Lawrence Berkeley National Laboratory
Biological Systems & Engineering

Title

Tolerance Characterization and Isoprenol Production of Adapted Escherichia coli in the Presence of Ionic Liquids

Permalink

https://escholarship.org/uc/item/32g3t39t

Journal

ACS Sustainable Chemistry & Engineering, 7(1)

ISSN

2168-0485

Authors

Wang, Shizeng
Cheng, Gang
Dong, Jie
et al.

Publication Date

2019-01-07

DOI

10.1021/acssuschemeng.8b05144

Peer reviewed

Tolerance Characterization and Isoprenol Production of Adapted *Escherichia coli* in the Presence of Ionic Liquids

Shizeng Wang,[†] Gang Cheng,[*,†] Jie Dong,[‡,§] Tian Tian,[‡,§] Taek Soon Lee,[‡,§] Aindrila Mukhopadhyay,[‡,§] Blake A. Simmons,[‡,§] Qipeng Yuan,[†] and Steven W. Singer[*,‡,§]

[†]State Key Laboratory of Organic−Inorganic Composites and College of Life Science and Technology, Beijing University of Chemical Technology, North 3rd Ring East, #15, Beijing 100029, China

[‡]Joint BioEnergy Institute, 5885 Hollis Street, Emeryville, California 94608, United States

[§]Biological Systems and Engineering Division, Lawrence Berkeley National Laboratory, 1 Cyclotron Road, Berkeley, California 94702, United States

Ⓢ *Supporting Information*

ABSTRACT: Ionic liquid (IL)-based pretreatment makes lignocellulosic biomass more accessible to enzymes and improves enzymatic digestibility. However, the ILs left in biomass slurry after pretreatment could inhibit activity of enzymes and microbial fermentation. Therefore, it is necessary to develop robust host strains that are IL-tolerant. In this study, we characterized IL tolerance and biofuel production of adapted *Escherichia coli* obtained by adaptive laboratory evolution (ALE). We found that IL-tolerant *E. coli* obtained via ALE by 1-butyl-3-methylimidazolium chloride ([C$_4$C$_1$Im]-Cl) showed improved growth in the presence of four ILs, [C$_4$C$_1$Im]Cl, 1-ethyl-3-methylimidazolium chloride ([C$_2$C$_1$Im]Cl), 1-butyl-3-methylimidazolium acetate ([C$_4$C$_1$Im][OAc]), and 1-ethyl-3-methylimidazolium acetate ([C$_2$C$_1$Im][OAc]) compared to that of the parent strain. The growth of adapted strain *E. coli* MG1655-A1 was even promoted by [C$_4$C$_1$Im]Cl and [C$_2$C$_1$Im]Cl of certain concentrations. The adapted strains were further transformed by introducing mevalonate-based metabolic pathway and they showed significantly increased isoprenol titer compared to parent strain *E. coli* MG1655. Furthermore, they were shown to use [C$_4$C$_1$Im][OAc] and [C$_2$C$_1$Im][OAc] as carbon sources and assimilate the acetate ions. These results indicated that ALE provided promising host strains for one-pot biofuel production.

KEYWORDS: *Ionic liquid pretreatment, Adaptive laboratory evolution, Tolerance, Isoprenol, E. coli*
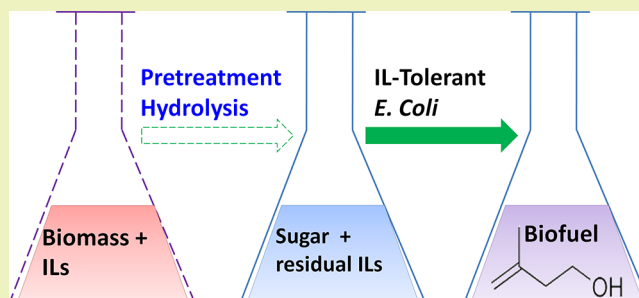
INTRODUCTION

Using lignocellulosic biomass for production of renewable biofuel and value-added chemicals provides a sustainable and promising solution to the depletion of fossil fuels and environmental pollution.[1,2] Conversion of lignocellulosic biomass into sugars relies on efficient pretreatment techniques to improve enzymatic digestibility of biomass.[3−5] Recently, ionic liquid (IL)-based pretreatment processes have emerged and gained much attention for their effectiveness to process a variety of biomasses.[6−9] Among the ILs investigated for biomass pretreatment, [C$_4$C$_1$Im]Cl, [C$_4$C$_1$Im][OAc], [C$_2$C$_1$Im]Cl, and [C$_2$C$_1$Im][OAc] are the most popular ones because of their remarkable capability to dissolve cellulose.[10−15] However, a certain amount of ILs would be left in the biomass slurry after pretreatment. ILs' toxicity to cellulases and microbes is one of the major challenges in the application of IL-based biomass pretreatment.[6] A previous study showed that multiple washing processes were required to remove ILs from biomass.[16] However, extensive washing is not economically feasible on an industrial scale. Therefore, it is of

great importance to develop IL-tolerant microbes that could accommodate high levels of residual ILs (for example, 0.2−5% w/v[16−18] ILs in hydrolysate) during saccharification and fermentation.[18−21] In addition, integration of pretreatment, saccharification, and fermentation into one process, known as "one-pot" process,[22,23] also depends on the development of IL-tolerant strains.

Progress has been made on developing IL-tolerant strains by searching for IL-efflux pumps and IL-assimilating strains from extreme environments, like tropical forest soil and marine environment with high level of salinity and hydrocarbon.[7,18,23] The mechanism of autoregulation of an IL-efflux pump by *EilR* repressor in *Enterobacter lignolyticus* was revealed by Ruegg et al.[18] The autoregulation system was successfully introduced into *Escherichia coli* to produce bisabolane in the presence of [C$_2$C$_1$m]Cl. Frederix et al. found that *ybjJ* and *rcdA* were

involved in IL transportation and the corresponding repressor in a spontaneous IL-tolerant *E. coli* mutant.[22] On the basis of this mechanism, *E. coli* DH1rcdA mutant was used as host for one-pot limonene production in the presence of $[C_2C_1Im]$-$[OAc]$.[22] However, the IL-tolerant strains still face some challenges. The plasmids used to express IL transporters would lead to an extra metabolic burden in cells.[24] The transporters themselves could also be toxic to cells.[18] The extra selection markers for plasmids would also increase the cost of fermentation. In addition, the application of these IL-tolerant strains was limited by the type and concentrations of ILs.

