# Biodegradation of pyridinium-based ionic liquids by an axenic culture of soil *Corynebacteria*

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We investigated the biodegradation of ionic liquids *N*-ethylpyridinium tetrafluoroborate $[EtPy]^+[BF_4]^-$, *N*-ethylpyridinium trifluoroacetate $[EtPy]^+[CF_3COO]^-$, and 1-butyl-3-methylimidazolium hexafluorophosphate $[BMIM]^+[PF_6]^-$ by a soil bacterium isolated by an enrichment-culture technique. The bacterium identified as *Corynebacterium* sp. degraded the *N*-ethylpyridinium cation in the first two compounds when present as its sole carbon and nitrogen source without any obvious effects of the anion; however, $[BMIM]^+[PF_6]^-$ was not metabolized. We observed cleavage of the pyridinium ring and identified the resulting metabolites by ESI/MS/MS. We propose a degradation pathway.



## Introduction

The many unique properties of ionic liquids (ILs) have attracted intensive attention in the past decade; they include low melting point, low to non-measurable vapor pressure, tunable physical-chemical properties, and high solvation for many organic and inorganic materials.[1,2] Therefore, they offer tremendous potential applications in various fields, such as the extraction and separation of metals,[3–6] as solvents in organic synthesis,[7,8] and as catalysts and biocatalysts.[9–13] Since their impacts on the environment have not been evaluated fully, and their fates are not known, the environmental risks posed by ILs have become a major concern that must be resolved before they can be categorized as "green".

A growing number of biological and biochemical studies attempted to assess the toxicity and biodegradability of ILs. The results of toxicity studies of ILs on bacteria,[14] fungi, algae,[15] fish,[16] terrestrial plants,[17] and higher classes of organisms, such as freshwater snails[18] indicated that they could induce adverse effects. Several studies focused on the biodegradability of imidazolium-based ILs. Thus, Kumar *et al.*[19] exploring the breakdown of 1-butyl-3-methyl imidazolium tetrafluoroborate [BMIM][$BF_4$] by soil- and waste-water microorganisms, and by *Escherichia coli* reported that this compound is biodegradable. Gathergood *et al.*[20] designed new ILs containing ester- or amide-groups in the alkyl side chain, and studied their biodecomposition; they demonstrated that introducing a group susceptible to enzymatic hydrolysis greatly improves biodegradation compared with the commonly used dialkylimidazolium ILs. However, in these studies, only the alkyl groups bound to the imidazolium ring were biodegradable, while the ring remained intact.

Compared with imidazolium-based ILs, few studies have examined the biodegradability of pyridinium-based ones. Pyridinium-based ionic liquids are heterocyclic aromatic compounds proven to have great potential in organic synthesis and biocatalysis.[21–23] Although the aerobic- and anaerobic-decomposition pathways for pyridine and pyridine-derivative compounds are well known,[24–26] the pyridinium salt may differ significantly from ring-substituted pyridines because of the distribution of electron-density around the heterocyclic nucleus. These salts have many unique properties, such as antimicrobial-, anti-electrostatic-, and surface-activity, along with adsorption onto negatively charged solids.[27,28]

Pyridinium-based surfactants reportedly exhibit strong resistance to biodegradation.[29] Recently, Grabinska-Sota and Kalka[30] undertook research to assess this resistance. They investigated the metabolism of 1-dodecylthiomethyl pyridinium chloride by activated sludge communities, and reported complete mineralization of the ring. By manipulating the structures, Harjani *et al.*[31] demonstrated that inserting an ester side chain into the pyridinium cation improved the biodegradation of ILs in an aerobic aquatic environment. Docherty *et al.*[32] examined the biodegradability of 1-butyl-3-methyl pyridinium bromide [BMPy][Br], 1-hexyl-3-methyl pyridinium bromide [HMPy][Br], and 1-octyl-3-methyl pyridinium bromide [OMPy][Br]) using several approaches, *viz.*, the standard Organization for Economic Cooperation and Development's dissolved organic carbon Die-Away Test, the changes in total dissolved nitrogen concentrations, and the $^1$H-nuclear magnetic resonance analysis of the initial- and final-chemical structures. They found that hexyl- and octyl-substituted pyridinium-based ILs were fully mineralized, but bmpyrBr was not biodegradable, while imidazolium-based ILs were only partially mineralized. Additionally, the length of the alkyl chain may affect the rate of pyridinium biodegradation, and the presence of linear alkyl

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chains, particularly those containing four or more carbons was an important factor in determining biodegradation. In those studies, adaptations of the $CO_2$ headspace test (ISO 14593), the closed bottle test (OECD 301D), and modified Sturm test (OECD 301B) often were used in evaluating the biodegradability of ILs. However, the degradation pathway and metabolites were not examined. Pham *et al.*[33] described the intermediates in, and the pathway of degradation of 1-butyl-3-methylpyridinium bromide by activated sludge microorganisms, suggesting that it was catalyzed by cytochrome P450, and that oxidation occurred at different positions on the alkyl side-chains. Nonetheless, the pyridinium ring remained intact throughout their experiments, and the N-substituted methylpyridinium metabolites persisted after 28 days of incubation.

