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Biodegradation and toxicity of emerging contaminants: Isolation of an exopolysaccharide-producing *Sphingomonas* sp. for ionic liquids bioremediation

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Declarations of interest: none

Highlights

- A microorganism metabolizing three major classes of ionic liquids was isolated
- MKIV achieved 91% removal for 100 mg L^{-1} of an imidazolium-based ionic liquid
- *D. magna* and algae were more sensitive to ionic liquids compared to *A. fischeri*
- *Sphingomonas* sp. MKIV produced 19.29 g L^{-1} of biopolymer from 25 g L^{-1} of glucose
- Biotreatment increased the environmental impact of tetrabutylammonium iodide

Abstract

Ionic liquids (ILs) have been characterized as contaminants of emerging concern (CEC) that often resist biodegradation and impose toxicity upon environmental release. *Sphingomonas* sp. MKIV has been isolated as an extreme microorganism capable for biodegradation of major classes of ILs. Six imidazolium-, pyridinium- and ammonium-based ILs (pyridinium trifluoromethanesulfonate [Py][CF_3SO_3], 1-(4-pyridyl)pyridinium chloride [1-4PPy][Cl], 1-butyl-3-methylimidazolium bromide [BMIM][Br], 1-butyl-3-methylimidazolium methanesulfonate [BMIM][$MeSO_4$], tetrabutylammonium iodide [n-Bu_4N][I] and tetrabutylammonium hexafluorophosphate [n-Bu_4N][PF_6]) were used for microbial growth. The strain achieved 91% and 87% removal efficiency for cultures supplemented with 100 mg L^{-1} of [BMIM][$MeSO_4$] and [n-Bu_4N][I] respectively. The metabolic activity of MKIV was inhibited following preliminary stages of cultures conducted using [BMIM][$MeSO_4$], [BMIM][Br], [Py][CF_3SO_3] and [n-Bu_4N][PF_6], indicating potential accumulation of inhibitory metabolites. Thus, a comprehensive toxicological study of the six ILs on *Aliivibrio fischeri*, *Daphnia magna* and *Raphidocelis subcapitata* was conducted demonstrating that the compounds impose moderate and low toxicity. The end-products from [BMIM][$MeSO_4$] and [n-Bu_4N][I] biodegradation were assessed using *Aliivibrio*

fischeri, exhibiting increased environmental impact of the latter following biotreatment. MKIV produced 19.29 g L^{-1} of biopolymer, comprising mainly glucose and galacturonic acid, from 25 g L^{-1} of glucose indicating high industrial significance for bioremediation and exopolysaccharide production. [1]

Keywords: Ionic liquid, Biodegradation, Toxicity assessment, Extracellular polymeric substances, Emerging contaminants

1. Introduction

ILs constitute organic salts comprising organic cations and organic/inorganic anions that remain in liquid form at room temperature. They have attracted substantial attention as emerging greener alternatives to traditional solvents mainly due to unique properties that include, negligible volatility, good performance as reaction media, low flammability and easily modified structures [1-4]. A wide range of compounds has been synthesized using cations such as imidazolium, pyridinium, pyrrolidinium, ammonium, cholinium and phosphonium [5], while varying the combination of cations and anions has led to numerous applications entailing exceptional impact [6]. Therefore, ILs comprise several uses as green solvents in (bio)catalysis and (bio)organic synthesis [7,8], biomass pretreatment [9], bioreactor technology [10], as well as extraction, absorption and degradation processes [11].

Abbreviations: Ionic liquids (ILs), contaminants of emerging concern (CEC), 1-butyl-3-methylimidazolium [bmim], pyridinium [py], tetrabutyl ammonium [Bu_4N], extracellular polymeric substances (EPS), mineral salts medium (MSM), metabolic activity inhibition (I), tightly bound extracellular polysaccharides (TB-EPS), loosely bound extracellular polysaccharides (LB-EPS).

Although ILs exhibit lower vapor pressure in relation to common solvents, their environmental impact can be significant [1] and they are not always aligned with "green chemistry" principles compared to other natural counterparts [12]. The length and branching of alkyl side chains of cations, as well as the presence of polar head groups, nitrogen in cationic aromatic rings and positively charged atoms in anions can exert toxicity [13]. Moreover, although studies regarding the occurrence of ILs in the environment are still scarce, the high solubility of many ILs [14] could result in environmental release via processing effluents and potentially impact the terrestrial and aquatic ecosystems. Current data support the toxic nature of ILs, which varies substantially between different organisms [15]. However, although they have been characterized as "*contaminants on the horizon*", environmental studies of these persistent molecules are limited highlighting the need for toxicity and biodegradability assessment [16].

The development of treatment technologies for IL-polluted wastewater is in agreement with the EU Water Framework Directive (2000/60/EC) stimulating establishment of efficient systems for CEC removal [17]. The 1-butyl-3-methylimidazolium [bmim], pyridinium [py] and tetrabutyl ammonium [Bu_4N] classes constitute some of the most common industrial choices of ILs [18]. [bmim]-ILs exhibit various applications as alternatives for VOC electrolytes due to high chemical stability and inertness [19]. However, early biodegradation data demonstrate that [bmim]-ILs are resistant to biodegradation accumulating in the environment [20]. Although [py]-ILs comprise similar inertness to [bmim]-ILs and include non-biodegradable compounds [22], some [py]-derivatives can be successfully metabolized [23]. Moreover, [Bu_4N]-

based compounds are widely used and evidence exists that they cannot biodegrade [24], highlighting the need to identify viable strains that improve the removal of IL-cations.

Herein, the toxicity posed by different [bmim]-, [py]-, and [Bu_4N]-ILs and the isolation of extreme microorganisms mineralizing several of these neoteric contaminants was investigated. The toxicity of six ILs (Table 1) on marine bacteria, green algae and crustaceans, was evaluated. One microbial isolate was identified and used for biodegradation of all six environmental pollutants. Moreover, the toxicity of end-products from biodegradation of [n-Bu_4N][I] and [BMIM][$MeSO_4$] was tested, while the capacity of the strain to produce extracellular polymeric substances (EPS) was also assessed.

2. Experimental

2.1 Toxicity tests

2.1.1 Acute toxicity assessment using marine bacteria

The toxicity of six ILs (Table 1) against *Aliivibrio fischeri* (NRRL B11177) was evaluated using an acute toxicity microscale test adapted from Parmaki et al. [25]. Stock solutions of 1000-2000 mg L^{-1} were serially diluted into nine different IL concentrations using 2% NaCl. The pH was maintained between 7-8 and the salinity was set at approximately 2% using an osmotic adjustment buffer. Lyophilized bacteria were reconstituted and kept at 15 °C throughout the test. A toxicity range-finding experiment was conducted for concentrations between 10-2000 mg L^{-1} and using the data generated definite concentrations were set for each IL based on individual toxicity.

