Degradation of ionic liquids by a UV/H₂O₂ process and CMCase from novel ionic liquid-tolerant alkaliphilic Nocardiopsis sp. SSC4

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ABSTRACT
We demonstrated the degradation of two ionic liquids (1-butyl-3-methylimidazolium chloride, [BMIM][Cl]), and 1-ethylpyridinium bromide, [EtPy][Br]) that are useful for the solubilization of wood components. [BMIM][Cl] and [EtPy][Br] were detected by thin-layer chromatography (TLC) and electrospray ionization–mass spectrometry (ESI-MS). [BMIM][Cl] was harder to degrade than [EtPy][Br]. Ultraviolet (UV) irradiation with 0.2% (v/v) H₂O₂ for 16 h degraded 1 mmol/L [BMIM][Cl], whereas UV irradiation alone degraded 1 mmol/L [EtPy][Br]. Additionally, we isolated an ionic liquid-tolerant alkaliphilic actinomycete, Nocardiopsis sp. SSC4. Strain SSC4 produced carboxymethylcellulase (CMCase) in the presence of 1.0% (v/v, 48.1 mmol/L) 1-ethyl-3-methylimidazolium trifluoromethanesulphonate ([EMIM]CF₃SO₃), which is useful for the extraction of cellulose-rich materials from wood. In the case of strain SSC4, CMCase was inducibly synthesized by more than 0.5% CMC. The addition of 0–10% tryptone or 0–20% yeast extract decreased the CMCase activity in a concentration-dependent manner. After cultivation of strain SSC4 with 1.0% (w/v) CMC medium (pH 9.0) for 48 h at 37 °C, the culture supernatant exhibited CMCase activity at 0.03 U/mg. The optimum reaction temperature of CMCase was 45 °C. CMCase was stable up to 37 °C for 20 h incubation. The degradation characteristics of [BMIM][Cl] and [EtPy][Br] and the activity of CMCase in the presence of [EMIM]CF₃SO₃ may be useful for the development of a bioconversion system for biomass resources.

INTRODUCTION
Ionic liquids are a great variety of molten organic salts that are composed of a bulky asymmetric cation and a small anion. Cation and anion combinations can be modified, and hydrophilic and hydrophobic ionic liquids can be prepared [1,2]. Unlike conventional organic solvents applied for biocatalytic reactions, ionic liquids dissolve many compounds, remain liquid over a wide range of temperatures, and possess no vapour pressure. Ionic liquids have prominent physical properties for use as reaction solvents, and we have developed effective biocatalytic reactions using ionic liquids [3,4]. To solve the problems of growth inhibition of microorganisms and denaturation of enzymes by ionic liquids, we isolated the ionic liquid-tolerant Bacillus amyloliquefaciens CMW1 [5,6]. Although organic solvents are indispensable as reaction solvents, in the development of bioconversion systems, ionic liquids are focused on as potential ‘green’ replacements for organic solvents.

In recent years, biomass resources have attracted much attention as a substitute for fossil resources. Wood contains cellulose, hemicellulose, and lignin, and is considered a promising biomass source of energy [7,8]. Treatment of wood with ionic liquids is under consideration. For instance, [BMIM][Cl] (1-butyl-3-methylimidazolium chloride; Figure 1(A)) and [EtPy][Br] (1-ethylpyridinium bromide; Figure 1(B)) have been found to liquefy these wood components [9,10]. [EMIM]CF₃SO₃ (1-ethyl-3-methylimidazolium trifluoromethanesulphonate; Figure 1(C)) has been employed to dissolve lignin for the extraction of cellulose from wood [11]. These findings are expected to be applied to wood-processing technology in consideration of the effects of ionic liquids on the environment. Although the degradation of some ionic liquids by the ultraviolet (UV)/H₂O₂ process has been analysed with the aim of practical use of ionic liquids [12,13], little information is available about the degradation of 1-butyl-3-methylimidazolium cation ([BMIM][Cl]) and 1-ethylpyridinium cation ([EtPy][Br]). Additionally, in order to develop a process of bioconversion of the cellulose-rich material extracted from wood with [EMIM]CF₃SO₃ to fermentable reducing sugars, acquisition of a novel [EMIM]CF₃SO₃-tolerant
Degradation of [BMIM]+ and [EtPy]+ by UV/H₂O₂ process

