BNL-107565-2015-JA

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

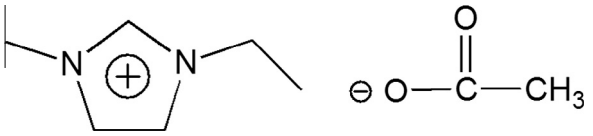
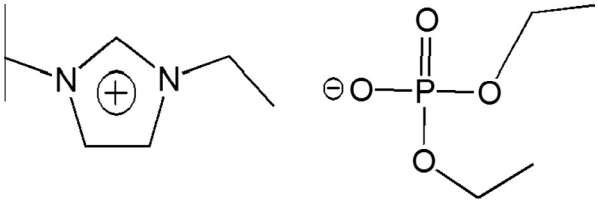
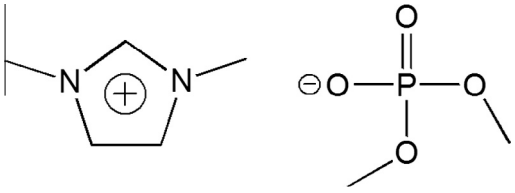
To determine the effect of ILs on *Clostridium* sp. different concentrations (0.5–10 g L^{-1} w/v) of ILs were added to serum bottles containing sterile mineral salts medium. Sterile mineral salts medium without ionic liquids was used as control. The serum bottles with and without ionic liquids in mineral 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*

Table 1
The structure of ionic liquids used in the present study.

IL name	Abbreviation	Structure
1-Ethyl-3-methylimidazolium acetate	[EMIM][AC]	
1-Ethyl-3-methylimidazolium diethylphosphate	[EMIM][DEP]	
1,3-Dimethylimidazolium dimethylphosphate	[MMIM][DMP]	

respectively and incubated as described above. All experiments were setup in triplicate serum bottles or culture flasks. The liquid samples were collected at regular time intervals for monitoring growth and pH.

2.6. Analytical methods

Bacterial growth was monitored by measuring turbidity at 600 nm using Spectronic 20 spectrophotometer (Thermo Scientific, USA) or UV-3600 UV–Vis spectrophotometer (Shimadzu, Japan). Total gas produced by *Clostridium* sp. was measured using a pressure gauge connected to a syringe (Francis and Dodge, 1987). An aliquot of the culture sample was filtered through 0.45 μm Millex filter and the pH was determined using a Beckman 350 pH meter with a Beckman 511275-AB combination electrode. Glucose was analyzed by HPLC. The HPLC system consisted of a SCL-10A controller, a SIL-10A sample autoinjector, and a LC-10AS liquid chromatograph (Shimadzu, Japan). The HPLC system was fitted with a Bio-Rad HPX-87H column (Bio-Rad labs, USA) and mobile phase (0.003 N H_2SO_4) was pumped at flow rate of 0.7 mL min^{-1}. Glucose concentration was determined using RID-6A refractive index detector (Shimadzu, Japan).

3. Results and discussion

3.1. Effect of ionic liquids on Clostridium sp. growth

The effect of water miscible imidazolium ILs with short alkyl chains on the cation (-ethyl and -methyl) and three different anion groups (acetate, diethylphosphate and dimethylphosphate) on *Clostridium* sp. growth indicated that the toxicity was dependant with the type of IL and its concentration. The growth curves of *Clostridium* sp. could be characterized with distinct rapid exponential and extended stationary phases. *Clostridium* sp. grew rapidly in the first 24 h by fermenting the glucose. This rapid exponential growth has caused a significant drop in the medium pH to ~2.8 (Fig. 1), which has lowered the subsequent growth of *Clostridium* sp. The growth of *Clostridium* sp. was higher by almost 40% in mineral salts medium in the presence of lower concentrations of [EMIM][Ac]. Delay and complete inhibition in the growth of *Clostridium* was observed in the presence of moderate and higher concentrations of [EMIM][Ac], respectively. The growth of