Ten µl of inoculum was exposed to varying concentrations of test compounds. Each independent experimental was performed in triplicate and sampling included at least two technical replicates. Phenol was applied as positive control, whereas 2% NaCl was employed as negative control. The bioluminescence of bacteria was assessed at 0, 5 and 15 min of exposure. Bioluminescence inhibition was calculated by normalizing the inhibition percentage to Γ values followed by linear regression as described in Parmaki et al. [25].

2.1.2 Chronic toxicity testing using algae

The green algae *Raphidocelis subcapitata* was used for chronic toxicity testing of ILs. At least ten concentrations ranging between 3-909 mg L^{-1} were assessed in triplicate for each IL, while a given concentration was tested three times within each experiment. All solutions were prepared in algal cultivation media following manufacturer's instructions (Microbiotests, Belgium).

A miniaturized protocol was developed based on the 201 OECD guidelines [27] and Blaise and Férard [28]. *Raphidocelis subcapitata* was purchased as algal beads immobilized in inert matrix, which were dissolved using matrix dissolving media. Free cells were transferred into algal cultivation media, following removal of the matrix dissolving medium by discarding the supernatant twice after centrifugation. The initial inoculum concentration was 10^4 cells ml^{-1}, while 10 µl of inoculum and 200 µl of test solution supplemented with 10 µl algal test enrichment medium were transferred to 96-well polystyrene flat bottom microplates. Only central wells were used, whereas

peripheral cells were filled up with water to reduce evaporation. At least 10 wells were used as positive ($K_2Cr_2O_7$) and negative (algal cultivation media) control. Microplates were maintained at 24 °C under continuous gentle agitation and lighting. Optical density at 670 nm as well as fluorescence at excitation and emission wavelengths of 430 and 680 nm respectively were determined using a microplate reader (Tecan-Infinite 200 Pro, Switzerland).

Data were expressed as the total number of cells ml^{-1} using conversion equations correlating the total number of algal cells ml^{-1} to different algal density populations. A microscope at 200-400× magnification (SZ61, Olympus Corp., Japan) and Neubauer chambers were used to measure the total number of algal cells. Growth inhibition was calculated through comparison of the specific growth rate during exposure to test compounds against that of cells exposed to the negative control. Inhibition percentages were plotted against the concentration of test compounds and linear regression was used to calculate EC_{50} values. The validity criteria for the test to be acceptable included achieving: (i) an EC_{50} value for $K_2Cr_2O_7$ of 1.19 ± 0.27 mg L^{-1}, and (ii) an increase in the number of algal cells in the control by at least a factor of 16 over 72 h.

2.1.3 Acute toxicity assessment with crustaceans

Acute toxicity assessment of ILs was conducted determining the immobilization of *Daphnia magna* [29]. Each concentration was tested in triplicate with four internal replicates, while ephippia were hatched for 72 h and fed with *Spirulina* 2 h prior use. At least five concentrations ranging between 0.5-100 mg L^{-1} were employed for each IL. Positive and negative controls were conducted using $K_2Cr_2O_7$ and osmotically adjusted water respectively. Five neonates (<24 h) were placed in separate wells of a multiwell plate and exposed to IL-containing solutions as well as the control. Immobilization was

expressed as the total number of neonates not capable of swimming after gentle agitation for 15 s. Immobilization in the control was not significant (<5%), whereas the EC_{50} value for $K_2Cr_2O_7$ was acceptable (0.8-1.6 mg L^{-1}). The immobilization percentage was transformed into Probit values and plotted against the logarithmized concentration to calculate EC_{50} values as presented before [25].

2.1.4 Toxicity assessment of biotransformation products

The toxicity following biotreatment of [n-Bu_4N][I] and [BMIM][$MeSO_4$] using *Sphingomonas* sp. MKIV was evaluated through the *Aliivibrio fischeri* acute toxicity test. MKIV cultures were conducted under conditions described in section 2.4 using 10 mg L^{-1} as initial IL concentration. Two different stirring speeds (0 and 75 rpm) were applied and biomedium samples were withdrawn prior addition of the inoculum as well as following 5 d of biotreatment. *Aliivibrio fischeri* inhibition was determined as described above.

2.2 Pure strain isolation

The mineral salts medium (MSM) used for subcultures during the isolation procedure was adapted from [26] and comprised (g L^{-1}): $(NH_4)_2SO_4$ 0.5, K_2HPO_4 0.5, $MgCl_2$ 0.2, $CaCl_2 \cdot H_2O$ 0.007 and NaCl 0.5. MSM was autoclaved, the pH was adjusted to 7 and 100 mg L^{-1} of each IL was added in separate flasks following filter sterilization (Ahlstrom, Germany, 0.2 μm pore size). The environmental samples applied for IL-degraders' isolation included primary, secondary and activated sludge obtained from a municipal wastewater treatment plant (Sewerage Board of Limassol – Amathus, Moni, Cyprus). Samples were passed through 0.45 μm filters (Whatman, UK) and remaining solids were applied as inoculum (1% w/v).

All enrichment cultures were conducted aerobically using 25 ml working volume in 100 ml gas-tight flasks. Cultures contained inoculums and MSM, supplemented with a different IL each time, under sterile conditions and were incubated at 30 ^{o}C and 100 rpm for 5 d. Following three sequential enrichments 1 μl was withdrawn from flasks exhibiting high turbidity and plated on solid media (MSM supplemented with 1.5% (w/v) agar). The concentration of each IL in solid media was increased to 400 mg L^{-1} aiming to isolate the most tolerant strains. Each culture was incubated for 5 d at 30 ^{o}C and several sequential enrichments were required to observe pure identical colonies. Single colonies were re-cultivated into liquid MSM, while grown cells were stored in media containing 15% glycerol, at -80 ^{o}C. Only a single strain could tolerate 400 mg L^{-1} for all ILs tested, which was characterized and applied in the experiments.

2.3 Strain identification by 16S rRNA sequence analysis

The isolated strain was cultivated for 72 h in MSM supplemented with 10 g L^{-1} of glucose at 100 rpm and 30 ^{o}C. Samples of 1.5 ml were transferred into sterile tubes and centrifuged twice for 4 min at 13,500 rpm. The cell pellet was resuspended in 1% sodium dodecyl sulphate and 0.2 M NaOH for DNA extraction. PCR amplification and purification as well as sequencing alignment and comparison for homology of the 16S rRNA was performed as previously described [26]. The neighbor-joining method employing MEGA 6.06 software as well as the most similar sequences according to the NCBI database by BLASTn and boot strapping in 10000 replicates was used for phylogenetic tree construction.

