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Rapid screening of toxicity to thermotolerant yeasts: inhibition of growth and fermentation by ionic liquids and zwitterions†

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For extremely efficient bioethanol production, simultaneous pretreatment, saccharification, and fermentation in the same reaction pot (called a one-pot process) is necessary. Thermotolerant yeast *Kluyveromyces marxianus* can ferment at around 50 °C and is thus suitable for this process. We have developed cellulose-dissolving zwitterionic liquids, which are suitable pretreatment solvents to enable a one-pot process. On the other hand, there are no studies of the toxicity to yeasts including *Kluyveromyces marxianus*. We here studied the toxicity after establishing the screening methods applicable to high temperature. The zwitterion was confirmed to be low-toxic in most cases, compared to the most famous cellulose-dissolving ionic liquid. We further subjected two natural zwitterions, trimethylglycine and L-carnitine, to the same screening. Trimethylglycine, especially, was low-toxic, while it does not dissolve cellulose. The inhibition of growth and fermentation depended on the ion species, concentration, microorganism species, and temperature.

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Sustainability spotlight

Inedible cellulosic biomass-derived ethanol is a solution for the competition between food and energy, which is a problematic issue in starch-derived bioethanol. However, cellulosic bioethanol has not been practically industrialised for decades. One of the reasons which prevent its industrialisation is the production energy cost. An ideal bioethanol production process includes simultaneous pretreatment, saccharification, and fermentation at 50 °C; which requires biocompatible cellulose solvents and thermotolerant yeasts in addition to cellulase. This study revealed the relation between a biocompatible cellulose solvent (zwitterionic liquid) and a thermotolerant yeast (*Kluyveromyces marxianus*), contributing to achieving SDGs 2 (zero hunger), 7 (affordable and clean energy), and 13 (climate action).

Introduction

Ethanol from cellulosic biomass is a potential alternative to fossil fuels.[1] However, the recalcitrant and complex structure of cellulosic biomass makes chemical/biological conversion difficult.[2] Therefore, pretreatment is necessary to relax the structure. A typical pretreatment method is dissolution in solvents, which requires high temperature (low temperature in some cases) and/or high pressure; in other words, it demands a high energy cost.[3–6] Because the heat of combustion of ethanol is only approximately 60% that of petroleum, the high energy cost is a critical issue for industrialisation.

Although ionic liquids have high cost, they dissolve cellulose at ambient temperature and pressure, thus reducing the energy cost of bioethanol production.[7–12] For the industrialisation of cellulosic bioethanol, successive pretreatment, saccharification, and fermentation in a single vessel are required to reduce energy costs further.[13–16] However, typical cellulose-dissolving ionic liquids, except for few exceptions,[17,18] are highly toxic to microorganisms, and this successive process is not feasible with these ionic liquids.

A well-known mechanism of ionic liquid toxicity is the disruption of cell membranes, as detailed below:[17,19–22] (1) the cations of ionic liquids are attracted by electrostatic interactions with the phospholipids of the cell membrane. (2) The hydrophobic alkyl chains of the cations are inserted into the cell membrane *via* hydrophobic interactions. Based on these mechanisms, in 2017, we designed a low-toxicity zwitterionic liquid lacking hydrophobic alkyl chains (OE_2imC_3C; Fig. 1).[13,19,23–28] OE_2imC_3C dissolves cellulose because of its

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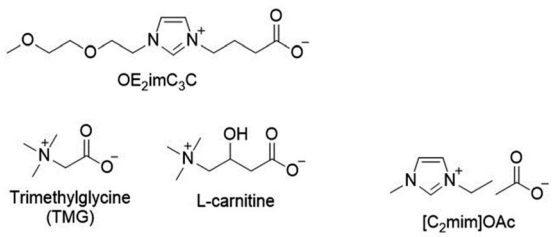

Fig. 1 The structures of zwitterions and ionic liquids used in this study.