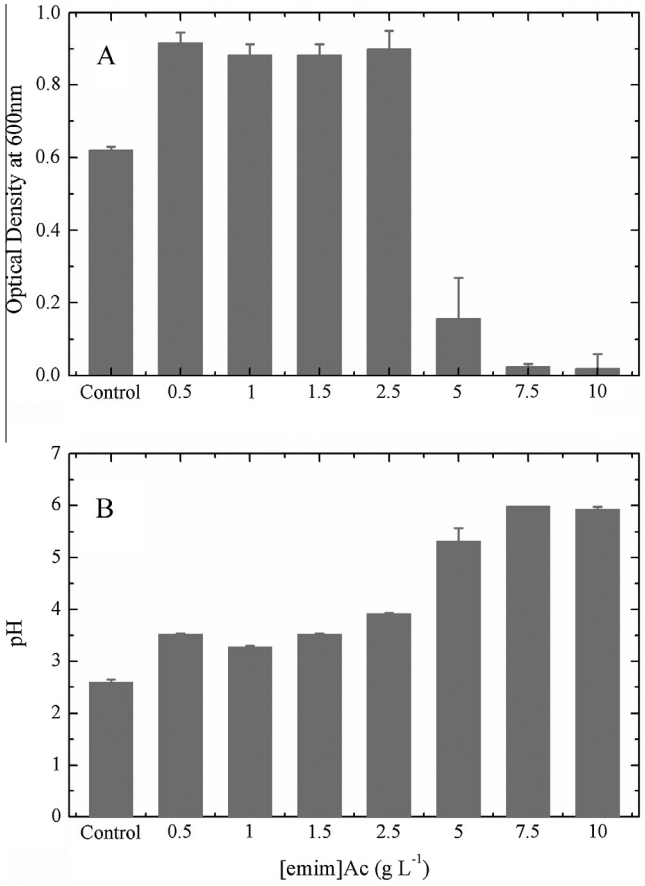


Fig. 1. Growth (A) and medium pH (B) of *Clostridium* sp. BC1 in mineral salts medium supplemented with different concentrations of 1-ethyl-3-methylimidazolium acetate ([EMIM][Ac]). Growth measured at 96 h of inoculation is shown. Symbols and error bars represent averages and standard deviations from triplicate experiments.

Clostridium sp. was not significantly altered in the presence of [EMIM][DEP] up to 2.5 g L^{-1}. However, the final growth was decreased in the range of 9–60% in the presence of higher concentrations of [EMIM][DEP]. The growth was not significantly altered by [EMIM][DMP] up to 4 g L^{-1}. The growth of *Clostridium* sp. was either retarded or completely inhibited at higher concentrations of [MMIM][DMP]. Overall, ionic liquids used in the present study did not cause inhibition in the growth and fermentative metabolism of *Clostridium* sp. up to 2.5 g L^{-1} (Fig. 1, Figs. SM-1 and SM-2). The toxicity of ionic liquids was evident in either delaying the growth or causing complete growth inhibition, which is seen only at higher concentrations above >2.5 g L^{-1}. Interestingly, [EMIM][Ac] exhibited hormesis, thereby increasing the growth of *Clostridium* sp. at lower concentrations ranging from 0.5 to 2.5 g L^{-1} (Fig. 1). At higher concentrations (>2.5 g L^{-1}) of [EMIM][Ac], the growth of *Clostridium* sp. was inhibited significantly. The growth of *Clostridium* sp. was completely inhibited at 7.5 g L^{-1} and higher concentrations of [EMIM][Ac]. Whereas the other two ionic liquids, [EMIM][DEP] and [MMIM][DMP] caused a concentration dependent inhibition in the growth (Fig. SM-1) and fermentative metabolism (Figs. SM-2 and SM-3) of *Clostridium* sp.

3.2. Hormetic effect of [EMIM][Ac]

In the presence of [EMIM][Ac], the pH of mineral salts medium was always found to be higher than that of control (Figs. 1 and 2).