2.4 Microbial cultures

IL biodegradation was performed using MSM supplemented with a different IL each time and conditions according to the requirements of each experiment. Samples of 5 ml were withdrawn from cultures pre-grown using MSM supplemented with 10 g L^{-1} of glucose, centrifuged at 15,000 rpm for 5 min and the cell pellet was used for inoculation. Abiotic controls were conducted supplementing 50 mg L^{-1} of IL to MSM, while biotic controls were performed incubating IL-free media inoculated with the microorganism. All chemicals used were at least 98% pure (Sigma-Aldrich Company Ltd, UK).

2.5 Analytical methods

2.5.1 CO_2 production and inhibition of metabolic activity

The concentration of CO_2 in the headspace of cultures was determined by gas chromatography (Agilent technologies 7820A GC system, USA). A ShinCarbon ST packed column (Restek Corporation, USA) was employed. Column temperature was maintained at 60 °C for 2 min, followed by an increase (using a rate of 20 °C min^{-1}) to 160 °C where it remained constant for 1 min. A thermal conductivity detector was used, applying argon as carrier gas, while the temperature of the injector and detector were maintained at 125 and 250 °C respectively. The concentration of CO_2 was used to calculate the percentage of metabolic activity inhibition (I) imposed by each IL as follows (C_c: CO_2 concentration in biotic control experiments, C_i: CO_2 concentration in IL-biodegradation experiments):

$$I = \frac{C_c - C_i}{C_c} \times 100 \quad (1)$$

2.5.2 Determination of [n-Bu_4N][I] and [BMIM][$MeSO_4$] concentration

Culture samples were centrifuged at 13,000 rpm for 5 min and the supernatant was filtered using 0.2 μm syringe filters. The concentration of [n-Bu_4N][I] and [BMIM][$MeSO_4$] was determined by absorbance at 226 nm and 216 nm respectively, on a UV/VIS spectrophotometer (JENWAY 7315, Staffordshire, UK) interpolating from calibration curves prepared using MSM supplemented with IL concentrations ranging between 1-100 mg L^{-1}. The coefficient of variation for 3 samples was 4.4% and 3.6% at a concentration level of 15 mg L^{-1} for [n-Bu_4N][I] and [BMIM][$MeSO_4$] respectively.

2.6 EPS production

2.6.1 Production of EPS on glucose media

The isolated strain was inoculated into 150 ml of MSM, supplemented with 25 g L^{-1} of glucose (a stock solution was sterilized separately) and 0.01 g L^{-1} of yeast extract, in 500 ml flasks. The pH was adjusted to 5.5 and cultures were incubated at 30 ^{o}C and 100 rpm for 48 h. Cells were harvested through centrifugation (6,000 rpm for 20 min) followed by washing and re-suspension in fresh media. 28 ml of re-suspended cells was applied as inoculum in 1 L flasks containing 400 ml of MSM.

2.6.2 Extraction of EPS

EPS was extracted as described in Bezawada et al. [30] following modification. Culture samples of 15 ml were centrifuged at 6,000 rpm for 20 min at 4 ^{o}C. Given that the tightly bound extracellular polysaccharides (TB-EPS) remain in the cell pellet and the loosely bound extracellular polysaccharides (LB-EPS) remain in the supernatant, the

latter was withdrawn and mixed in 1:1 ratio with 95% ethanol. The mixture was maintained overnight at -20 ^{o}C for LB-EPS precipitation. The cell pellet was re-suspended using 15 ml of deionized water and maintained at 65 ^{o}C for 30 min. Samples were centrifuged for 20 min at 6,000 rpm and the supernatant was treated with 95% ethanol as described above for precipitation of TB-EPS. Subsequently, both LB-EPS and TB-EPS was collected through centrifugation for 45 min at 6,000 rpm and 4 ^{o}C.

2.6.3 EPS dry weight, protein and carbohydrate content

Extracted EPS was passed through GF/C filters (Whatman glass microfiber filters, 47 mm diameter) followed by overnight drying at 105 ^{o}C. The dry weight of LB-EPS and TB-EPS was determined calculating the increase of the filter's dry mass following filtration. The protein content in LB-EPS and TB-EPS was measured through re-suspension of the biopolymer in deionized water (15 ml) and application of the Bradford assay [31]. The carbohydrate content of each type of EPS was analyzed using the phenol-sulfuric acid method [32].

2.6.4 Analysis of EPS composition

Extracellular polysaccharides were hydrolyzed according to Freitas et al. [33]. Samples were precisely weighted, following addition of 5 ml deionized water and 0.1 ml trifluoroacetic acid. The effect of polysaccharide concentration on chemical composition was evaluated during hydrolysis using four different concentrations (0.4, 1, 2 and 4 mg ml^{-1}). Hydrolysis was performed at 120 °C for 2 h and the hydrolysate's chemical composition was determined by HPLC (Prominence, Shimadzu, Japan) equipped with a Shimadzu RID-10A detector, a Shimadzu SIL-20AXR autosampler and a Shimadzu CTO-10ASvp column oven. Samples were eluted isocratically from a ROA organic acids H+ column (Phenomenex, USA) using 10 mM H_2SO_4 as eluent, flow rate

of 0.6 ml min^{-1}, temperature of 65 °C and 10 μl injection volume. Identification and quantification were conducted using various high-purity external standards (glucose, fructose, rhamnose, galactose, arabinose, mannose, xylose, raffinose, galacturonic acid, glucuronic acid, succinic acid, acetic acid, pyruvic acid, formic acid, fumaric acid and lactic acid).

2.7 Statistical analysis

One-way analysis of variance (ANOVA) was performed to evaluate significant differences in *Aliivibrio fischeri* inhibition values obtained following exposure to IL-containing MSM and samples obtained following IL-biotreatment. SigmaStat (version 3.5, Systat Software UK Ltd, UK) was employed, while post hoc comparisons were performed at P-values <0.05.

3. Results and discussion

3.1 Toxicological assessment of [bmim]-, [py]- and [Bu_4N]-ILs

Two exposure periods were investigated in *Aliivibrio fischeri*, *Daphnia magna* and *Raphidocelis subcapitata* assays (Table 2), demonstrating decrease in ecotoxicity of ILs in the following order [Bu_4N]>[py]>[bmim]. The long side-chains incorporated in [Bu_4N]-ILs could impose the high toxicity of these compounds, as previously shown [34,35]. [n-Bu_4N][I] was more toxic towards freshwater organisms compared to [n-Bu_4N][PF_6], whereas [n-Bu_4N][PF_6] demonstrated higher toxicity to marine bacteria. [Py][CF_3SO_3] was more toxic towards all endpoints tested given the higher toxicity of the [CF_3SO_3]-moiety compared to [Br] [36]. Moreover, [BMIM][$MeSO_4$] imposed higher toxicity than [BMIM][Br] towards all tested organisms. The elevated toxicity of [$MeSO_4$] compared to [Br] could be due to the long alkyl chain present facilitating

penetration of the cellular membrane, followed by morphological transformations and defects [37].