Adaptive laboratory evolution (ALE) has also been shown to be a useful tool to obtain strains which could tolerate ILs.[25,26] In our recent study, we obtained adapted strains *E. coli* MG1655-A1 (named as MG 4.7 in ref 27) and *E. coli* MG1655-A2 (named as MG 3.1 in ref 27) via ALE using $[C_4C_1Im]Cl$.[27] Here, we characterized the IL tolerance of *E. coli* MG1655-A1 and *E. coli* MG1655-A2. The adapted strains show increased tolerance to four extensively studied imidazolium-based ILs ($[C_4C_1Im]Cl$, $[C_4C_1Im][OAc]$, $[C_2C_1Im]Cl$, and $[C_4C_1Im][OAc]$) compared to the parent strain *E. coli* MG1655.

The rapid development of C4−C5 alcohols as biofuels has significantly expanded the biofuel contents and could potentially substitute ethanol in future gasoline mixtures.[28] 3-Methyl-3-buten-1-ol (isoprenol) is a promising biofuel for its octane numbers and combustion properties.[29] Both methylerythritol phosphate pathway and mevalonate pathway can be used to produce isoprenol.[30,31] To evaluate adapted *E. coli*'s potential in biofuel production, we transformed mevalonate-based metabolic pathway of isoprenol into *E. coli* MG1655-A1, *E. coli* MG1655-A2, and *E. coli* MG1655.[32] The transformed strains, named as *E. coli* MG1655-A1-KG1R10, *E. coli* MG1655-A2- KG1R10, and *E. coli* MG1655-KG1R10, showed increased isoprenol yield in the presence of the ILs compared to the parent strain. They could even grow up and produce biofuel when using $[C_4C_1Im][OAc]$ and $[C_2C_1Im][OAc]$ as carbon sources. The adapted *E. coli* showed potential application in reusing and recycling of wastewater containing $[C_4C_1Im][OAc]$ and $[C_2C_1Im][OAc]$ for biofuel production. The development of IL-tolerant *E. coli* by ALE provides a promising host for discovering a novel mechanism of IL tolerance, integrating biofuel fermentation with pretreatment and saccharification by a one-pot process, and recycling wastewater containing ILs.

■ MATERIALS AND METHODS

Experimental procedures and materials characterization are provided in the Supporting Information.

The strains and plasmids are listed in Table 1.

■ RESULTS AND DISCUSSION

Increased IL Tolerance and Isoprenol Production of Adapted *E. coli*. *E. coli* MG1655-A1 and *E. coli* MG1655-A2 in this study were obtained via ALE by $[C_4C_1Im]Cl$. Figure 1 presents growth profiles of the strains without the presence of ILs. The culture volume in 96-well plates was 200 μL. Both of the adapted strains grew faster and showed greater biomass (*E. coli* cells) yield than *E. coli* MG1655 in 10 h after inoculation (Figure 1). However, they showed less biomass yield after 36 h of cultivation compared to the parent strain. MG1655-A2 and *E. coli* MG1655 showed a stationary phase after the exponential phase. In contrast, *E. coli* MG1655-A1 came into

Table 1. Strains and Plasmid Used in This Study

| strains/plasmids	description	reference or source
	Strains	
E. coli MG1655	parent strain	ATCC 700926
E. coli MG1655-A1	IL-tolerant strain obtained by ALE	27
E. coli MG1655-A2	IL-tolerant strain obtained by ALE	27
E. coli MG1655-A1-KG1R10	pJBEI-6829 + JPUB-004507 in *E. coli*MG1655-A1	this study
E. coli MG1655-A2-KG1R10	pJBEI-6829 + JPUB-004507 in *E. coli*MG1655-A2	this study
MG1655-KG1R10	pJBEI-6829 + JPUB-004507 in *E. coli*MG1655	this study
	Plasmids	
pJBEI-6829	pBbA5c-MevTsa-MK-PMK	33
JPUB-004507	pTrc99A-nudB$_{RBS10}$-PMD	32
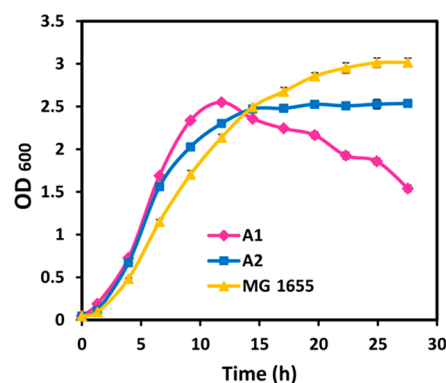
Figure 1. Growth profiles of *E. coli* MG1655-A1, *E. coli* MG1655-A2, and *E. coli* MG1655. The error bars represent standard deviation calculated from each experiment run in triplicate.

the decline phase as soon as the exponential phase ended. It is noted that *E. coli* MG1655-A1 possessed a stationary phase with the ILs in the medium, as shown later in Figures 2 and 3.

The tolerances of *E. coli* MG1655-A1, *E. coli* MG1655-A2, and *E. coli* MG1655 to $[C_4C_1Im]Cl$, $[EMIM]Cl$, $[C_4C_1Im]$-$[OAc]$, and $[C_2C_1Im][OAc]$ were evaluated by growth assay and IC_{50}. Figure 2 presents growth profiles of them in the presence of $[C_4C_1Im]Cl$ and $[C_2C_1Im]Cl$. The growth profiles of *E. coli* MG1655-A1 showed a stationary phase which was in contrast to that of *E. coli* MG1655-A1 in the absence of the ILs in the media. When the $[C_4C_1Im]Cl$ concentration was in the range between 50 and 250 mM, the final biomass yield (after 36 h of cultivation) of *E. coli* MG1655-A1 was greater than that obtained without the $[C_4C_1Im]Cl$) (Figure 2A). It was noted that the biomass yield of *E. coli* MG1655-A1 in the presence of 250 mM $[C_4C_1Im]Cl$ was greater than those in the presence of 50 and 150 mM $[C_4C_1Im]Cl$. This suggested that $[C_4C_1Im]Cl$ may stimulate biomass production of *E. coli* MG1655-A1.