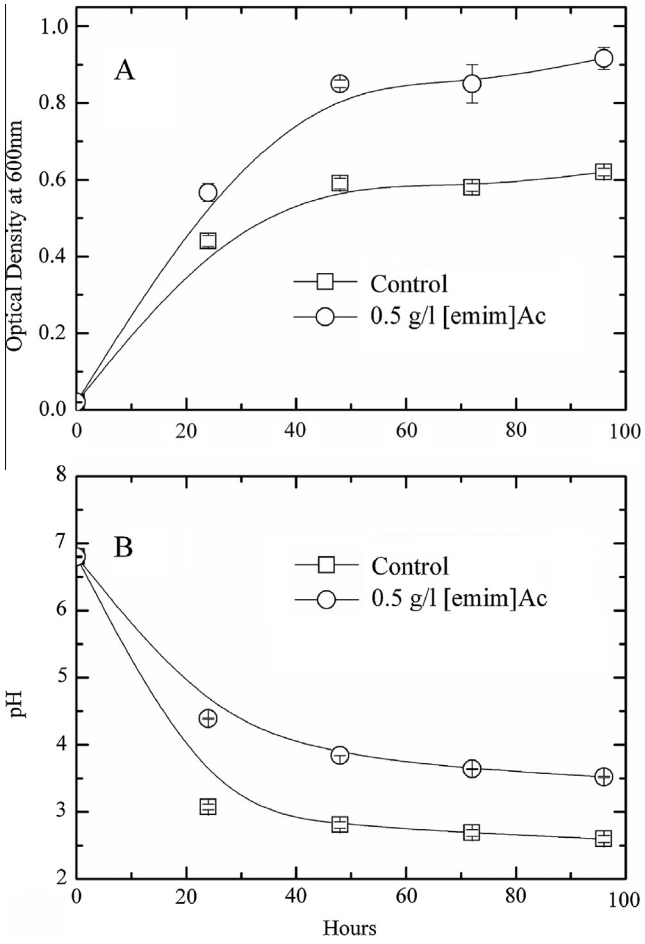


Fig. 2. *Clostridium* sp. BC1 growth (A) and medium pH (B) in mineral salts (MS) medium (control), and MS medium amended with 0.5 g L^{-1} [EMIM][Ac]. Symbols and error bars represent averages and standard deviations from triplicate experiments.

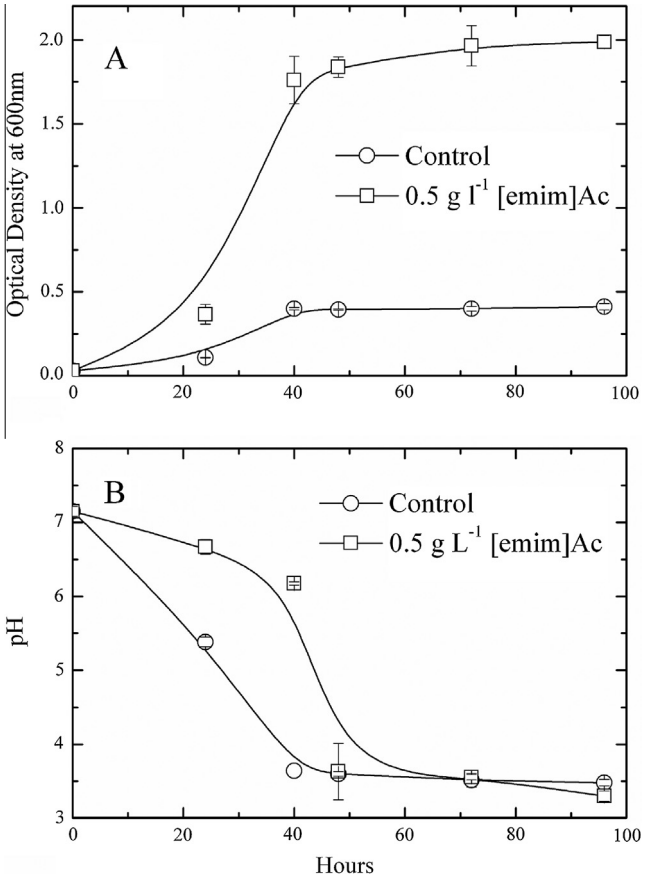


Fig. 3. *Clostridium* sp. BC1 growth (A) and medium pH (B) in phosphate buffered mineral salts (PMS) medium (control), and PMS medium amended with 0.5 g L^{-1} [EMIM][Ac]. Symbols and error bars represent averages and standard deviations from triplicate experiments.

The pH of MS medium remained at 4 or higher in the presence of ionic liquid that facilitated growth. *Clostridium* sp. growth was almost 40% higher in mineral salts medium supplemented with 0.5 g L^{-1} of [EMIM][Ac] as compared to the growth observed in mineral salts medium without ionic liquid (Fig. 2). Enhanced growth of *Clostridium* sp. was corroborated with higher glucose utilization (Fig. SM-3) and total gas production (Fig. SM-2).

