KEYS FOR BIOETHANOL PRODUCTION PROCESSES BY FERMENTATION AND IONIC LIQUID EXTRACTION

Antonia Pérez de los Ríos, Francisco José Hernández-Fernández,

Patricio Alfredo Zapata Henríquez, Fatiha Missoun, Jesús Hernández-Fernández,

Víctor Ortiz-Martínez, María José Salar-García,

Luis Javier Lozano Blanco, and Carlos Godínez

ABSTRACT:

Bioethanol is produced by fermenting biomass. However, the main drawback of this process is the high purification costs involved, which are due to the energy-intensive distillation step and a drying step if high ethanol purity is required. This work evaluates an alternative separation−purification process for the production of ethanol, in which an ionic liquid is used to develop an integrated extraction−fermentation process. The capacity of ionic liquids to extract ethanol from an ethanol aqueous solution and their biocompatibility with Saccharomyces cerevisiae was analyzed. Ethanol extraction percentages ranging from 33% to 62% were obtained with the following water insoluble ionic liquids: [EPy+][NTf2−] < [MTOA+][NTf2−] < [OMIM+][BF4−] < [Hex3TDP+][dca−] < [Hex3TDP+][Br−] < [Hex3TDP+][Cl−]. These ionic liquids could be used for in the postfermentation step of extraction. To evaluate the potential use of these ionic liquids as extraction phase in an integrated reaction/separation process, their biocompatibility with S. cerevisiae was analyzed. Of the above ionic liquids, [MTOA+][NTf2−], [BMIM+][NTf2−], [OMIM+][NTf2−], [BMIM+][PF6−] and [Hex3TDP+][Cl−] were found to be biocompatible with S. cerevisiae because they allowed bacterial growth. From the extraction and biocompatibility tests, it can be concluded that [MTOA+][NTf2 −] and [Hex3TDP+][Cl−] would be suitable for an integrated extraction-fermentation process for ethanol production.

KEYWORDS: Bioethanol, Ionic liquids, Liquid−liquid extraction, Fermentation, Saccharomyces cerevisiae.

INTRODUCTION

The world is facing a gradual decline of energy resources, mainly due to the use of non-renewable fuels. At the same time, energy consumption is expected to grow by 46% over the next 20 years [1]. The solution to this problem depends mostly on the development and application of technologies based on renewable energy. In particular, the biofuels industry has a unique role to play in climate policy as a low carbon emission alternative to fossil fuels. However, the success of biofuels as a viable option depends on both the production process and the way emissions related to the use of land for the required biomass production are managed [2]. Among possible biofuels, ethanol has attracted considerable attention and is considered one of the most important renewable fuels for contributing to the reduction of the negative environmental impacts generated by the use of fossil fuels. The main types of feedstock used for the production of ethanol are (i) raw materials containing fermentable sugars (cane, beet and sweet sorghum), (ii) polysaccharides, which can be hydrolyzed to obtain fermentable sugars (starch contained in grains such as corn and wheat) and (iii) lignocellulosic biomass [3]. Additionally, in recent years, there has been an ongoing effort to diversify and adapt new sources of biomass for its production [4,5]. Moreover, such materials do not contribute to the world food supply, so their use avoids the constant debate about soil exploitation for the production of fuels. However, ethanol production is a complex process, and the transformation of such biological resources requires their conditioning or pretreatment to make it possible for the microorganisms to convert them into ethanol. Next, aqueous solutions of ethanol need to be concentrated to obtain hydrousethanol and this product must be dried for it to be used as an oxygenate in gasoline [6]. The complexity of this process partly explains why ethanol has not played a major role as fuel compared to the cheapest petroleum-derived fuels. Only in recent years, due to increasing environmental concerns and periodic crises in the major oil exporting countries, has bioethanol become to be regarded as a viable and real alternative in the energy market. In bioethanol production, the separation of alcohols in aqueous solutions has usually been achieved by distillation, often using multiple columns to achieve the desired purity. However, conventional distillation is an energy-intense process and the degree of alcohol purity achievable by distillation is limited due the existence of a homogeneous azeotrope (as in the case of ethanol). For these reasons, alternative and energetically less expensive separation methods, such as membrane technology and in situ product recovery, have

69 attracted increasing interest is recent years. However, the liquid−liquid extraction of
70 ethanol from aqueous media is difficult because the extractor solvent needs to be water
71 immiscible and highly polar in order to extract polar molecules like ethanol. As is well-
72 known, it is difficult to find both properties in conventional organic solvents.

73

74 Ionic liquids (ILs), for their part, have are shown themselves to be capable of extracting
75 polar molecules from water. A few examples can be found in the literature dealing with
76 the separation of butanol from water using ionic liquids [7], whereas fewer deal with the
77 separation of ethanol from water [8]. Ionic liquids consist of an organic cation and an
78 inorganic polyatomic anion, which are liquid at temperatures close to environmental
79 ranges. Their most important characteristic is their non-detectable vapour pressure, which
80 makes them environmentally benign compared with volatile organic compounds (VOCs).
81 Moreover, due to their ionic nature, by selecting different combinations of cations and
82 anions, there is an almost limitless possibility to synthesize new ionic liquids, and thus
83 tailor their physical−chemical properties [9,10]. To develop an integrated
84 fermentation/extraction process, itis of great importance to consider solvent toxicity
85 toward the microorganisms involved because the microbial activity necessary for the
86 production of the desire product must be allowed to proceed. Despite their many
87 disadvantages, conventional organic solvents have been commonly used in a range of
88 multiphase bioprocess operations. However, they have also been shown to be toxic to
89 microbial cells, causing membrane damage and reducing the long-term operational
90 viability of catalysts. Furthermore, the main concerns for the industrial biotransformation
91 processes are their toxicity toward the process operators and the environment, and the
92 explosion hazard due their volatile and flammable nature [11]. Ionic liquids need to
93 overcome this inconvenience. Indeed, they could be designed to be highly selective for a
94 given compound and to show low toxicity during successful in situ product recovery [12]-
95 This work describes key aspects of an integrated fermentation/separation process using
96 ionic liquids as extraction agents, in this case for extracting ethanol from aqueous
97 solutions, the results of which should help establish structure−activity relationships. Until
98 now, very few examples of the extraction of ethanol with ionic liquids have been reported
99 [7]. Furthermore, the biocompatibility of these ionic liquids with Sacharomyces
100 cerevisiae (S. cerevisiae) was analyzed to assess their potential use as extracting phase in
101 an integrated fermentation/separation system.

