered as biocompatible at 20% (v/v). However the initial inoculum concentration ($OD_{600\ nm}$ = 1), in addition to the criteria of growth determination, was different. As discussed in literature, contrasting results can be explained by different biomass concentrations[2].

These salts inhibited mainly bacteria growth. This trend can be asserted in a work where the toxic effect of [$C_n$mim][NTf_2] (n=2-8) toward the bacteria *Escherichia coli* was evaluated, and this strain could not grow in the presence of 2% of all these salts.[31]

In the present work, it was demonstrated a trend of increase in the toxicity of [NTf_2]-based ionic liquids, when associated with short alkyl chain in quaternary ammonium cation, in other words, more water miscible. It was opposite with the idea of as

short is the cation alkyl chain, less toxic is the IL. However it is suggested that due to the presence of a hydrophilic cation, migration of anion to aqueous phase may be facilitated, increasing the toxic effect.[31] As a way of error decrease, during the transfer of IL from stock solution to the medium was used dimethylsulfoxide (DMSO) as detergent to homogenize the solution. Thus there was an alteration in IL solubility in the medium, though it was not possible to obtain a totally homogeneous solution. Therefore, the toxicity of ILs water immiscible could be enhanced by DMSO addition, since this substance provided the interaction of [NTf_2]anion with the aqueous medium as well as with the microorganisms. Another important observation was the formation of a precipitate in the assay realized with *L. delbrueckii* and the hydrophobic ILs. This could be a result of interaction between MRS medium, which was used only to this bacteria, and the ILs, causing precipitation of medium components. More studies are needed to evaluate the influence of this event.

Toxic effect of [NTf_2] anion also have been reported in a work where the anions [NTf_2], [PF_6], [BF_4], trifluoroacetate [CF_3COO] and methane sulfonate [OMS], exhibited, in this order, reduction of inhibition intensity, being [NTf_2] considered the more toxic anion to bacteria *Clostridium* sp.[52] This inhibitory effect was linearly related with the number of fluorine atoms presents in the anionic portion, [NTf_2], [PF_6] with 6, and the other with 3 and 4 atoms, respectively, except [OMS], which have not fluorine in the molecule.[52]

Even though the toxic effect reported for this anion, filamentous fungi were quite tolerant to ILs with [NTf_2], which makes them promising for future investigations in processes where the use of water immiscible solvents is required, as in the case of extractive fermentation. The use of filamentous fungi species as a model for the investigation of eukaryotic organism toxicity of ionic liquids is quite recent. The first work with this claim was published in 2009, where the tolerance of ten *Penicillium* species at 16 ILs among them some used in the present study, such as [C_2mim][Cl], [C_2mim][$EtSO_4$], [Ch][Cl] e [Ch][NTf_2].[51] The authors fixed a concentration of 50 mM, as a high value according to them, and those species which grew were considered as quite resistant. Making a comparison on the some unit with results described on Tables 1, 2 and 3, *A. brasiliensis* and *R. oryzae* were not inhibited at even higher concentrations in contact with ILs [C_2mim][Cl] (426 mM e 852.5 mM), [C_2mim][$EtSO_4$] (540 mM), [Ch][Cl] (448 mM e 896 mM) and [Ch][NTf_2] (74 mM), showing the potential of application ILs in process involving these group of microorganisms.

Hydrophobic ionic liquids usually studied are those compounded by [NTf_2]and [PF_6]. The first have been considered toxic to organisms, and the last one is known for hydrolyzing in water generating hydrofluoric acid (HF). Due to this, the application of this kind of ILs could not be a good idea in bioprocesses. Recent study [53] sought direct the design of ionic liquids assembling low toxicity and high hydrophobicity, evaluating the effects on growth of different trophic level organisms, such as *Vibrio fischeri* (bacteria), *P. subcapitata* (marine algae) and *D. magna* (crustacea). The authors of this work observed that is possible to decrease the toxic effect of the anions, when cations more benign are used. In this case, the non-aromatic cyclic cation piperidinium and pyrrolidinium which increased the hydrophobicity and decreased the toxicity.[53] Choline cation also can be a good choice (Table 3), especially considering its biocompatibility and low costs.

Experimental

Ionic liquids

1-ethyl-3-methylimidazolium chloride [C_2mim][Cl], 1-ethyl-3-methylimidazolium ethylsulfate [C_2mim][$EtSO_4$], 1-ethyl-3-methylimidazolium ethylsulfonate [C_2mim][$EtSO_3$], choline acetate [Ch][CH_3COO], choline chloride [Ch][Cl],tributylmethylphosphoniummethylsulfate [P_{4441}][$MetSO_4$], 1-ethyl-3-methylimidazolium bis(trifluoromethylsulfonyl)imide [C_2mim][NTf_2], 1-butyl-3-methylimidazolium bis(trifluoromethylsulfonyl)imide, [C_4mim][NTf_2] were provided by Isabel M. Marrucho (ITQBq) Universidade Nova de Lisboa, Portugal, and choline bis(trifluoromethylsulfo-nyl)imide [Ch][NTf_2] was purchased from Iolitec with (purity >99%).

