Exploring ionic liquids based on pyrrolidinium and imidazolium cations with low toxicity towards *Escherichia coli* for designing sustainable bioprocesses

F.J. Hernández-Fernández[a,b,c*], A. P. de los Ríos[a,b,c*], P. Licence [b], G. Stephens [c]

[a]Department of Chemical Engineering, Faculty of Chemistry, University of Murcia (UMU), P.O. Box 4021, Campus de Espinardo, E-30100, Murcia, Spain

[b]School of Chemistry, The University of Nottingham, University Park, Nottingham, UK NG7 2RD.

[c]Department of Chemical and Environmental Engineering, The University of Nottingham, University Park, Nottingham, UK NG7 2RD.

*** Correspondence:**

Corresponding Authors: fjhernan@um.es

Keywords: *Ionic Liquid, Toxicity, Escherichia coli, Sustainable material, Biocompatibility*

Abstract

Ionic liquids (ILs) are widely applied in many bioprocesses involving microorganisms due to their unique properties. In this work, the toxicity of imidazolium and pyrrolidinium ionic liquids towards *E. coli*., a bacterium for which there are limited toxicity data in the literature, was determined. For its simplicity, the nephelometry method was used to estimate ionic liquid toxicity values. The influence of the cation and the alkyl chain length of the cation and anion was analysed. Pyrrolidinium cations were seen to be less toxic than imidazolium cations, while an increase in the alkyl chain length of both pyrrolidinium and imidazolium cations increased the toxicity. Among the anions studied, dimethylphosphate ($[Me_2PO_4]$) was the less toxic, while the EC50 for the ionic liquid 1-butyl-3-methylpyrrolidinium dimethylphosphate ($[C_1C_4Pyr][Me_2PO_4]$) was close to 200 mM. Furthermore, a dicationic ionic liquid based on imidazolium and pyrrolidinium cations was synthetized and its toxicity toward *E. coli* was analysed, maintaining a growth rate of 100% in the range 0-0.76 mM. The methodology used in this work allows to easily find the less toxic ionic liquids that are biocompatible with *E. coli* to be used in new bioprocesses.

1. Introduction

The bacterium *Escherichia coli* is crucial in modern biotechnology as it is an important host that is used in the biopharmaceutical industry (Castiñerias *et al*., 2018). *Escherichia coli* is the most used microorganism for expressing heterologous proteins for therapeutic use due to its rapid growth, high productivity and the easy to scale-up processes involved (Baeshen *et al.,* 2015). *E. coli* strain can also be engineered to produce chemical intermediates, such as citramalate (Webb *et al.,* 2018), amino acids, antibiotics, succinic acid, ethanol, L-tryptophan, and, also of novel chemicals, such as 1,3- propanediol, octanoate, indigo and polyhydroxy alkanoate (Balbas *et al.,* 2001). Furthermore, *E. coli*, can be used as a good model system in systems biological studies (Lee *et al.,* 2009).

In biotechnological processes such as those mentioned, the solvent toxicity towards microorganisms is one of the main parameters that must be considered. Conventional organic solvents have been shown to be toxic to microbial cells, causing membrane damage and so decreasing the operational stability of the biocatalyst. Furthermore, organic solvents are toxics and flammable, explosive and bioavailable due to its high volatility. On the other hand, ionic liquids are combinations of cations (e.g., imidazolium, pyrrolidinium, phosphonium) and anions (e.g., hexafluorophosphate, halides, bis[(trifluoromethyl)sulfonyl]imide), which remain liquid at temperatures below 100 °C (de los Ríos et al., 2017). In the context of its use in bioprocess, the most important properties of ionic liquids include their negligible vapour pressure, chemical and thermal stability, non-flammability and their solvent power. ILs are considered friendly alternative solvents to organic solvents, mainly due to their low vapour pressure, which prevents the atmospheric pollution. In addition, their physical and chemical properties can be tailored to specific applications by tuning the cation and/or anion composition of the ionic liquid. For this, ionic liquids are also called green designer solvents. However, considering the wide variety of ion liquid which could be synthesized by different cation and anion combination, we can find different toxicity in them. For that, it would be of great interest to identify rational guidelines for developing bio-technologically suitable but also environmentally harmless ILs.

Very few examples have been reported on the use of ionic liquids with *E. coli*, making it a still unexplored field. In order to improve substrate solubility and to prevent substrate/product enzymatic, $[C_1C_4Im][NTf_2]$ was selected to create a biphasic system with water where *E. coli*-catalysed production of (S)-3-chloro-1-phenyl-1-propanol from 3-chloro-1-phenyl-1-propanone. The substrate and product were dissolved in the ionic liquid phase and *E. coli* stayed in the aqueous phase (Choi *et al.,* 2011). In another example, $[C_1C_4Im][PF_6]$, $[C_1C_4Im][NTf_2]$ and $[OMA][NTf_2]$ were used as substrate reservoir

