Exploring fungal activity in the presence of ionic liquids

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In this work, the toxicological assessment towards filamentous fungi (*Penicillium* sp.) as model eukaryotic organisms of sixteen ionic liquids (containing an imidazolium, pyridinium, or cholinium cation) is presented. Amongst these fungi are members which show much higher tolerance towards ionic liquids than any other microorganism so far studied. Furthermore, guided by the paradigm that the choice of an ionic liquid as catalyst can alter the outcome of a given chemical reaction, the ability of ionic liquids to alter the metabolic profile in fungi was studied. The metabolic footprint, as investigated by electrospray ionisation mass spectrometry, revealed that fungal cultures respond to specific ionic liquids by changing their cell biochemistry, resulting in an altered pattern of secondary metabolites.

Introduction

In the last two decades, ionic liquids have attracted a lot of scientific and commercial interest, as demonstrated in numerous publications and patents. They are, by definition, salts that are liquid at, or near, room temperature, completely composed of ions.[1,2] Their negligible vapour pressure and usual non-flammability are the basis for them sometimes being classified as "green" solvents.[3] In addition, ionic liquids can be, by design, chemically and thermally stable, recyclable, and with tunable physical and chemical properties. Integration with their outstanding solvation ability opens doors for numerous industrial applications as replacements for conventional organic solvents.[1] Further to remarkable developments in their core chemical properties and applications, ionic liquids are now providing unexpected opportunities at the interface of chemistry with the life sciences, *e.g.* acting as solvents in enzymatic[4] and whole-cell[5] biocatalysis. In order to move ionic liquids beyond being an academic curiosity, their environmental, health, and safety impacts must be fully determined.[6] Although this field is still in its infancy, significant efforts are being made to obtain ecotoxicological data and design ionic liquids composed of naturally-derived materials for reduced toxicity and increased biodegradability.[7] Reports that ionic liquids can be used as active pharmaceutical ingredients (APIs) serve to further emphasise their potential for *in vitro* biochemical studies.[8,9]

Filamentous fungi are significant members of the Mycota kingdom, widespread in nature, especially in soil.[10] They play an important role in the carbon cycle on Earth, food spoilage, and as pathogens. On the other hand, due to the enormous diversity of species and their rich enzymatic systems, resulting in a broad range of secondary metabolites, they are widely used in biotechnological processes for production of chemicals, pharmaceuticals, food ingredients, and enzymes.[11] Many secondary metabolites of filamentous fungi have found application as therapeutic and/or bioactive compounds (*e.g.* antibiotics, cholesterol-lowering agents, anti-tumour agents, and immunosuppressors).[12,13] Their ability to adapt to extreme environmental conditions and to degrade some of the most recalcitrant materials (such as lignin and aromatic xenobiotics) makes them a very attractive model for screening the toxicity of chemicals with an unknown risk factor. In this work, for the first time, filamentous fungi were used as model eukaryotic organisms to assess the possible impact of ionic liquids whenever they are released into the environment.

Metabolic profiling using mass spectrometry (MS) has become one of the most important techniques in fields ranging from diagnostics to functional genomics,[14] from taxonomy of filamentous fungi,[15] to screening of new compounds for biological activity. Here, metabolic footprinting is focussed on qualitative scanning of metabolites secreted by the cells. Components of the substrate that were transformed by the organism are also a part of the footprint.[14]

The cell chemodiversity strongly depends on inherited phylogenetic information (genomics), as well as on environmental conditions. Here, a relationship is proposed between the phylogenetic origin of fungal species, their specific response to the presence of ionic liquids, and the modification of the metabolic profile caused by it.