highly polar anion. Successive bioethanol production was achieved with OE_2imC_3C; however, the reduction in energy cost was still insufficient. The application of simultaneous saccharification and fermentation is the next potential means to reduce energy costs further.[29] Simultaneous saccharification and fermentation are generally performed at around 30 °C using *Saccharomyces cerevisiae*; however, this is inefficient because the optimum temperature for cellulase is around 50 °C. The heat-resistant yeast *Kluyveromyces marxianus* can ferment at 40–45 °C, allowing efficient simultaneous saccharification and fermentation.[30,31] Until now, fermentation in OE_2imC_3C has been studied only with recombinant *Escherichia coli* KO11 (optimum temperature: 37 °C),[13] and the effects of OE_2imC_3C on other microorganisms have not been investigated. Therefore, this study examined the effects of OE_2imC_3C on the growth and fermentation of *K. marxianus* at around 40–50 °C. The relationship between temperature and toxicity was also examined since *K. marxianus* can be cultured even at 30 °C, which means it can be grown over a relatively wide temperature range. To the best of our knowledge, the relationship between the toxicity of ionic/zwitterionic liquids and the growth and fermentation temperatures has not yet been investigated.

In studies using ionic/zwitterionic liquids, the number of samples is often very large as there may be several conditions, such as ion species, ion concentration, incubation time, and temperature. Therefore, it is important to establish a method for rapidly evaluating growth and fermentation. Therefore, establishing a rapid evaluation method applicable to high-temperature cultures is also one of the purpose of this study. High temperatures induce evaporation of water and ethanol from the media, causing critical problems for analyses. Screening methods at high temperature, which have not been well-established, were developed in this study.

Experimental

Materials

K. marxianus DMKU3-1042 and NBRC1777 were purchased from the National Institute of Technology and Evaluation Biological Resource Center (NBRC). OE_2imC_3C was synthesised as previously reported, with minor modifications.[13,32] The modification is changing the starting material from benzenesulfonyl chloride to *p*-toluenesulfonyl chloride. Trimethylglycine and L-carnitine were purchased from Tokyo Chemical Industry Co., Ltd. (Chuo-ku, Tokyo, Japan) and used as received. $[C_2mim]OAc$ was purchased from Iolitech GmbH (Heilbronn, Germany) and was used as received. D (+)-Glucose, meat peptone, and yeast extract (dried yeast extract) were purchased from Nacalai Tesque, Inc. (Nakagyo-ku, Kyoto, Japan) and used as components of the YPD media.

Methods

Inhibition of *K. marxianus* growth by OE_2imC_3C. YPD media (10 g L^{-1} yeast extract, 20 g L^{-1} peptone, and 20 g L^{-1} glucose) were prepared and autoclaved (121 °C, 20 minutes). *K. marxianus* was precultured aerobically at 40 °C for 24 h in 27 mL test tubes containing 6 mL of the YPD medium. After preculturing, the optical density of the media at 600 nm (OD_{600}) after a 20-fold dilution was measured using 96-well plates (100 μL of solution in each well) and a microplate spectrophotometer (Epoch, BioTek). *K. marxianus* was inoculated into 100 μL of the OE_2imC_3C/YPD mixtures (OE_2imC_3C concentration: 0.01, 0.05, 0.1, 0.5, and 1 M) at an OD_{600} of 0.2. The inoculated media were incubated at various temperatures for 6 h in a rotary shaker (DWMax MBR-032P, Taitec Corporation, Saitama, Japan) at 700 rpm, and the OD_{600} of the solutions was measured. This experiment was conducted three times to ensure reproducibility. To prevent the media from drying up during high-temperature incubation, wet paper towels were placed in the incubator at temperatures above 50 °C to reduce the amount of volatilisation during incubation (Fig. S1†). The paper towels were moistened every two hours. In addition, the 96-well plates were covered with sheets which allowed air to permeate but not moisture (SureSeal Breathable, Sterile, BMF-BS-25, BMBio ECO) during incubation. We did not use the outermost wells and added distilled water (100 μL) to all the empty wells to prevent drying.