The OD_{600} of *E. coli* MG1655-A1 reached 3.5 in the presence of 250 mM $[C_4C_1Im]Cl$, but *E. coli* MG1655-A1 showed no growth in the presence of 350 mM $[C_4C_1Im]Cl$, indicating that 350 mM $[C_4C_1Im]Cl$ was lethal for *E. coli* MG1655-A1. Figure 2B shows that the presence of $[C_4C_1Im]$Cl inhibited growth of *E. coli* MG1655-A2. The OD_{600} reached 1.5 in the presence of 350 mM $[C_4C_1Im]Cl$ and *E. coli* MG1655-A2 has a higher IC_{50} value than that of *E. coli* MG1655-A1. The parent strain *E. coli* MG1655 could be tolerant only to 50 mM $[C_4C_1Im]Cl$, reaching a final OD_{600} of 1.2 (Figure 2C). After adaptive evolution, the values of the
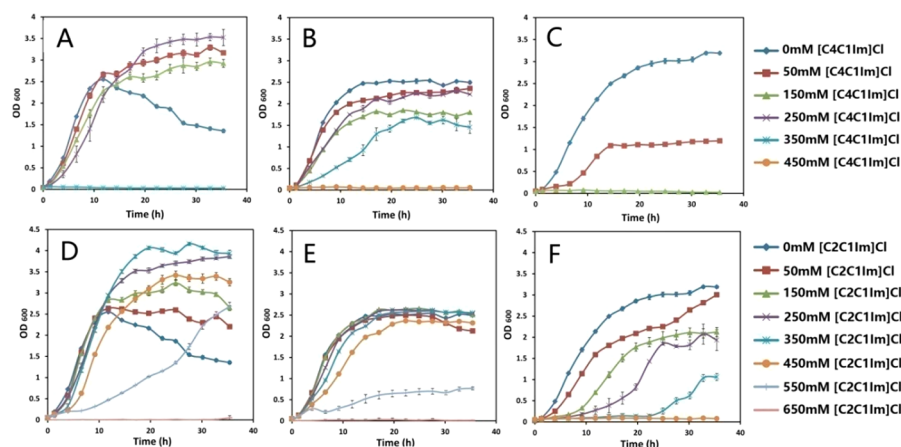
Figure 2. Growth profiles of *E. coli* in the presence of $[C_4C_1Im]Cl$ and $[C_2C_1Im]Cl$. *E. coli* MG1655-A1 (A), *E. coli* MG1655-A2 (B), and *E. coli* MG1655 (C) in the presence of 0, 50, 150, 250, 350, and 450 mM $[C_4C_1Im]Cl$; *E. coli* MG1655-A1 (D), *E. coli* MG1655-A2 (E), and *E. coli* MG1655 (F) in the presence of 0, 50, 150, 250, 350, 450, 550, and 650 mM $[C_2C_1Im]Cl$. ILs were added into M9 medium before fermentation. The error bars represent standard deviation calculated from each experiment run in triplicate.

IC_{50} of *E. coli* MG1655-A1 and *E. coli* MG1655-A2 for $[C_4C_1Im]Cl$ were increased 4.5-fold and 6.1-fold compared to that of the parent strain (Table 2), respectively.

**Table 2. IC_{50} (mM) for ILs in Different Microorganisms**

organism	IC_{50} for $[C_4C_1Im]Cl$	IC_{50} for $[C_4C_1Im][OAc]$	IC_{50} for $[C_2C_1Im]Cl$	IC_{50} for $[C_2C_1Im][OAc]$
E. coli MG1655-A1	207	142	551	176
E. coli MG1655-A2	321	191	530	286
E. coli MG1655	46	48	225	169

As shown in Figure 2D, the presence of $[C_2C_1Im]Cl$ increased the biomass yield of *E. coli* MG1655-A1 when the $[C_2C_1Im]Cl$ concentration was in the range of 50−550 Mm. The biomass yield decreased with further increase of the $[C_2C_1Im]Cl$ concentration. *E. coli* MG1655-A1 showed no growth in the presence of 650 mM $[C_2C_1Im]Cl$. Interestingly,

the biomass yield increased with the increasing $[C_2C_1Im]Cl$ concentration in the range of 50−350 mM. This shows the stimulation effect of $[C_2C_1Im]Cl$ on the growth of *E. coli* MG1655-A1, similar to that of $[C_4C_1Im]Cl$.

E. coli MG1655-A2 did not show significant differences in growth curves in the presence of 0−450 mM $[C_2C_1Im]Cl$ (Figure 2E), suggesting that this strain was not very sensitive to $[C_2C_1Im]Cl$ within this concentration range. The biomass yield of *E. coli* MG1655-A2 dropped significantly when the $[C_2C_1Im]Cl$ concentration was increased to 550 mM. The IC_{50} value for $[C_2C_1Im]Cl$ of *E. coli* MG1655-A1, *E. coli* MG1655-A2, and *E. coli* MG1655 was 551, 530, and 225 mM, respectively. These results indicated that *E. coli* MG1655-A1's and *E. coli* MG1655-A2's tolerances to $[C_2C_1Im]Cl$ were improved significantly after adaptive evolution. What's more, a certain amount of $[C_2C_1Im]Cl$ or $[C_4C_1Im]Cl$ was shown to simulate the growth of *E. coli* MG1655-A1.