Experimental

Chemicals

All ionic liquids used in this study (see Fig. 1) were prepared by QUILL (Queen's University Ionic Liquids Laboratory, Belfast, UK), except for cholinium chloride (Sigma, Germany), 1-ethyl-3-methylimidazolium thiocyanate, [C_2mim][SCN]

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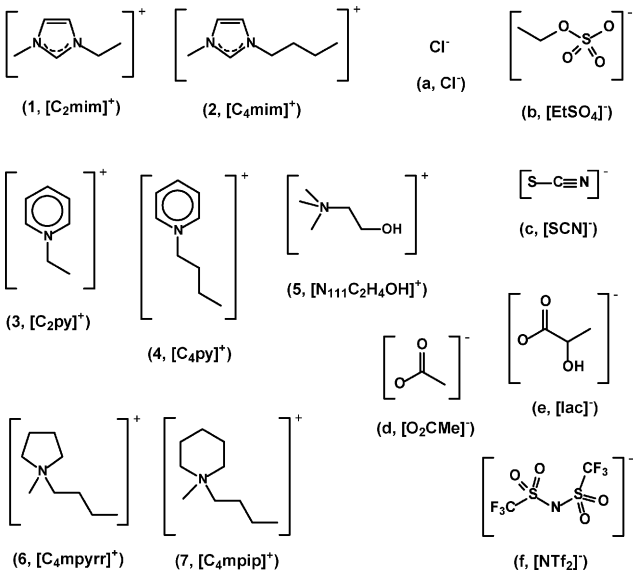

Fig. 1 Chemical structures of all ionic liquids used (cations and anions, numbers and letters, respectively). (1a) 1-ethyl-3-methylimidazolium chloride ([C_2mim]Cl); (1b) 1-ethyl-3-methylimidazolium ethyl sulfate [C_2mim][$EtSO_4$]); (1c) 1-ethyl-3-methylimidazolium thiocyanate ([C_2mim][SCN]); (1d) 1-ethyl-3-methylimidazolium ethanoate ([C_2mim][O_2CMe]); (1e) 1-ethyl-3-methylimidazolium DL-lactate ([C_2mim][lac]); (2a) 1-butyl-3-methylimidazolium chloride ([C_4mim]Cl); (4a) 1-butylpyridinium chloride ([C_4py]Cl); (3d) 1-ethylpyridinium ethanoate ([C_2py][O_2CMe]); (3e) 1-ethylpyridinium DL-lactate ([C_2py][lac]); (5a) cholinium chloride ([$N_{111}C_2H_4OH$]Cl); (5d) cholinium ethanoate ([$N_{111}C_2H_4OH$][O_2CMe]); (5e) cholinium DL-lactate ([$N_{111}C_2H_4OH$][lac]); (5f) cholinium bis((trifluoromethyl)sulfonyl)amide ([$N_{111}C_2H_4OH$][NTf_2]); (6a) 1-butyl-1-methylpyrrolidinium chloride ([C_4mpyrr]Cl); (6e) 1-butyl-1-methylpyrrolidinium DL-lactate ([C_4mpyrr][lac]); (7d) 1-butyl-1-methylpiperidinium ethanoate ([C_4mpip][O_2CMe]).

(Fluka, Switzerland) and 1-ethyl-3-methylimidazolium ethyl sulfate, [C_2mim][$EtSO_4$] (Solvent Innovation, Germany). D(+)-glucose, $K_2[HPO_4]$, $ZnSO_4{\cdot}7H_2O$, $CuSO_4{\cdot}5H_2O$, $FeSO_4{\cdot}7H_2O$, KCl, and ethyl ethanoate were purchased from Sigma Aldrich (Germany). CsCl, $MgSO_4{\cdot}7H_2O$, $NaNO_3$, 1-methylimidazole, and pyridine were purchased from Fluka (Switzerland). NaCl was purchased from Panreac (Spain), and ethanenitrile from Merck (Germany).

Ionic liquids prepared at QUILL were characterised by a combination of ^{1}H NMR, ^{13}C NMR, electrospray ionisation mass spectrometry, halide content, CHN elemental analysis, and water content. Typical halide content was <0.1%, and the maximum amount in one sample was 1.2%.