Inhibition of *K. marxianus* fermentation by OE_2imC_3C. *K. marxianus* was precultured aerobically at 40 °C for 24 h in a 200 mL baffled flask containing 60 mL YPD medium. *K. marxianus* was collected by centrifugation (15 000 rpm, 5 min) and inoculated into OE_2imC_3C/YPD (OE_2imC_3C concentration: 0.01, 0.05, 0.1, 0.5, and 1 M). The initial OD_{600} was 20, measured using a cuvette with 1 cm path length. A high initial OD was set so that glucose was not used for growth. The inoculated medium (1 mL) was incubated in 2 mL cryotubes (TR7001, NIPPON Genetics Co., Ltd., Tokyo, Japan), used to prevent the evaporation of ethanol, at various temperatures for 3 h using a rotator (ASONE, ACR-100) at 10 rpm. The ethanol concentrations were analysed using high-performance liquid chromatography (HPLC). 100 μL of solution was taken and filtered through a small-dead-volume syringe filter (Millex®-LG, SLLGH04NK, Merck) for HPLC analysis. Glass inserts (TORAST vial insert, GLCTV-I01; Shimadzu GLC, Ltd.) were placed in the HPLC vials. The sample injection volume for HPLC was 20 μL. HPLC analyses were conducted under the following conditions: refractive index detector (Shimadzu Co.); column oven, 70 °C; mobile phase, 4.25 mM H_2SO_4; flow rate, 0.7 mL min^{-1}. Coregel ION-300 column (Tokyo Chemical Industry Co., Ltd). This experiment was conducted three times to ensure reproducibility.

Inhibition of *S. cerevisiae* growth and fermentation by OE_2imC_3C. *S. cerevisiae* BY4741 (ref. 33) was incubated and fermented at 30 °C. The other experimental methods were the same as those used for *K. marxianus*. This experiment was conducted three times to ensure reproducibility.

Results & discussion

In this study, we employed *K. marxianus* and *S. cerevisiae*. *K. marxianus* has excellent thermostability, and *S. cerevisiae* has an optimum temperature of around 30 °C and critical growth inhibition occurring below 40 °C.[30] In contrast, *K. marxianus* DMKU3-1042 is capable of growing at least at 49 °C and fermenting at 45 °C.[30]

In addition to OE_2imC_3C, trimethylglycine (TMG) and L-carnitine (Fig. 1) were examined as references in this study, whereas they have been reported to have low biomass pretreatment potential.[25] TMG and L-carnitine are natural zwitterions containing ammonium cations. A typical cellulose-dissolving ionic liquid ([C_2mim]OAc) was also used as a reference control (Fig. 1).

Growth inhibition of *K. marxianus* by OE_2imC_3C

Experimental design for quick screening. Owing to the several variable conditions, such as time, temperature, ion species, and ion concentration, an experimental system suitable for rapid screening was established. The suitable screening method requires a small sample volume because the typical syntheses of ionic liquids/zwitterions in laboratories are of the gram order (*e.g.* 5–10 g) and OE_2imC_3C, specifically, requires multiple synthetic steps. We planned to proceed with the experiment using 96-well plates because the OD_{600} can be measured directly after incubation, and only a 100 μL sample volume is required.

The culture was performed in a 96-well plate incubator under agitation. However, the medium evaporates quickly when cultured at high temperatures. Therefore, an air-permeable but non-moisture-permeable sheet was used. Furthermore, we placed wet paper towels in the incubator, wetted them again every 2 hours when incubated at 50 °C or higher, and poured 100 μL of water into unused wells. The outermost wells were not used because the water in these wells is prone to evaporation (the effects are shown in Fig. S1†).

The OD_{600} is generally proportional to the density of bacteria at low densities, but the sensitivity of spectrophotometers becomes poor at high densities and is not proportional to the bacterial density. Therefore, initially the OD_{600} was properly diluted to a range proportional to the bacterial density and measured. The correct OD_{600} value was calculated by multiplying it with the dilution factor (described below as "accurate OD_{600}"). On the other hand, in this study, OD_{600} measured directly in 96-well plates without dilution (defined as "directly measured OD_{600}") is preferable to rapidly screen a large number of samples. We assumed that there is a difference between "directly measured OD_{600}" and "accurate OD_{600}" at high OD_{600}, and the relation is shown in Fig. 2.

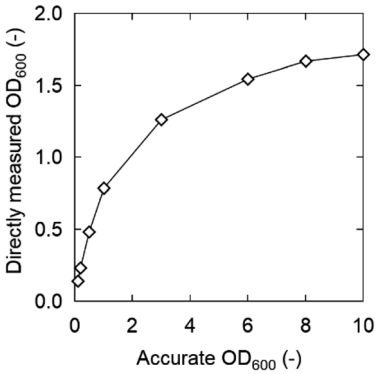

Fig. 2 Relation between directly measured and accurate OD_{600} values measured in 96-well plates. Strain: *K. marxianus* DMKU3-1042.