In this study, we investigated the ability of a soil bacterium isolated by an enrichment culture technique to degrade *N*-ethylpyridinium tetrafluoroborate $[EtPy]^+[BF_4]^-$, *N*-ethylpyridinium trifluoroacetate $[EtPy]^+[CF_3COO]^-$, and 1-butyl-3-methylimidazolium hexafluorophosphate $[BMIM]^+[PF_6]^-$ as their sole source of carbon and nitrogen (Fig. 1); also, we characterized the degradation products. To the best of our knowledge, this is the first report demonstrating ring cleavage in pyridinium-based ILs and its complete degradation to innocuous products.

## Experimental

### Ionic liquids

*N*-Ethylpyridinium tetrafluoroborate $[EtPy]^+[BF_4]^-$, *N*-ethylpyridinium trifluoroacetate $[EtPy]^+[CF_3COO]^-$, and 1-butyl-3-methylimidazolium hexafluorophosphate $[BMIM]^+[PF_6]^-$ were synthesized and purified according to the methods of Zhao and Malhotra.[23] The ILs used were > 98% pure as tested by NMR and FTIR.[23]

### Isolation of ILs-utilizing bacteria

Ten grams of a sample obtained from a sandy soil near Brookhaven National Laboratory, Long Island, NY was placed in a 250 ml Erlenmeyer flask containing 100 ml of mineral salt (MS) medium and 10 µl of $[EtPy][BF_4]$, $[EtPy][CF_3COO]$, or $[BMIM][BF_4]$ as the sole source of carbon and nitrogen. After several serial transfers of the soil-enrichment culture solution to the mineral-salts medium containing the respective ILs, we isolated a pure culture of bacteria from the $[EtPy][BF_4]$ enrichment able to degrade $[EtPy][BF_4]$. The culture was then maintained in the MS medium containing $[EtPy][BF]_4$. The bacterium, a rod-shaped pleomorphic, gram- positive, aerobic, and catalase- and urease-positive, was identified as *Corynebacterium* sp.

### Mineral salts medium

The mineral salt (MS) medium used to culture the bacteria contained the following ingredients:[34] $K_2HPO_4$, 1 g $L^{-1}$; KCl, 0.25 g $L^{-1}$; $MgSO_4 \cdot 7H_2O$, 0.25 g $L^{-1}$; and a trace element solution, 1 ml. The latter solution contained (per litre): $FeSO_4 \cdot 7H_2O$, 40 mg; $MnSO_4 \cdot 4H_2O$, 40 mg; $ZnSO_4 \cdot 7H_2O$, 20 mg; $CuSO_4 \cdot 5H_2O$, 5 mg; $CoCl_2 \cdot 7H_2O$, 4 mg; $Na_2MoO_4 \cdot 2H_2O$, 5 mg; $CaCl_2 \cdot 6H_2O$, 0.5 mg; and NaCl, 1 g. Several drops of concentrated HCl added to the solution prevented precipitation of the metals. The final pH was adjusted to 6.5 with HCl or NaOH. Fifty millilitres of MS was dispensed into 125 ml Erlenmeyer flasks fitted with cotton plugs, and then autoclaved for 30 min.

### Biodegradation of ionic liquids

We added ten microlitres of the ionic liquids $[EtPy][BF_4]$, $[EtPy][CF_3COO]$, or $[BMIM][BF_4]$ to 50 ml of MS medium in 125 ml Erlenmeyer flasks, and inoculated them with 2 ml of an early-log-phase of the bacterial culture isolated from the $[EtPy][BF_4]$ enrichment. Triplicate samples were incubated at 28 °C on a rotary shaker (200 rpm) in the dark. Periodically, we withdrew 5 ml of the culture sample, and measured the growth of bacteria *via* optical density (O.D.) at 600 nm. The culture medium was filtered through a 0.45 µm syringe filter, the pH was determined by Mettler Toledo MP 220 pH meter, and the concentration of the *N*-ethylpyridinium cation by UV-vis spectroscopy.

### UV-vis spectrophotometry

The pyridinium ring has a characteristic absorbance at 259 nm. Biodegradation of the *N*-ethylpyridinium cleaved the ring, with the concomitant disappearance in its absorbance. We monitored the UV-vis spectra with a Hewlett Packard 8453 diode array scanning UV-vis spectrophotometer, using a 1 cm square quartz cuvette.