Fungal isolates

The following fungal isolates were used, all belonging to the Instituto de Biologia Experimental e Tecnológica (IBET) culture collection: *Penicillium brevicompactum* Dierckx (IBETPeA), *P. olsonii* Bainier and Sartory (DSM 16515), *P. janczewskii* K.M. Zalessky (IBETF5), *P. glandicola* (Oudem) Seifert and Samson (IBETPeB), *P. corylophilum* Dierckx (IBETF6), *P. glabrum* (Wehmer) Westling (DSM 16516), *P. restrictum* J.C. Gilman and E.V. Abbot (IBETF4), *P. adametzii* Zaleski (IBETF2), *P. variabile* Sopp (IBETF2), *P. diversum* Raper and Fennell (IBETPeE). They had previously been isolated from cork samples purchased from several Portuguese cork industries.[16,17]

Toxicity tests

The toxicity of ionic liquids towards filamentous fungi was determined by measuring the absorbance of the culture medium at 600 nm.

The minimal culture medium containing glucose (1.0 g l^{-1}), K_2HPO_4 (1.0 g l^{-1}), $NaNO_3$ (3.0 g l^{-1}), $ZnSO_4{\cdot}7H_2O$, (0.01 g l^{-1}), $CuSO_4{\cdot}5H_2O$ (0.005 g l^{-1}), $MgSO_4{\cdot}7H_2O$ (0.5 g l^{-1}), $FeSO_4{\cdot}7H_2O$ (0.01 g l^{-1}), and KCl (0.5 g l^{-1}) was dissolved in distilled water and sterilised in an autoclave (20 min; 121 °C), and supplemented with ionic liquids in order to obtain a final concentration of 50 mM (corresponding to 7–20 g l^{-1}, depending on the molecular weight of the ionic liquid).

Each liquid medium (2 cm^3) was inoculated with a suspension of fungal spores, prepared as previously described,[18] in order to obtain the final concentration of 10^5 spores per cm^3, and divided into eight wells (0.25 cm^3 each) of a 96 well microtitre plate. Cultures were incubated in the dark at 25 °C.

The control samples (inoculated) and the blanks (non-inoculated) were produced and incubated under the same conditions: negative control (an ionic liquid free medium), osmotic stress controls (media supplemented with either aqueous NaCl (50 mM) or aqueous CsCl (50 mM): high charge density and low polarisability, or low charge density and high polarisability, respectively), organic function controls (media with aqueous 1-methylimidazole (50 mM) or aqueous pyridine (50 mM)), and blank samples (media with addition of the selected substances).

Fungal growth (or lack thereof) was followed daily by measuring the absorbance (600 nm) of the medium. Increase of absorbance was taken as indication of growth. Spore formation (gauged by eye) and/or the absorbance approaching a constant value indicated that the culture entered a stationary growth phase. The culture supernatant liquid was separated from the mycelium by centrifuging (4 °C) and filtration (0.2 μm nylon membrane), and stored at −20 °C awaiting further analysis.

Toxicity data analysis

The toxicity data were analysed using scripts written in the R language, version 2.7.1 (for review see ref. 19 and 20). The relation between any two toxicity profiles was quantified by the Jaccard distance, which measures dissimilarity between sample sets consisting of binary data. The obtained distance matrix was used to assess the existence of groups of fungal species with similar toxicity profiles by means of hierarchical cluster analysis (HCA). Results are presented based on Ward's method for cluster linkage, which is designed to minimise the variance of distances within each cluster.[20] The robustness of the resulting dendrogram was validated using several other linkage methods as well. Given the binary nature of the data (growth or its inhibition), the distance measured between species is equivalent to the number of different cases between two fungal species.

Metabolites extraction

Fungal secondary metabolites (fSM, ionic liquid free) were extracted from the fungal cultures. The mycelium-free cultures (1 cm^3) were lyophilised (freeze-dried) in order to remove water, and ethyl ethanoate (1 cm^3) was added to the residue. Ethyl ethanoate was selected, due to its common use for extraction of fSM and to its limited miscibility with the ionic liquids studied. The upper layer (ethyl ethanoate) contains the fSM. This extraction was repeated once more and the combined ethyl ethanoate extracts were evaporated in a vacuum concentrator. The residue was dissolved in ethanenitrile (0.4 cm^3) and ultrasonicated (10 min). The same procedure was applied on blank samples previously prepared (see Toxicity Tests).