EC_{50} values indicate that immobilization of *Daphnia magna* constitutes the most sensitive endpoint followed by algal growth inhibition, while *Aliivibrio fischeri* inhibition was less sensitive. Based on a classification by Passino and Smith [38], providing a hazard ranking for 19 families of pollutants, tested ILs can be classified primarily as compounds of moderate (i.e. [n-Bu_4N][I], [n-Bu_4N][PF_6]) and low toxicity (i.e. [Py][CF_3SO_3], [1-4PPy][Cl], [BMIM][Br], [BMIM][$MeSO_4$]). Interestingly, the toxicity of [n-Bu_4N][I] was significantly lower towards *Aliivibrio fischeri* compared to *Daphnia magna* and *Raphidocelis subcapitata*, indicating a potential specific mode-of-action. This finding is reinforced by Dickinson et al. [39] demonstrating that mitochondria can be affected by [bmim]-ILs. Although a trend can be observed between the various chemical groups of ILs, the degree of toxicity was not uniformly expressed for each endpoint, indicating that the toxicity is organism-specific and relevant battery assays should be used to understand potential adverse environmental effects.

3.2 Isolation and characterization of IL-degraders

Environmental samples were supplemented with a different IL each time and cultures exhibiting substantial growth following sequential enrichments were used for solid media inoculation. One strain, which could grow on 400 mg L^{-1} of all ILs tested, was purified, while considering its phylogenetic and physiological properties, it was identified as *Sphingomonas* sp. MKIV (MH367522).

The 16S rDNA gene sequence was aligned and compared with other 16S rRNA sequences in GenBank using the NCBI Basic Local Alignment Search Tools, nucleotide

(BLASTn) program (http://www.ncbi.nlm.nih.gov/BLAST/). Analysis involved 10 nucleotide sequences (Fig. 1). Reliability branch contains a percentage value of more than 60% indicated. *Nocardia asteroides* (X53205.1), *Nocardia shimofusensis* YZ-96 (NR_028650.1) and *Rhodococcus rhodochrous* ATCC 12674 (NG_047662.1) were used as outgroups to root the tree (database accession numbers presented in parentheses). The 16S rRNA sequence of MKIV forms a single cluster with high reliability (100%) and possesses 99% sequence similarity with *Sphingomonas* sp. VITPTHJ (KP305914.1).

3.3 EPS production using glucose

Extreme microorganisms acclimated in the presence of ILs may present important industrial applications [40]. MKIV, which was isolated in the presence of ILs, formed sticky colonies in solid media and large cell aggregates in liquid cultures (Fig. 2A) indicating high potential for EPS production. Thus, liquid cultures were conducted using 25 g L^{-1} of glucose as the sole carbon source to assess EPS generation. MKIV formed 1.16 g L^{-1} of protein tightly bound to cell aggregates, 0.35 g L^{-1} of loosely bound protein and 17.78 g L^{-1} of total carbohydrates (Fig. 2B-D). Thus, considering both types of EPS (carbohydrate and proteinic) the total quantity of biopolymer extracted using 25 g L^{-1} of glucose reached 19.29 g L^{-1}. Other *Sphingomonas* strains form elevated contents of polysaccharides, such as *Sphingomonas paucimobilis* producing 13.2 g L^{-1} of gellan from 20 g L^{-1} of starch which was increased to 35.7 g L^{-1} under optimal conditions [41]. Moreover, *Sphingomonas* sp. CGMCC 6833 generated 18.56 g L^{-1} of rhamsan using 40 g L^{-1} of glucose under two-stage pH control batch conditions [42], which was elevated to 19.58 g L^{-1} using statistical optimization [43].

Thus, the isolated strain holds great potential for EPS synthesis considering that EPS production was achieved without optimization of conditions.

The EPS produced were heteropolysaccharides, consisting mainly carbohydrates and traces of organic acids (Table 3). Galacturonic acid and glucose were the main carbohydrates formed, while a small amount of mannose was also present. Organic acids comprised a considerable quantity of acetate and a lower amount of succinate. All EPS components comprise typical biopolymer constituents (i.e. monosaccharides, amino sugars, uronic acids, organic acids), while the high uronic acid quantity could indicate favourable gelifying properties [44]. *Sphingomonas* strains produce various structurally relevant biopolymers, including gellan, welan, diutan and rhamsan, known as sphingans [45]. These biopolymers entail similar tetrasaccharide linear structures (consisted of glucose, glucuronic acid, glucose and rhamnose/mannose) and divergent side chains [46]. Sphingans have recently entered the market receiving increasing demand due to novel properties for manufacturing of thermo-reversible gels with high applicability in environmental bioremediation, pharmaceutical and food industries [47]. Thus, future research of EPS production from MKIV should investigate the physical and structural properties of the biopolymer to identify potential applications.

3.4 Biodegradation of ILs by Sphingomonas sp. MKIV

The effect of IL concentrations (ranging between 10-100 mg L^{-1}) on the metabolic activity of MKIV was determined in batch experiments. Control experiments were additionally conducted to determine CO_2 production in the absence of IL (biotic control) and in the absence of microorganism (abiotic control). Although the latter did not produce CO_2, in biotic controls CO_2 formation occurred potentially due to biodegradation of the EPS content in cell aggregates. Thus, the production of CO_2 was

used to calculate the metabolic activity inhibition (I) imposed on MKIV during IL-biodegradation (Fig. 3).