102

103   MATERIALS AND METHODS

104

105   Chemicals.

106

107   The ionic liquids 1-ethyl-3-methylimidazolium dicyanamide [EMIM+][dca−], 1-butyl-3-

108   methylimidazolium   hexafluorophosphate   [BMIM+][PF6−],   1-butyl-3-

109   methylimidazolium chloride [BMIM+][Cl−], 1-butyl-3-methylimidazolium dicyanamide

110   [BMIM+]-[dca−], 1-butyl-3-methylimidazolium acetate [BMIM+][acetate−], 1-butyl-3-

111   methylimidazolium   glycolate   [BMIM+][glycolate−],   1-ethyl-3-methylimidazolium

112   tetrafluoroborate,   [EMIM+][BF4−],   1-butyl-3-methylimidazolium   tetrafluoroborate

113   [BMIM+][BF4−],   1-butyl-3-methylimidazolium   {bis(trifluoromethyl)sulfonyl}imide

114   [BMIM+]-[NTf2−],   1-butyl-2,3-dimethylimidazolium   tetrafluoroborate

115   [BDIMIM+][BF4−], 1-hexyl-3-methylimidazolium chloride [HMIM+]-[Cl−], 1-octyl-3-

116   methylimidazolium   chloride   [OMIM+][Cl−],   1-hexyl-3-methylimidazolium

117   dicyanamide   [HMIM+][dca−],   1-octyl-3-methylimidazoliumtetrafluoroborate

118   [OMIM+][BF4−],   1-octyl-3-methylimidazolium{Bis(trifluoromethyl)sulfonyl}imide

119   [OMIM+][NTf2−], 1-octyl-3-methylimidazolium dicyanamide [OMIM+][dca−], 1-

120   methoxymethyl-3-methylimidazolium   tetrafluoroborate   [MOMMIM+][BF4−],   1-

121   methoxymethyl-3-methylimidazolium   {Bis(trifluoromethyl)-sulfonyl}imide

122   [MOMMIN+][NTf2−],   1-methoxymethyl-3-methylimidazolium   dicyanamide

123   [MOMMIM+][dca−], 1-methoxymethyl-3-methylimidazoliumtrifluoromethanesulfonate

124   [MOMMIM+]-[CF3SO3−],   1-methoxyethyl-3-methylimidazolium   tetrafluoroborate

125   [MOEMIM+][BF4−],   1-methoxyethyl-3-methylimidazolium   dicyanamide

126   [MOEMIM+][dca−],   1-(3-hydroxypropyl)-3-methylimidazoliumdicyanamide

127   [HOPMIM+][dca−],   1-(3-hydroxypropyl)-3-methylimidazoliumchloride

128   [HOPMIM+][Cl−],   1-ethoxyethyl-3-

129   methylimidazolium{Bis(trifluoromethyl)sulfonyl}imide [EOEMIM+][NTf2−], 1-octyl-

130   3-methylpyridinium   tetrafluoroborate   [MOPy+][BF4−],   1-ethoxyethyl-3-

131   methylimidazolium   hexafluorophosphate   [EOEMIM+][PF6−],   1-ethoxyethyl-3-

132   methylimidazolium dicyanamide [EOEMIM+][dca−], 1-butyl-3-methylimidazolium

133   nitrate   [BMIM+][NO3−],   ethylpyridinium   ethylsulfate   [EPy+][EtSO4−],

134   ethylpyridinium   methylsulfate   [MPy+][MeSO4−],   1-butyl-3-methylpyridinium

135   tetrafluoroborate [BMPy+][BF4−], ethylammonium nitrate [CH3CH2NH3+][NO3−], 1-

136   ethyl-1-methyl   morpholinium   dicyanamide   [EMMor+][dca−]   and

137 methyltrioctylammonium {bis(trifluoromethyl)sulfonyl}imide [MTOA+][NTf2−] were
138 purchased from Solvent Innovation GmbH (Cologne, Germany).
139 Tetradecyl(trihexyl)phosphonium chloride [Hex3TDP+][Cl−],
140 tetradecyl(trihexyl)phosphonium dicyanamide [Hex3TDP+][dca−],
141 triisobutyl(methyl)phosphonium tosylate [iBu3MeP+][TOS−] and
142 tetradecyl(trihexyl)phosphonium bromide [Hex3TDP+][Br−] were purchased from
143 CYTEC GmbH (Germany). Ethylpyridinium {bis(trifluoromethyl)sulfonyl}imide
144 [EPy+][NTf2−],[MOPy+][dca−] and 1-butyl-3-methylpyridinium dicyanamide
145 [BMPy+][dca−] were purchased from LONZA GmbH (Germany). Methyloxazolinium
146 methylsulfate [MOxa+][MeSO4−] and 1-butyl-3-methyl-2-phenylimidazolium
147 methylsulfate [BMPhIM+][MeSO4−] were purchased from DEGUSSA GmbH
148 (Germany). Other substrates, solvents and other chemicals were purchased from Sigma-
149 Aldrich Chemicals Co. (Spain), and were of the highest purity available.
150
151 Water Solubility of Ionic Liquids.
152
153 The solubility of the IL in water was determined by using the cloud point method. For
154 that, increasing 2 μL volumes of ionic liquid were added to 1 mL of water contained in
155 10 mL glass test tubes, homogenizing by vigorously stirring for 1 min. Each time, a new
156 portion of ionic liquid was added until a complete dissolution occurred. The additions
157 stopped when more than 100 μL had been added and saturation was still not reached. In
158 this case, the ionic liquid was considered water-soluble. The solubility of the ionic liquid
159 was calculated on the basis of the amount of ionic liquid added to the formation of the
160 saturated solution.
161
162 Extraction of Ethanol with Ionic Liquids.
163
164 Extraction was performed at 303.15 K. One milliliter of a 7% v/v ethanol solution (usual
165 concentration in fermentation medium) was brought into contact with 1 mL of pure water-
166 immiscible IL in 10 mL glass test tubes. The mixture was shaken vigorously for 2 min to
167 facilitate the transfer of ethanol into the ionic liquid phase before being left at a constant
168 temperature to complete the phase separation. Samples were taken from the aqueous
169 phase at 24, 48 h and 21 days. Extraction percentages were calculated by the following
170 equation:

171
$$E = \frac{C_{IL}}{C_{IL} + C_W} \times 100 \qquad [1]$$

172

173 where CIL and CW refer to the initial and equilibrium concentration of ethanol in the

174 ionic liquid and the aqueous phase, respectively. Determinations were made in triplicate

175 to ensure the repeatability of the tests and the standard deviations were calculated.