Figure 3 shows growth profiles of the three strains in the presence of $[C_4C_1Im][OAc]$ and $[C_2C_1Im][OAc]$. In the presence of low concentrations of ILs (50 and 100 mM for $[C_4C_1Im][OAc]$, 50 mM for $[C_4C_1Im][OAc]$), the biomass yield was greater than that of *E. coli* MG1655-A1 in the absence of the ILs because of the decline phase. This is similar
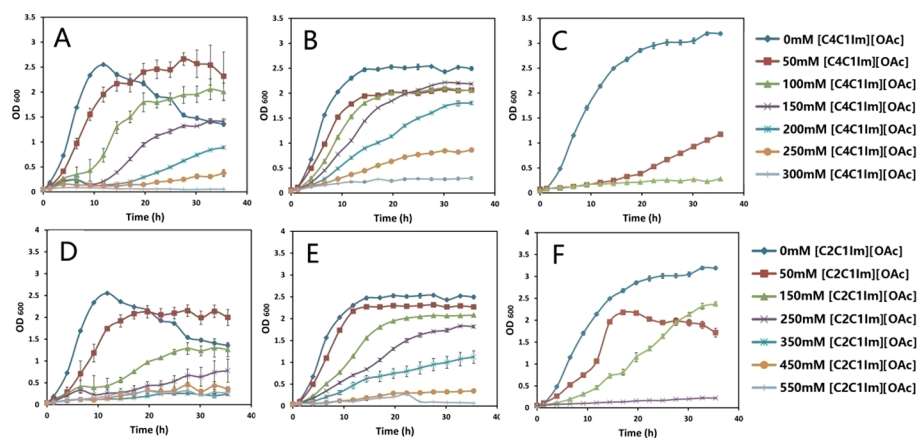
**Figure 3.** Growth profiles of *E. coli* in the presence of $[C_4C_1Im][OAc]$ and $[C_2C_1Im][OAc]$. *E. coli* MG1655-A1 (A), *E. coli* MG1655-A2 (B), and *E. coli* MG1655 (C) in the presence of 0, 50, 100, 150, 200, 250, and 300 mM $[C_4C_1Im][OAc]$; *E. coli* MG1655-A1 (D), *E. coli* MG1655-A2 (E), and *E. coli* MG1655 (F) in the presence of 0, 50, 150, 250, 350, 450, and 550 mM $[C_2C_1Im][OAc]$.
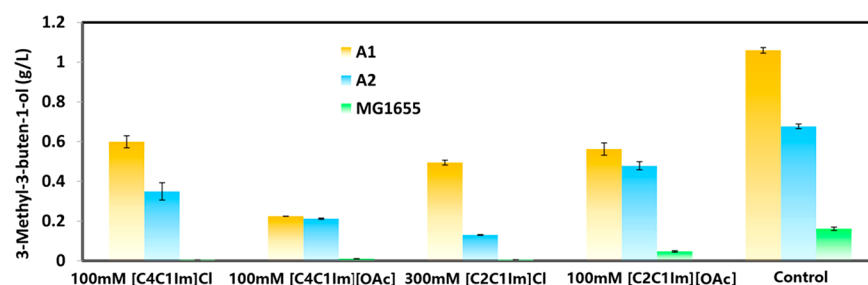
Figure 4. Isoprenol production from *E. coli* MG1655-A1, *E. coli* MG1655-A2, and *E. coli* MG1655 with or without the presence of ILs in EZ-rich medium. The medium of control groups contains no IL. The error bars represent standard deviation calculated from each experiment run in triplicate.
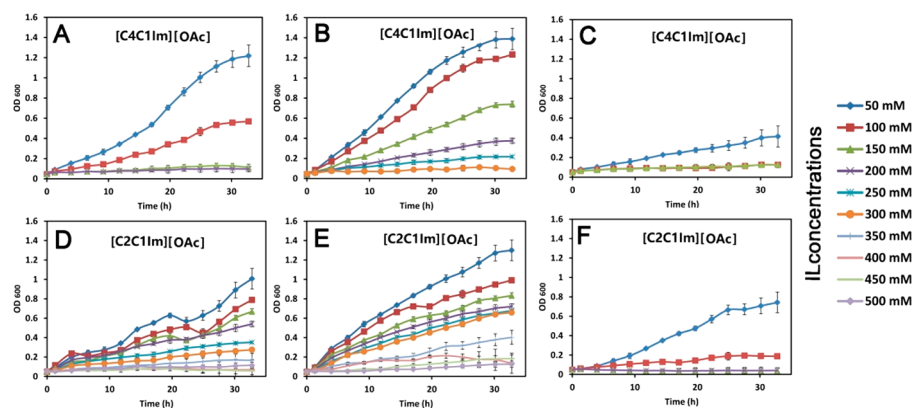
Figure 5. Growth profiles of *E. coli* using ILs as carbon sources. (A) *E. coli* MG1655-A1 treated by $[C_4C_1Im][OAc]$; (B) *E. coli* MG1655-A2 treated by $[C_4C_1Im][OAc]$; (C) *E. coli* MG1655 treated by $[[C_4C_1Im][OAc]$; (D) *E. coli*MG1655-A1 treated by $[C_2C_1Im][OAc]$; (E) *E. coli* MG1655-A2 treated by $[C_2C_1Im][OAc]$; (F) *E. coli* MG1655 treated by $[C_2C_1Im][OAc]$. The error bars represent standard deviation calculated from each experiment run in triplicate.

to the cases of $[C_4C_1Im]Cl$ and $[C_2C_1Im]Cl$. With increasing of the concentration of $[C_4C_1Im][OAc]$ or $[C_2C_1Im][OAc]$, the final biomass yield decreased for all three strains. This is different from the growth profiles of the *E. coli* MG1655-A1 in the presence of $[C_4C_1Im]Cl$ or $[C_2C_1Im]Cl$ where a certain amount of ILs promoted its growth. The *E. coli* MG1655 could tolerate only 50 mM $[C_4C_1Im][OAc]$ with a final OD_{600} of 1 (Figure 3C). The *E. coli* MG1655-A1's and *E. coli* MG1655-A2's IC_{50} values for $[C_4C_1Im][OAc]$ were increased 3.0-fold and 4.0-fold compared to the parent strain, respectively (Table 2).

Table 2 shows that values of IC_{50} of the three strains for $[C_2C_1Im][OAc]$ and $[C_2C_1Im]Cl$ are larger than those for $[C_4C_1Im][OAc]$ and $[C_4C_1Im]Cl$, respectively. This suggests that the toxicity of the ILs is related to the alkyl chain length where longer chains lead to higher toxicity. This has been reported in the literature for a variety of ILs.[34] The results shown here also suggest that the Cl^- ion is less toxic than the $[OAc]^-$ ion. This also has been reported before.[34]

The significantly increased IL tolerance in adapted strains *E. coli* MG1655-A1 and *E. coli* MG1655-A2 made them promising hosts for biofuel production from IL-pretreated biomass. To further evaluate whether the adapted strains could improve biofuel production in the presence of ILs, we transformed mevalonate-based metabolic pathway of isoprenol into *E. coli* MG1655-A1, *E. coli* MG1655-A2, and *E. coli* MG1655.[32] The metabolic pathway of isoprenol consists of two plasmids: one plasmid contains five mevalonate pathway genes and the other plasmid contains two isoprenol synthase genes. In the EZ-rich medium without ILs, the isoprenol titer of *E. coli* MG1655-A1-

KG1R10, *E. coli* MG1655-A2-KG1R, and *E. coli* MG1655 reached 1.06, 0.68, and 0.16 g/L (Figure 4), respectively. Compared to that in *E. coli* MG1655, the isoprenol titer of *E. coli* MG1655-A1-KG1R10 and *E. coli* MG1655-A2-KG1R10 was increased 6.6-fold and 4.3-fold, respectively. This result indicated that ALE increased not only *E. coli* MG1655's IL tolerance but also *E. coli* MG1655's isoprenol production without the presence of ILs. The increased isoprenol production in tolerant strains could be due to the spontaneous mutations which were beneficial to the metabolic pathway of isoprenol.