ESI-MS analysis

The electrospray mass spectra were recorded on a Bruker Esquire 3000 plus ion trap mass spectrometer in the positive and negative polarity modes (Bruker Daltonics, Billerica, MA, USA). The samples (fSM in ethanenitrile) were injected at a rate of 0.1 cm^3 h^{-1} into the electrospray ionisation (ESI) probe. The capillary temperature was set to 250 °C. All spectra were acquired using the Esquire Control Programme (Bruker Daltonics, Billerica, MA, USA) and then converted to ASCII file format for computational interpretation.

Computational interpretation of MS data

Mass spectrometry data were analysed with the same software as the toxicity data. After binning the intensities of mass spectra to integral m/z values, spectra pre-processing, peak detection and alignment of the spectra were performed using the msProcess package.[21] Peak intensities were normalised by their total, and values below 0.001 were set to zero. Peaks of the sample spectra were compared with the spectra from corresponding solvents and blanks, and coinciding peaks were eliminated from the peak matrix. To eliminate ionic liquid cations or anions with $z = 1$, only m/z values between 150 and 1100 were considered in the analysis. Ionic liquid solubility in ethyl ethanoate (see Metabolites Extraction) is very limited but its nature may lead to an intense m/z peak even at a negligible concentration. The resulting binary matrices, peaks present or absent, were further analysed by HCA in an analogous manner to the toxicity data.

Results and discussion

Toxicological assessment

In this work, an examination of the toxicity of ionic liquids to filamentous fungi, belonging to the *Penicillium* genus, is presented. The selection of sixteen ionic liquids as test compounds was made by combining seven different cations and six different anions (see Fig. 1). This approach enabled us to investigate *inter alia* the toxic effect of the head group in the cation. Focussing on chloride, ethanoate, and DL-lactate anions (herein depicted as lactate) enabled the toxic effect of the anion to be studied.

In order to prove that the ionic liquids are not causing a toxic effect solely due to osmotic stress, the effects of sodium chloride and caesium chloride were tested. The latter loosely approximates the low-charge density and high polarisability of ionic liquids. Its addition to the growth medium has inhibited fungal growth (except for the case of *P. adametzii*), causing a stronger growth inhibitory effect than all the tested ionic liquids. These data were not used in the hierarchical clusters presented (see Fig. 2 and 3), but their inclusion does not alter the cluster profile. 1-Methylimidazole and pyridine, as commonly used building blocks in ionic liquid chemistry, were also tested to assess whether their unsaturated nature had a deciding role to play in the antifungal activity of the ionic liquids.

The HCA of the inhibitory effect of twenty selected compounds at 50 mM upon the growth of ten *Penicillium* species is illustrated in Fig. 2. The ionic liquid growth inhibitory effect was simplified to a binary matrix of fungal growth inhibition or no inhibition. It became evident that the chemical nature of the cationic head group influenced the overall toxicity of the ionic liquid, which has been demonstrated in numerous other studies.[22,23,24,25,26]

The imidazolium-based ionic liquids have the highest toxicity rankings of those studied, with an average of three toxic cases. Comparison of $[C_2mim]Cl$ and $[C_4mim]Cl$ showed the expected tendency[25] of increased toxicity with increasing alkyl side chain length. The $[N_{111}(C_2H_4OH)]^+$ cation elicited the lowest toxic effect, and choline chloride, $[N_{111}(C_2H_4OH)]Cl$, failed to inhibit growth in any of the fungal species. With the exception of $[C_2py][lac]$, which showed no toxicity, the remaining ionic liquids (containing pyridinium, pyrrolidinium, or piperidinium cations) lay in between these extremes.

The control compounds, 1-methylimidazole and pyridine, inhibited growth in 100% and 60% of the cases, respectively, proving to be significantly more toxic to fungi than any of their derived ionic liquids (studied herein).