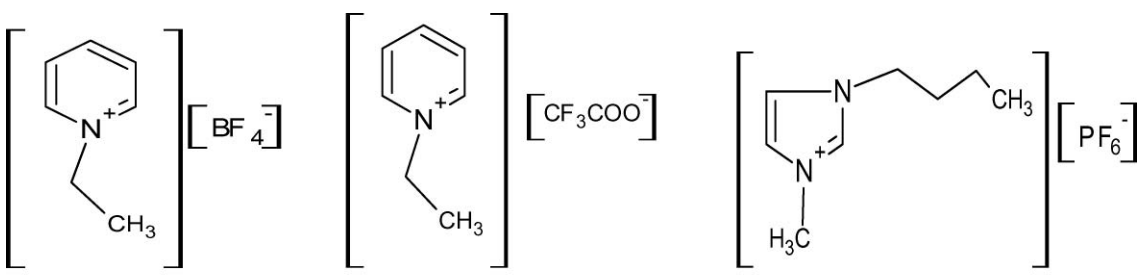


**Fig. 1** Molecular structures of ionic liquids $[EtPy][BF_4]$, $[BMIM][PF_6]$, and $[EtPy][CF_3COO]$.

 

### High performance liquid chromatography (HPLC)

The biodegradation products of *N*-ethylpyridinium were analyzed by HPLC (Shimadzu Inc.), using a Bio-Rad organic-acid analysis column (300 × 7.8 mm). The mobile phase consisted of 0.003 M sulfuric acid, with a flow rate of 0.7 ml $min^{-1}$. Biodegradation products were monitored by a UV detector at 210 nm.

### Liquid chromatography-mass spectrometry (ESI-MS)

The biodegradation products in the samples were identified with the Thermo-Finnigan LCQ Advantage LC-MS system. The sample was introduced into the instrument at a rate of 10 μL $min^{-1}$ using a microsyringe pump and analyzed by electrospray ionization mass-spectrometry (ESI-MS) under the following conditions: sheath gas, nitrogen; spray voltage, 4.5 kV; capillary temperature, 325 °C; and, capillary voltage 35 V. The operating parameters for the ESI, including gas flow rate, operating voltage, and current were optimized using an equimolar 1 mM Cu(II)–citrate complex. The ESI source was operated in both the negative- and positive-ion modes.

## Results and discussion

### Bacterial growth

The bacteria rapidly metabolized both [EtPy][$BF_4$] and [EtPy][$CF_3COO$] as the sole carbon- and nitrogen-source, as evidenced by an increase in turbidity of the liquid, with no discernable effect of the anions. However, the imidazolium-based ionic liquid, [BMIM][$PF_6$], was not metabolized by the bacterium (Fig. 2). We examined the growth of the bacterium at various concentrations of [EtPy][$BF_4$]. The bacteria metabolized 1.2, 2.4, 3.6, and 4.8 mM [EtPy][$BF_4$], and the OD reached 0.8 at the highest concentration without showing any inhibitory effects (Fig. 3). During incubation, the pH of the medium dropped slightly from 6.7 to 6.6.

### Degradation of [EtPy][$BF_4$]

Fig. 4A shows the decrease in $EtPy^+$ concentration as a function of bacterial growth. An optical density of 0.25 was attained after