The hormetic effect of [EMIM][Ac] was even observed on growth of a Gram negative bacterium *P. putida*. In fact, the enhancement in the growth of *P. putida* was much higher in mineral salts medium supplemented with [EMIM][Ac] (Fig. 3). The growth of *P. putida* was almost 400% higher in mineral salts medium supplemented with 0.5 g L^{-1} of [EMIM][Ac]. The pH of the mineral salts medium decreased to ~3.5 within 40 h of inoculation and remained stable thereafter. In the presence of ionic liquid, pH of the medium was maintained above 6.0 until 40 h and subsequently decreased to ~3.5. Based on the data, it was hypothesized that the hormetic effect of [EMIM][Ac] was mediated via medium pH regulation. This was verified by determining the growth of *Clostridium* sp. and *P. putida* in phosphate buffered mineral salts medium (PMS) with and without 0.5 g L^{-1} [EMIM][Ac]. The growth of *Clostridium* sp. or *P. putida* was much higher in PMS medium compared to the growth observed in MS medium. The higher growth was possible because the pH of PMS medium maintained at ~6.5 throughout growth phase. However, the growth of *Clostridium* sp. and *P. putida* were not enhanced by the addition of [EMIM][Ac] to PMS medium (Figs. 4 and 5). These results were in agreement with earlier findings that the

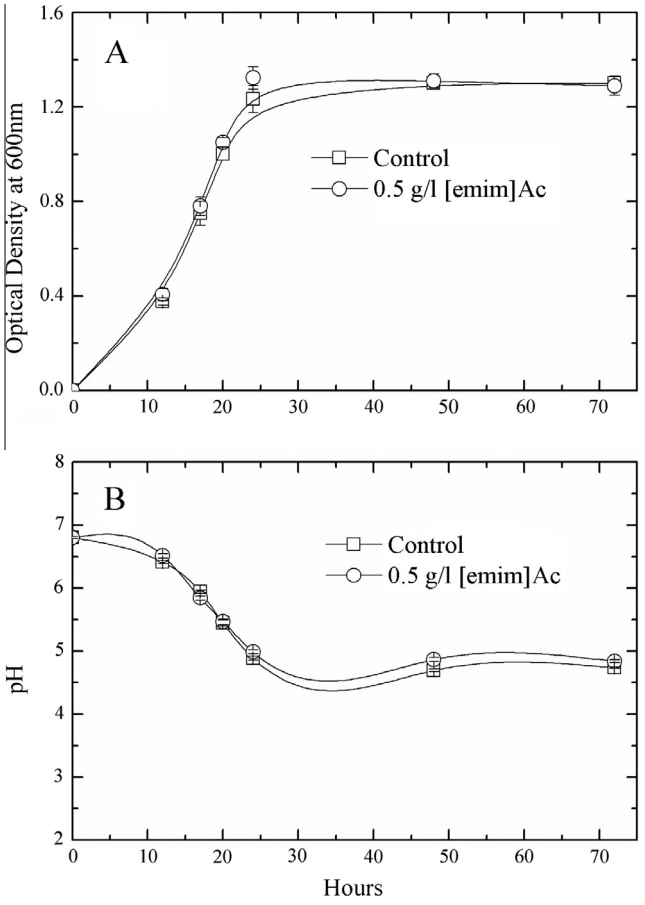


Fig. 4. *P. putida* growth (A) and medium pH (B) in mineral salts (MS) medium (control), MS medium amended with 0.5 g L^{-1} [EMIM][Ac]. Symbols and error bars represent averages and standard deviations from triplicate experiments.