In *K. marxianus*, these values did not match above an accurate OD_{600} of 0.5 (directly measured $OD_{600} = 0.34$ at an accurate OD_{600} of 0.5), whereas they were still similar when the accurate OD_{600} was 0.2 (directly measured $OD_{600} = 0.17$). The slope became very small, especially when the OD_{600} exceeded 1.3. However, the directly measured OD_{600} increased slightly even at a highly accurate OD_{600}, indicating that the 96-well plate can be used for qualitative/semi-quantitative evaluation. Therefore, the directly measured OD_{600} values were adequate for rapid screening.

When *K. marxianus* was cultured without OE_2imC_3C at an initial OD_{600} of 0.2, growth was complete within 6 h at appropriate temperatures (Fig. S2†). Since the general method requires incubation for at least 24 h,[13] the duration of the experiment was highly conserved. This also means that midnight sampling and repeated wetting of paper towels (at temperatures above 50 °C) can be avoided, which are also significant advantages.

Confirmation of the thermotolerance of *K. marxianus* DMKU3-1042. Previously, thermotolerance upon growth only up to 49 °C has been studied.[30] In this study, we explored the effects of incubation temperatures. The results indicate that the growth rate was better at 40 °C (optimal growth temperature) compared to 30 °C. Notably, at 45 °C, the OD_{600} was similar to

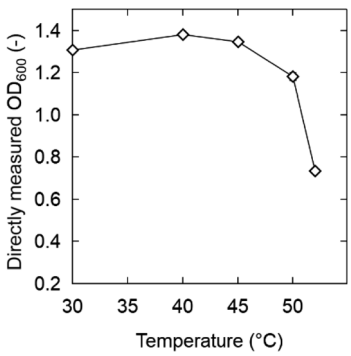

Fig. 3 OD_{600} of the media without zwitterion/ionic liquid after 6 h of incubation. The time courses of OD_{600} at specific temperatures are shown in Fig. S3.† Strain: *K. marxianus* DMKU3-1042.

that at 40 °C (Fig. 3). *K. marxianus* DMKU3-1042 also grew well at 30 °C, but it is worth noting that in the high OD_{600} region, a small change in OD_{600} means a relatively large difference. The growth rate at 50 °C is similar to that at 40–45 °C ($OD_{600} = 1.22$) but lower, indicating that significant growth inhibition begins at 50 °C. Our results suggest that *K. marxianus* DMKU3-1042 can grow up to 50 °C without critical inhibition. Thus, the impact of OE_2imC_3C up to 50 °C was evaluated in this study.

Inhibition by OE_2imC_3C, other zwitterions, and an ionic liquid. The concentration-dependent growth inhibition by OE_2imC_3C was also examined (Fig. 4). *K. marxianus* grew up to an OE_2imC_3C concentration of 0.5 M ($OD_{600} = 0.64$ after 6 h incubation) but was completely inhibited at 1.0 M OE_2imC_3C ($OD_{600} = 0.13$) at the optimum temperature of 40 °C. These results were compared with those of a typical ionic liquid that dissolves cellulose, $[C_2mim]OAc$. In short-chain alkyl cations such as $[C_2mim]OAc$, strong insertion into the cell membrane does not occur.[24] Therefore, they are known to be less toxic than long-chain alkyl imidazolium-based ionic liquids, but their toxicity is still higher than that of common organic solvents.[19,24] The reported mechanism of toxicity is the entry of cations into the cell *via* transporters on the cell membrane.[34] It alters mitochondrial membrane potential, leading to apoptosis. In the case of $[C_2mim]OAc$, even at 0.1 M, there was no growth. These results indicate that OE_2imC_3C is less toxic to *K. marxianus* than $[C_2mim]OAc$, not only in prokaryotes such as *E. coli* but also in eukaryotes. Unlike $[C_2mim]OAc$, OE_2imC_3C did not enter animal cells,[23] which is consistent with our toxicity results.

Natural ammonium-based zwitterions, TMG and L-carnitine, were less toxic than OE_2imC_3C (Fig. 4; 40 °C). The growth of *K. marxianus* was inhibited depending on the concentration of the zwitterions; notably, *K. marxianus* grew even in 1.0 M TMG and L-carnitine solutions. In particular, TMG showed very low toxicity.