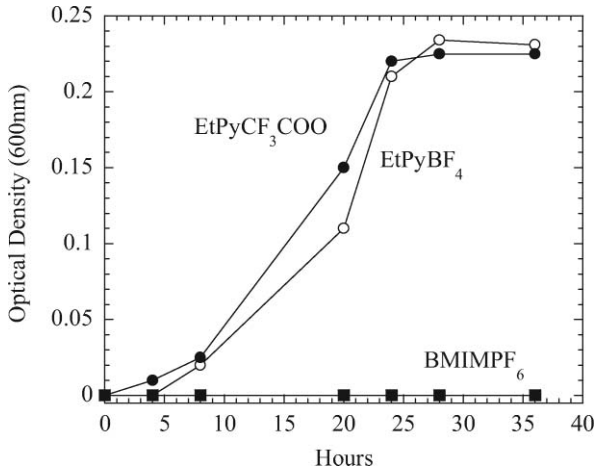


**Fig. 2** Degradation of ethylpyridinium- and imidazolium-based ionic liquids.

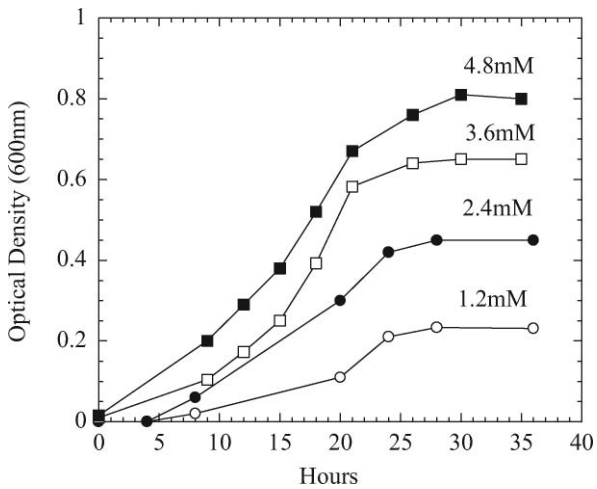


**Fig. 3** Biodegradation of different concentrations of [EtPy][$BF_4$].

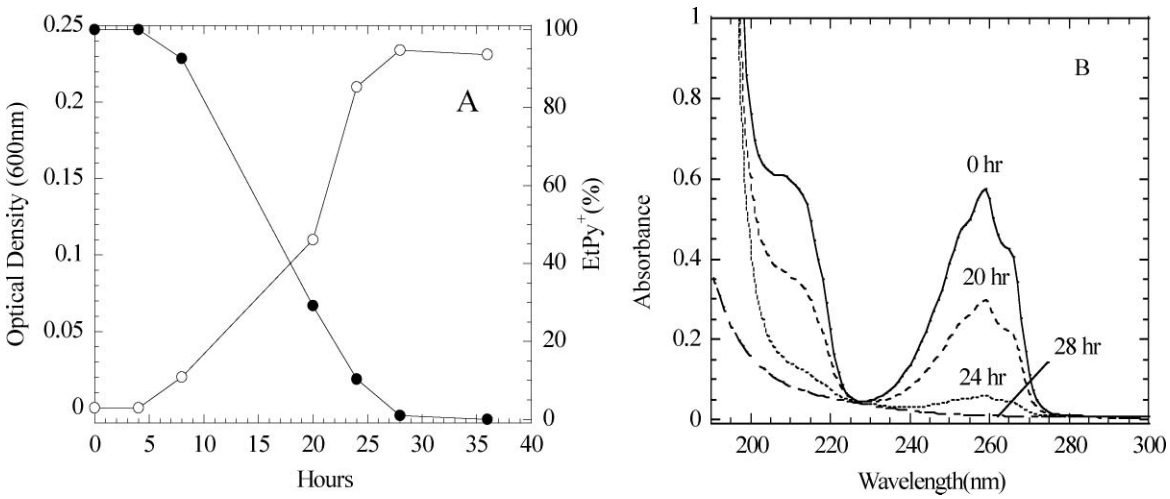


**Fig. 4** Degradation of 1.2 mM [EtPy][$BF_4$] by *Corynebacterium* sp. (A) Growth of bacteria–increase of O.D with the decrease of $EtPy^+$ concentration; (B) UV-vis spectra showing the disappearance of $EtPy^+$.