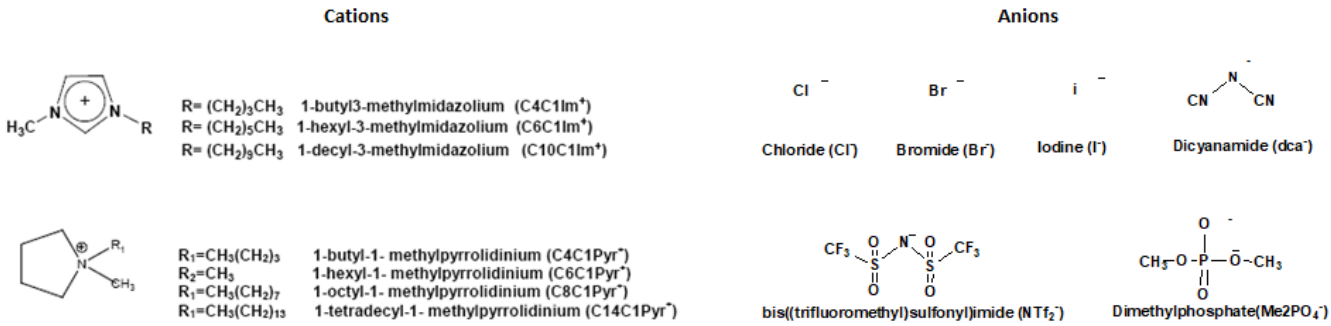
and extracting agent for the asymmetric reduction of ketones. These biphasic systems allowed to increase in chemical yield from <50% to 80–90% in a batch process (Pfruender *et al.*, 2006). As regards the toxicity of ionic liquids towards *E. coli*, Cornmell *et al*. (2008) proposed to use Fourier transform infrared (FT-IR) spectroscopy to study the same, first growing *E. coli* in the presence of $[P_{6,6,6,14}][NTf_2]$, $[N_{1,8,8,8}]$ $[NTf_2]$, $[P_{6,6,6,14}][Cl]$ and $[N_{1,8,8,8}][Cl]$. Bistriflimide-based ionic liquids were classified as biocompatible, while chloride-based ionic liquids were classified as not biocompatible since did not allow any growth. Their FT-IR demonstrated that they were accumulating toxic ionic liquids within the cells more rapidly than the biocompatible ionic liquids. More recently, the toxicity of twelve piperazinium- and guanidinium-based ionic liquids towards *E. coli* were measured by J. Yu *et al*. (2016). The ILs exhibited low toxicity, with minimum inhibitory concentration values ranging from 1.20 to higher than 200 mg mL^{-1}. The ionic liquids based on tetrafluoroborate anion and those with a benzene ring on cation showed the greatest toxicity among the studied ILs. The length of the alkyl chain involved an increase in IL toxicity. Since properties like toxicity can be tuned by modifying cation and anion substituents, ionic liquids of lower toxicity can be designed, for which purpose more toxicity data and chemical design tools are needed.

In this context, this work studies the biocompatibility of 15 ionic liquids containing imidazolium and pyrrolidinium cations combined with different anions with *E. coli*, since few data are available on the toxicity of pyrrolidinium towards *E. coli*. For that, the toxicity of ionic liquids towards *E. coli* MG 1655 was measured by growing experiments in LB medium in the presence and absence of ionic liquid using the nephelometry method. Furthermore, a dicationic ionic liquid based on both imidazolium and pyrrolidinium cation was synthesized and its toxicity toward *E. coli* was studied. The results obtained are discussed in-depth and a qualitative structure-toxicity relationship is established. The key factors for designing biocompatible ILs for biotechnology applications involving *E. coli* were identified.

2. Materials and Methods

2.1. Ionic liquids

The ILs studied are based on imidazolium and pyrrolidinium ionic liquids. Monocationic ionic liquids were supplied by IoLiTec of the highest available purity. The dicationic ionic liquid was synthetized. Figure 1 includes the complete and abbreviated name of the monocationic ionic liquids analysed and their structures.



Figur**e 1. Complete and abbreviated name of the monocationic ionic liquids analysed and their structures.**

2.1.1. Ionic liquids synthesis

Synthesis of 3-(6-bromohexyl)-1-methylimidazolium bromide: [C_1ImC_6Br][Br]

1,6-dibromohexane (42.47 mL, 0.28 moles) was dried over 3 Å molecular sieves, dissolved in dry dichloromethane (75 mL) and added to a dry purged round bottomed flask via a dry purged cannula and stirred. Distilled 1-methylimidazole (7.33 mL, 0.092 moles) was dissolved in dry dichloromethane (25 mL) and added very slowly drop-wise to the reaction via a dry purged cannula. The reaction was heated to 65 °C and stirred for 72 hours. The crude product was purified by column chromatography using an eluent system of 7:1 DCM: methanol. The column was monitored by TLC. The first elution band was identified as unreacted 1,6 dibromohexane by NMR. The second elution band was identified as product. The product was heated at 65 °C under vacuum (6 x 10^{-2} mbar) for 12 hours (18.25 g, 20 %). ^{1}H NMR (270 MHz, Chloroform-d) δ 10.54 - 10.58 (m, 1H), 7.41 - 7.45 (m, 1H), 7.37 - 7.40 (m, 1H), 4.33 - 4.41 (m, 2H), 4.10 - 4.14 (m, 3H), 3.38 - 3.45 (m, 2H), 1.71 - 2.03 (m, 4H), 1.33 - 1.58 (m, 4H)

Synthesis of [1-(1-imidazolium-yl-hexyl)methylpyrrolidinium] dibromide: [C_1ImC_6PyrC_1][Br]$_2$

[C_1Im(C_6Br)][Br] (1.9938 g, 6.08 mmol) was dissolved in acetonitrile (10 mL) and refluxed in a two-neck round bottomed flask. 1-Methyl pyrrolidine (0.57 g, 6.69 mmol) was added very slowly drop-wise to the reaction via a dry purged cannula. The mixture was stirred for 2 days at 60 ºC. The excess of 1-methyl pyrrolidine was removed by rotary evaporation to yield a slightly yellow ionic liquid. The product was kept under vacuum (6 x 10^{-2} mbar) for 5 hours (2.50 g, 99.4 %). ^{1}H NMR (270 MHz, DMSO-d_6): δ 9.21 – 9.44 (m, 1H), 7.80 - 7.89 (m, 1H), 7.72 - 7.78 (m, 1H), 4.09 - 4.25 (m, 2H), 3.83 – 3.90 (m, 3H), 3.44 - 3.65 (m, 4H), 3.26 - 3.38 (m, 2H), 3.01 (s, 3H), 1.93 – 2.23 (m, 4H), 1.55 – 1.93

(m, 4H), 1.00 – 1.55 (m, 4H). ^{13}C NMR (270 MHz, DMSO-d_6): δ 21.62, 23.28, 25.50, 25.75, 29.62, 36.35, 49.11, 49.28, 55.05, 63.29, 63.93, 122.82, 124.15, 137.13.