We further investigated the reasons behind the lower toxicities of TMG and L-carnitine. A significant pH effect on growth was not observed (Fig. S3, Table S1†). The molecular weights of OE_2imC_3C and TMG/L-carnitine are different (OE_2imC_3C: 256, TMG: 117 and L-carnitine: 161), and the difference in molecular weight might have an effect. Similar trends in the three zwitterions were observed when the abscissa concentration was g L^{-1} instead of the molar concentration, which might indicate that the unit of g L^{-1} is important in the toxicity (Fig. S4†). To explore the scientific meaning of the unit of g L^{-1}, the ion-to-water molar ratio was calculated (Table S2†). When ions are present at high concentrations, the zwitterion/water molar ratio changes even at the same molar concentration, depending on the molecular weight. We here hypothesised that the consequent difference, the amount of free water, was reflected in the toxicity. However, the molar ratios of zwitterion/water in the mixtures were similar. Even at 1.0 M, the zwitterion/water molar ratio was approximately 1 : 40–1 : 50, which did not seem to indicate the lack of free water. Furthermore, in the case of *E. coli*, the EC_{50} values of OE_2imC_3C (M_w: 256) and a smaller zwitterion (M_w: 168, an analogous zwitterion with a methyl cation side chain) were 158 and 141 g L^{-1},[24] respectively, suggesting that the molecular weight itself is not a critical factor and the observation of similar trends in the g L^{-1} unit may be coincidental.

Biologically, TMG and L-carnitine, which are natural zwitterions, are known to be biocompatible solutes that act as osmolytes in microorganisms.[35,36] Some yeasts can take up TMG,[37] and they may alleviate osmotic pressure through the uptake. In other words, the difference between non-uptakable OE_2imC_3C and uptakable TMG and carnitine is assumed to be a possible reason for growth inhibition although further investigation is needed.

To interpret it chemically in molecular design, we studied which is more important, being natural or ammonium-based. A similar artificial ammonium-based zwitterion ($N_{2,2,2}C_3C$, Fig. S5†) was synthesised and subjected to the same experiments. The results indicated that the presence of an ammonium cation was important for developing a less toxic zwitterion (Fig. S5†). In summary, ammonium-based zwitterions, including TMG and carnitine, were found to be superior zwitterions in terms of toxicity; however, TMG and carnitine showed no cellulose solubility and had a lower pretreatment capacity for biomass compared to OE_2imC_3C. The search for ammonium-based ZIs with superior pretreatment capacities will be an important design guideline in the future.

Considering simultaneous saccharification and fermentation, the effect of zwitterions at 50 °C is also important (Fig. 4). OE_2imC_3C was confirmed to be less toxic than $[C_2mim]OAc$; growth was observed at OE_2imC_3C concentrations below 0.5 M. The effect of OE_2imC_3C concentration on growth at 50 °C was greater than that at 40 °C. The effects of OE_2imC_3C concentration were similar between 30 and 45 °C (Fig. S6†) but the effect of TMG and carnitine was the mildest at 40 °C. These results are summarized in Fig. 5 and indicate that temperature and growth inhibition are related and that the inhibition is minimal at around the optimum temperature.

Inhibition against a different strain: *K. marxianus* NBRC1777. To further examine the growth inhibition by OE_2imC_3C, *K. marxianus* NBRC1777 was used. When we performed a growth experiment at 30–50 °C in a medium without OE_2imC_3C, 40 °C was found to be optimal for growth (Fig. S7†). When we examined inhibition by OE_2imC_3C and other ions at

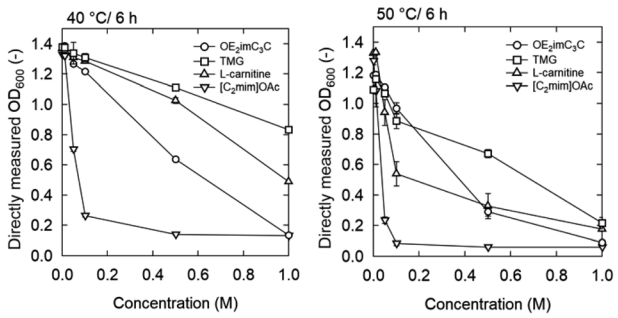

Fig. 4 OD_{600} after 6 h incubation in media supplemented with OE_2imC_3C, TMG, L-carnitine, or $[C_2mim]OAc$ at 40 and 50 °C. Strain: *K. marxianus* DMKU3-1042. Initial OD_{600}: 0.2. The figures for 30 and 45 °C are shown in Fig. S4.†