176

177 Microorganism, Medium, and Culture Conditions.

178

179 The yeast S. cerevisiae was used throughout this study. A suspension of 1 g/L was

180 prepared in physiological water (9 g L^{-1}), and 200 μL of this suspension was transferred

181 to YPD agar plates and incubated for 48 h. The inoculum was prepared by transferring a

182 single colony to a 250 mL flask with 100 mL of liquid YPD medium. The flask was

183 placed on an orbital shaker (shaking diameter 5 cm and shaking frequency 170rpm), and

184 incubated at 30 °C for 24 h. This solution was used as the inoculum for ethanol

185 fermentation, which was carried out in a 100 Ml flask with 45 mL of ethanol fermentation

186 medium and 1% (v/v) inoculum at 30 °C and 200 rpm for 48 h. During the fermentation,

187 small samples were taken at regular intervals to measure growth rates by

188 spectrophotometer. The compositions of the culture media were as follows (g L^{-1}): The

189 YPD agar medium: D-glucose 20, peptone 20, yeast extract 10, agar 20. The inoculum

190 medium: D-glucose 20, peptone 20, yeast extract 10. The fermentation medium: D-

191 glucose 20, peptone 20, yeast extract10. Each medium was autoclaved at 121 °C for 20

192 min. After that, ionic liquid was added, and the mixture was vigorously shaken manually

193 for 30 s and then placed in a water bath to be heated at 30°C for 24 h.

194

195 Ionic Liquid Toxicity to S. cerevisiae Testing in Liquid Media.

196

197 The growth rates of S. cerevisiae were measured in the presence and absence of ionic

198 liquids. It should be noted that ionic liquids were added directly to the culture medium

199 and shaken for 24 h at 30 °C, thus forming a biphasic system of 3% (v/v) ionic liquid.

200 The cultures were inoculated with a 1% (v/v) sample from a 24 h S. cerevisiae culture

201 grown in the same medium. The Erlenmeyer flasks were incubated at 30 °C with

202 continuous, intensive shaking and samples were taken regularly to measure the OD660

203 using a 1650 PC ShimadzuUV−vis spectrophotometer. The OD660 was used to plot a

204 growth curve and specific growth rates for each condition. Specific growth rates (μ, h−1)

205 were calculated by selecting two time points, t_1 and t_2, in the exponential growth phase.

206

$$\mu = \frac{\ln\left(\frac{O2_2}{OD_1}\right)}{t_2 - t_1} \qquad [2]$$

207

208

209 The residual activity (a_r) was also calculated as follows:

210

$$a_r(\%) = \left(\frac{\mu_{IL}}{\mu_o}\right) \times 100 \qquad [3]$$

211

212

213 where μIL and μo are the specific growth measured in the presence ofionic liquid and in

214 the control, respectively. The morphological structure was observed using an OLYMPUS

215 CX41 microscope (Olympus Corporation, Tokyo, Japan). Yeast concentration was

216 determined by the dry weight method at 48 h [13].

217

218 Agar Diffusion Test for Ionic Liquid Toxicity to S. cerevisiae.

219

220 The agar diffusion test was also used to test ionic liquid toxicity toward S. cerevisiae.

221 Pure ionic liquids and 3% (v/v) ionic liquid concentrations were used. Wells of 6 mm

222 diameter were punched under sterilized conditions (by heating with a Bunsen burner in a

223 laminar flow chamber) with a sterile glass tube. Samples of each IL (50µL total volume)

224 were then placed in the wells [14,15] using physiological water for the control wells. The

225 radius of the inhibition zone around the wells was recorded.

226

227 GC Analysis.

228

229 Ethanol content in the aqueous phase was determined by gas chromatography (450 GC

230 Bruker) using a Beta DEXTM capillary column (30 m × 0.25 mm × 0.25 µm, Tmax 230

231 °C, Supelco), and propanoic acid as internal standard. The chromatographic conditions

232 were as follows: carrier gas (N2) at 1.89 psi (51 mL/min total flow); temperature program,

233 200 °C, 15 min; split ratio, 1/50; detector, 260 °C. The retention times of the peaks were

234 as follows: ethanol, 5.1 min; propanoic acid, 13.2 min. Concentrations were calculated

235 from calibration curves using stock solutions of pure ethanol.

236

237

238   RESULTS AND DISCUSSION

239

240   Solubility of Ionic Liquids in Water.

241

242   The aim of this work was to use ionic liquids for the in situ extraction of ethanol in

243   aqueous solutions. The first condition for an ionic liquid to be used was that it must be

244   immiscible with water, thus forming the biphasic system in the aqueous solution required

245   for the extraction of ethanol. For this reason, we first determined the solubility of a wide

246   range of ionic liquids with different anionic and cationic compositions. The solubility

247   values are reported in units of % volume (ionic liquid)/volume (water) (Table 1). Ionic

248   liquids with a solubility lower than 10% in water (volume/volume) were considered

249   water-immiscible. As can be seen in Table 1, certain trends between the ionic liquid

250   compositions and their solubility were observed. The solubility of ionic liquids in water

251   strongly depends on the type of anion in the ionic liquid because the same cation and

252   different anions may produce significant differences in their solubility values, as can be

253   observed,   for   example,   for   the   pairs   [BMIM+][PF6−]/[BMIM+][Cl−]   and

254   [BMIM+][acetate−]/[BMIM+][NTf2−].   The   solubility   of   the   anions   followed   the

255   sequence: [dca−], [Cl−] > [BF4−] > [PF6−] > [NTF2−]. This sequence can be clearly

256   observed for ionic liquids containing the cation [omim+]: [omim+] [dca−] > [omim+]

257   [BF4−] >[omim+] [PF6−] > [omim+] [NTf2−]. With respect to the type of cation, the

258   solubility in water decreased as the alkyl chain length of the cation increased. This

259   tendency was observed when the following ionic liquids were compared: [bmim+]-

260   [NTF2−] > [omim+] [NTF2−], [bmim+] [PF6−] > [omim +][PF6−] and [bmim +] [BF4−]

261   > [omim +] [BF4−]. The trihexyltetradecyl phosphonium cation was seen to be more

262   water insoluble than 1-butyl-3-methyl imidazolium because the water solubility of the

263   ionic liquid [BMIM+][Cl−] was much higher than that of [Hex3TDP+][Cl−]. Because the

264   presence of ethanol might affect the solubility of the ionic liquid in the aqueous solution,

265   solubility tests with the water-immiscible ionic liquids were performed in a 7% v/v

266   ethanol solution. No significant changes were observed in the solubility values of the

267   ionic liquids selected (data not shown), except for [MOPy+][dca−], which increased its

268   solubility and became miscible in the ethanol solution (<10% volume ionic liquid/water