Given that negative I values represent increased CO_2 formation compared to the control, the metabolic activity of cultures conducted using [BMIM][$MeSO_4$], [BMIM][Br], [Py][CF_3SO_3] and [n-Bu_4N][PF_6] was overall enhanced during the first day of experiments indicating the onset of pollutant biodegradation. Nevertheless, the metabolic activity was slightly inhibited thereafter in [BMIM][$MeSO_4$] cultures reaching maximum I of 15%. Moreover, although the metabolic activity of cells grown on 30-100 mg L^{-1} of [BMIM][Br] and [Py][CF_3SO_3] was marginally inhibited between 2-14 d, the use of 10 mg L^{-1} of each IL resulted in enhanced CO_2 production for the whole experiment compared to the biotic control. [n-Bu_4N][PF_6] cultures demonstrated the lowest I in concentrations of 10-50 mg L^{-1}, exhibiting 2%-22% more CO_2 production than the control (2-14 d). However, 100 mg L^{-1} of [n-Bu_4N][PF_6] resulted in inhibition of 25% at 5 d, which was subsequently reduced to 2% at 14 d. Feeding of [1-4PPy][Cl] and [n-Bu_4N][I] demonstrated similar response, where the metabolic activity was generally inhibited at almost all concentrations. *Sphingomonas paucimobilis* has been successfully applied for biodegradation of [bmim]-ILs, achieving 75% and 39% removal following 28 d in cultures fed with 456 mg L^{-1} [BMIM][Br] and 575 mg L^{-1} [BMIM][Cl] respectively [48]. Nevertheless, no removal was detected at a primary biodegradation experiment of [BMIM][Cl] after 28 d [23]. [py]-ILs biodegradation has been also recently demonstrated in *Rhodococcus rhodochrous* cultures fed with 402 mg L^{-1} of 1-propylpyridinium bis(trifluoromethylsulfonyl)imide achieving 76% removal in 14 d [49]. However, although [Bu_4N]-ILs have been regarded as non-biodegradable

[24], MKIV metabolized the specific class of pollutants exhibiting significant potential for application in IL-bioremediation.

The enhanced CO_2 production at the beginning of cultures fed with [BMIM][$MeSO_4$], [BMIM][Br], [Py][CF_3SO_3] and [n-Bu_4N][PF_6] as well as the subsequent metabolic activity inhibition indicate that although ILs could be readily biodegraded producing more CO_2 compared to the biotic control, IL-bioconversion could accumulate reactive inhibitory metabolites. Similarly, although *Rhodococcus rhodochrous* removed more than 80% of pyridinium-ILs in 28 d, metabolites with potential toxic activity were accumulated [49]. Moreover, even though complete mineralization of [py]-ILs can be achieved, biodegradation of N-ethylpyridinium tetrafluoroborate by *Pseudomonas fluorescens* generated N-hydroxylethylpyridinium and pyridinium as metabolic products [50]. Nevertheless, 1-butyl-3-methylimidazolium chloride was completely mineralized by a *Bacteroidetes*-enriched activated sludge community [51]. Therefore, based on the inconsistent conclusions of the literature, the toxicity and biodegradability of metabolic products should be carefully considered to determine whether biotreatment can form compounds with higher environmental impact [52].

3.5 Assessment of metabolic products toxicity from [BMIM][$MeSO_4$] and [n-Bu_4N][I] biodegradation

Biotreatment of [BMIM][$MeSO_4$] and [n-Bu_4N][I] was monitored in conjunction with toxicological tests to simultaneously assess the biodegradability and environmental impact of metabolic products formed by MKIV. Thus, IL biodegradation was monitored at different temperatures, pH and initial pollutant concentrations to determine the capacity of the strain for IL removal. *Sphingomonas* sp. MKIV could degrade both ILs

at initial concentrations of 10 mg L^{-1} and 100 mg L^{-1} (Fig. 4). However, although minor differences were observed in IL-removal at 10 mg L^{-1} for different temperatures, the increase of the pollutants' concentration to100 mg L^{-1} demonstrated that biotreatment kinetics were substantially enhanced at 30 ºC. The removal of 100 mg L^{-1} [BMIM][$MeSO_4$] was 91% following 23 d of incubation at 30 ºC, while 25 ºC and 37 ºC resulted in removal efficiency of 50% and 17% respectively. Similarly, cultures conducted using 100 mg L^{-1} of [n-Bu_4N][I] and 30 ºC exhibited 87% removal at 28 d, which was reduced to 72% and 24% at 25 ºC and 37 ºC respectively. MKIV achieved 93% of [BMIM][$MeSO_4$] removal following 140 h and 95% of [n-Bu_4N][I] removal at 100 h for the lower pollutant concentration tested. Moreover, varying pH values between 5-7 were also evaluated using 10 mg L^{-1} of [BMIM][$MeSO_4$] and [n-Bu_4N][I] (Fig. 5), demonstrating that both compounds were biodegraded more rapidly at a pH of 5.5.

Although several publications have focused on identifying pathways of IL metabolism [51,53-55] the number of studies evaluating the toxicity of IL-biodegradation products is limited. Nevertheless, the end-products generated might not necessarily constitute molecules of lower toxicity and their accumulation should be avoided according to "green chemistry" principles [56]. Bioconversion products of 1-butyl-3-methylpyridinium bromide and 1-hexyl-3-methylpyridinium bromide from a mixed culture posed substantially lower toxicity to *Daphnia magna* compared to initial molecules [57]. Moreover, *Rhodococcus hoagii* VRT1 biodegraded 1-butyl-3-methylimidazolium bromide producing metabolites of lower genotoxicity than the parental IL [58]. Nevertheless, *Raphidocelis subcapitata* and *Daphnia magna* exposed to several imidazolium-based ILs and the products formed following UV_{254}/H_2O_2

treatment demonstrated that the molecules generated entail increased toxicity compared to target compounds [59]. Thus, assessing the toxicity of metabolic products from IL biotreatment is necessary to determine the fate of bioprocess derivatives in the environment.

MKIV was employed for the treatment of 10 mg L^{-1} of [n-Bu_4N][I] and [BMIM][$MeSO_4$]. The microorganism was incubated under optimal conditions (30 ^oC, pH 5.5) using two different stirring speeds (0 and 75 rpm), while following 5 d of biotreatment samples were withdrawn to test the toxicity of bioconversion end-products on *Aliivibrio fischeri* (Fig. 6). Statistical analysis confirmed that inhibition was increased following exposure to [n-Bu_4N][I]-biodegradation products as compared to the control ($P<0.05$) for both stirring speeds tested. However, there was no significant change in the toxicity of [BMIM][$MeSO_4$] before and after the biotreatment, remaining non-toxic at all times. Therefore, although MKIV could remove both compounds, in the case of [n-Bu_4N][I] the toxicity hazards associated to the parental pollutant did not decrease. This effect has been previously reported for similar CEC demonstrating that biotransformation products can be more toxic, persistent and hazardous [49].

4. Conclusions

A new microorganism thriving in the presence of ILs was isolated. *Sphingomonas* sp. MKIV metabolized [bmim]-, [py]- and [Bu_4N]-based ILs, constituting a versatile strain that holds great potential for EPS production as added-value metabolite. The microorganism formed 19.29 g L^{-1} of biopolymer with high content in glucose and galacturonic acid from 25 g L^{-1} of glucose. However, although MKIV is suitable for

development of IL-biotreatment and EPS production processes, test compounds expressed moderate and low toxicity to aquatic organisms, while end-products from [n-Bu_4N][I] biodegradation increased the environmental impact compared to the parental pollutant. Future research should characterize the IL-biodegradation products generated as well as the physical properties and chemical structure of EPS.