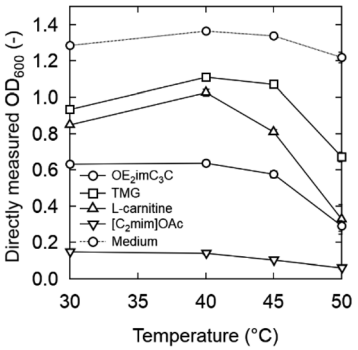

Fig. 5 OD_{600} after 6 h incubation in 0.5 M OE_2imC_3C, TMG, L-carnitine, or $[C_2mim]OAc$ solutions. Strain: *K. marxianus* DMKU3-1042.

30–50 °C, only meagre differences were observed between the two strains (Fig. S8†). This suggested that the effects of zwitterion addition are universal in *K. marxianus* strains.

Inhibition against a different yeast: *S. cerevisiae*. For comparison, we also studied the growth inhibition by OE_2imC_3C against a general yeast, *S. cerevisiae* BY4741 (Fig. 6). Similar to *K. marxianus*, OE_2imC_3C inhibited cell growth in a concentration-dependent manner. Thus, we compared the impact of zwitterions/ILs on the three yeast species studied. Because the size and shape of each microbial species differed, comparison was conducted based on relative OD_{600}, not absolute OD_{600} (Fig. 7). The effects of the zwitterion/IL concentration at the optimal temperatures were similar for all yeast species used, except for the combination of $[C_2mim]OAc$/*S. cerevisiae* BY4741.

Fermentation inhibition by OE_2imC_3C against *K. marxianus*

Experimental design for rapid screening. In this case, 96-well plates were not applicable because of ethanol evaporation. In our previous report,[13] we used glass vials and a magnetic stirrer for multiple samples (6 samples can be tested simultaneously with a single stirrer). However, only one or two magnetic stirrers could be placed in the incubator; therefore, the sample number was too small. A smaller stirrer for 10 samples was also tried,

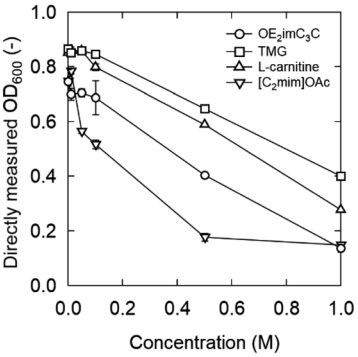

Fig. 6 OD_{600} after 6 h incubation in media supplemented with OE_2imC_3C, TMG, L-carnitine, and $[C_2mim]OAc$ at 30 °C. Strain: *S. cerevisiae* BY4741. Initial OD_{600}: 0.2.

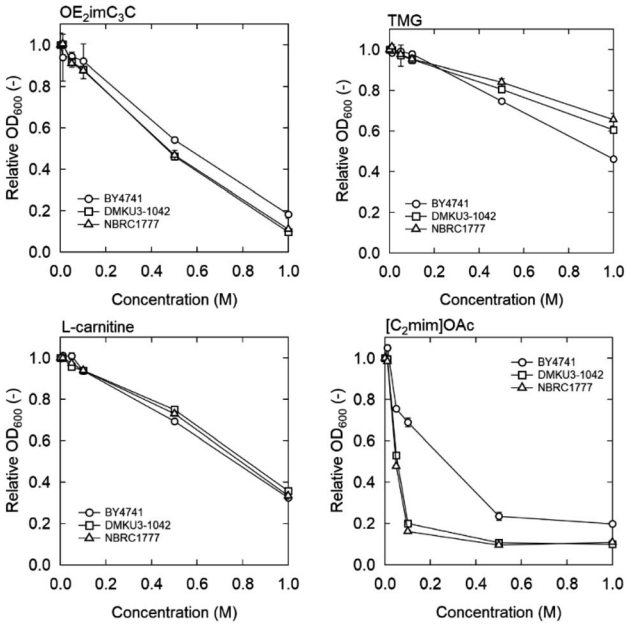

Fig. 7 Comparison of relative OD_{600} after 6 h incubation in media supplemented with OE_2imC_3C, TMG, L-carnitine, and $[C_2mim]OAc$. Strain: *K. marxianus* DMKU3-1042, NBRC1777 at 40 °C and *S. cerevisiae* BY4741 at 30 °C.