Next, 100 mM $[C_4C_1Im]Cl$, 100 mM [BMIM]Ac, 300 mM $[C_2C_1Im]Cl$, and 100 mM $[C_2C_1Im][OAc]$ were added in EZ-rich medium (containing 10 g/L glucose) to simulate residual ILs in hydrolysate after IL pretreatment and saccharification.[16] In the presence of 100 mM $[C_4C_1Im]Cl$, 100 mM [BMIM]Ac, 300 mM $[C_2C_1Im]Cl$, and 100 mM [EMIM]Ac, the isoprenol titer of the *E. coli* MG1655 was lower than 0.1 g/L (Figure 4). However, the *E. coli* MG1655-A1-KG1R10 and *E. coli* MG1655-A2-KG1R10 showed significantly increased isoprenol titers compared to parent strain *E. coli* MG1655 (Figure 4). Furthermore, *E. coli* MG1655-A1-KG1R10s performance in isoprenol production was better than *E. coli* MG1655-A2-KG1R10. The *E. coli* MG1655-A1-KG1R10s isoprenol titer in the presence of 100 mM $[C_4C_1Im]Cl$, 100 mM [BMIM]Ac, 300 mM $[C_2C_1Im]Cl$, and 100 mM $[C_2C_1Im][OAc]$ reached 0.60, 0.22, 0.49, and 0.56 g/L, respectively. These results indicated that ALE provided promising host strains for one-pot biofuel production integrating IL pretreatment with saccharification and fermentation.
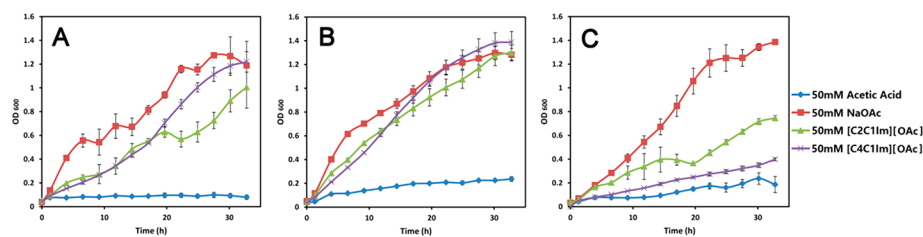
Figure 6. Growth profiles of *E. coli* MG1655-A1 (A), *E. coli* MG1655-A2 (B), and *E. coli* MG1655 (C) using [C$_4$C$_1$Im][OAc], [C$_2$C$_1$Im][OAc], NaOAc, and acetic acid as carbon sources in M9 medium. The error bars represent standard deviation calculated from each experiment run in triplicate.

[C$_4$C$_1$Im][OAc] or [C$_2$C$_1$Im][OAc] was used as the sole carbon source for growth and isoprenol fermentation of adapted *E. coli*. During IL-based pretreatments, multiple washing processes were needed to precipitate cellulose[35,36] and wash off ILs from biomass.[16] Because of their high cost,[37] resistance to biodegradation[38] and toxicity to microbes[39] of certain ILs, they have to be recycled and reused. The ILs containing a certain amount of water need be regenerated by rotary evaporation,[40,41] which was a commonly used approach. However, it is difficult to use rotary evaporation to recycle the ILs of low concentration; the ILs remained in fermentation liquor of one-pot biofuel production[22] and the ILs in other complex industrial wastewater. Therefore, it is necessary to develop an approach to reuse these ILs in wastewater.

Thus, we studied the feasibility of using [C$_4$C$_1$Im][OAc] and [C$_2$C$_1$Im][OAc] as carbon sources for microbe growth and biofuel fermentation. Figure 5 presents the growth curve of *E. coli* MG1655-A1, *E. coli* MG1655-A2, and *E. coli* MG1655 using [C$_4$C$_1$Im][OAc] and [C$_2$C$_1$Im][OAc] as carbon sources in M9 medium. When 50 mM [C$_4$C$_1$Im][OAc] and 50 mM [C$_2$C$_1$Im][OAc] are used as carbon sources, *E. coli* MG1655's OD$_{600}$ reached 0.41 and 0.74, respectively. When the IL concentration was greater than 50 mM, *E. coli* MG1655 could not grow. The adapted strains *E. coli* MG1655-A1 and *E. coli* MG1655-A2 showed better growth performance with [C$_4$C$_1$Im][OAc] and [C$_2$C$_1$Im][OAc] as carbon sources in M9 medium compared to *E. coli* MG1655. The maximum OD$_{600}$ of *E. coli* MG1655-A1 and *E. coli* MG1655-A2 using 50 mM [C$_4$C$_1$Im][OAc] as carbon source reached 1.2 and 1.4, respectively. The maximum OD$_{600}$ of *E. coli* MG1655-A1 and *E. coli* MG1655-A2 using 50 mM [C$_2$C$_1$Im][OAc] as the carbon source reached 1 and 1.2, respectively. When using 100 mM [C$_4$C$_1$Im][OAc] and 100 mM [C$_2$C$_1$Im][OAc] as carbon sources, the OD$_{600}$ of *E. coli* MG1655-A2 could even reach 1.2 and 1.0, respectively. These results suggested that *E. coli* MG1655-A1 and *E. coli* MG1655-A2 could potentially reuse wastewater containing [C$_4$C$_1$Im][OAc] and [C$_2$C$_1$Im][OAc] as the carbon source for microbe growth. High-performance liquid chromatography (HPLC) analysis (by comparing peak area in the chromatogram) showed that [C$_4$C$_1$Im]$^+$ or [C$_2$C$_1$Im]$^+$ could not be assimilated by *E. coli* MG1655-A1, *E. coli* MG1655-A2, or *E. coli* MG1655 because the imidazolium ring was resistant to biodegradation.[38] These results suggested that the acetate ion in ILs was used as the carbon source. Singer et al. also found that *Aspergillus fumigatus* and *Aspergillusustus* isolated from lignocellulose-deconstructing environments could use acetate ion in [C$_2$C$_1$Im][OAc] as the carbon source.[42] During the fermentation process, [C$_4$C$_1$Im][OAc] and [C$_2$C$_1$Im][OAc] were transformed into [C$_4$C$_1$Im]X and [C$_2$C$_1$Im]X (X represents various anions), respectively. [C$_4$C$_1$Im]X and

