Chemosphere 128 (2015) 178–183

Contents lists available at ScienceDirect

Chemosphere

journal homepage: www.elsevier.com/locate/chemosphere

Hormetic effect of ionic liquid 1-ethyl-3-methylimidazolium acetate on bacteria

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HIGHLIGHTS

- Among three ILs tested, only [EMIM]Ac exhibited hormesis in Gram −ve and Gram +ve bacteria.
- Growth of Gram −ve aerobic bacterium *Pseudomonas putida* was increased by 4-fold in presence of 0.5 g L^{-1} of [EMIM]Ac.
- Growth of Gram +ve anaerobic bacterium *Clostridium* sp. was increased by 0.4-fold in presence of 0.5 g L^{-1} of [EMIM]Ac.
- Hormesis of [EMIM]Ac on bacterial growth was mediated via regulation of medium pH.

ARTICLE INFO

Article history:
Received 19 March 2014
Received in revised form 18 January 2015
Accepted 24 January 2015
Available online 19 February 2015

Handling Editor: Shane Snyder

Keywords:
1-Ethyl-3-methylimidazolium acetate
Biomass pretreatment
Clostridium sp.
Hormesis
Imidazolium ionic liquids
Pseudomonas putida

ABSTRACT

The biological effect of ionic liquids (ILs) is one of the highly debated topics as they are being contemplated for various industrial applications. 1-ethyl-3-methylimidazolium acetate ([EMIM][Ac]) showed remarkable hormesis on anaerobic *Clostridium* sp. and aerobic *Pseudomonas putida*. Bacterial growth was stimulated at up to 2.5 g L^{-1} and inhibited at >2.5 g L^{-1} of [EMIM][Ac]. The growth of *Clostridium* sp. and *P. putida* were higher by 0.4 and 4-fold respectively, in the presence of 0.5 g L^{-1} [EMIM][Ac]. Assessment of the effect of [EMIM][Ac] under different growth conditions showed that the hormesis of [EMIM][Ac] was mediated via regulation of medium pH. Hormetic effect of [EMIM][Ac] was evident only in medium with poor buffering capacity and in the presence of a fermentable substrate as the carbon source. The hormetic effect of [EMIM][Ac] on bacterial growth is most likely associated with the buffering capacity of acetate anion. These observations have implications in ILs toxicity studies and ecological risk assessment.

1. Introduction

Ionic liquids (ILs) are novel class of organic salts with low melting points (<100 °C), increasingly considered as green replacements for volatile organic compounds (Patel and Lee, 2012; Passos et al., 2014). Ionic liquids are typically made up of two components, a bulky organic cation (i.e. N,N′-dialkylimidazolium, N-alkylpyridinium, alkylammonium, alkylphosphonium, alkylsulphonium and triazolium) and an organic or inorganic anion (i.e. halides, tetrafluoroborate, hexafluorophosphate, alkylphosphates, acetate) (Bubalo et al., 2014). These compounds have been in the spotlight of scientific and industrial community as novel green solvents for replacement of conventional volatile solvents (Bubalo et al., 2014). Ionic liquids are attractive due to their low vapor pressure, non-flammability, and high thermal stability. Importantly, ionic liquids offer unprecedented flexibility in designing several classes of compounds with novel physical and chemical properties by means of tuning cation and anion structure (Earle and Seddon, 2000). Ionic liquids are extensively studied for applications in organic synthesis, separation technology, biocatalysis, corrosion inhibitors, biomass pretreatment and in use as corrosion inhibitors and antimicrobials (Plechkova and Seddon 2008; Pham et al., 2010; Nancharaiah et al., 2012a,b; Brandt et al., 2013).