but it has insufficient magnetic force and the issue of heat generation, shifting the sample temperature (and it was easy to breakdown). Therefore, we decided to use a rotator in this study. The rotator tested 18 samples simultaneously. Furthermore, the rotator is compact; thus, multiple units can be placed in a single incubator. The used glass vials did not fit the rotator. When we choose suitable sample vials/tubes, generally commercialized microtubes are preferable. Therefore, in this study, we utilised a pressure-resistant cryotube (2 mL) to prevent evaporation of water and ethanol at 50 °C.

To reduce the sample amount, which is important when using non-commercial or expensive compounds, the sample volume was set to 1 mL. Each time, 100 μL of solution was taken and filtered through a small-dead-volume syringe filter for HPLC analysis. The HPLC sample volume was significantly reduced by placing glass inserts (TORAST vial insert, GLCTV-I01) in the HPLC vials. The same company sells another type of glass insert (GLCTV-I02), which has 60% volume of GLCTV-I01 (0.25 *vs.* 0.15 mL). However, even when GLCTV-I02 was used, the amount of solution required for HPLC analysis was almost the same. Therefore, we decided not to use it because it was four times expensive (reuse by washing is difficult because it is too fine). The sample injection volume for HPLC was 20 μL.

The initial OD_{600} value was set to 20. A higher initial OD_{600} value allows fermentation to be completed rapidly, which is favourable for screening. As a result, ethanol was produced within 1 h at most of the tested temperatures (Fig. 8). Fermentation, which typically takes 12–72 hours,[13] can be reduced to 1 h. Furthermore, increasing the initial OD_{600} increases the yield because sugars are not used for yeast growth.[38]

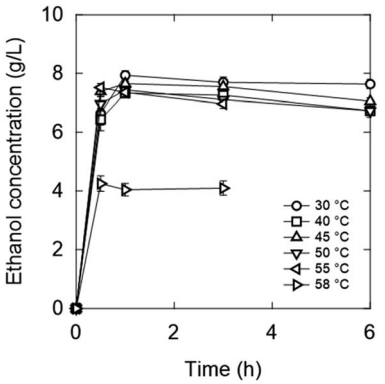

Fig. 8 Ethanol concentrations in the media without zwitterion/ionic liquid during fermentation. Strain: *K. marxianus* DMKU3-1042. Initial OD_{600}: 20.

Confirming the thermotolerance of *K. marxianus* DMKU3-1042. *K. marxianus* DMKU3-1042 was reported to ferment up to at least 45 °C.[30] In this study, the relationship between temperature and ethanol production was investigated from 30 °C to over 50 °C. Ethanol was produced at 68–78% of the theoretical yield from 30 °C to 55 °C (Fig. 9). Previous reports have shown that the rate of ethanol production is slower at 45 °C than at 30 °C,[30] but no such results were obtained in this study. The reason is presumably the high fermentation rate because of the high initial OD_{600} for rapid screening. Ethanol was also produced at 58 °C, but the yield was approximately 50%. Based on these results, 55 °C was considered the upper limit.

Inhibition by OE_2imC_3C, other zwitterions, and an ionic liquid. Although the optimal temperature for fermentation was not clear from this study, we initially assumed it to be 30 °C based on the previous report[30] (Fig. 10). Ethanol concentration decreased depending on the concentration of OE_2imC_3C. There was almost no effect up to 0.1 M, but when 0.5 and 1.0 M were added, the ethanol concentration decreased by 27 and 82%, respectively.

$[C_2mim]OAc$ also decreased the ethanol concentration in a concentration-dependent manner at a concentration of 0.5 M or higher. Specifically, when 0.5 and 1.0 M were added, the ethanol concentration decreased by 25% and 67%, respectively. Under our experimental conditions, $[C_2mim]OAc$ and OE_2imC_3C inhibited fermentation to the same extent. In the ethanol fermentation by *E. coli* KO11, $[C_2mim]OAc$ showed very