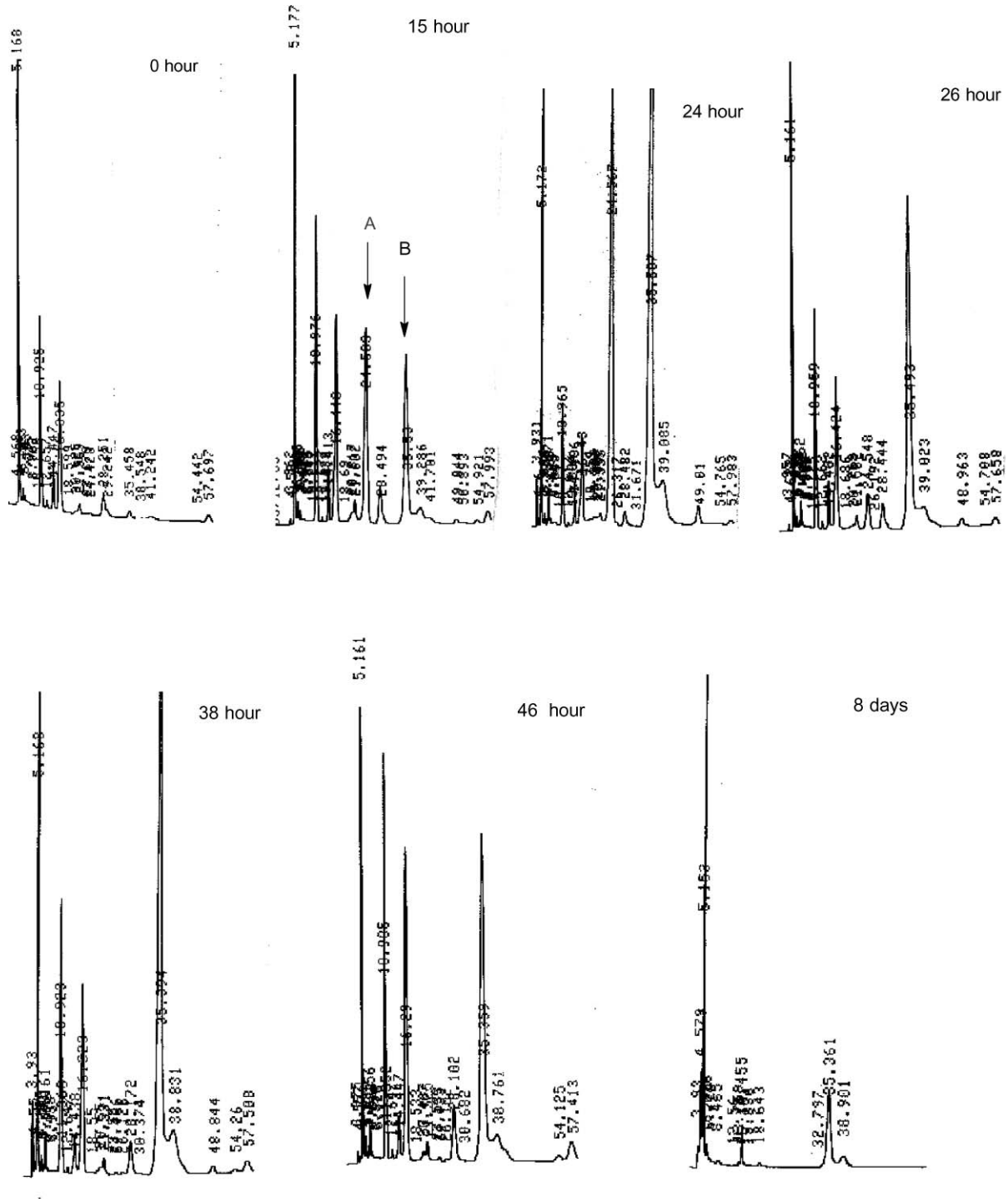


**Fig. 5** HPLC analyses of $EtPy^+$ degradation products from 0 h to 8 days.

24 h, and the bacteria metabolized the added 1.2 mM of $EtPy^+$. Fig. 4B displays the UV absorption of [EtPy][$BF_4$] between 190–300 nm during biodegradation. There are two characteristic absorption bands, one at 210 nm and the other at 259 nm, reflecting the presence of the pyridinium ring. Initially, the absorption at 259 nm was strong, and then started to decrease. After 24 h, the peaks at 210 nm and 259 nm disappeared, thereby demonstrating the ring cleavage of $EtPy^+$. UV-vis absorption spectrophotometry of the *N*-ethylpyridinium ring disclosed that the ring cleavage was completed in about 28 h; no additional peaks were detected.

### Identification of degradation products

HPLC analysis demonstrated the accumulation in the medium of two intermediate degradation products at 24.4 min (A) and 35.5 min (B) (Fig. 5). After reaching the highest con-

 

centration, product A quickly disappeared in 4 h, whereas product B disappeared much more slowly over the course of one week (Fig. 6) and no increase in growth was observed.

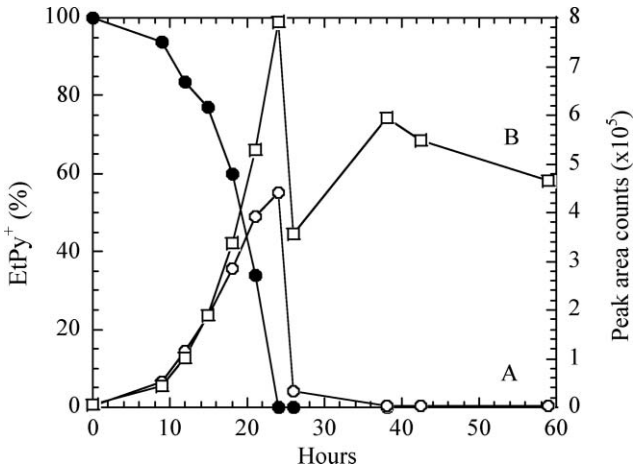


**Fig. 6** Degradation of $EtPy^+$, and the formation and degradation of intermediate products A and B.

ESI/MS analyses showed that the molecular weight of product A and B, respectively, was 157.9 and 143.9 (Fig. 7). These products are generated from the C2–C3 ring-opening. The ring-opening step seemingly is fast since the absorbance of the ring disappeared in 24 h. Apparently, intermediate product A was converted into product B as evidenced by the increase in the peak area of B between 24 and 40 h.