Within the two groups of ionic liquids containing $[C_2mim]^+$ or $[N_{111}(C_2H_4OH)]^+$ combined with Cl^-, $[O_2CMe]^-$ or $[lac]^-$, the $[O_2CMe]^-$ anion was the most toxic. None of the other ionic liquids tested showed any significant correlation with the nature of the selected anion. This suggests that a more extensive study would be of great value. Here, it is noted that, while the cation has the more predictable effect, the anion also contributes toward the antifungal activity (*cf.* with rat leukaemia cell line).[24]

The HCA of the toxicity profiles (Fig. 2) revealed four groups of fungal species with similar growth behaviours. The group {A, B} showed the highest ionic liquid susceptibility. The group {C, D} showed intermediate, and the remaining groups {E, F, G} and {H, I, J} low susceptibility. It was interesting to compare and correlate these data with the clusters in a phylogenetic study on *Penicillium* species by Wang and Zhuang, which they based on calmodulin gene partial sequences (about 600 nucleotides).[27] Their study distinguished eleven clusters and covered all the species featured in our work, except {A} and {I}, and a high degree of correlation between the phylogenetic background of the species and their response to the ionic liquid environment was observed (see Table 1).

This detected correlation between the genetic proximity of the species and their susceptibility to different environmental conditions can be used in the rationalisation of toxicological studies, and in the prediction of the behaviour of different species.