5. Acknowledgements

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imidazole and N,N'-alkyl-imidazolium chlorides in water, Water Res. 106 (2016) 450–460.

7. Figure Captions

Fig. 1. Phylogenetic position of *Sphingomonas* sp. MKIV. Evolutionary history was inferred using the Neighbor-Joining method. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (10000 replicates) are shown next to the branches. Evolutionary distances were computed using the Maximum Composite Likelihood method.

Fig. 2. EPS production by *Sphingomonas* sp. MKIV using 25 g L^{-1} of glucose. **A:** MKIV cell aggregates, **B:** protein content of cell aggregates, **C:** total carbohydrate content, and **D:** protein content in bulk media.

Fig. 3. Metabolic activity inhibition (I) imposed on *Sphingomonas* sp. MKIV during biodegradation of each IL at 30 ºC. I percentages have been calculated through equation 1. **A:** [BMIM][$MeSO_4$], **B:** [BMIM][Br], **C:** [1-4PPy][Cl], **D:** [Py][CF_3SO_3], **E:** [n-

Bu_4N][I], and **F:** [n-Bu_4N][PF_6]. Initial IL concentration: 10 mg L^{-1} (diamonds), 30 mg L^{-1} (squares), 50 mg L^{-1} (triangles), and 100 mg L^{-1} (circles).

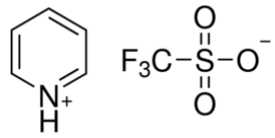
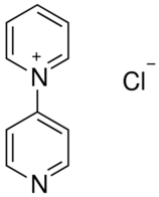
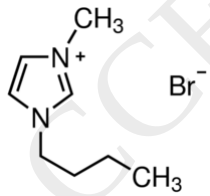
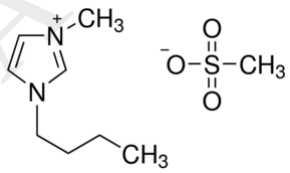
Fig. 4. Biodegradation of [BMIM][$MeSO_4$] and [n-Bu_4N][I] at different temperatures and concentrations. Initial IL concentration: **A:** 100 mg L^{-1} [BMIM][$MeSO_4$], **B:** 100 mg L^{-1} [n-Bu_4N][I], **C:** 10 mg L^{-1} [BMIM][$MeSO_4$], and **D:** 10 mg L^{-1} [n-Bu_4N][I]. Temperature: 25 ºC (squares), 30 ºC (diamonds), and 37 ºC (triangles).

Fig. 5. Biodegradation of ILs at different pH values. **A:** [n-Bu_4N][I], and **B:** [BMIM][$MeSO_4$].

Fig. 6. IL toxicity imposed on *Aliivibrio fischeri* following biotreatment. Cultures were conducted at 0 and 75 rpm, while inhibition was determined following 5 and 15 min of exposure. **A:** [n-Bu_4N][I], and **B:** [BMIM][$MeSO_4$]. Control: abiotic media supplemented with 10 mg L^{-1} of IL, MKIV: biomedium samples withdrawn following 5 d of IL biotreatment. Asterisks indicate statistical significance.

8. Tables and Figures

Table 1. Abbreviations of ILs used in this work.

Structure	Chemical name	Abbreviation
	Pyridinium trifluoromethanesulfonate	[Py][CF_3SO_3]
	1-(4-pyridyl)pyridinium chloride	[1-4PPy][Cl]
	1-butyl-3-methylimidazolium bromide	[BMIM][Br]
	1-butyl-3-methylimidazolium methanesulfonate	[BMIM][$MeSO_4$]

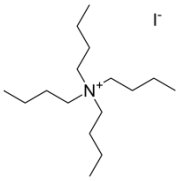
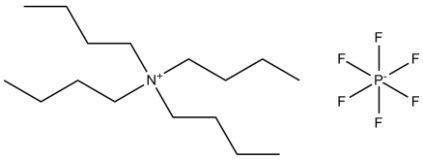
	tetrabutylammonium iodide	[n-Bu_4N][I]
	tetrabutylammonium hexafluorophosphate	[n-Bu_4N][PF_6]

Table 2. EC_{50} values obtained from toxicity assessment of the ILs.

Ionic liquid	***Aliivibrio fischeri*** **(mg L^{-1})**		***Daphnia magna*** **(mg L^{-1})**		***Raphidocelis subcapitata*** **(mg L^{-1})**	
	5 min	**15 min**	**24 h**	**48 h**	**72 h**	**96 h**
[Py][CF_3SO_3]	75.53 (6.31)	76.31 (15.97)	12.16 (2.94)	10.07 (1.44)	174.71 (26.80)	66.41 (22.80)
[1-4PPy][Cl]	363.18 (54.35)	290.22 (32.17)	24.82 (7.71)	17.98 (5.76)	166.37 (19.28)	99.52 (12.49)
[BMIM][Br]	410.45 (69.85)	281.72 (36.20)	30.00 (3.53)	25.93 (1.02)	212.19 (1.48)	138.89 (14.41)
[BMIM][$MeSO_4$]	359.65 (46.48)	276.74 (0.29)	13.98 (4.46)	11.61 (3.17)	94.83 (9.19)	60.98 (11.99)
[n-Bu_4N][I]	685.55 (73.48)	312.16 (23.83)	2.74 (1.26)	2.25 (0.88)	6.99 (0.47)	5.03 (1.95)
[n-Bu_4N][PF_6]	108.75 (20.11)	86.41 (4.10)	23.60 (5.40)	20.51 (3.38)	<3	<3

□ : very low toxicity; □ : low toxicity; ■ : moderate toxicity; ■ : highly toxic. Numbers in parenthesis correspond to standard deviation.

Table 3. Chemical composition of EPS produced from glucose fermentation using *Sphingomonas* sp. MKIV.

Compound	Content (%, w/w)				
	C_1 [a]	C_2 [a]	C_3 [a]	C_4 [a]	Average
Galacturonic acid	52.76	46.74	45.28	44.34	47.27 ± 3.8
Glucose	35.81	41.81	44.60	44.56	41.69 ± 4.1
Mannose	7.24	5.56	6.19	7.25	6.56 ± 0.8
Succinic acid	1.14	1.46	0.64	0.72	0.99 ± 0.4
Acetic acid	3.05	4.44	3.29	3.15	3.48 ± 0.6

[a] concentration of polysaccharide sample used in hydrolysis, C_1: 0.4 mg ml^{-1}, C_2: 1 mg ml^{-1}, C_3: 2 mg ml^{-1}, C_4: 4 mg ml^{-1}.

Fig. 1.