269 volume). For this reason, [MOPy+] [dca−] was discarded for the following extraction
270 steps.
271
272 Liquid−Liquid Extraction of Ethanol with Ionic Liquids.
273
274 Ethanol was extracted from a 7% v/v solution of this compound ionic liquids with the
275 common characteristic of being immiscible in both water and 7% v/v ethanol solution in
276 water. Table 2 shows the results for the different ionic liquids at several extraction times
277 (24, 48 h and 21 days). The increase in the extraction percentage from 24 h to 21 days
278 could be explained by the low extraction kinetics considering not continuous stirring was
279 applied during all over experiment (see section Extraction of Ethanol with Ionic Liquids).
280 The highest extraction percentages was achieved using the ionic liquids based on the
281 phosphonium and ammonium cations for which extraction ranged between 51.3 and
282 39.4% at 24 h. Regarding the relationship between the cation and anion composition of
283 the ionic liquids and their respective capacity as extracting solvents, it seems that the
284 extraction percentage could be related with localized charges and small volumes ions,
285 which would permit strong interactions between the negative charges of the anion and the
286 dipole formed in the ethanol molecule. The positive charge of phosphonium and
287 ammonium cations are located in the phosphorus and ammonia atoms, respectively.
288 However, in the imidazolium cation the positive charge is delocalized. In the same way,
289 the chloride, bromide and dicyanamide anions are small and present a more localized
290 negative charge with respect to NTf2−, whose charge is localized in five atoms. A
291 reduction in the extraction percentage was observed when the anion [NTf2−] was used,
292 as can be seen, for example, by comparing[BMIM+][PF6−] with [BMIM+][NTf2−] at
293 the beginning of the experiment and [OMIM+][BF4−] vs [OMIM+][NTf2−]. The
294 pyridinium-based ionic liquids showed extraction percentages between those of
295 phosphonium- and imidazolium-based ionic liquids. Neves et al. (2011) [7] evaluated the
296 potential use oftetradecyltrihexylphosphonium-based ILs by means of ternary phase
297 diagrams. For 20 wt % of ethanol, the maximum ethanol extraction followed the
298 sequence: [Hex3TDP+][NTF2−] (87%)> [Hex3TDP+][dca−] (82%) >
299 [Hex3TDP+][Br−] (78%) >[Hex3TDP+][Cl−] (72%) >
300 [Hex3TDP+][phosphinate−](72%) > [Hex3TDP+][decanoate−] (70%) > [Hex3TDP+]-
301 [CH3SO3−] (65%). Similarly, a high extraction percentage was obtained by using a
302 phosphonium-based ionic liquid. Furthermore, the extraction percentage decreased when

the size of the anion and the delocalization of the charge of the anion increased, as in the case of the methanesulfonate anion. In the above study, the use of bistriflimide anion led to good extraction behaviour, which does not agree with the results of the present work. The higher extraction percentage reached withthe phosphonium cation than in the present work may have been due to the different ethanol concentrations used: 20 wt %compared with the 7 wt %% used in the present work. Furthermore, the extraction percentages reported by Neves et al. were those obtained in the best extraction conditions. Garcia-Chavez et al. (2012) [8] studied the extraction of butanol (10% aqueous solution) with ionic liquids based on ammonium cations and carboxylate and phosphinate anions. High extraction percentages (around 90%) were obtained using ammonium ionic liquids, while the use of [HMIM+][NTf2−] (imidazolium cation and bistriflimide anion) significantly reduced the extraction percentage to 52.6%, which agrees with the results of the present work. Hernández-Fernández et al. (2010) carried out the extraction of butanol from n-hexane solutions using 13 ionic liquids based on 1-n-alkyl-3-methylimidazolium and n-alkylpyridinium cationsand a wide range of anions (hexafluorophosphate,bis{(trifluoromethyl)sulfonyl}imide, tetrafluoroborate, methylsulfate,2(2-methoxyethoxy)ethylsulfate, ethylsulfate, n-octylsulfate,dicyanamide, nitrate, tetrafluoroborate and chloride) [16]. These authors reported that the highest extraction percentages of butanol from hexane solution for the same cation was obtained with the small anions and localized charges, which is also in agreement with the results of the present work. It is important to point out that ethanol extraction from aqueous phase is more complicated than butanol extraction from hexane phase, because in the first case a very polar compound (ethanol) is extracted from a very polar phase (water).

Biocompatibility of Ionic Liquids to S. cerevisiae.

After the extraction capability of ionic liquids was measured, the biocompatibility of these solvents with S. cerevisiae was evaluated by (i) measuring the growth rates of S. cerevisiae in culture media in the presence of 3% (v/v) ionic liquid and (ii) using the agar diffusion test.

Effect of Ionic Liquids on the Growth of the Yeast Saccharomyces cerevisiae.

The biocompatibility of ionic liquids with S. cerevisiae was analyzed by measuring the growth rates in the presence and absence of the ionic liquids. Growth curves were obtained in YPD culture media. In the case of ionic liquid biphasic systems, we chose a 3% (v/v) ionic liquid because many authors [17,18] have shown that ILs are significantly toxic at between 2% and 5% (v/v) concentrations. Figure 1 shows the growth of the yeast S. cerevisiae at 3% (v/v) of the studied ionic liquids.

To evaluate the effect of ionic liquids on the morphological structure of the micoorganism, the yeast S. cerevisiae was observed at 48 h by microscope using the methylene blue staining method (see Figure 2). As illustrated in Figure 1, the growth curves of the yeast S. cerevisiae in the absence and presence of ionic liquids fitted typical batch bacterial growth curves in the case of [MTOA+][NTf2−], [BMIM+][NTf2−], [OMIM+][NTf2−], [BMIM+]-[PF6−], [Hex3TDP+][Cl−] and the control, the specific growth rate (μ) in the log phase being between 0.08 and 0.47 h⁻1 (see Table 3). For [Hex3TDP+][Cl−], the culture started growing after an adaptation time, whereas in the case of [MTOA+]-[NTf2−] and [BMIM+][NTf2−] the growth curves were similar to the control growth curve. The final yeast concentration of S. cerevisiae in [BMIM+][NTf2−] and [MTOA+][NTf2−] was slightly higher than in the control at 48 h (see Table 3), after which growth continued, whereas it stopped in the control. This may be explained by the extraction of ethanol or other fermentation products by the ionic liquids which can inhibit the yeast growth. Furthermore, no cell death was observed (see Figure 2) in the above ionic liquids. [BMIM+][PF6−] and [OMIM+][NTf2−] were relatively nontoxic for S. cerevisiae, but the yeast took longer to adapt to the medium and the decline phase occurred earlier in the case of the [BMIM+][PF6−] than in [OMIM+][NTf2−], in which the yeast continued to grow at 48 h. In the case of [Hex3TDP+][Cl−], following total inhibition during the first 12 h without any growth; after that, cells began to bud and growth continued at 48 h, as can be observed in Figure 2, probably due to the complete adaptation of cells by this time. No growth or near zero growth was observed in the ionic liquids [Hex3TDP+][Br−], [EPy+][NTf2−], [OMIM+][BF4−] and [Hex3TDP+][dca−], with μ ranging from 0 to 0.03 h⁻¹ (see Table 3). For these ionic liquids, cells became smaller and turned blue (dead cells) and no budding was observed (see Figure 2). It was observed that yeast maintained higher activity in ionic liquids based on imidazoulim cations combined with hydrophobic anions, such as [PF6−] and [NTf2−], as well as in ammonium cation combined with hydrophobic anions, such as [MTOA+][NTf2−].