Products A and B were identified, respectively, as *N*-ethyl-(4-(carboxyamino)but-3-enoic acid semialdehyde, and (4-(carboxyamino)but-3-enoic acid by ESI/MS/MS analysis, as shown in Fig. 8 and 9. The fragment of 139.8 (*m*/*z*) from A might result from the loss of an OH (*m*/*z* 17) group from the carboxyl group. The fragment at 130.0 (*m*/*z*) is attributable to the loss of a –CHO or $C_2H_5$ group (*m*/*z* 29). The fragment at 112 (*m*/*z*) is due to loss of the –COOH group (*m*/*z* 45). Product B had fewer fragments. The 125.8 (*m*/*z*) fragment could result from the loss of an –OH group (*m*/*z* 17), while that of 74 (*m*/*z*) may reflect cleavage between the double bonds (–$CH_2$–NHCOOH).

We tried to identify the final products, but due to their low molecular weights and volatile properties, no major peaks were detected by LC-MS between 5 and 20 minutes. However, peaks were observed at 73 (*m*/*z*) and 60 (*m*/*z*) when the sample was injected into the instrument directly without any separation (Fig. 10). Glyoxylate (*m*/*z* 73) can be rapidly metabolized by the glyoxylate pathway,[35] and therefore its accumulation was not observed. The peak for acetic acid (*m*/*z* 60) was prevalent, especially in the negative mode (Fig. 11), confirming our hypothesis that acetic acid is the final product which can be further metabolized to carbon dioxide and water. Our proposed degradation pathway is shown in Fig. 12. It is to be noted that other pathways may also be involved.

### Proposed degradation mechanism

Our results demonstrated that an N-substituted pyridinium compound without any alkyl side chain on the ring is mineralized completely by *Corynebacterium* sp. isolated from the soil by an enrichment-culture technique. Ring cleavage between C2–C3 is proposed as the primary degradation pathway. The intermediate products identified by ESI/MS/MS were ethyl [(1*Z*)-4-oxobut-1-en-1-yl carbamic acid or (3*Z*)-4-[ethyl (formayl)amino]but-3-enoic acid, and (4-(carboxyamino)but-3-enoic acid. These metabolites subsequently were oxidized, to acetic acid and glyoxylate, as confirmed by MS. Wright and Cain[36] suggested similar decomposition pathways from their investigation of the transformation of 4-carboxy-1-methylpyridinium chloride by *Achromobacter* Strain D, which uses this compound as its sole carbon- and nitrogen-source. Succinic acid, formic acid, methylamine, and carbon dioxide were identified as the end-products. These authors' radio-labeling experiments indicated that the heterocyclic ring was cleaved between carbon 2 and 3 by multi-enzyme reactions. The N-formyl group was hydrolyzed to formic acid, while γ-(*N*-formyl-*N*-methylamino)vinylacetaldehyde was hydrolyzed to its corresponding acid. The subsequent hydrolytic processes lead to the formation of succinic acid, semialdehyde, and methylamine. A similar process of pyridine ring cleavage was recorded in the biotransformation of pyridine by *Bacillus* strain.[37]

We note that the presence of a different anion, *viz*. $BF_4^-$ and $CF_3COO^-$ in this study does not inhibit the degrading activity of the soil microorganisms. As suggested by Harjani *et al.*[31] and Garcia *et al.*,[38] biodegradability does not depend significantly on the anion, although some toxic effects are apparent. However, in this study, we observed that the bacterium did not break down 1-butyl-3-methylimidazolium-based IL, suggesting that different microorganisms may be involved in degrading different ILs. This explanation is supported by the work of Docherty *et al.*[32] who showed that different microbial communities may be responsible for metabolizing two distinct pyridinium-based ILs with different alkyl-chain lengths.

## Conclusions

To our knowledge, this is the first report showing complete biodegradation by an axenic culture of an N-substituted pyridinium to low molecular organic acid degradation products. We suggested that the cleavage between C2 and C3 is the degradation pattern of the pyridinium ring, and we identified the intermediate products by ESI-MS/MS. Considering that ionic liquids continue to shift from being academic curiosities to commercial realities, the data we present here may well provide fundamental information on the fate of these chemicals.