Synthesis of [1-(1-imidazolium-yl-hexyl)methylpyrrolidinium] di[bis{(trifluoromethane)sulfonyl}imide]: [C_1ImC_6PyrC_1][NTf_2]$_2$

[C_1ImC_6PyrC_1][Br]$_2$ (2.50 g, 6.08 mmol) was dissolved in water (20 mL) and lithium bis {(trifluoromethyl)sulfonyl}imide (1.92 g, 6.69 mmol) was added. The mixture was stirred for 3 days at 40 ºC. The resulting mixture was washed with water (4×10mL) to give the corresponding ionic liquid. The product was kept under vacuum (6 x 10^{-2} mbar) for 5 hours, yielding a slightly yellow ionic liquid (2.0382 g, 41.3 %). ^{1}H NMR (270 MHz, DMSO-d_6) δ 9.10 (s, 1H), 7.73 - 7.79 (m, 1H), 7.68 - 7.73 (m, 1H), 4.09 - 4.23 (m, 2H), 3.85 (s, 3H), 3.38 - 3.68 (m, 4H), 3.20 - 3.32 (m, 2H), 2.96 (s, 3H), 1.99 – 2.12 (m, 4H), 1.56 – 1.89 (m, 4H), 1.18 – 1.42 (m, 4H). ^{13}C NMR (270 MHz, DMSO-d_6): δ 21.63, 23.34, 25.63, 25.88, 29.69, 36.31, 48.04, 49.18, 55.30, 63.46, 63.98, 122.82, 124.24, 137.03. The synthetic route for obtaining the dicationic ionic liquids is shown in Figure 2.

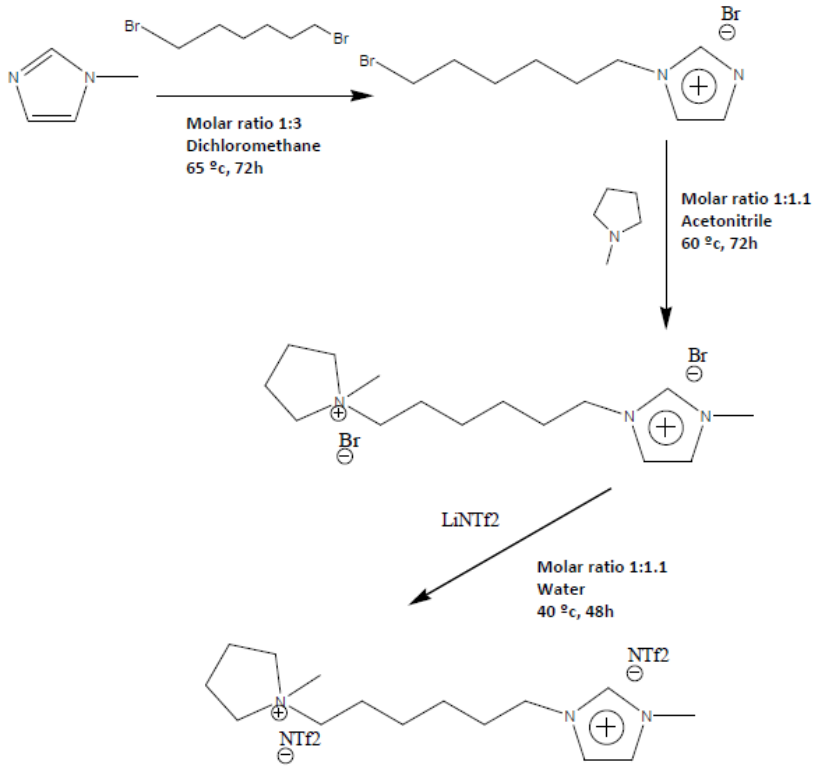


Figure 2. Synthetic route and conditions for obtaining the dicationic ionic liquid $[C_1ImC_6PyrC_1][NTf_2]_2$.

2.2 Solubility of ionic liquids in water

2.2.1. Solubility test

The solubility in water of bis[{trifluoromethyl}sulfonyl]imide-based ionic liquids was measured. For that, 0.1 mL of ionic liquid was coming into contact with 10mL of ultrapure water. The mixture was stirred for 7 days at 25 ºC to facilitate the solubilisation of the ionic liquid in the water. Samples were taken from the aqueous phase in three sampling events occurring over a 4h to 7 days. The composition of the aqueous phase was analysed by ion chromatography, as described in the next section.

2.2.2. Ion chromatography analysis

The concentration of the anion bis[{trifluoromethyl}sulfonyl]imide in aqueous solutions was determined by ion chromatography using a Dionex ICS-3000 instrument equipped with a conductivity detector and a Chromeleon® SE data management software. The chromatographic conditions were as follows: eluent composition, water + NaOH(100mM) + acetonitrile (60:15:25); flow rate, 0.25 mL min^{-1}; column temperature, 40 ºC; detector temperature, 35 ºC; suppressor current 10mA; injection volume, 5 µL. The retention time of the peak was 27.8 min. Ionic liquid concentrations in aqueous solutions were calculated from a calibration curve using stock solutions of lithium bis[{trifluoromethyl}sulfonyl]imide.

2.3. Toxicity analysis by nephelometry

The growth rates of cultures of *E. coli* MG 1655 were measured in 96-well plates in the presence and absence of ionic liquids. A solution containing 50% (v/v) of the ionic liquid in MilliQ water was prepared. The solution was then serially diluted in milliQ water and aliquots of 40 µl were added to

96-well plates. *E. coli* was inoculated into LB medium (2% v/v), and aliquots (200 ml) were added to the wells. The cultures were then sealed with breath easy film and transferred to a plate reader (Nephelostar; BMG Labtech Ltd.). A graphical abstract of the protocol is presented in Figure 3. The culture was incubated and shaken in the plate reader at 37 ªC. Every 20 minutes the shaking was stopped in order to measure the light scattering. The conditions for measuring the light scattering were: 2 s per well with a period delay set at 0.5; gain set at 40; laser beam focus seat at 2mm. Maximum specific growth rates were calculated in the exponential growth phase, using the equation, ln Nt/No = mt, where Nt is light scattering units at time t (h), and m is the growth rate (h-1). The assays were performed in triplicate. The growth rates in the presence of the ionic liquids were calculated as a percentage of the growth rate in control cultures in the absence of ionic liquid and the mean values are reported. EC50 values were calculated from the plots of percentage growth inhibition.