370   Furthermore, the combination of imidazolium cations with more hydrophilic anions

371   inhibited S. cerevisiae growth, as in the case of [OMIM+][BF4−], which completely

372   inhibited growth, revealing its toxic character toward the microorganism. Low

373   biocompatibility was obtained with pyridinium and phosphonium cations such as

374   [EPy+][NTf2−] and [Hex3TDP+][dca−], [Hex3TDP+]Br−] [Hex3TDP+][Cl−],

375   respectively. In the last case, a degree of adaptation seemed to have been reached by the

376   end of the experiment. Such behaviour seems to be related with the ionic liquid solubility

377   (see Table1). Ionic liquids with low water-solubility, such as [MTOA+]-[NTf2−],

378   [BMIM+][NTf2−], [OMIM+][NTf2−] and [BMIM+]-[PF6−], presented a higher degree

379   of yeast compatibility than the more water-soluble ionic liquids, such as

380   [Hex3TDP+][Cl−], [Hex3TDP+][Br−], [EPy+][NTf2−], [OMIM+][BF4−] and

381   [Hex3TDP+][dca−]. The better behaviour of [BMIM+][NTf2−]vs [BMIM+][PF6−]

382   could be also related with the higher water solubility of ILs containing [PF6−] anion

383   respect to [NTf2−].The greater the solubility of the ionic liquids in water, the greater the

384   concentration in the culture medium and the greater the interaction of the ionic liquid with

385   the yeast. Not only the water solubility affect to ionic liquids toxicity to yeast but also the

386   ionic liquids structure since [OMIM+][NTf2−] is more hydrophobic than

387   [BMIM+][NTf2−] (higher alkyl chain length)and better biocompatibility was obtained

388   with [BMIM+]-[NTf2−] than [OMIM+][NTf2−] based on growth rates of S. cerevisiae

389   (Table 2) and (ii) using the agar diffusion tests (Table3). That behaviour was found in S.

390   cerevisiae [19] and even other microorganism [20]. In the case of S. cerevisiae [19] an

391   increased in the toxicity was found from [BMIM+][Cl−] to [HMIM+][Cl−] and

392   [OMIM+][Cl−] at 1000 ppm concentration of ionic liquid. Regarding water insoluble

393   ionic liquids, [MTOA+][NTf2−],[BMIM+][NTf2], [BMIM+][PF6−] resulted to be

394   biocompatible by using a viability assays conducted after the incubation of 20 g DCW

395   L^{-1} S. cerevisiae FasB His6 at 27 °C and 300 rpm for 20 h [21]. Water-immiscible ionic

396   liquids have been also used in a biphasic system to enhance the 2-phenylethanol

397   concentration by means of in situ product removal catalyst by S. cerevisiae. A correlation

398   between the IL structure and the effect on yeast growth was investigated. [NTf2−] anions

399   were found to be the most biocompatible in comparison to [PF6−] and [BF4−].

400   Furthermore, it was also found that the longer the alkyl side chain on the imidazolium

401   ring, the lower is its biocompatibility [22]. The relationship between ionic liquids

402   structure− activity found in previous work are in agreement with the results found in the

403   present work. On the other hand, water-soluble ionic liquid has recently received attention

as suitable candidates for lignocellulosic biomass pretreatment prior enzymatic saccharification and, obviously, for second-generation bioethanol production. Consequently, their impacts on downstream S. cerevisiae growth and biofuel production have been studied. Specifically, the ionic liquids 1-ethyl-3-methylimidazolium acetate23 ([BMIM+][OAc−]), 1-butyl-3-methylimidazolium chloride24 ([BMIM+][Cl−]) and 1-ethyl-3-methylimidazolium methylphosphonate25 ([EMIM+][MeO(H)PO2−]) were studied. Both the growth and ethanol production using S. cerevisiae is strongly influenced for the above ionic liquids. However, low ionic liquid concentrations shown to have a minimal impact on S. cerevisiae. Those limit concentrations were 0.1% for [EMIM+][OAc−] [23], 1 ppm for [BMIM+][Cl−] [24] and 5% for [EMIM+][MeO(H)PO2−] [25]. In the latter case, the addition of ILs to the growth medium inhibited the oxygen transfer rate (OTR) and switched the metabolism from respiration (conversion of glucose into biomass) to fermentation (conversion of glucose to ethanol). This behaviour was observed at low IL concentrations (≤5% IL), whereas above this value there is no significant growth or ethanol production. Very recently, the mechanism of toxicity was investigated for 1-ethyl-3-methylimidazolium chloride ([EMIM+][Cl−]), [BMIM+] [Cl−] and [EMIM+][OAc−]. It has been found that some ILs likely target mitochondria. ILs induced abnormal mitochondrial morphology, as well as altered polarization of mitochondrial membrane potential [26]. Those studies corroborate our results in which solubility of ionic liquids in water could negatively affect the growth and ethanol production using S. cerevisae.

Determination of the Toxic Effect of Ionic Liquids on the Yeast S. cerevisiae Using the Agar Diffusion Test.

The agar diffusion test is an inexpensive method, requires little preparation and no specialized equipment, uses small quantities of the test compound [27,28] and only basic microbiological skills are needed. This test was used in order to confirm the results in liquid medium. The experiments were carried out with two concentrations, pure ionic liquids and 3% (v/v) ionic liquid concentration. The results showed that [MTOA+][NTf2−] and[BMIM+][NTf2−] had no effect on the growth of the yeast S. cerevisiae because the diameter of the growth inhibition zone was zero in the concentration of 3%(v/v) and for the pure ILs.

438   Furthermore, the same toxic behaviour was obtained in the liquid medium with respect to
439   the agar diffusion test at 3% (v/v) ionic liquids (i.e., good behaviour for
440   [MTOA+][NTf2−] and[BMIM+][NTf2−] and high inhibition for [OMIM+][BF4−]
441   and[Hex3TDP+][dca−]), which confirm the results obtained in the growing experiment
442   (see Table 3).