[C$_2$C$_1$Im]X left in wastewater could be further regenerated by bipolar membrane electrodialysis into hydroxide-based precursors for IL syntheses.[43,44]

In this study, we also compared the growth behavior of the three strains using the 50 mM [C$_4$C$_1$Im][OAc] and [C$_2$C$_1$Im][OAc] as carbon sources to that using 50 mM sodium acetate (NaOAc) and acetic acid as carbon sources. The biomass yields of adapted strains using [C$_4$C$_1$Im][OAc] and [C$_2$C$_1$Im][OAc] as carbon sources did not show significant differences compared to that using NaOAc as the carbon source (Figure 6A,B), even though [C$_4$C$_1$Im][OAc] and [C$_2$C$_1$Im][OAc] slightly prolonged lag phase. However, for *E. coli* MG1655, using [C$_4$C$_1$Im][OAc] and [C$_2$C$_1$Im]-[OAc] as carbon sources caused a significant inhibition to growth compared to that using NaOAc as the carbon source (Figure 6C). The biomass yields of *E. coli* MG1655 using [C$_4$C$_1$Im][OAc], [C$_2$C$_1$Im][OAc], and NaOAc as carbon sources were in the following order: NaOAc > [C$_2$C$_1$Im]-[OAc] > [C$_4$C$_1$Im][OAc]. For both *E. coli* MG1655 and adapted *E. coli*, acetic acid was most toxic among the four tested carbon sources. These results indicated that within a certain range the toxicity of [C$_4$C$_1$Im][OAc] and [C$_2$C$_1$Im]-[OAc] as the carbon source for adapted strains was similar to that of NaOAc, which could be used as the carbon source for lipid production.[45]

To further evaluate whether [C$_4$C$_1$Im][OAc] and [C$_2$C$_1$Im][OAc] could be used as carbon sources for biofuel production, we used 50 mM [C$_4$C$_1$Im][OAc] and 50 mM [C$_2$C$_1$Im][OAc] as carbon sources to produce isoprenol. The parent strains *E. coli* MG1655-KG1R10s isoprenol titer using 50 mM [C$_4$C$_1$Im][OAc], 50 mM [C$_2$C$_1$Im][OAc], and 50 mM NaOAc as carbon source was lower than 14 mg/L (Figure 7). However, the strains *E. coli* MG1655-A1-KG1R10 and *E. coli* MG1655-A2-KG1R10 showed significantly increased isoprenol titer using the ILs or NaOAc as carbon sources
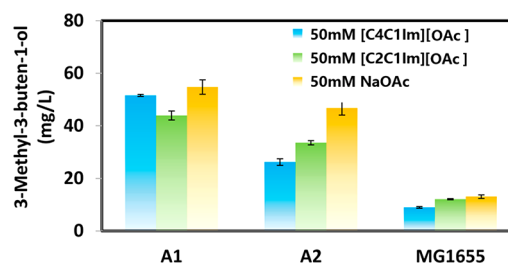
Figure 7. Isoprenol production of *E. coli* MG1655-A1-KG1R10, *E. coli* MG1655-A2-KG1R10, and *E. coli* MG1655-KG1R10 using [C$_4$C$_1$Im][OAc], [C$_2$C$_1$Im][OAc], and NaOAc as carbon source in EZ-rich medium. The error bars represent standard deviation calculated from each experiment run in triplicate.

(Figure 7). Furthermore, *E. coli* MG1655-A1-KG1R10's performance in isoprenol production was better than *E. coli* MG1655-A2-KG1R10's (Figure 7). *E. coli* MG1655-A1-KG1R10s isoprenol titer using 50 mM $[C_4C_1Im][OAc]$ and 50 mM $[C_2C_1Im][OAc]$ as carbon source reached 52 and 44 mg/L, respectively. *t* test showed that the isoprenol titer using $[C_4C_1Im][OAc]$ and $[C_2C_1Im][OAc]$ as carbon sources in *E. coli* MG1655-A1-KG1R10 did not show significant differences compared to that using NaOAc as the carbon source ($p >$ 0.05), indicating that *E. coli* MG1655-A1-KG1R10 was a promising host to reuse acetate of $[C_4C_1Im][OAc]$ and $[C_2C_1Im][OAc]$ in wastewater for biofuel production.

CONCLUSIONS

In this study, we characterized the growth and biofuel production of IL-tolerant *E. coli* obtained by adaptive laboratory evolution. Adapted *E. coli* showed increased biomass yield, increased IC_{50}, and increased biofuel production compared to the parent strain. The growth of *E. coli* MG1655-A1 was even promoted by $[C_4C_1Im]Cl$ and $[C_2C_1Im]Cl$ of certain concentrations. The adapted *E. coli* provided new host strains for one-pot biofuel production integrating IL-based pretreatment with saccharification and fermentation. We also found that *E. coli* MG1655-A1 was a promising host for using the acetate ion of $[C_4C_1Im][OAc]$- and $[C_2C_1Im][OAc]$ in wastewater for biofuel production, providing an approach to reuse and recycle ILs in wastewater.

ASSOCIATED CONTENT

ⓢ Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acssuschemeng.8b05144.

> Bacterial strains, medium and culture conditions, characterization of adapted *E. coli*'s IL tolerance, growth assay using $[C_4C_1Im][OAc]$ and $[C_2C_1Im][OAc]$ as carbon sources, biodegradation assay, isoprenol fermentation, and production assay (PDF)

AUTHOR INFORMATION

Corresponding Authors
*E-mail: SWSinger@lbl.gov. Mailing address: Deconstruction Division, Joint BioEnergy Institute, 5885 Hollis Street, Emeryville, CA 94608, USA. (S.W.S.).
*E-mail:chenggang@mail.buct.edu.cn. Mailing address: Science and Technology Building, West Wing 404, Beijing University of Chemical Technology, North 3rd Ring East, #15, Beijing 100029, China. (G.C.)

ORCID ⓘ
Gang Cheng: 0000-0003-2014-7838

Notes
The authors declare no competing financial interest.

ACKNOWLEDGMENTS

REFERENCES

(1) De Bhowmick, G.; Sarmah, A. K.; Sen, R. Lignocellulosic biorefinery as a model for sustainable development of biofuels and value added products. *Bioresour. Technol.* **2018**, *247*, 1144−1154.