Lignocellulosic materials (i.e. wood, agricultural or forest residues) are most abundant on our planet and available at a much lower cost than starch and sucrose based materials for production of biofuels (Brandt et al., 2013). However, major obstacle in using the lignocellulosic materials is the non availability of cost-effective pretreatment technologies for hydrolysis and deconstruction to

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http://dx.doi.org/10.1016/j.chemosphere.2015.01.032
0045-6535/

readily fermentable products (Datta et al., 2010). Ionic liquid based pretreatment methods show promise for cellulose dissolution and biomass deconstruction (Zavrel et al., 2009; Brandt et al., 2013). Ionic liquids with chloride, acetate, and phosphate anions showed good cellulose dissolution capacities (Vitz et al., 2009). The cellulose dissolving abilities of 1-ethyl-3-methylimidazolium acetate ([EMIM][Ac]), 1-ethyl-3-methylimidazolium dimethylphosphate ([EMIM][DEP]) and 1-methyl-3-methylimidazolium dimethylphosphate ([MMIM][DMP]) were reported to be in the range of 8%, 10% and 12–14% (w/v) respectively (Vitz et al., 2009). Moreover, the pretreatment methods should neither introduce nor generate compounds that would negatively impact the downstream processes such as fermentation. Interestingly, the growth and fermentative metabolism of *Clostridium* sp. was not inhibited by 1-ethyl-3-methyl imidazolium and 1-methyl-3-methyl imidazolium ionic liquids with anions such as acetate, dimethylphosphate or diethylphosphate up to 2.5 g L^{-1} (Nancharaiah and Francis, 2011).

High volume production and wide applications of ionic liquids could lead to pollution of aquatic environments due to water solubility of ILs. Many studies have shown that ILs are persistent in the environment and exhibit toxicity towards prokaryotic and eukaryotic organisms (Pham et al., 2010; Bubalo et al., 2014). However, the toxicity of IL is dependent on cation, alkyl chain length of substituent of cation, and anion. Recently, hormesis was observed in case of certain ionic liquids, particularly those with short alkyl chains (Ge et al., 2010; Nancharaiah and Francis 2011; Wang et al., 2011a,b; Zhang et al., 2013a; Zhang et al., 2013b). Hormesis was originally applied to describe the effect of low doses of ionizing radiation, but now it is generally used to describe biphasic dose–response of biological systems to environmental conditions or stress (Davies et al., 2006). In toxicology, hormesis is defined as a biphasic dose–response phenomenon primarily characterized by stimulation of biological response at lower concentrations while inhibition at higher concentrations. The hormetic response of ionic liquids is a poorly understood phenomenon and the chemical and biochemical mechanisms are unknown. Among the three ionic liquids (i.e. [EMIM][Ac], [EMIM][DEP], [MMIM][DMP]), tested for their influence on the growth and fermentative metabolism of *Clostridium* sp. BC1, only [EMIM][Ac] showed hormetic effect. Consequently, the aim of the present study was to investigate the mode of action of hormesis by determining the hormetic effect of [EMIM][Ac] on anaerobic Gram +ve and Gram −ve bacteria under different growth conditions.

2. Materials and methods

2.1. Ionic liquids

The structures of ILs [EMIM][Ac], [EMIM][DEP] and [MMIM][DMP] used in the present study are shown in Table 1. All the ionic liquids were obtained from Sigma–Aldrich and used as received.

2.2. Ionic liquid stock solutions

Stock solutions of ionic liquids were prepared in de-ionized water as described earlier (Nancharaiah and Francis, 2011). The ionic liquid solutions were sterilized by filtering through 0.22 μm Millex filter. The ionic liquid solutions were transferred to serum bottles, closed with butyl rubber stoppers, aluminum crimp sealed and deoxygenated by purging with ultra high purity (UHP) N_2 gas. The ionic liquid stock solutions were stored at room temperature.