443

444   New Extraction-Fermentation Process for BioethanolProduction.

445

446   Several ionic liquids were found to be water insoluble and capable of a high ethanol
447   extraction efficiency: [Hex3TDP+][Cl−], [Hex3TDP+][dca−], [Hex3TDP+][Br−],
448   [MTOA+][NTf2−] and [OMIM+][BF4−]. The extraction percentage was mainly related
449   to the ionic liquid composition, increasing with localized charges and small volumes ions.
450   It is important to note that it is very difficult to find conventional organic solvents which
451   extract ethanol with a high efficiency because they would need to be water-insoluble and
452   polar, two properties that are very difficult to find in conventional organic solvents.
453   However, we have shown that it is possible to find water-insoluble ionic liquids with the
454   capacity to extract the ethanol, because ionic liquids are polar compounds due to the
455   charge of the anion and the cation. Furthermore, an integrated fermentation−extraction
456   process requires that the insoluble ionic liquid be biocompatible with the yeast at saturated
457   ionic liquid concentrations in water. It has been seen that the ionic liquids
458   [MTOA+][NTf2−], [BMIM+][NTf2−], [BMIM+][PF6−] and, to a lesser extent,
459   [Hex3TDP+][Cl−] are biocompatible with S. cerevisiae because they allow the bacteria
460   to grow. The ionic liquids [MTOA+][NTF2−] and [Hex3TDP+][Cl−] were suitable for
461   ethanol extraction and allowed the growth of S. cerevisiae, meaning that they can be used
462   in an integrated ethanol fermentation and extraction process. Both [Hex3TDP+][dca−]
463   and [Hex3TDP+] [Br−] showed a good ethanol extraction capacity but were toxic to S.
464   cerevisiae, so that cannot be considered suitable for extractive fermentation, although
465   they could be used for the postfermentation-extraction of ethanol. Once the ethanol has
466   been extracted with the ionic liquids, both can be easily separated by distillation due to
467   the very low vapour pressure of the ionic liquid. All the energy of this distillation process
468   would be used in evaporating the ethanol (not ethanol and water, as in the case of
469   ethanol−water distillation). For that reason, the new processes could save energy since
470   conventional distillation is an energy-intense process. In the case of some of water can be
471   retained in the ionic liquid phase, the amount of water will below because we are using

472 water insoluble ionic liquids and easily separable from ethanol since ionic liquids have
473 been shown to break azeotropes [29]. Because of easy separation of the little amount of
474 water from ethanol, it is possible not to need an expensive drying process after ionic
475 liquids distillation, reducing the capital cost of the process. Furthermore, in the case of
476 integrated fermentation and separation process, besides saving capital cost the
477 fermentation efficiency could be improved because the ethanol, which could inhibit S.
478 cerevisiae, continuously relies from the growth medium. Regarding the sustainability of
479 the process, it is worthy to be highlighted that the ionic liquids could be recycled along
480 the process after separation in the distillation step and then reused in the extraction step.
481 That is possible thanks to the thermal and chemical stability of ionic liquids [30].

482

483 CONCLUSIONS

484

485 This work evaluates the use of ionic liquids as extraction agentsin an integrated
486 extraction−fermentation process for bioethanolproduction. The ethanol extraction
487 percentages ranged from33% to 62%, increasing in the sequence [EPy+][NTf2−]
488 <[MTOA+][NTf2−] < [OMIM+][BF4−] < [Hex3TDP+][dca−] <[Hex3TDP+][Br−] <
489 [Hex3TDP+][Cl−]. Of these, [MTOA+][NTf2−] and [Hex3TDP+][Cl−] can be
490 considered suitable for an integrated extraction−fermentation process for ethanol
491 production. This work has clearly demonstrated the exciting potential of combining ionic
492 liquid extraction with fermentation for the bio-production of polar molecules like ethanol.
493 The results of this study are encouraging and suggest that this new process may lead the
494 way toward new alternatives for the biosynthesis of polar compounds using
495 microorganisms.

496

497 Notes
498 The authors declare no competing financial interest.

499

505

511

512 DEDICATION

513

514 Dedicated to Mateo Pérez Bernal, a beloved father, for his lifelong lessons.

515

516 REFERENCES

517

518 (1) Monti, A.; Fazio, S.; Venturi, G. Cradle-to-farm gate life cycle assessment in perennial
519 energy crops. Eur. J. Agron. 2009, 31, 77−84.

520

521 (2) Borjesson, P.; Tufvesson, L. M. Agricultural crop-based biofuels resource efficiency
522 and environmental performance including directl and use changes. J. Cleaner Prod. 2011,
523 19, 108−120.

524

525 (3) Sánchez, O. J.; Cardona, C. A. Trends in biotechnological production of fuel ethanol
526 from different feedstocks. Bioresour. Technol.2008, 99, 5270−5295 2010.

527

528 (4) Sánchez, S.; Lozano, L. J.; Godínez, C.; Juan, D.; Pérez, A.; Hernández, F. J. Carob
529 pod as a feedstock for the production of bioethanol in Mediterranean areas. Appl. Energy
530 2010, 87, 3417−3424.

531

532 (5) Sánchez, S.; Lozano, L. J.; Pérez, A.; Hernández, F. J.; Godínez,C.; Juan, D. Process
533 design and economic analysis of a hypothetical bioethanol production plant using carob
534 pod as feedstock. Bioresour. Technol. 2012, 104, 324−328.

535

536 (6) Cardona, C. A.; Sánchez, O. J. Fuel etanol production: Process design trend and
537 integration opportunities. Bioresour. Technol. 2007,98, 2415−2457.

538

539 (7) Neves, C. M. S. S.; Granjo, J. F. O.; Freire, M. G.; Robertson, A.; Oliveira, N. M. C.;

540 Coutinho, J. A. P. Separation of ethanol−water mixtures by liquid−liquid extraction using

541 phosphonium-based ionic liquids. Green Chem. 2011, 13, 1517−1526.

542

543 (8) García-Chavez, L. Y.; Garsia, C. M.; Schuur, B.; de Haan, A. B. Biobutanol Recovery

544 Using Non fluorinated Task-Specific Ionic Liquid. Ind. Eng. Chem. Res. 2012, 51,

545 8293−8301.

546

547 (9) Ortiz-Martínez, V. M.; Salar-García, M. J.; Hernández-Fernández,F. J.; Pérez de los

548 Ríos, A. Development and characterization of a new embedded ionic liquid based

549 membrane-cathode assembly for its application in single chamber microbial fuel cells.