(2) Rodionova, M. V.; Poudyal, R. S.; Tiwari, I.; Voloshin, R. A.; Zharmukhamedov, S. K.; Nam, H. G.; Zayadan, B. K.; Bruce, B. D.; Hou, H. J. M.; Allakhverdiev, S. I. Biofuel production: Challenges and opportunities. *Int. J. Hydrogen Energy* **2017**, *42* (12), 8450−8461.

(3) Kumari, D.; Singh, R. Pretreatment of lignocellulosic wastes for biofuel production: A critical review. *Renewable Sustainable Energy Rev.* **2018**, *90*, 877−891.

(4) Yang, B.; Tao, L.; Wyman, C. E. Strengths, challenges, and opportunities for hydrothermal pretreatment in lignocellulosic biorefineries. *Biofuels, Bioprod. Biorefin.* **2018**, *12* (1), 125−138.

(5) Zhao, Y.; Damgaard, A.; Christensen, T. H. Bioethanol from corn stover - a review and technical assessment of alternative biotechnologies. *Prog. Energy Combust. Sci.* **2018**, *67*, 275−291.

(6) Konda, N. M.; Shi, J.; Singh, S.; Blanch, H. W.; Simmons, B. A.; Klein-Marcuschamer, D. Understanding cost drivers and economic potential of two variants of ionic liquid pretreatment for cellulosic biofuel production. *Biotechnol. Biofuels* **2014**, *7* (1), 86.

(7) Megaw, J.; Busetti, A.; Gilmore, B. F. Isolation and characterisation of 1-alkyl-3-methylimidazolium chloride ionic liquid-tolerant and biodegrading marine bacteria. *PLoS One* **2013**, *8* (4), No. e60806.

(8) Rastogi, M.; Shrivastava, S. Recent advances in second generation bioethanol production: An insight to pretreatment, saccharification and fermentation processes. *Renewable Sustainable Energy Rev.* **2017**, *80*, 330−340.

(9) Brandt, A.; Gräsvik, J.; Hallett, J. P.; Welton, T. Deconstruction of lignocellulosic biomass with ionic liquids. *Green Chem.* **2013**, *15* (3), 550−583.

(10) Zavrel, M.; Bross, D.; Funke, M.; Büchs, J.; Spiess, A. C. High-throughput screening for ionic liquids dissolving (ligno-) cellulose. *Bioresour. Technol.* **2009**, *100* (9), 2580−2587.

(11) Mora-Pale, M.; Meli, L.; Doherty, T. V.; Linhardt, R. J.; Dordick, J. S. Room temperature ionic liquids as emerging solvents for the pretreatment of lignocellulosic biomass. *Biotechnol. Bioeng.* **2011**, *108* (6), 1229−1245.

(12) Kilpeläinen, I.; Xie, H.; King, A.; Granstrom, M.; Heikkinen, S.; Argyropoulos, D. S. Dissolution of wood in ionic liquids. *J. Agric. Food Chem.* **2007**, *55* (22), 9142−9148.

(13) Gupta, K. M.; Hu, Z.; Jiang, J. Mechanistic understanding of interactions between cellulose and ionic liquids: A molecular simulation study. *Polymer* **2011**, *52* (25), 5904−5911.

(14) Yuan, X.; Duan, Y.; He, L.; Singh, S.; Simmons, B.; Cheng, G. Characterization of white poplar and eucalyptus after ionic liquid pretreatment as a function of biomass loading using X-ray diffraction and small angle neutron scattering. *Bioresour. Technol.* **2017**, *232*, 113−118.

(15) Zhang, X.; Zhao, W.; Li, Y.; Li, C.; Yuan, Q.; Cheng, G. Synergistic effect of pretreatment with dimethyl sulfoxide and an ionic liquid on enzymatic digestibility of white poplar and pine. *RSC Adv.* **2016**, *6* (67), 62278−62285.

(16) Li, C.; Tanjore, D.; He, W.; Wong, J.; Gardner, J. L.; Sale, K. L.; Simmons, B. A.; Singh, S. Scale-up and evaluation of high solid ionic liquid pretreatment and enzymatic hydrolysis of switchgrass. *Biotechnol. Biofuels* **2013**, *6* (1), 154.

(17) Li, C.; Tanjore, D.; He, W.; Wong, J.; Gardner, J. L.; Thompson, V. S.; Yancey, N. A.; Sale, K. L.; Simmons, B. A.; Singh, S. Scale-Up of Ionic Liquid-Based Fractionation of Single and Mixed Feedstocks. *BioEnergy Res.* **2015**, *8* (3), 982−991.

(18) Ruegg, T. L.; Kim, E.-M.; Simmons, B. A.; Keasling, J. D.; Singer, S. W.; Lee, T. S.; Thelen, M. P. An auto-inducible mechanism for ionic liquid resistance in microbial biofuel production. *Nat. Commun.* **2014**, *5*, 3490.

(19) Dickinson, Q.; Bottoms, S.; Hinchman, L.; McIlwain, S.; Li, S.; Myers, C. L.; Boone, C.; Coon, J. J.; Hebert, A.; Sato, T. K.; Landick, R.; Piotrowski, J. S. Mechanism of imidazolium ionic liquids toxicity in Saccharomyces cerevisiae and rational engineering of a tolerant, xylose-fermenting strain. *Microb. Cell Fact.* **2016**, *15*, 17.

(20) Xu, J.; Wang, X.; Hu, L.; Xia, J.; Wu, Z.; Xu, N.; Dai, B.; Wu, B. A novel ionic liquid-tolerant Fusarium oxysporum BN secreting ionic liquid-stable cellulase: Consolidated bioprocessing of pretreated lignocellulose containing residual ionic liquid. *Bioresour. Technol.* **2015**, *181*, 18−25.

(21) Yu, C.; Simmons, B. A.; Singer, S. W.; Thelen, M. P.; VanderGheynst, J. S. Ionic liquid-tolerant microorganisms and microbial communities for lignocellulose conversion to bioproducts. *Appl. Microbiol. Biotechnol.* **2016**, *100* (24), 10237−10249.

(22) Frederix, M.; Mingardon, F.; Hu, M.; Sun, N.; Pray, T.; Singh, S.; Simmons, B. A.; Keasling, J. D.; Mukhopadhyay, A. Development of an E. coli strain for one-pot biofuel production from ionic liquid pretreated cellulose and switchgrass. *Green Chem.* **2016**, *18*, 4189.