Materials and methods

Detection and quantification of [BMIM]+ and [EtPy]+

Isolation of [EMIM]CF₃SO₃-tolerant bacterium exhibiting CMCase activity

Strain SSC4 was isolated from a soil and lake water mixture from the Chubu region, Japan, and cultivated aerobically at 37 °C for 3 d in 1.0% (v/v) 48.1 mmol/L [EMIM]CF₃SO₃ medium containing 1.0% (w/v) CMC (Nacalai Tesque), 0.25% Bacto tryptone (Difco Laboratories, Detroit, MI, USA), 1.0% Bacto yeast extract (Difco), 1.0% MgSO₄ 7H₂O, 0.5% KCl, 1.0% NaHCO₃, and 1.0% (v/v) 48.1 mmol/L [EMIM] CF₃SO₃ (Kanto Chemical, Tokyo, Japan) (pH 9.0). A colony of strain SSC4 was repeatedly purified and separated, suspended in 15% glycerol, and stored at −80 °C. For detection of CMCase activity by a cup-plate diffusion assay, 5 mL of the culture supernatant of strain SSC4 was prepared after cultivation, followed by centrifugation at 4 °C at 17 400 g for 10 min. Using stainless steel cups (8 × 6 × 10 mm), 100 μL of the culture supernatant was spotted on a plate containing 1.0% (w/v) CMC (Nacalai Tesque), 0.25% Bacto tryptone (Difco Laboratories), 1.0% Bacto yeast extract (Difco), 1.0% MgSO₄ 7H₂O, 0.5% KCl, 1.0% NaHCO₃, 0.005% trypan blue (Sigma, Munich, Germany), and 1.5% agar (pH 9.0). The cell growth was measured based on the optical density at 660 nm (OD₆₆₀).
Phylogenetic analysis of bacterial isolate

The bacterial isolate strain SSC4, which exhibited 1.0% (v/v, 48.1 mmol/L) [EMIM]CF₃SO₃ tolerance and high CMC activity, was evaluated. The DNA fraction was extracted from a bacterial cell pellet of strain SSC4 using an Illustra bacteria genomic Prep Mini Spin Kit (GE Healthcare, Munich, Germany). To determine the phylogenetic classification, 1520 bp of the 16S rRNA gene fragment was amplified from the DNA fraction by PCR with eubacterial primers 27f and 1525r and Blend Taq (Toyobo Co. Ltd., Osaka, Japan). The DNA sequencing was carried out by FASMAC DNA Sequencing Services (Kanagawa, Japan). Nucleotide substitution rates (Knuc) were determined, and a distance matrix tree was constructed by using the neighbour-joining method with the CLUSTAL_X program [14–16]. Alignment gaps and unidentified base positions were not taken into consideration for the calculations. The topology of the phylogenetic tree was evaluated by performing a bootstrap analysis with 1000 replications.