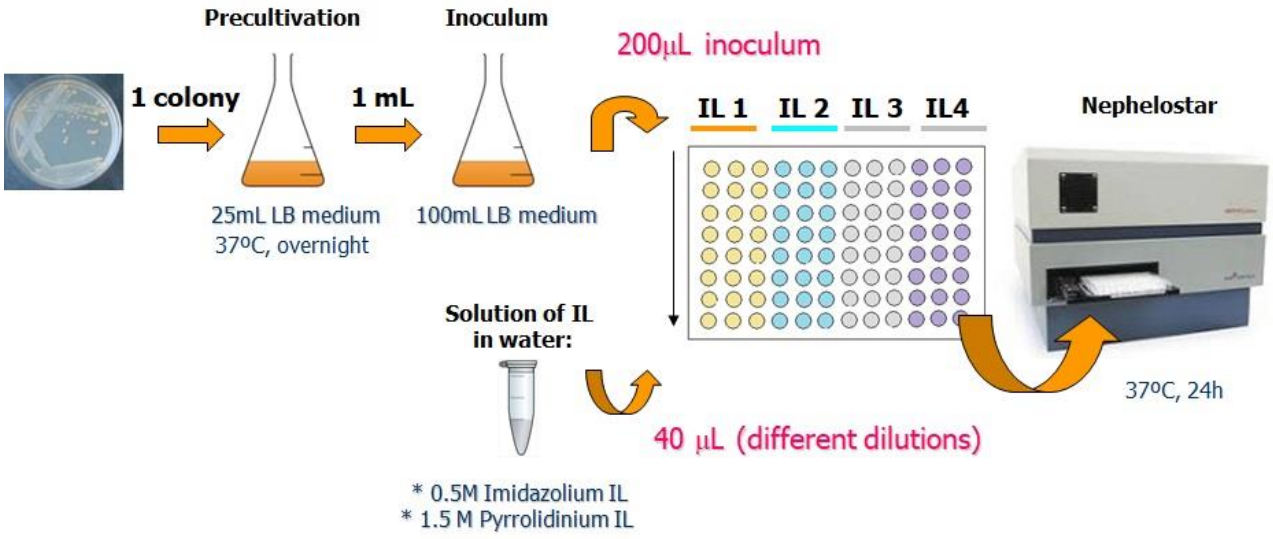
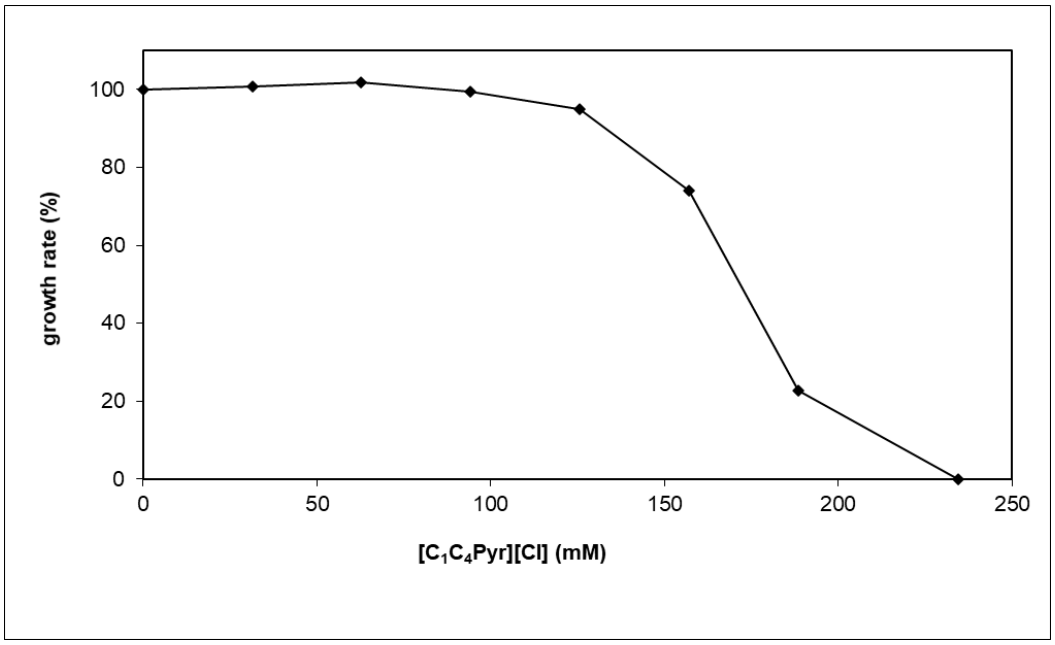


Figure 3. Graphical abstract of the protocol for analysing of toxicity of ionic liquids by nephelometry.