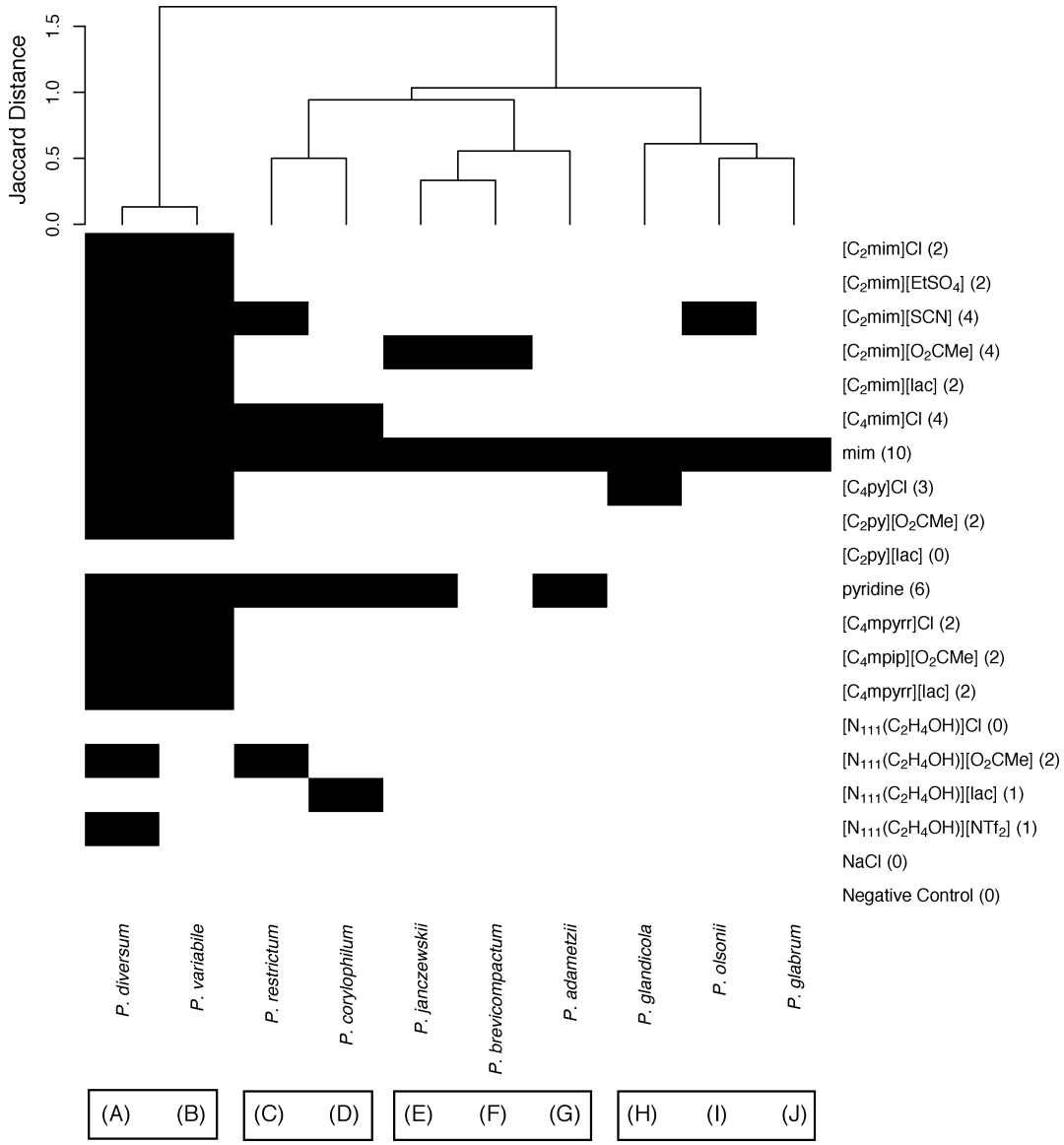

Fig. 2 Hierarchical cluster analysis of the growth behaviour of *Penicillium* species in the ionic liquid containing media and controls (see Experimental section for details). Black fields show cases of growth inhibition; row labels indicate the tested ionic liquids (ordered by common cation group) and control substances; the number in brackets corresponds to the toxicity ranking (from 0 to 10).

Table 1 Comparison of the results of this work with those of Wang and Zhuang[27]

Toxicity study (this work)	Phylogenetic study
{B}	{XI}
{C, D}	{VIII}
{E, F, G}, {H, J}	{I, II}

Metabolic footprinting

In order to assess the ability of ionic liquids to alter the metabolism of fungi, fungal culture extracts (fSM extracts, ionic liquid free) were analysed by ESI-MS, the spectra being interpreted qualitatively according to the presence or absence of peaks.

The selection of samples (combinations of fungal species and ionic liquids) was made based on the analysis of the toxicological data presented above. The species {B}-{H} were used, including one or more species from each cluster. The ionic liquids, $[C_2mim]Cl$, $[C_2mim][lac]$, $[C_4py]Cl$, $[C_2py][lac]$, $[N_{111}(C_2H_4OH)]Cl$ and $[N_{111}(C_2H_4OH)][lac]$, were selected according to Fig. 2, comprising all cationic groups which have shown distinct effects, imidazolium and cholinium, as the most and the least toxic, respectively, and pyridinium, with intermediate toxic effect. In addition, negative and sodium chloride controls were included.

After growth, extraction of fungal metabolites, and collection of their ±ESI-MS spectra, the HCA of these "metabolic footprints" for all ionic liquids tested per species showed no discernable pattern (data not shown). However, the metabolic footprints induced by $[C_2mim]Cl$ and $[C_2mim][lac]$ were

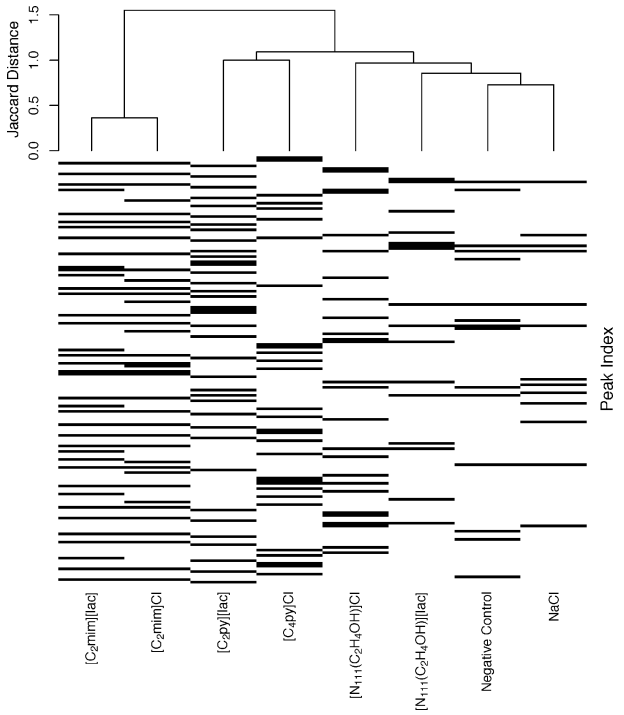

Fig. 3 Hierarchical cluster analysis of the joint peak lists, uniting all individual peak matrices of positive and negative mode, detected by ESI-MS after fungal growth in the ionic liquid containing media and controls (see Experimental section for details). Black fields indicate the presence of a peak at a given m/z value.