Strains and growth media

The following strains were used in this study:bacteria *Pseudomonas aeruginosa* ATCC 9027 and *Bacillus subtilis* ATCC 6633 were maintained in brain heart infusion (BHI) agar, lactic acid bacteria *Lactobacillus delbrueckii* subsp. *Delbrueckii* ATCC 9649 was routinely cultured in deMan, Rogosa and Sharpe (MRS) agar, and actinobacteria *Streptomyces drozdowiczii* M7a in a yeast extract-malt extraxt medium. [54] The fungal strains: *Saccharomyces cerevisiae* ATCC 2601, *Yarrowia lipolytica* IMUFRJ 506822 (Instituto de Microbiologia/Universidade Federal do Rio de Janeiro), *Kluyveromyces marxianus* IMUFRJ 508152, *Aspergillus brasiliensis* ATCC 16404, *Rhizopus oryzae* UCP 15063 (Universidade Católica de Pernambuco) were kept in Sabouraud agar.

Microdilution method

The microdilution method used to determine the maximum non toxic concentration (MNTC) was based on international standard methodology M27-A2 (yeast),[55] M38-A (filamentous fungi),[56] M7–A6 (bacteria),[57] M24-A2 (actinobacteria),[58] M45 A (fastidious bacteria) [59] CLSI/NCCLS (Clinical and Laboratory Standards Institute). The MNTC assay was realized in 96-well plates by eight successive dilutions (1:2) of a stock solution at 500 mg/mL of ionic liquids in 100 µL of culture medium: Müeller Hinton for bacteria and actinobacteria, MRS broth for lactobacilli and RPMI-MOPS pH 7.2 for fungi. Wells were inoculated with 10 µL of bacterial suspension or 100 µL of fungal suspension. The microplates were incubated overnight at 37º C for bacteria, at room temperature (28-30ºC) for actinobacteria; and during 48 h at room temperature for fungi. Pure medium was used as the negative control, and positive controls comprised inoculated growth medium. After incubation time the determination of MNTC was based on visual growth (turbidity), which was confirmed with 30 µL of resazurin (Sigma-Aldrich) added aseptically to the microplate wells and incubated at 37 °C for 1 h. MNTC values were expressed as average of at least two independent assays, performed in triplicate, as showed in Figure 1.

The hydrophobic ionic liquids ([C_2mim][NTF_2], [C_4mim][NTF_2], [Ch][NTF_2]) were diluted in dimethyl sulfoxide (DMSO) in order to allow pipetting a homogenous aliquot from stock solution.Where the highest concentration of DMSO used, at the first well of microplate, was 4 % for bacteria and 2 % for fungi. In order to assure the non-influence of this solvent on obtained results was realized a control with the concentration of DMSO used in the assayand in all cases the microorganisms showed growth in the applied concentrations.

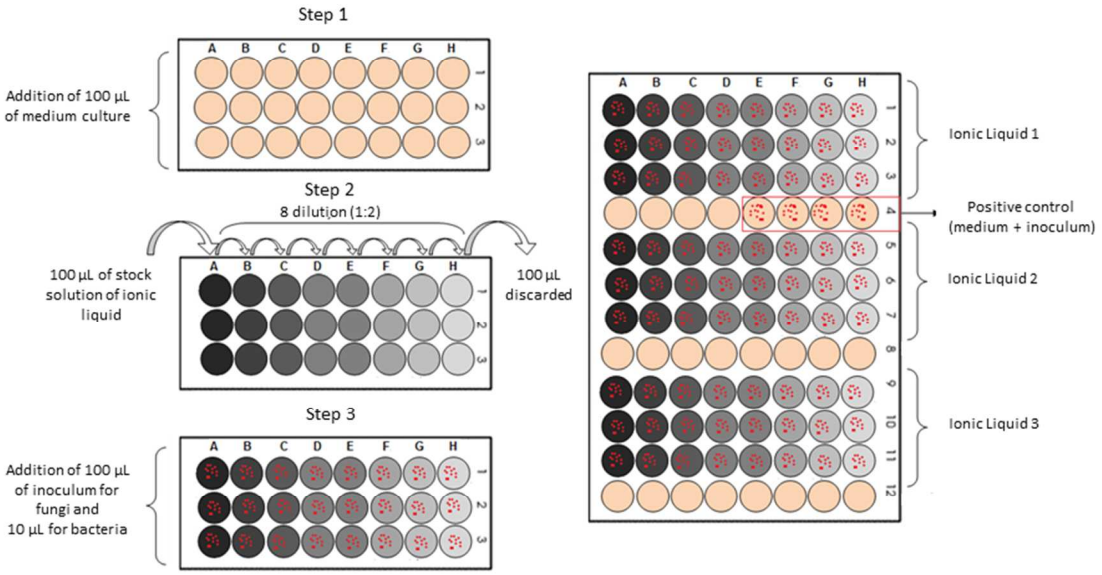

Figure 1 Scheme of MNTC determination assay

Conclusions

By using the microdilution test, it was possible determine the toxicity of some ionic liquids toward a range of microorganisms, some of them tested for the first time, with a qualitative and quantitative result. Choline cation was promising to be used in a processes involving microorganisms, since it exhibited low toxicity to most microorganisms. Filamentous fungi herein tested were in general the more tolerant microorganisms to ionic liquids, followed by the yeast *Yarrowia lipolytica*. The anion bis(trifluoromethylsulfonyl)imi-de [NTf_2] was quite toxic for the tested microorganisms. Thus the study of other anions to obtain hydrophobic ionic liquids is interesting to application of them in biphasic systems of fermentation.

Therefore this study has revealed the promising application of biocompatible ionic liquids in process involving microorganisms in biochemical's production.

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Notes and references

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