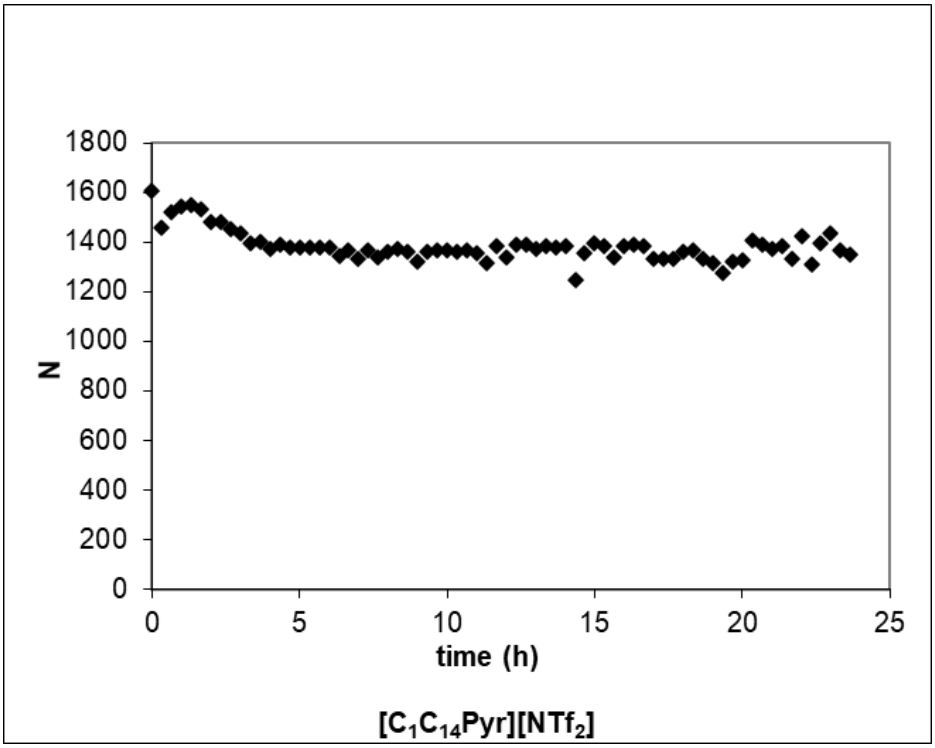
3. Results and Discussion

The toxicity of 15 ionic liquids based on imidazolium and pyrrolidinium cations towards *E. coli* was measured by nephelometry. Thirteen of the fifteen ionic liquids are soluble in water and two of them, those based on bistriflimide anions, are water-insoluble (the solubility values for $[C_1C_{14}Pyr][NTf_2]$ and $[C_1C_4Im]$ $[NTf_2]$ were14.6 mM and < 5.0 mM, respectively). Typical growth rate (%) curves *vs*. ionic

liquid concentration for the water-soluble ionic liquids are presented in Figure 4a. The EC50 values were obtained as explained in the Materials and Methods section. The concentration of ionic liquids in which *E. coli* maintains 100% growth (100% GR) can be inferred from the growth rate (%) curves. In the case of the water-insoluble ionic liquids, the typical N curves *vs.* time (Figure 4b) did not permit growth rate percentages to be inferred. In these cases, the maximum solubility values of the ionic liquids in water were obtained and the toxicity tests were carried out below the maximum solubility values.



(a)



(b)

Figure 4. (a) Typical growth rate (%) curves *versus* ionic liquid concentration ([C_1C_4Pyr][Cl].

8. (b) Typical growth rate (%) curves for water insoluble ionic liquids. [C_1C_{14}Pyr][NTf_2].

Table 1 shows the EC50 values obtained by nephelometry for water-soluble and water-insoluble ionic liquids below water saturation concentration. The concentration range in which *E. coli* maintains a 100% growth rate with respect to a medium of free ionic liquids is presented. A growth rate value of 100 ± 5% was considered as 100% GR. The influence of the cation, anion and the alkyl substituent of the cation are systematically analysed below in order to elucidate toxicity-structure relationships.

Table 1. EC50 values of *E. coli* in different pyrrolidinium and imidazolium ionic liquids, and concentration range in which *E. coli* maintains a 100% growth rate (100% GR) compared with a medium of free ionic liquids. * *Range of concentration analysed.*

199

	[dca](mM)		[Cl] (mM)		[Br] (mM)		[I] (mM)		[NTf_2](mM)		[Me_2PO_4] (mM)	
	EC_{50} (mM)	100% GR	EC_{50} (mM)	100% GR	EC_{50} (mM)	100% GR	EC_{50} (mM)	100% GR	EC_{50} (mM)	100% GR	EC_{50} (mM)	100% GR
[C_1C_4Pyr]	98.4	25.0	169.1	100		138.7	50.0					
[C_1C_6Pyr]						<34.7						
[C_1C_8Pyr]						<33.0						
C_1C_{14}Pyr]						119.8	60.0			0-1.4*		
[C_1C_4Im]	43.0	20.0	58.9	40					<5.0			
[C_1C_6Im]			<11.4									
[C_1C_{10}Im]	33.0	20.0				<11.5						
[C_1PyrC_6ImC1]										0-0.8*		

200

201

202 3.1. Influence of the alkyl substituent of the ionic liquid cation on toxicity towards *E. coli*

203 Studies using other microorganisms have shown that the toxicity of ionic liquids is directly correlated
204 with the chain length of the cation alkyl substituent (Couling *et al*., 2006; Luis *et al*., 2007; Pretti *et*
205 *al*., 2009; Romero *et al*., 2008; Pérez de los Ríos *et al.,* 2017; Ranke *et al.*, 2007; Stepnowski *et al.*,
206 2004). This effect is known as 'side-chain effect' (Matzke *et al.*, 2010). It is known that alkyl chain
207 length relationships with toxicity are linear over a restricted range and the toxicity could even decrease
208 with increasing the alkyl chain length (Pernak et al., 2003). Similar results were found in our work for
209 *E. coli* in pyrrolidinium ionic liquids combined with bromide anion (see Table 1). The toxicity of
210 pyrrolidinium ionic liquids increased with increased alkyl chain length, in the following order:
211 [C_1C_4Pyr][Br], [C_1C_6Pyr][Br], [C_1C_8Pyr][Br] (considering the EC_{50} values). No activity was found at
212 lower concentrations than 34.7 and 33 mM in the case of [C_1C_6Pyr][Br] and [C_1C_8Pyr][Br],
213 respectively. A decrease in toxicity with increasing alkyl chain length was observed in [C_1C_{14}Pyr][Br].
214 In the case of imidazolium cation, an increase of the toxicity with increasing alkyl chain length was
215 also observed from [C_1C_4Im][Cl] to [C_1C_6Im][Cl] and from [C_1C_4Im][dca] to [C_1C_{10}Im][dca].