clustered together for all fungal species, and were disassociated from the metabolic footprints induced by the other ionic liquids (data not shown). This observation is confirmed in the integrated assessment of the joint peak lists, uniting all individual peak matrices (depicted in Fig. 3), and this result was robust against variations of the clustering algorithm. The remaining metabolic footprints produced a single cluster divided into two subclusters: those induced by $[C_4py]Cl$ and $[C_2py][lac]$, and those induced by $[N_{111}(C_2H_4OH)]Cl$, $[N_{111}(C_2H_4OH)][lac]$ and controls. In the latter, the metabolic footprints induced by the cholinium-based ionic liquids showed weak correlation with controls and between themselves.

Distinct groups are also revealed when one merely accounts for the number of the peaks in the metabolic profiles. The correlation between the number of detectable mass species in the extracts and the toxicity of the corresponding ionic liquids is apparent. $[C_2mim]Cl$ and $[C_2mim][lac]$ induced the highest number of peaks (≥38), significantly different from the controls (≤15). Interestingly, despite the fact that $[C_2py][lac]$ and $[N_{111}(C_2H_4OH)]Cl$ showed no inhibition to fungal growth, both have still induced significant metabolic alterations.

In order to provide a more complete picture of the specific events in the metabolic network (species and ionic liquid dependency), quantitative analysis will be attempted in the future.

Conclusion

This is the first time that filamentous fungi have been used in a toxicological study on ionic liquids. The most significant observation is the very high tolerance of *Penicillium* species towards the ionic liquids. Complete inhibition of growth was noted in only 20% of ionic liquids/species cases. The concentration of ionic liquids that was tested in this study (50 mM) is much higher, by several orders of magnitude, than in any other testing system reported so far.[22,26] In addition, we have observed that fungi can tolerate, in some cases, even higher ionic liquid concentrations, *e.g. P. olsonii* can grow in the presence of 0.375 M of $[C_2mim]Cl$ (data not shown).

Testing a vast number of ionic liquids and organisms is rather time-consuming and costly, but the cluster strategy presented here, along with the concept of QSAR (quantitative structure–activity relationship), which refers to estimation of the hazard potential of ionic liquids based on structure, simplifies their risk evaluation.

These data show that the ionic liquid induced effects on fungal metabolism cannot be simply explained by the ionic liquid toxicity. Moreover, the metabolic footprints induced by the ionic liquids do not correlate with those induced by common salts, such as sodium chloride. Even caesium chloride, in the specific case of *P. adametzii*, clustered closer to the controls than to the ionic liquids. This is supported by preliminary data on the fungal secretome (bidimensional electrophoresis analysis) induced by the different ionic liquids.[28] Results suggest that some ionic liquids, *e.g.* $[N_{111}(C_2H_4OH)]Cl$ and $[C_4mpyrr]Cl$, have induced the expression of a distinct set of fungal extracellular proteins, which could be grouped into ionic liquid responsive, ionic liquid specific or non-specific, and inorganic salt responsive proteins. In addition, these metabolic alterations cannot be, in the case of all ionic liquids, a result of their co- or direct metabolism by the fungi. That would certainly not be the case of imidazolium cation, which is resistant to microbial degradation.[29,30]

The behaviour of fungi, and any other organisms, is highly linked to different omes (genome, transcriptome, proteome and metabolome) and influenced by environmental conditions. Transcriptional profiling and proteomic analysis are becoming routine techniques and, joined with metabolite analysis, could build a platform applicable in many areas. Going beyond ecotoxicological studies to describe the effect of toxic compounds on living cells, could be useful in the discovery of novel natural compounds and in the development of efficient and environmentally friendly bioprocesses.

The test systems described in this work are highly promising, especially considering that filamentous fungi have the ability to produce biologically active secondary metabolites.[13,12] Further studies are necessary to prove whether or not ionic liquids have the ability to induce a tailored metabolic alteration. Should this be observed, it would be a significant breakthrough in whole-cell biocatalysis, creating a new concept: an ionic liquid controlled bio-tool for designing targeted end products.

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