550 Energy 2015, 93,1748−1757.

551

552 (10) Pérez de los Ríos, A.; Hernández-Fernández, F. J.; Presa, H.;Gómez, D.; Víllora, G.

553 Tailoring supported ionic liquid membranes for the selective separation of

554 transesterification reaction compounds. J.Membr. Sci. 2009, 328, 81−85.

555

556 (11) Cull, S. G.; Holbrey, J. D.; Vargas-Mora, V.; Seddon, K. R.; Lye, G. J. Room

557 temperature ionic liquids as replacements for organic solvents in multiphase bioprocess

558 operations. Biotechnol. Bioeng. 2000,69, 227−233.

559

560 (12) Hernández-Fernández, F. J.; Bayo, J.; Pérez de los Ríos, A.; Vicente, M. A.; Bernal,

561 F. J.; Quesada-Medina, J. Discovering less toxic ionic liquids by using the Microtox®

562 toxicity test. Ecotoxicol. Environ. Saf. 2015, 116, 29−33.

563

564 (13) Atala, D. I. P.; Costa, A. C.; Filho, M. R.; Maugeri, F. Kinetics of Ethanol

565 Fermentation with High Biomass Concentration Considering the Effect of Temperature.

566 Appl. Biochem. Biotechnol. 2000, 91, 353−365.

567

568 (14) Ismail Hossain, M.; El-Harbawi, M.; Noaman, Y. A.; Bustam, M.A. B.; Alitheen, N.

569 A.; Affandi, N. B. M.; Hefter, G.; Yin, C.-Y. 2011.Synthesis and anti-microbial activity

570 of hydroxyl ammonium ionic liquids. Chemosphere 2011, 84, 101−104.

571

572 (15) Silva, K.; Rodrigues, S.; Filho, L.; Lima, A. Antimicrobial Activity of Broth
573 Fermented with Kefir Grain. Appl. Biochem. Biotechnol. 2009,152, 316−325.

574

575 (16) Hernández-Fernández, F. J.; Pérez de los Ríos, A.; Gómez, D.; Rubio, M.; Víllora,
576 G. Selective extraction of organic compounds from transesterification reaction mixtures
577 by using ionic liquids. AIChE J.2010, 56, 1213−1217.

578

579 (17) Wood, N.; Ferguson, J. L.; Nimal Gunaratne, H. Q.; Seddon, K.R.; Goodacre, R.;
580 Stephens, G. M. Screening ionic liquids for use in bio-transformations with whole
581 microbial cells. Green Chem. 2011, 13,1843−1851.

582

583 (18) Zhu, S.; Yu, P.; Lei, M.; Tong, Y.; Zheng, L.; Zhang, R.; Ji, J.;Chen, Q.; Wu, Y.
584 Investigation of the toxicity of the ionic liquid 1-butyl-3-methylimidazolium chloride to
585 Saccharomyces cerevisiaeAY93161 for lignocellulosic ethanol production. Pol. J. Chem.
586 Technol.2013, 15, 94−98.

587

588 (19) Docherty, K. M.; Kulpa, C. F., Jr. Toxicity and antimicrobial activity of imidazolium
589 and pyridinium ionic liquids. Green Chem.2005, 7, 185−189.

590

591 (20) Hernández-Fernández, F. J.; Bayo, J.; Pérez de los Ríos, A.; Vicente, M. A.; Bernal,
592 F. J.; Quesada-Medina, J. Discovering less toxic ionic liquids by using the Microtox®
593 toxicity test. Ecotoxicol. Environ. Saf. 2015, 116, 29−33.

594

595 (21) Pfruender, H.; Jones, R.; Weuster-Botz, D. Water immiscible ionic liquids as
596 solvents for whole cell biocatalysis. J. Biotechnol. 2006,124, 182−190.

597

598 (22) Sendovski, M.; Nir, N.; Fishman, A. Bioproduction of 2-phenylethanol in a biphasic
599 ionic liquid aqueous system. J. Agric. FoodChem. 2010, 58, 2260−2265.

600

601 (23) Ouellet, M.; Datta, S.; Dibble, D. C.; Tamrakar, P. R.; Benke, P.I.; Li, C.; Singh, S.;
602 Sale, K. L.; Adams, P. D.; Keasling, J. D.; Simmons, B. A.; Holmes, B. M.;
603 Mukhopadhyay, A. Impact of ionic liquid pretreated plant biomass on Saccharomyces
604 cerevisiae growth and biofuel production. Green Chem. 2011, 13, 2743−2749.

605

606 (24) Zhu, S.; Yu, P.; Tong, Y.; Chen, R.; Lv, Y.; Zhang, R.; Lei, M.; Ji,J.; Chen, Q. Effects

607 of the Ionic Liquid 1-Butyl-3-methylimidazoliumChloride on the Growth and Ethanol

608 Fermentation of Saccharomyces cerevisiae AY92022. Chem. Biochem. Eng. Q. 2012, 26,

609 105−109.

610

611 (25) Mehmood, N.; Husson, E.; Jacquard, C.; Wewetzer, S.; Büchs,J.; Sarazin, C.;

612 Gosselin, I. Impact of two ionic liquids, 1-ethyl-3-methylimidazolium acetate and 1-

613 ethyl-3-methylimidazolium methylphosphonate, on Saccharomyces cerevisiae:

614 metabolic, physiologic and morphological investigations. Biotechnol. Biofuels 2015, 8,

615 1−13.

616

617 (26) Dickinson, Q.; Bottoms, S.; Hinchman, L.; McIlwain, S.; Li, S.; Myers, C. L.; Boone,

618 C.; Coon, J. J.; Hebert, A.; Sato, T. K.; Landick, R.; Piotrowski, J. S. Mechanism of

619 imidazolium ionic liquids toxicity in Saccharomyces cerevisiae and rational engineering

620 of a tolerant, xylose fermenting strain. Microb. Cell Fact. 2016, 15, 1−13.

621

622 (27) Rebros, M.; Gunaratne, H. Q. N.; Ferguson, J.; Seddon, K. R.; Stephens, G. A high

623 throughput screen to test the biocompatibility of water-miscible ionic liquid. Green

624 Chem. 2009, 11, 402−408.

625

626 (28) Wood, N.; Stephens, G. Accelerating the discovery of biocompatible ionic liquids.

627 Phys. Chem. Chem. Phys. 2010, 12,1670−1674

628

629 (29) Jork, C.; Seiler, M.; Beste, Y. A.; Arlt, W. Influence of Ionic Liquids on the Phase

630 Behavior of Aqueous Azeotropic Systems. J. Chem. Eng. Data 2004, 49, 852−857.

631

632 (30) Endres, F.; El Abedin, S. Z. Air and water stable ionic liquids in physical chemistry.