216 Similar behaviour towards *E. coli* has also been recorded for ionic liquids based on imidazolium and
217 phosphonium cations. Pernak *et al.* (2003) studied the antimicrobial activity of 3-alkoximethyl-1-
218 methylimidazolium ionic liquids of different alkyl chain lengths (from 3 to 16 atom carbons) combined
219 with chloride, tetrafluoroborate and hexafluoroborate anions. They calculated the minimal inhibitory

concentration (MIC) of ionic liquids for *E. coli*, finding that the MIC decreased as the cation alkyl chain length increased from C_3 to C_{12}, after which the MIC increased again from C_{14} to C_{16}. Stephen`s group (2011) screened the toxicity of ionic liquid towards *E. coli* K-12, using both agar diffusion tests and growth inhibition tests in liquid cultures. In the case of imidazolium halides [Cxmim][Br] , they found an increase in the inhibition zone from 0 cm, for C_2 and C_4 to 1.1 cm for C_{10}. However, in liquid medium no growth was observed from C_4 to C_{10}. They also established a positive relationship between ionic liquid toxicity and their membrane accumulation using the FT-IR method (Cornmell *et al*, 2008).

More recently, Coutinho`s group (2019) studied the disruption of *E. coli* cells by analysing the green fluorescent proteins (GFP) released in the presence of ionic liquids. An interesting result was the observation that two long alkyl chains in an ionic liquid hinder its interaction with the cell membrane. For instance, disruption was greater with $[C_1C_{14}Im][Br]$ than with $[C_{14}C_{14}Im][Br]$ and with $[N_{1,1,1,14}][Br]$ compared to $[N_{1,1,14,14}][Br]$. According to these results, the greater the hydrophobic character of an IL, the greater its possibility of interacting with the cell membrane, disrupting the membrane's physiological functions and, consequently, killing the cell. (Latała *et al.*, 2005; Ranke *et al*., 2004; Stepnowski *et al.*, 2004; Hernández-Fernández *et al.*, 2015). However, one very long alkyl chain or several long alkyl chains could hinder the interaction with the membrane or result in steric hindrance. Recently studies on quaternary alkylammonium ionic toxicity on *E. coli* strains revealed that intracellular damage to DNA was also correlated with alkyl chain length due to interaction with the membrane and the generation of oxidative stress. So, DNA damage was only observed when bacteria were treated with ionic liquids and was not observed in vitro assay with isolated DNA (Kowalczyk et al., 2018).

3.2.Effect of the ionic liquid cation on toxicity towards *E. coli*

In general, ionic liquids compose of aromatic cations, such as imidazolium, have shown higher toxicity than those containing non-aromatic cations, like pyrrolidinium (Hakima *et al*., 2020; Missoun *et al*., 2020). The higher hydrophobic nature of aromatic cations favors interaction with the cell membrane (Latała *et al.*, 2005; Ranke *et al.*, 2007; Stepnowski *et al*., 2004). Furthermore, the lower steric hindrance of the aromatic cations, due to their planarity, may favor their interaction with the lipid membrane (Viboud *et al.*, 2012).

There are few data on the toxicity towards *E. coli* of ionic liquids based on pirrolidinium cations. For this reason and the possible interest of this cation due to its apparent reduced toxicity compared with

other aromatic cations, this study focuses on ionic liquids based on pyrrolidinium cations and compares their toxicity with ILs based on imidazolium ionic liquids. In order to study the effect of the cation, ILs with the same anion and alkyl chain lengths in the cation and different cation groups are compared. As expected, the EC_{50} values were higher in pyrrolidinium cation than in imidazolium cations, as in the following comparisons: $[C_1C_4Pyr][dca]$ *vs* $[C_1C_4Im][dca]$ and $[C_1C_4Pyr][Cl]$ *vs* $[C_1C_4Im][Cl]$. Indeed, the values were more than twice as high in pyrrolidinium than in imidazolium cation. 100GR values were also higher in pyrrolidinium cation than in imidazolium cation. These results confirm the above-described studies on pyrrolidinium toxicity towards microorganisms.

In this work, a dicationic ionic liquid based on imidazolium and pyrrolidinium cations combined with bistriflimide anion ($[C_1PyrC_6ImC_1][NTf_2]_2$) was synthesized and its toxicity towards *E. coli* was measured. This ionic liquid was water insoluble, with a water solubility of 6.60 mM. The toxicity toward *E. coli* was analyzed in the range 0-0.76 mM ionic liquid concentration. Within this range *E. coli* maintained a 100% growth rate. The ionic liquid $[C_1C_{14}Pyr][NTf_2]$ was also water insoluble, its water solubility being 14.72 mM. The toxicity toward *E. coli* was around 100% GR in the range 0-1.35 mM.

To date, the toxicity of ammonium, imidazolium and phosphonium toward *E. coli* has been the main object of study. Florio *et al.* (2019) evaluated the antimicrobial activity of different types of ionic liquids, including 1-dodecyl-3-methyl-imidazolium bromide ($[C_1C_{12}Im][Br]$) and 1-dodecyl-3-methylpyrrolidinium bromide ($[C_1C_{12}Pyr][Cl]$), finding that the MIC values for these ILs were four times higher for ($[C_1C_{12}Pyr][Cl]$) than for ($[C_1C_{12}Im][Cl]$), which agrees with our findings herein. In the same way, Mester *et al.* (2015) measured the MIC value for ($[C_1C_4Pyr][Cl]$) and ($[C_1C_4Im][Cl]$) toward *E. coli* and, again, a higher value (three times more) was obtained for pyrrolidinium ionic liquids. These results corroborated the results obtained in the present work by nephelometry.

3.3. Effect of the ionic liquid anion on toxicity towards *E. coli*

For studying the effect of the anion composition on ionic liquids toxicity, the toxicity of the ionic liquids with different anions and the same cation was analyzed. However, we should consider that synergy effects between anion and cation that may occur make it difficult to identify the contributions of individual anions. In studies involving different microorganisms, it has been reported that the toxicity is usually directly correlated with the nature of the cation, while the anion seems to modulate

the toxicity to a lesser extent (Ranke *et al*., 2004; Couling *et al.,* 2006; Luis *et al.,* 2007; Pretti *et al*., 2009; Romero *et al*., 2008).