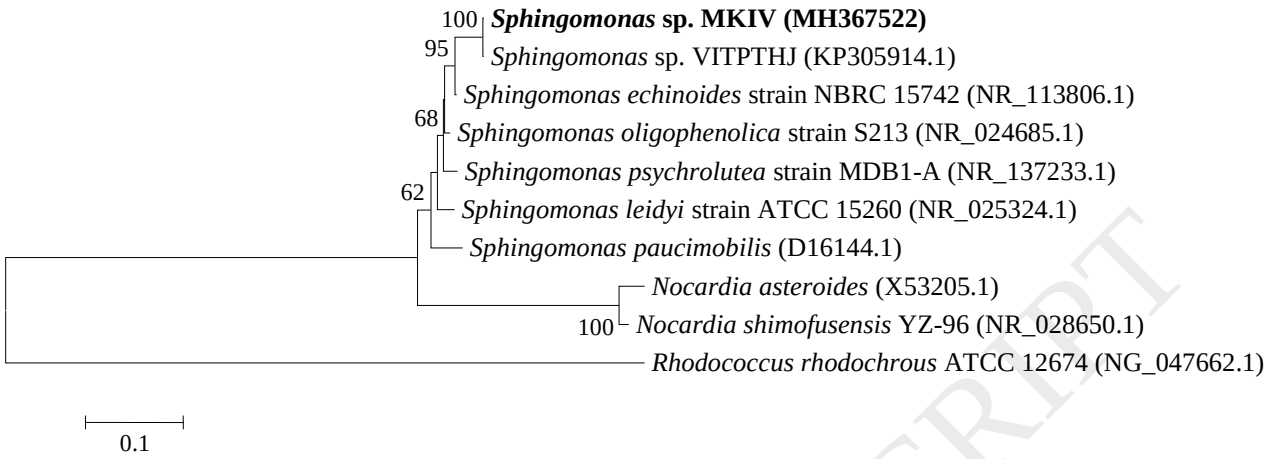


Fig. 2.

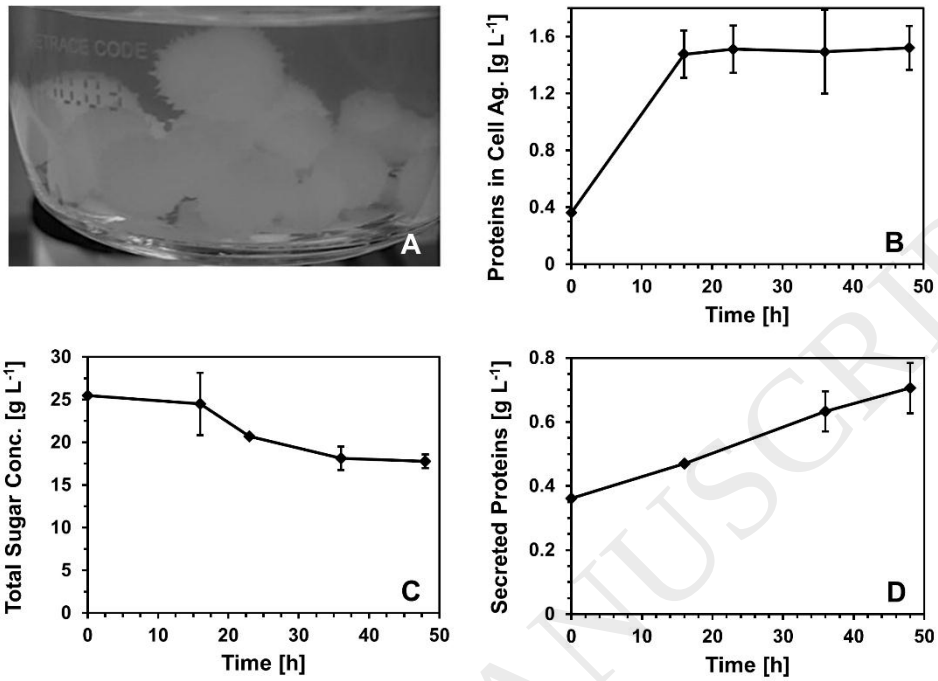


Fig. 3.

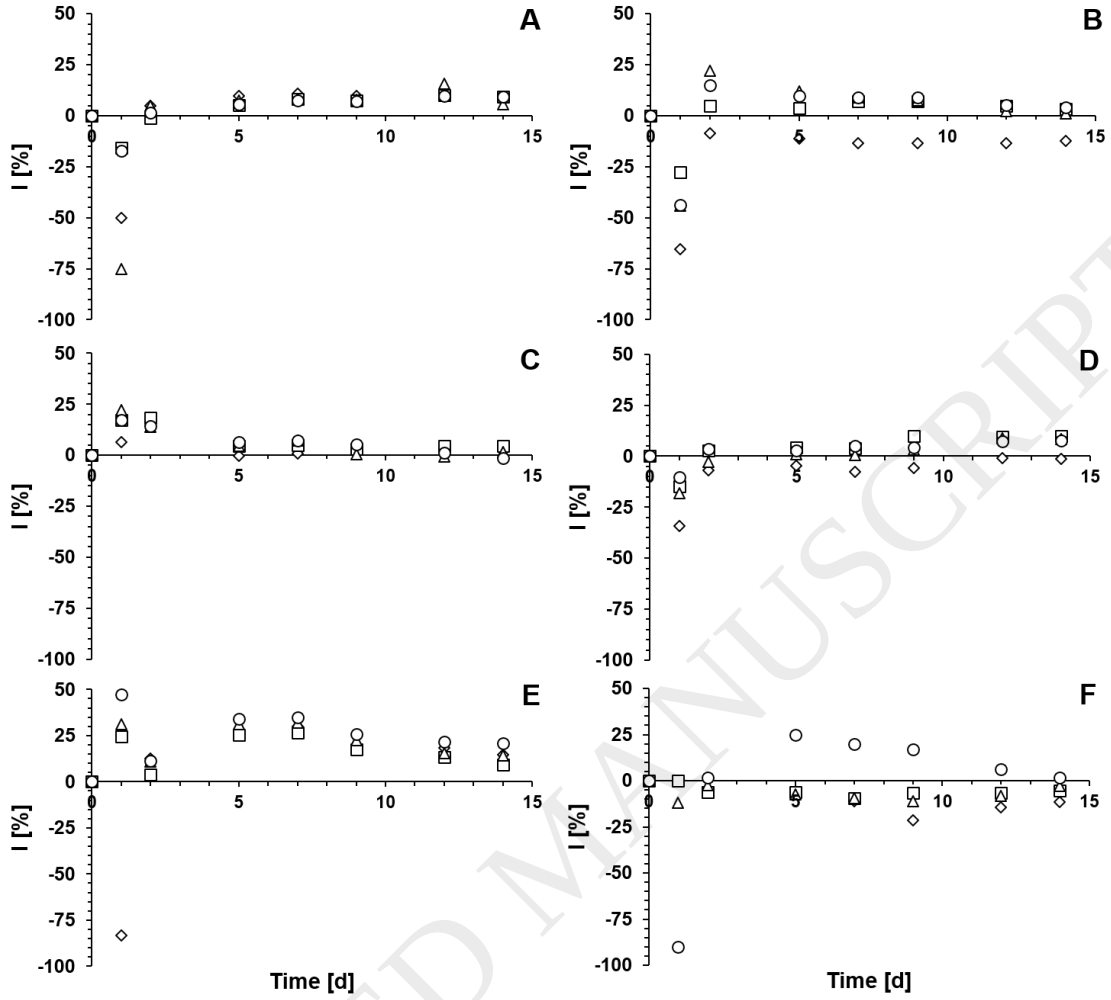


Fig. 4.