Evaluation of CMCase activity with culture supernatant

Strain SSC4 was grown in 1.0% (w/v) CMC medium (1.0% (w/v) CMC (Nacalai Tesque), 0.25% Bacto tryptone (Difco), 1.0% Bacto yeast extract (Difco), 1.0% MgSO₄ 7H₂O, 0.5% KCl and 1.0% NaHCO₃, pH 9.0) at 37 °C for 48 h to obtain 25 mL of the culture supernatant by the centrifugation at 4 °C at 20 000 g for 15 min. CMCase activity in the culture supernatant was measured by the release of reducing sugars from CMC by the Somogyi–Nelson method using a glucose standard [17]. The standard assay mixture (500 μL) contained 0.5% (w/v) CMC (Nacalai Tesque), 10 mmol/L glycine–NaOH buffer (pH 9.0) and 10 μL of culture supernatant. After incubation for 18 h at 37 °C, 100 μL of the reaction mixture was used for the quantification of reducing sugars by the Somogyi–Nelson method. One unit (1 U) of CMCase activity was defined as the enzyme necessary to catalyse the production of 1 μmol of glucose equivalent per min at 37 °C and pH 9.0. To determine the optimum temperature for hydrolysis of CMC, the activity of CMCase was evaluated. The culture supernatant (2.1 μg) was added to 0.5% (w/v) CMC in 10 mmol/L glycine-NaOH buffer (pH 9.0). The reaction was done for 18 h at 30–60 °C. To assess the thermal stability of CMCase, the culture supernatant (2.1 μg) was preincubated for 20 h at 4–60 °C in 10 mmol/L glycine-NaOH buffer (pH 9.0). Heat treatment was stopped by cooling on ice. The residual activity was measured at 37 °C for 18 h.

Figure 2. Degradation of ionic liquids by UV/H₂O₂ process: TLC (A) and ESI-MS (B) analysis.
Protein concentration

The protein concentration was determined with a protein assay kit (Nacalai Tesque) according to the manufacturer’s instructions.

Results and discussion

Degradation of [BMIM]Cl and [EtPy]Br by UV/H₂O₂ process

Ionic liquids, being polar solvents with very low vapour pressure and low flammability, are often considered as promising ‘green’ substitutes for volatile organic solvents. However, in order to convincingly demonstrate the ‘green’ nature of ionic liquids, the degradation process must be investigated. After 16 h reaction, [BMIM]⁺ and [EtPy]⁺ were completely degraded by the UV/H₂O₂ process (Figure 2(A)). The degradation of [BMIM]⁺ required the addition of 0.2% (v/v) H₂O₂ and UV irradiation (254 nm), while the degradation of [EtPy]⁺ was achieved by UV irradiation alone. As shown in Figure 2 (B), [BMIM]⁺ was harder to degrade than [EtPy]⁺. Although it was reported that more than 55% of [BMIM]⁺ is degraded after 6 h of UV irradiation alone with a 1000 W Xenon arc lamp [13], we demonstrated that the addition of H₂O₂ was necessary for the degradation of [BMIM]⁺.

Biodegradation intermediates and pathways for 1-octyl-3-methylimidazolium chloride and 1-butyl-3-methylpyridinium bromide with activated sludge have been reported [18,19]. In each degradation pathway, the much further broken structures of methylimidazolium and methylpyridinium were not measured. We examined the biodegradation of [BMIM]⁺ and [EtPy]⁺ using 27 samples (e.g. activated sludges, soils, and seawater) as sources for microorganisms. Although reaction mixtures (5 mL) containing 0.1% (v/v) of each sample, 0.0001%–0.5% (v/v) of each ionic liquid and microbial nutrients were incubated with aerobic shaking for 2–4 weeks, decreases in [BMIM]⁺ and [EtPy]⁺ were not detected by the TLC and ESI-MS analyses. Thus, it was suggested that [BMIM]⁺ and [EtPy]⁺ are hard to biodegrade. Because the [BMIM]⁺ and [EtPy]⁺ concentrations were immediately decreased with the UV/H₂O₂ treatment (Figure 2), this process was shown to be suitable for the degradation of cations.