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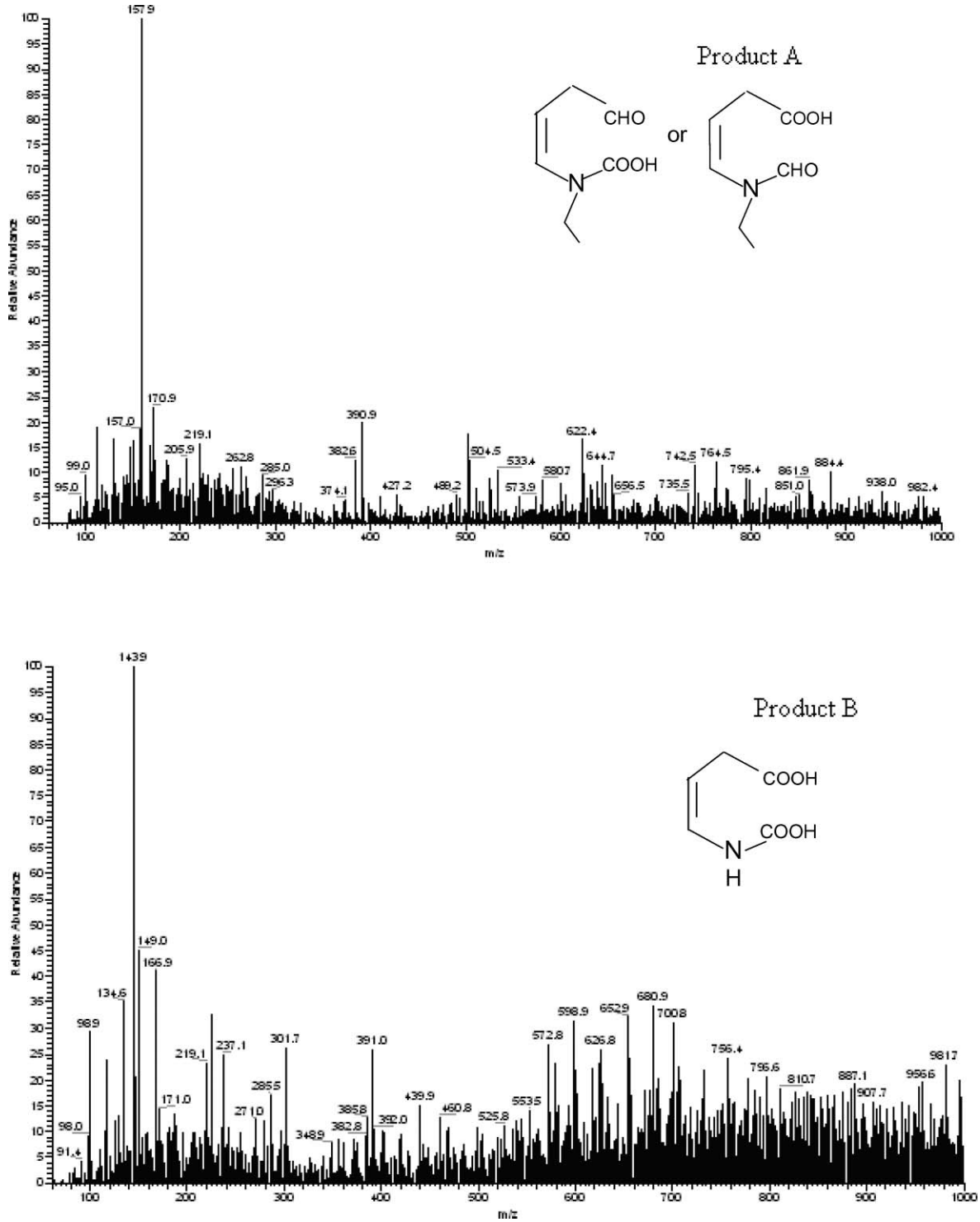


**Fig. 7** ESI/MS analysis of products A and B under positive mode.

 