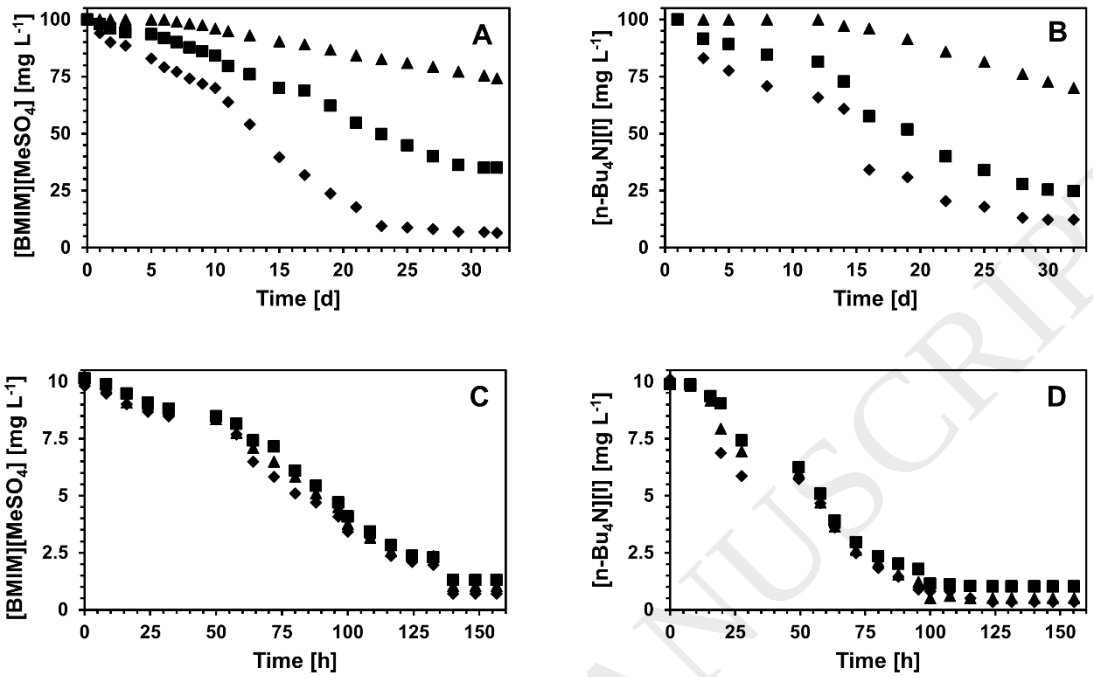


Fig. 5.

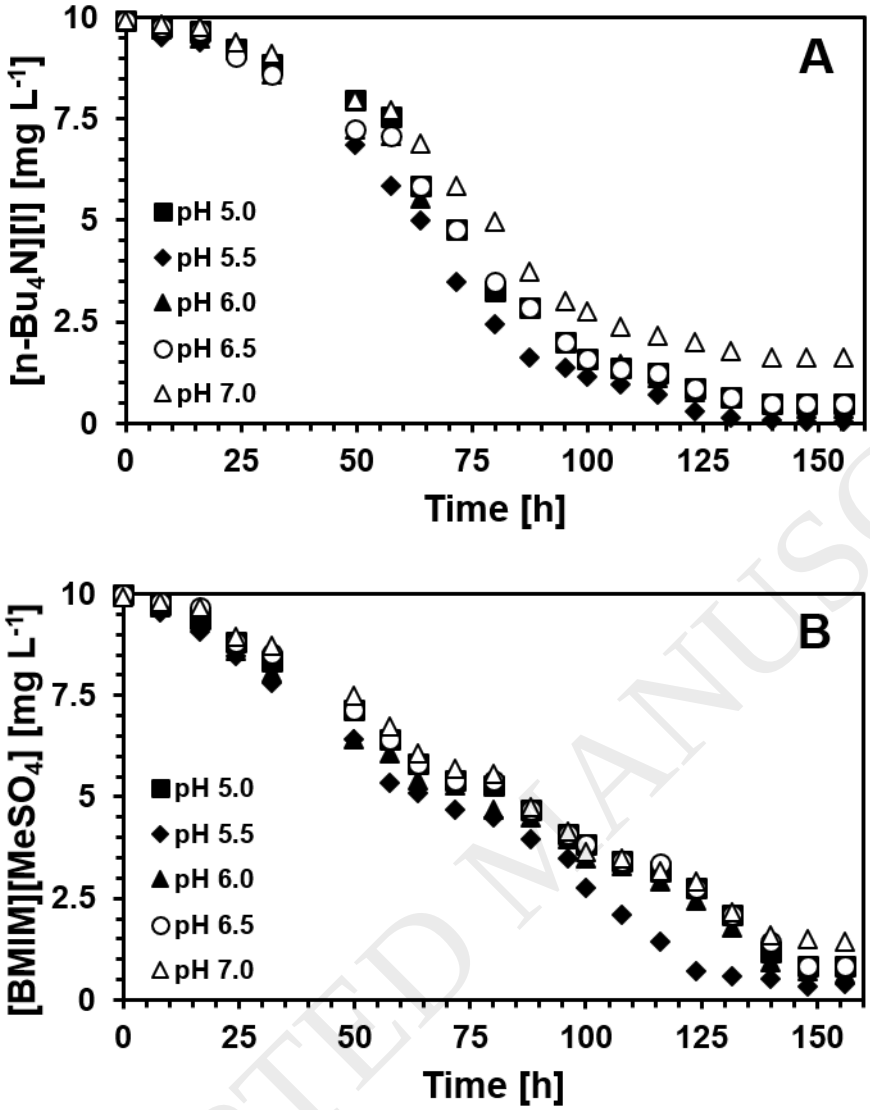


Fig. 6.