In our work with *E. coli*, we observed the same behavior as other microorganisms, as commented above, since differences were more pronounced after changing the cation (with the same anion) than by changing the anion (with the same cation). For example, comparing $[C_1C_4Pyrr][dca]$ *vs* $[C_1C_4Im][dca]$ and $[C_1C_4Pyrr][Cl]$ *vs* $[C_1C_4Im][Cl]$ (see Table 1), the difference in EC50 values were much higher than 100%. However, comparing the EC50 for pirrolidinium cation with different anions, the differences were smaller.

Furthermore, comparing the same cation with different anions the following sequence was found for the EC50: $[Me_2PO_4^-]$> $[Cl^-]$> $[Br^-]$> $[dca^-]$ for $[C_1C_4Pyr^+]$ cation and $[Cl^-]$> $[dca^-]$> $[I^-]$> for $[C_1C_4Im^+]$ cation. Wood *et al.* (2011) analysed the toxicity of ionic liquids based on imidazolium cations and halides anions ($[Cl^-]$, $[Br^-]$ and $[I^-]$) towards *E. coli*. They observed that $[C_1C_2Im^+]$ and $[C_1C_4Im^+]$ chlorides and bromides did not produce inhibition zones in the agar diffusion test. Inhibition zones were found for 6 and 8 atom carbons alkyl chain. In these later cases, bromides showed higher toxicity than chlorides. Iodides also showed higher toxicity than bromides. In another study, the MIC value for $[C_1C_4Im][Cl]$ was higher than the MIC value for $[C_1C_4Im][dca]$. Hence, the anion sequence for imidazolium agrees with the anion sequence for pyrrolidinium observed in the present work (Meste *et al*., 2015).

4. Conclusions

This work assesses the toxicity of several ionic liquids based on pirrolidinium and imidazolium cation towards *E. coli* in order to analyze their biocompatibility for designing bioprocess based on *E. coli*.For this, nephelometry was used as an easy to use and rapid methodology to test the toxicological properties of ionic liquids. The method also has the advantage of providing results that are comparable with those obtained using other methodologies, as it has been corroborated in this work. The only limitation of this methodology is that it is not possible to obtain data regarding ionic liquids that are water insoluble with ionic liquid concentration above their solubility in water. In the last case, the ionic liquids could be mixture with an organic solvent which helps ionic liquids solubilization in water. If we supposed that the toxicity is additive parameter for both organic solvents and ionic liquids, the toxicity of ionic liquids for water insoluble ionic liquids could be determined by using an organic cosolvent. The results

obtained allowed several toxicity-structure relationships to be established. Pyrrolidinium cations are less toxic than imidazolium cations. Among pyrrolidinium cations, those whose alkyl substitutions are as short as possible combined with anions of low toxicity like [Me_2PO_4] are of the greatest interest. In this way, an EC_{50} of almost 200 mM with a GR value of 100 mM can be reached with the ionic liquid [C_1C_4Pyr] [Me_2PO_4]. It can be seen then that the suitable combination of cations and anions can provide biocompatible *E. coli*-ionic liquid systems for application in new bioprocesses.

Acknowledgments

A.P. de los Ríos and F.J. Hernández-Fernández thank the Seneca Foundation and Jose Castillejo Spanish Program for the fellowship received for exchange visitor professors.

References

Baeshen, M.N., Al-Hejin, A.M., Bora, R.S., Ahmed, M.M.M., Ramadan, H.A.I., Saini, K.S., Baeshen, N.A., Redwan, E.M. 2015. Production of Biopharmaceuticals in *E. coli*: Current Scenario and Future Perspectives. J. Microbiol. Biotechnol. 25(7), 953-962.

Balbás, P. 2001. Understanding the art of producing Protein and Nonprotein Molecules in *Escherichia coli*. Mol. Biotechnol. 19, 251-267.

Choi, H.J., Uhmb, K.N., Kima H.K. 2011. Production of chiral compound using recombinant Escherichia coli cells co-expressing reductase and glucose dehydrogenase in an ionic liquid/water two phase system. J. Mol. Catal. B: Enzym. 70, 114-118.

Cornmell, R. J., Winder, C. L., Tiddy, G. J. T., Goodacre, R., Stephens, G. 2008. Accumulation of ionic liquids in Escherichia coli cells. Green Chem. 10, 836–841.

Couling, D.J., Bernot, R.J., Docherty, K.M., Dixon, J.N.K., and Maginn, E.J. 2006. Assessing the factors responsible for ionic liquid toxicity to aquatic organisms via quantitative structure–property relationship modeling. Green Chem. 8, 82-90.

Kebaili, H., Pérez de los Ríos, A., Salar-García, M.J., Ortiz-Martínez, V.M., Kameche, M., Hernández-Fernández, J., Hernández-Fernández, F.J. 2020. Evaluating the Toxicity of Ionic Liquids on *Shewanella sp*. for Designing Sustainable Bioprocesses. Front. Mater. 7, 1-19.

Kowalczyk, P., Borkowski, A., Czerwonka, G., Cłapa, T., Cieśla, J., Misiewicz, A., Borowiec, M., Szala. M., 2018. The microbial toxicity of quaternary ammonium ionic liquids is dependent on the type

339 of lipopolysaccharide. J. Mol. Liq. 266, 540-547.

340 Florio, W., Becherini, S., D'Andrea, F., Lupettia, A., Chiappe, C., Guazzelli L. 2019. Comparative
341 evaluation of antimicrobial activity of different types of ionic liquids. Mat. Sci. Eng. C 104, 1-10.