633 Phys. Chem. Chem. Phys. 2006, 8, 2101−2116.

634

Table 1. Solubility of the assayed ionic liquids in pure water.

Ionic liquid	Solubility (% v/v)	Ionic liquid	Solubility (% v/v)
[OMIM+][NTf2-]	< 0.04	[HMIM+][dca-]	> 10
[MTOA+][NTf2-]	< 0.02	[OMIM+][dca-]	> 10
[BMIM+][NTf2-]	< 0.28	[MOMMIM+][dca-]	> 10
[BMIM+][PF6-]	< 0.50	[MOEMIM+][dca-]	> 10
[OMIM+][BF4-]	< 1.4	[HOPMIM+][dca-]	> 10
[MOMMIM+][NTf2-]	< 2.0	[BMPy+][dca-]	> 10
[EOEMIM+][PF6-]	< 2.0	[EMmor+][dca-]	> 10
[EOEMIM+][NTf2-]	< 2.0	[BMIM+][Cl-]	> 10
[Hex3TDP+][dca-]	< 2.0	[HMIM+][Cl-]	> 10
[Hex3TDP+][Br-]	< 2.0	[OMIM+][Cl-]	> 10
[Hex3TDP+][Cl-]	< 2.0	[Moxa+][MeSO4-]	> 10
[EPy+][NTf2-]	< 2.0	[MPy+][MeSO4-]	> 10
[MOPy+][dca−]	< 8.0	[BMPheIM+][MeSO4−]	> 10
[EMIM+][BF4-]	> 10	[EPy+][EtSO4-]	> 10
[BMIM+][BF4−]	> 10	[BMIM+][NO3−]	> 10
[BDIMIM+][BF4]	> 10	[CH3CH2NH3+][NO3−]	> 10
[MOMMIM+][BF4−]	> 10	[iBu3MeP+][TOS−]	> 10
[MOEMIM+][BF4−]	> 10	[MOMMIM+][CF3SO3−]	> 10
[BMPy+][BF4−]	> 10	[BMIM+][acetate−]	> 10
[MOPy+][BF4−]	> 10	[BMIM+][glycolate−]	> 10
[EMIM+][dca−]	> 10		
[BMIM+][dca−]	> 10		

Table 2. Extraction percentages of ethanol from aqueous solutions with ionic liquids

Ionic liquid	E_{24h} (%)	E_{48h} (%)	$E_{21\ days}$ (%)
[Hex3TDP+][Cl-]	51.3 ± 2.8	56.3 ± 2.8	61.7 ± 4.8
[Hex3TDP+][dca-]	47.3 ± 3.2	41.4 ± 2.8	45.2 ± 4.0
[Hex3TDP+][Br-]	46.0 ± 3.7	51.2 ± 2.8	52.0 ± 3.2
[MTOA+][NTf2-]	39.4 ± 1.7	35.4 ± 2.8	37.2 ± 3.1
[OMIM+][BF4-]	31.0 ± 1.1	36.8 ± 2.8	40.8 ± 4.2
[EPy+][NTf2-]	27.9 ± 2.0	30.2 ± 2.8	32.5 ± 4.2
[BMIM+][PF6-]	19.1 ± 2.4	24.9 ± 2.8	26.6 ± 1.5
[BMIM+][NTf2-]	12.6 ± 1.7	26.0 ± 2.8	26.2 ± 1.4
[OMIM+][NTf2-]	7.8 ± 1.8	23.7 ± 2.8	29.1 ± 0.4

Table 3. Effect of ionic liquids on the growth of the yeast S. cerevisiae [a].

ILs	Radius of inhibition (cm in solid medium)		In liquid medium	
	Pure ionic liquids	3% (v/v) ionic liquids	D_w (g L^{-1})	μ (h^{-1})
Control	0.00 ± 0.00	0.00 ± 0.00	5.33	0.43
[BMIM+][NTf2-]	0.00 ± 0.00	0.00 ± 0.00	6.44	0.47
[MTOA+][NTF2-]	0.00 ± 0.00	0.00 ± 0.00	5.81	0.30
[BMIM+][PF6-]	0.4 ± 0.10	0.00 ± 0.10	2.24	0.32
[OMIM+][NTf2-]	0.3 ± 0.10	0.20 ± 0.05	3.60	0.12
[Hex3TDP+][Cl-]	0.2 ± 0.05	0.40 ± 0.10	1.75	0.08
[Hex3TDP+][Br-]	0.9 ± 0.25	0.40 ± 0.10	0.34	0.03
[EPy+][NTf2-]	0.5 ± 0.15	0.40 ± 0.10	0.84	0.02
[OMIM+][BF4-]	1.3 ± 0.40	0.40 ± 0.10	0.64	0.00
[Hex3TDP+][dca-]	0.5 ± 0.10	0.40 ± 0.10	0.34	0.00

[a] Inhibition zones were evaluated using the agar diffusion test in the presence of pure ionic liquids and 3% (v/v) IL concentrations. D_w represents final dry weight concentration of yeast at 48 h, μ represents specific growth rates (h^{-1}). Data are the means of 3 replicates.
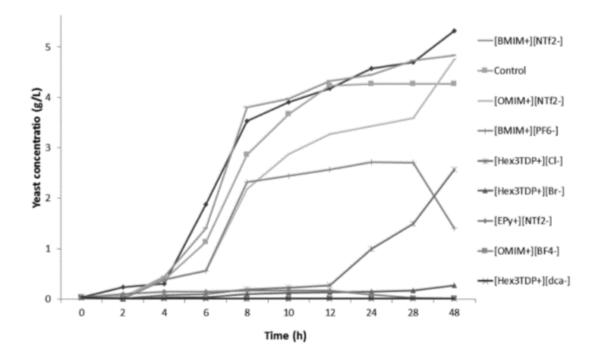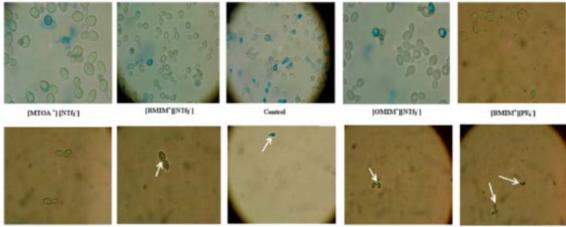
Figure 1. Growth curves of the yeast Saccharomyces cerevisiae in the presence of ionic liquids and control.

Figure 2. Morphological image of the yeast S. cerevisiae after 48 h of culture with different ionic liquids using the blue methylene staining (arrow: death of cells (small, dark blue cells).