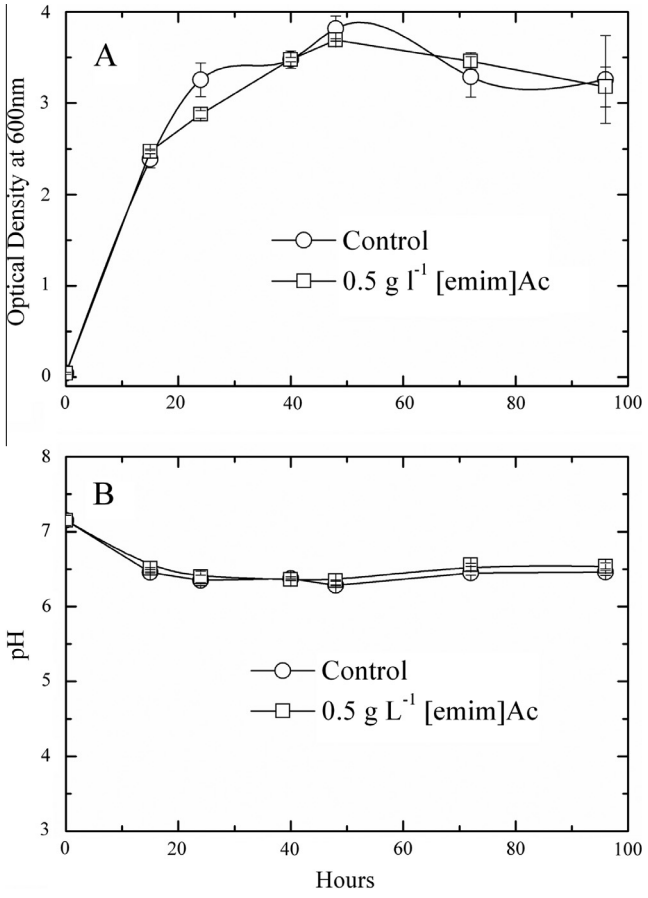


Fig. 5. *P. putida* growth (A) and medium pH (B) in phosphate-buffered mineral salts (PMS) medium (control), PMS medium amended with 0.5 g L^{-1} [EMIM][Ac]. Symbols and error bars represent averages and standard deviations from triplicate experiments.

stimulatory effect of [EMIM][Ac] on *Clostridium* sp. growth and fermentative metabolism could be simulated by the addition of bicarbonate to MS medium (Nancharaiah and Francis, 2011). Moreover, [EMIM][Ac] did not show hormesis on *P. putida* grown either in tryptic soy broth or in MS medium supplemented with a non-fermentable carbon source such as acetate (data not shown).

The toxicity of ionic liquids is associated with the type of cation, anion or the alkyl chain length of cation substituent's (Ranke et al., 2004; Nancharaiah and Francis, 2011; Wang et al., 2011a,b; Nancharaiah et al., 2012a,b). Certain ionic liquids, particularly those with short alkyl side chains have shown hormesis on bacteria (Ge et al., 2010; Nancharaiah and Francis 2011; Wang et al., 2011a,b; Zhang et al., 2013a,b;), microalgae (Cho et al., 2007, 2008), IPC-81 leukemia cells (Ranke et al., 2004) and HeLa cells (Stepnowski et al., 2004). The luminescence of *Vibrio qinghaiensis* sp. –Q67 was induced remarkably by 1-ethyl-3-methylimidazolium tetrafluoroborate (Wang et al., 2011a; Wang et al., 2011b). Ge et al. (2010) have predicted hormetic effects of ionic liquid mixtures on luciferase activity using concentration addition model. The mechanistic aspects of hormesis exhibited by ionic liquids are largely unknown. Dipeolu et al. (2009) hypothesized that N,N-dimethylethanolammonium acetate increased the growth rate of *Clostridium sporogenes* by either increasing the bioavailability of nutrients or by biodegradation of ionic liquid itself (Dipeolu et al., 2009). Based on the data obtained in this study, it can be concluded that the hormesis of [EMIM][Ac] on bacterial growth was is dependent on the medium buffering capacity and the type of fermentable and/or non-fermentable nature of the substrate. Bacterial growth was enhanced [EMIM][Ac] only in a poorly buffered medium that contained a fermentable substrate as the carbon source. It is apparent that the hormetic effect of [EMIM][Ac] on *Clostridium* sp. or *P. putida* in poorly buffered medium supplemented with glucose as carbon source is mostly associated with buffering action of IL in particular acetate anion group.

4. Conclusions

Among the three water miscible ionic liquids, 1-ethyl-3-methylimidazolium acetate, 1-ethyl-3-methylimidazolium diethyl phosphate and 1-methyl-3-methylimidazolium dimethylphosphate, tested only [EMIM][Ac] showed remarkable hormesis on *Clostridium* sp. and *P. putida* growth. The 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* increased by 0.4 and 4-times, respectively in mineral salts medium supplemented with 0.5 g L^{-1} of [EMIM][Ac]. The enhancement in bacterial growth due to [EMIM][Ac] was not evident in tryptic soy broth, phosphate buffered mineral salts medium, or mineral salts medium that contained a non-fermentable substrate. It is evident that the bacterial growth was enhanced only in poorly buffered medium that contained a fermentable substrate as carbon source. Therefore, the hormesis of [EMIM][Ac] could be alleviated by growing bacterial cultures in a phosphate buffered mineral salts medium or mineral salts medium supplemented with a non-fermentable substrate.

Acknowledgments

This research was in part supported by BK-21 plus program through the National Research Foundation of Korea funded by the Ministry of Education, Science and Technology (R31 – 30005) and by the Office of Biological and Environmental Research, Office of Science, US. Department of Energy, under contract No. DE-SC00112704.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.chemosphere.2015.01.032.

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