Isolation and phylogenetic analysis of bacterial isolate SSC4

For conversion of the cellulose-rich materials prepared from wood with the [EMIM]CF₃SO₃ treatment to fermentable reducing sugars, we explored a novel bacterium exhibiting cellulase activity in the presence of [EMIM]CF₃SO₃. To screen for an [EMIM]CF₃SO₃-tolerant bacterial isolate, 36 samples (e.g. soils, seawater, and lake water) were used as bacteria sources. After incubation of small amounts of these samples in 1.0% (v/v, 48.1 mmol/L) [EMIM]CF₃SO₃ medium at 37 °C for 3–4 d, several [EMIM]CF₃SO₃-tolerant microorganisms having CMCase activity were obtained. The bacterial isolate SSC4 was selected as the best CMCase producer in the presence of 1.0% (v/v, 48.1 mmol/L) [EMIM]CF₃SO₃. After 3-d cultivation of strain SSC4 with 1.0% (v/v, 48.1 mmol/L) [EMIM]CF₃SO₃ medium, good growth (OD₆₆₀ 6.5) was confirmed and the culture supernatant was prepared. As shown in Figure 3, the halo of the 1.0% (w/v) CMC degradation was detected by using the culture supernatant containing 1.0% (v/v, 48.1 mmol/L) [EMIM]CF₃SO₃.

The 16S rRNA gene of strain SSC4 consisted of 1520 nucleotides. The results from the phylogenetic analysis based on the 16S rRNA gene sequence data are shown in Figure 4. Strain SSC4 clearly falls within the genus Nocardiopsis and belongs to actinomycetes. The nucleotide sequence of the 16S rRNA gene of strain SSC4 was

Figure 3. Detection of CMCase activity in culture supernatant of strain SSC4.
most closely related to those of *Nocardiopsis alba* PCM2702 (JQ277723) and *Nocardiopsis valliformis* rsk9 (KX832933) with similarities of 99% for both. The next closest level of similarity was 98% similarity with the nucleotide sequences of *Nocardiopsis oceani* 10A08A\(^T\) (NR\_137 417) and *Nocardiopsis nanhaiensis* 10A08B\(^T\) (KF270095). The 16S rRNA sequence of strain SSC4 was submitted to the NCBI GenBank (Accession No. LC205716).

Related *Nocardiopsis* actinomycetes are found in terrestrial, marine, alkaline, and hypersaline environments [20]. The toxicity of ionic liquids towards microorganisms has been reported [21,22]. Some microorganisms, for example, *Bacillus subtilis* NBRC3134, *Escherichia coli* K12, *Haloarcula marismortui* ATCC43049\(^T\), and *Haloarcula japonica* NBRC101032\(^T\), do not grow in the presence of 1.0% (v/v) [BMIM]Cl [6]. In the case of strain SSC4, good growth was confirmed in the presence of 1.0% (v/v, 48.1 mmol/L) [EMIM]C\(_3\)SO\(_3\). A Gram-positive bacterium, *B. amyloliquefaciens* CMW1, exhibited tolerance against 10% (v/v) [EMIM]C\(_3\)SO\(_3\), 10% (v/v) [BMIM]Cl, and 10% (v/v) [BMIM]C\(_3\)SO\(_3\) [5,6]. A Gram-negative bacterium, *Enterobacter lignolyticus* SCF1, exhibited tolerance to 8.5% (v/v) 1-ethyl-3-methylimidazolium chloride [23,24]. In the case of the novel actinomycete *Nocardiopsis* sp. SSC4, the bacterial ionic liquid tolerance is expected to be elucidated as good as in the cases of *B. amyloliquefaciens* CMW1 and *E. lignolyticus* SCF1.

**Effect of nutrient concentration on production of CMCase**

To determine the optimum conditions for production of CMCase by strain SSC4, the effects of nutrient concentration on cell growth and CMCase activity were evaluated. Using 0.1%–1.0% (w/v) CMC or 0%–1.0% (w/v) tryptone, growth to OD\(_{660}\) of about 5.0–6.5 was detected (Figure 5(A,B)). Using 0%–2.0% (w/v) yeast extract, the growth increased from OD\(_{660}\) 2.3 to 7.3 in a yeast extract concentration-dependent manner (Figure 5(C)). Detection of the increase in CMCase activity after the addition of 0.5%–1.0% (w/v) CMC revealed that this enzyme was inducibly produced with the addition of CMC (Figure 5(A)). In the related *Nocardiopsis* actinomycetes [25,26], we clearly

![Figure 5.](image-url)
showed the induction of CMCase activity by the addition of CMC for the first time.