2.3. Bacterial cultures and growth conditions

Clostridium sp. BC1 (ATCC 53464), gram-positive, anaerobic, fermentative bacterium, is phylogenetically closely related to *C. pasteurianum*. It was grown in mineral salts medium in serum bottles as described earlier (Nancharaiah and Francis, 2011). The mineral salts medium contained the following: glucose, 10.0 g; NH_4Cl, 0.5 g; glycerol phosphate, 0.3 g; $MgSO_4{\cdot}7H_2O$, 0.2 g; $CaCl_2{\cdot}2H_2O$, 0.5 g; $FeSO_4{\cdot}7H_2O$, 0.005 g; peptone, 0.1 g; yeast extract, 0.1 g; deionized water, 1 L; pH, 6.8. The medium contained glycerol phosphate as the P source. The medium was pre-reduced by boiling for 10 min while purging with UHP nitrogen gas. The medium was dispensed as 40 mL aliquots into 60 mL serum bottles in an anaerobic chamber (Coy Laboratory products, USA). The serum bottles containing media were fitted with butyl rubber stoppers, crimp sealed with aluminum caps and autoclaved. The culture was maintained by repeated sub-culturing in serum bottles by inoculating autoclaved MS medium with 1 mL of log phase culture. The serum bottles were incubated at 26 °C.

Pseudomonas putida TUM-PP12 (Nancharaiah et al., 2003, 2008), a gram-negative bacterium, was maintained in Luria Bertani agar (Difco, USA) plates supplemented with 50 μg mL^{-1} kanamycin under aerobic conditions. For liquid cultures, *P. putida* was routinely grown in 250 mL Erlenmeyer flasks containing 100 mL sterile mineral salts medium by inoculating with log phase culture. The culture flasks were incubated at 30 °C in an orbital shaker set at 100 rpm.

2.4. Effect of ionic liquids on Clostridium sp. and P. putida

To determine the effect of ILs on *Clostridium* sp. different concentrations (0.5–10 g L^{-1} w/v) of ILs were added to serum bottles containing sterile mineral salts medium. Sterile mineral salts medium without ionic liquids was used as control. The serum bottles with and without ionic liquids in mineral salts medium were inoculated with 1 mL of 24 h-old *Clostridium* sp. culture (OD 0.4). The serum bottles were incubated at 26 °C. At periodic time intervals, total gas production was measured. After measuring the total gas production, a 4 mL of the culture sample was removed with a syringe for monitoring growth and medium pH.

To determine the effect of ILs on *P. putida*, Erlenmeyer flasks (250 mL volume) containing 100 mL of sterile mineral salts medium with and without [emim]Ac were inoculated with 1 mL of 24 h old culture of *P. putida*. The flasks were incubated at 30 °C in an orbital shaker set at 100 rpm. Liquid samples were collected at regular time intervals for measuring growth and pH. The effect of [EMIM][Ac] on the growth of *P. putida* was also determined in MS medium supplemented with acetate as sole carbon source and tryptic soy broth (Difco, USA) under the similar experimental conditions.

2.5. Effect of ionic liquids on growth in phosphate-buffered mineral salts medium

In order to understand the hormetic effect of [EMIM][Ac], the growth of *Clostridium* sp. and *P. putida* was determined in phosphate buffered mineral salts (PMS) medium (Nancharaiah et al., 2012a,b). The PMS medium consisted of the following: glucose, 10.0 g; NH_4Cl, 0.5 g; $MgSO_4{\cdot}7H_2O$, 0.2 g; KH_2PO_4, 5 g; $K_2HPO_4{\cdot}3H_2O$, 6.55 g; $FeSO_4{\cdot}7H_2O$, 0.005 g; peptone, 0.1 g; yeast extract, 0.1 g; deionized water to 1 L; pH, 6.8. The serum bottles and flasks were prepared with PMS medium and autoclaved as mentioned above. [EMIM][Ac] was added to serum bottles or culture flasks containing PMS medium. PMS medium without ionic liquid was used as the control. The serum bottles and flasks with and without ionic liquid were inoculated with *Clostridium* sp. and *P. putida*