(23) Khudyakov, J. I.; D'haeseleer, P.; Borglin, S. E.; DeAngelis, K. M.; Woo, H.; Lindquist, E. A.; Hazen, T. C.; Simmons, B. A.; Thelen, M. P. Global transcriptome response to ionic liquid by a tropical rain forest soil bacterium, Enterobacter lignolyticus. *Proc. Natl. Acad. Sci. U. S. A.* **2012**, *109* (32), E2173−E2182.

(24) Diaz Ricci, J. C.; Hernández, M. E. Plasmid effects on Escherichia coli metabolism. *Crit. Rev. Biotechnol.* **2000**, *20* (2), 79−108.

(25) Qin, D.; Hu, Y.; Cheng, J.; Wang, N.; Li, S.; Wang, D. An auto-inducible Escherichia coli strain obtained by adaptive laboratory evolution for fatty acid synthesis from ionic liquid-treated bamboo hydrolysate. *Bioresour. Technol.* **2016**, *221*, 375−384.

(26) Wang, S.; Sun, X.; Yuan, Q. Strategies for enhancing microbial tolerance to inhibitors for biofuel production: A review. *Bioresour. Technol.* **2018**, *258*, 302.

(27) Mohamed, E. T.; Wang, S.; Lennen, R. M.; Herrgard, M. J.; Simmons, B. A.; Singer, S. W.; Feist, A. M. Generation of a platform strain for ionic liquid tolerance using adaptive laboratory evolution. *Microb. Cell Fact.* **2017**, *16* (1), 204.

(28) Yang, Y.; Dec, J. E.; Dronniou, N.; Simmons, B. Characteristics of isopentanol as a fuel for HCCI engines. *SAE International Journal of Fuels and Lubricants* **2010**, *3*, 725−741.

(29) Hull, A.; Golubkov, I.; Kronberg, B.; Marandzheva, T.; Van Stam, J. An alternative fuel for spark ignition engines. *Int. J. Engine Res.* **2006**, *7* (3), 203−214.

(30) Liu, H.; Wang, Y.; Tang, Q.; Kong, W.; Chung, W.-J.; Lu, T. MEP pathway-mediated isopentenol production in metabolically engineered Escherichia coli. *Microb. Cell Fact.* **2014**, *13*, 135.

(31) Zheng, Y.; Liu, Q.; Li, L.; Qin, W.; Yang, J.; Zhang, H.; Jiang, X.; Cheng, T.; Liu, W.; Xu, X.; Xian, M. Metabolic engineering of Escherichia coli for high-specificity production of isoprenol and prenol as next generation of biofuels. *Biotechnol. Biofuels* **2013**, *6*, 57.

(32) George, K. W.; Thompson, M. G.; Kang, A.; Baidoo, E.; Wang, G.; Chan, L. J. G.; Adams, P. D.; Petzold, C. J.; Keasling, J. D.; Lee, T. S. Metabolic engineering for the high-yield production of isoprenoid-based C5 alcohols in E. coli. *Sci. Rep.* **2015**, *5*, 11128.

(33) George, K. W.; Chen, A.; Jain, A.; Batth, T. S.; Baidoo, E. E.; Wang, G.; Adams, P. D.; Petzold, C. J.; Keasling, J. D.; Lee, T. S. Correlation analysis of targeted proteins and metabolites to assess and engineer microbial isopentenol production. *Biotechnol. Bioeng.* **2014**, *111* (8), 1648−1658.

(34) Montalbán, M. G.; Hidalgo, J. M.; Collado-González, M.; Díaz Baños, F. G.; Víllora, G. Assessing chemical toxicity of ionic liquids on Vibrio fischeri: Correlation with structure and composition. *Chemosphere* **2016**, *155*, 405−414.

(35) Dadi, A. P.; Varanasi, S.; Schall, C. A. Enhancement of cellulose saccharification kinetics using an ionic liquid pretreatment step. *Biotechnol. Bioeng.* **2006**, *95* (5), 904−910.

(36) Singh, S.; Simmons, B. A.; Vogel, K. P. Visualization of biomass solubilization and cellulose regeneration during ionic liquid pretreatment of switchgrass. *Biotechnol. Bioeng.* **2009**, *104* (1), 68−75.

(37) Klein-Marcuschamer, D.; Simmons, B. A.; Blanch, H. W. Techno-economic analysis of a lignocellulosic ethanol biorefinery with ionic liquid pre-treatment. *Biofuels, Bioprod. Biorefin.* **2011**, *5* (5), 562−569.

(38) Docherty, K. M.; Kulpa, C. F., Jr. Toxicity and antimicrobial activity of imidazolium and pyridinium ionic liquids. *Green Chem.* **2005**, *7* (4), 185−189.

(39) Pernak, J.; Sobaszkiewicz, K.; Mirska, I. Anti-microbial activities of ionic liquids. *Green Chem.* **2003**, *5* (1), 52−56.

(40) Li, Q.; He, Y.-C.; Xian, M.; Jun, G.; Xu, X.; Yang, J.-M.; Li, L.-Z. Improving enzymatic hydrolysis of wheat straw using ionic liquid 1-ethyl-3-methyl imidazolium diethyl phosphate pretreatment. *Bioresour. Technol.* **2009**, *100* (14), 3570−3575.

(41) Li, B.; Asikkala, J.; Filpponen, I.; Argyropoulos, D. S. Factors affecting wood dissolution and regeneration of ionic liquids. *Ind. Eng. Chem. Res.* **2010**, *49* (5), 2477−2484.

(42) Singer, S. W.; Reddy, A. P.; Gladden, J. M.; Guo, H.; Hazen, T. C.; Simmons, B. A.; VanderGheynst, J. S. Enrichment, isolation and characterization of fungi tolerant to 1-ethyl-3-methylimidazolium acetate. *J. Appl. Microbiol.* **2011**, *110* (4), 1023−1031.

(43) Fukumoto, K.; Yoshizawa, M.; Ohno, H. Room temperature ionic liquids from 20 natural amino acids. *J. Am. Chem. Soc.* **2005**, *127* (8), 2398−2399.

(44) Himmler, S.; König, A.; Wasserscheid, P. Synthesis of [EMIM] OH via bipolar membrane electrodialysis−precursor production for the combinatorial synthesis of [EMIM]-based ionic liquids. *Green Chem.* **2007**, *9* (9), 935−942.

(45) Najafabadi, H. A.; Malekzadeh, M.; Jalilian, F.; Vossoughi, M.; Pazuki, G. Effect of various carbon sources on biomass and lipid production of Chlorella vulgaris during nutrient sufficient and nitrogen starvation conditions. *Bioresour. Technol.* **2015**, *180*, 311−317.