It was also revealed that the CMCase activity decreased as the concentrations of tryptone and yeast extract increased (Figure 5(B,C)). In the case of strain SSC4, the poor nutritional conditions were thought to be suitable for CMCase production. Using the 0.5% (w/v) CMC medium containing 0.1% yeast extract without tryptone, strain SSC4 was grown and the culture supernatant was prepared. When 0.5% (w/v) CMC medium was used, the total activity of CMCase in the culture supernatant decreased to 25% of that when 1.0% (w/v) CMC medium was used.

**Effect of temperature on activity and stability of CMCase**

To determine the optimum temperature for hydrolysis of CMC, the activity and stability of CMCase were evaluated at different temperatures with culture supernatant. The culture supernatant was prepared with 1.0% (w/v) CMC medium by the cultivation of strain SSC4 for 3 d at 37 °C. As shown in Figure 6(A), the optimal temperature for CMCase activity was around 45 °C at pH 9.0 in 10 mmol/L glycine–NaOH buffer. To assess the thermal stability of CMCase, the enzyme was incubated at various temperatures for 20 h in 10 mmol/L glycine–NaOH buffer (pH 9.0), and residual activity was measured (Figure 6(B)). The enzyme was stable up to about 37 °C. By using the standard assay, 0.03 U/mg of CMCase activity was detected in the culture supernatant of *Nocardiopsis* sp. SSC4.

Effect of temperature on activity and stability of CMCase

*Figure 6. Effects of temperature on CMCase activity (A) and stability (B).*

*Nocardiopsis* sp. KNU and *Nocardiopsis* sp. SES28 produce thermo-tolerant and alkali-tolerant cellulolytic enzymes and endo-β-1,4-D-glucanase, respectively [25,26]. To the best of our knowledge, the function of no CMCase from a *Nocardiopsis* actinomycete has been investigated in the presence of an ionic liquid. We demonstrated that strain SSC4 produces CMCase in the presence of 1.0% (v/v, 48.1 mmol/L) [EMIM]CF3SO3. CMCase activity was detected in the presence of 1.0% (v/v, 48.1 mmol/L) [EMIM]CF3SO3 and hydrolysed CMC at 37 °C. Using CMCase, development of a bioconversion system of cellulose-rich materials to produce fermentable reducing sugars at close to room temperature is expected. We plan to purify and elucidate the properties of CMCase from strain SSC4.

To produce wood-based biofuels, pretreatment of wood with ionic liquids has advantages over other methods currently used for degradation of lignocellulose [11]. Ionic liquids – due to their low volatility and low flammability – are intriguing as potentially ‘green’ solvents posing little environmental hazard. However, ionic liquids often inhibit the enzymes used subsequently for biomass conversion. Therefore, the [EMIM]CF3SO3-tolerant CMCase is considered to be useful for development of a novel bioconversion system.

**Conclusions**

Our study demonstrated the degradation of the ionic liquids [BMIM]Cl and [EtPy]Br and the acquisition of a cellulolytic enzyme that can be used with [EMIM]CF3SO3. Ionic liquids [BMIM]Cl, [EtPy]Br, and [EMIM]CF3SO3 are
considered to be ‘green’ solvents for conversion of wood to industrially useful compounds. Further study needs to be done to purify and characterize CMCase against ionic liquids that are able to develop novel efficient systems for bioconversion of wood with these ionic liquids.

**Disclosure statement**

No potential conflict of interest was reported by the authors.

**Funding**

This work was supported by Japan Society for the Promotion of Science London (JSPS) under Grant-in-Aid for Young Scientists (B) [grant number 26870731, to A.K.] and Grant-in-Aid for Scientific Research (C) [grant number 17K07736, to A.K.].

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