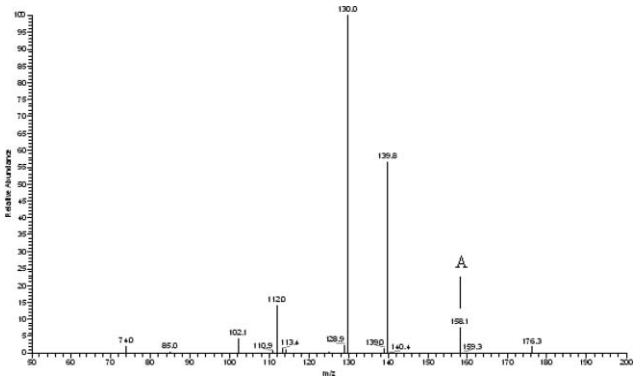


**Fig. 8** ESI/MS/MS analysis of product A at 40% collision energy under positive mode.

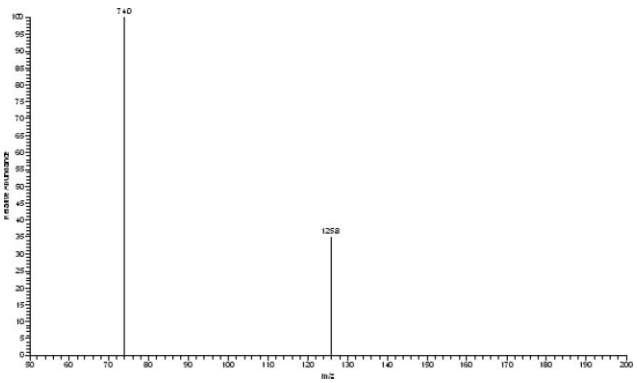


**Fig. 9** ESI/MS/MS analysis of product B at 40% collision energy under positive mode.

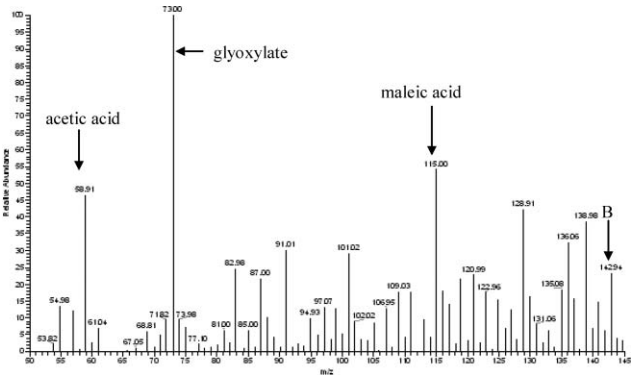


**Fig. 10** Mass spectroscopy of degradation products (after 35 h) under positive mode.

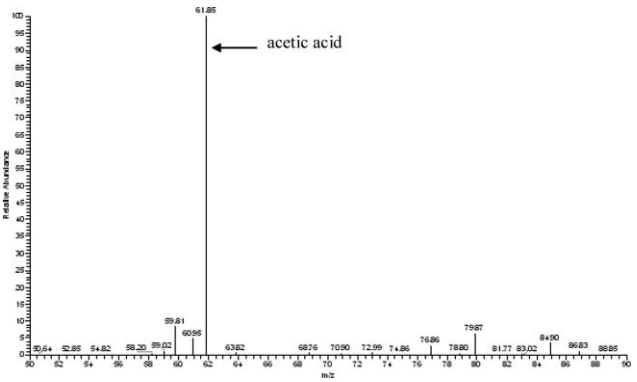


**Fig. 11** Mass spectroscopy of degradation products (after 35 h) under negative mode.



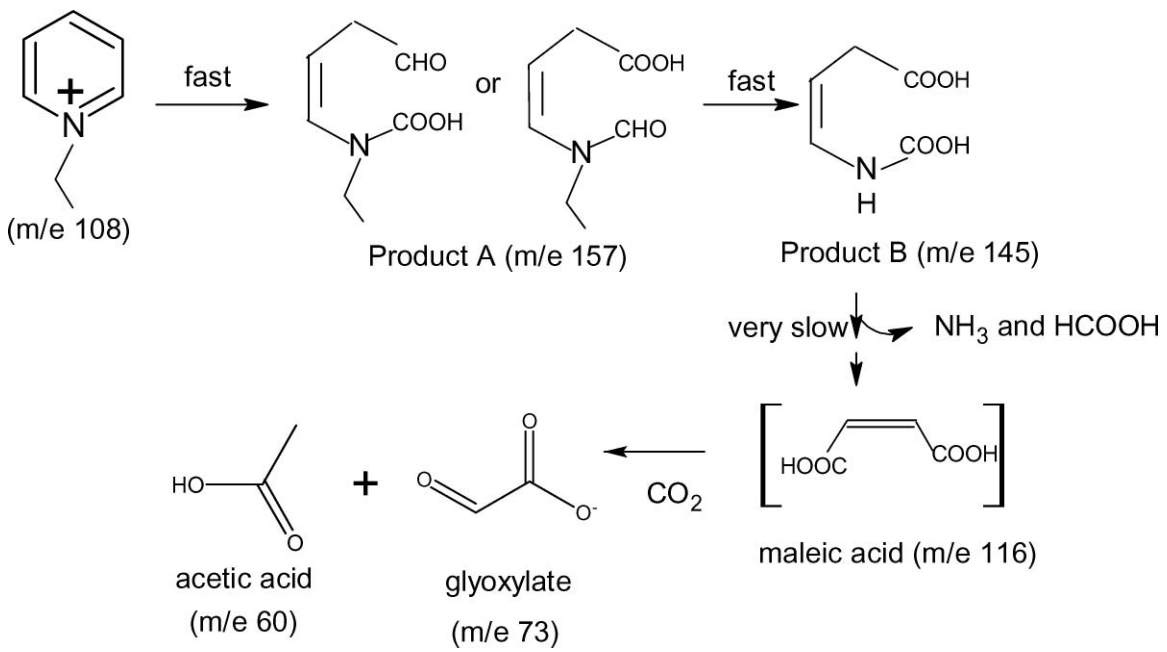


**Fig. 12** Proposed degradation pathway.

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