342 Hernández-Fernández, F.J., Bayo, J., Pérez de los Ríos, A., Vicente, M.A., Bernal, F.J., Quesada-
343 Medina, J. 2015. Discovering less toxic ionic liquids by using the Microtox® toxicity test. Ecotoxicol.
344 Environ. Saf. 116, 29-33.

345 Matzke, M., Arning, J., Ranke, J., Jastorff, B., Stolte, S. 2010. "Design of Inherently Safer Ionic
346 Liquids: Toxicology and Biodegradation," in *Handbook of Green Chemistry* (Weinheim, Germany:
347 Wiley-VCH Verlag GmbH & Co. KGaA), 233-298.

348 Missoun, F., Pérez de los Ríos, A., Ortiz-Martínez, V.M., Salar-García, M.J., Hernández-Fernández,
349 J., Hernández-Fernández, F.J. 2020. Discovering Low Toxicity Ionic Liquids for Saccharomyces
350 cerevisiae by Using the Agar Well Diffusion Test. Processes ,8, 1163-1181.

351 Mester, P., Wagner, M., Rossmanith, P., 2015. Antimicrobial effects of short chained imidazolium-
352 based ionic liquids—Influence of anion chaotropicity. Ecotoxicol. Environ. Saf. 111, 96-101.

353 Latała, A., Stepnowski, P., Nędzi, M., Mrozik, W. 2005. Marine toxicity assessment of imidazolium
354 ionic liquids: Acute effects on the Baltic algae *Oocystis submarina* and *Cyclotella meneghiniana*.
355 Aquat. Toxicol. 73, 91-98.

356 Lee, S.Y., 2009 Systems Biology and Biotechnology of *Escherichia coli.* ISBN 978-1-4020-9393-7.
357 Springer.

358 Ortiz, L.P., Aldaco, R., Irabien, A. 2007. A novel group contribution method in the development of a
359 QSAR for predicting the toxicity (*Vibrio fischeri* EC50) of ionic liquids. Ecotoxicol. Environ. Saf. 67,
360 423-429.

361 Peréz de los Ríos, A., Hernandez-Fernandez, F. J., Zapata Henríquez, P. A., Missoun, F., Hernández
362 Fernández, J., Ortiz Martínez, V.M., Salar-Garcia, M.J., Lozano-Blanco, L.J., Godinez, C., *et al.* 2017.
363 Keys for new bioethanol production processes by fermentation and ionic liquid extraction. ACS
364 Sustainable Chem. Eng. 5, 6986-6993.

365 Pernak, J., Sobaszkiewicz, K., Mirska, I. 2003. Anti-microbial activities of ionic liquids. Green Chem.

366 5, 52-56.

367 Pfruender, H., Jones, R., Weuster-Botz, D. 2006. Water immiscible ionic liquids as solvents for whole
368 cell biocatalysis. J. Biotechnol. 124, 182-190.

369 Pretti, C., Chiappe, C., Baldetti, I., Brunini, S., Monni, G., Intorre, L., 2009. Acute toxicity of ionic
370 liquids for three freshwater organisms: Pseudokirchneriella subcapitata, *Daphnia magna* and *Danio*
371 *rerio*. Ecotoxicol. Environ. Saf. 72, 1170-1176.

372 Ranke, J., Mölter, K., Stock, F., Bottin-Weber, U., Poczobutt, J., Hoffmann, J., Ondruschka, B., Filser,
373 J., Jastorff, B., 2004. Biological effects of imidazolium ionic liquids with varying chain lengths in acute
374 *Vibrio fischeri* and WST-1 cell viability assays. Ecotoxicol. Environ. Saf. 58, 396-404.

375 Ranke, J., Müller, A., Bottin-Weber, U., Stock, F., Stolte, S., Arning, J., Störmanna, R., Jastorffa, B.,
376 2007. Lipophilicity parameters for ionic liquid cations and their correlation to in vitro cytotoxicity.
377 Ecotoxicol. Environ. Saf. 67, 430-438.

378 Romero, A., Santos, A., Tojo, J., Rodríguez, A. 2008. Toxicity and biodegradability of imidazolium
379 ionic liquids. J. Hazard. Mater. 151, 268-273.

380 Selas Castiñeiras, T., Williams, S.G., Hitchcock A.G., Smith, D. C., 2018. *E. coli* strain engineering
381 for the production of advanced biopharmaceutical products. FEMS Microbiol. Lett. 365, 1-15.

382 Stepnowski, P., Składanowski, A.C., Ludwiczak, A., Łaczyńska, E. 2004. Evaluating the cytotoxicity
383 of ionic liquids using human cell line HeLa. Hum. Exp. Toxicol. 23, 513-517.

384 Sintra, T.E., Vilas M., Martins M., Ventura S.P.M., Lobo Ferreira Ana I.M.C., Santos L.M.N.B.F.,
385 Gonçalves, F.J.M., Tojo, E., Coutinho J.A.P. 2019. Liquids Used in the Disruption of *Escherichia coli*
386 Cells ChemPhysChem, 20, 727-735.

387 Viboud, S., Papaiconomou, N., Cortesi, A., Chatel, G., Draye, M., Fontvieille, D. 2012. Correlating
388 the structure and composition of ionic liquids with their toxicity on *Vibrio fischeri*: A systematic study.
389 J. Hazard. Mater. 215-216, 40–48.

390 Webb, J.P., Arnold, S.A., Baxter, S., Hall, S.J., Graham, E., Gill, S. 2018. Efficient bio-production of
391 citramalate using an engineered *Escherichia coli* strain. Microbiology. 164, 133-141.

392 Wood, N., Ferguson, J.L., Gunaratne, H.Q.N., Seddon, K.R., Goodacre, R., Stephens, G.M. 2011.

Screening ionic liquids for use in biotransformations with whole microbial cells. Green Chem. 13, 1843-1851.

Yu, J., Zhang, S., Dai, Y., Lu, X., Lei, Q., Fang, W. 2016. Antimicrobial activity and cytotoxicity of piperazinium- and guanidinium-based ionic liquids. J. Hazard. Mater. 307, 73-81.