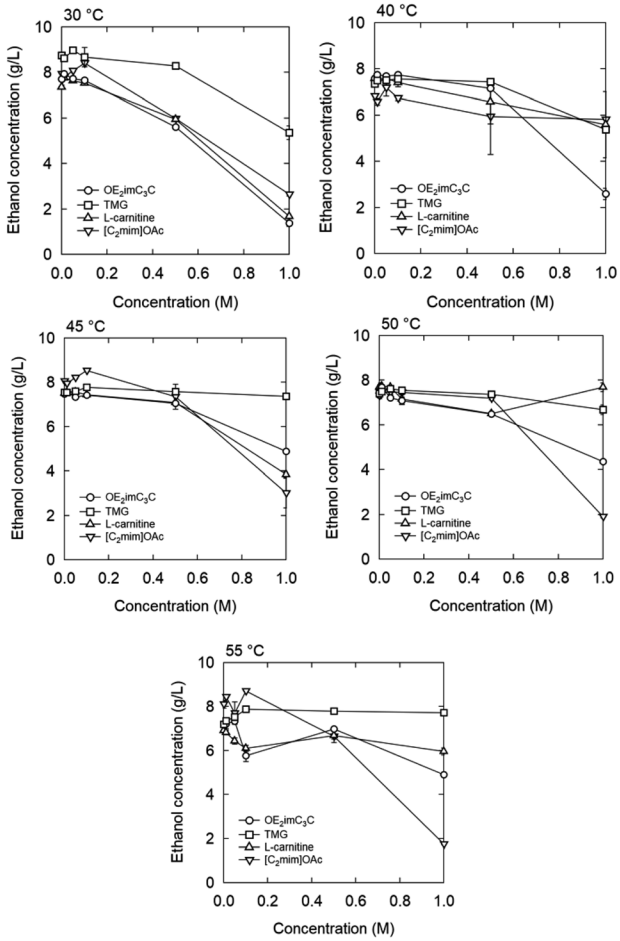

Fig. 10 Ethanol concentration after 1 h fermentation with zwitterion/ionic liquid. Strain: *K. marxianus* DMKU3-1042.

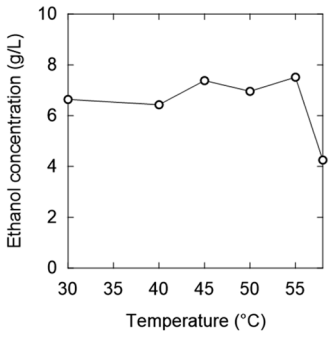

Fig. 9 Ethanol concentration after 1 h fermentation at various temperatures without zwitterion/ionic liquid. If glucose is completely converted to ethanol, 10.2 g L^{-1} of ethanol is yielded. Strain: *K. marxianus* DMKU3-1042.

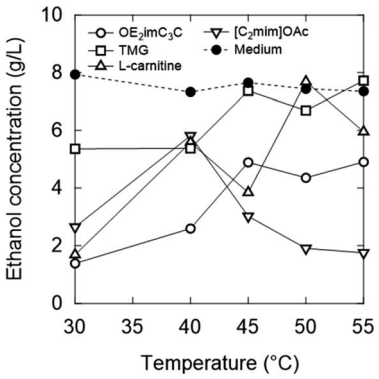

Fig. 11 Temperature dependence of ethanol concentration after 1 h fermentation in 1.0 M zwitterion/ionic liquid solutions. Strain: *K. marxianus* DMKU3-1042.

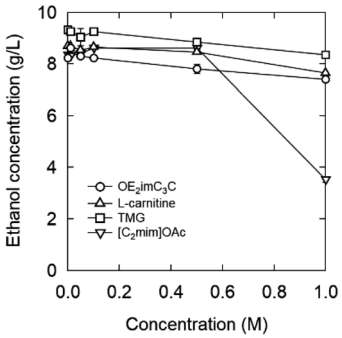

Fig. 12 Ethanol concentration at 30 °C after 1 h fermentation with zwitterion/ionic liquid. Strain: *S. cerevisiae* BY4741.

strong inhibition;[13,24] therefore, the microbial species may have an influence. However, the most plausible reason for the difference is based on the initial OD_{600}. An initial OD_{600} of 1 was applied to *E. coli* KO11, whereas 20 was adopted in this study. Bacterial cell density is also known to affect growth inhibition by drugs.[39] Additionally, since fermentation was completed within 30 minutes to an hour due to the high initial OD_{600}, it is possible that fermentation was completed before toxicity appeared. Thus, there is still room for improving the screening method, such as lowering the initial OD_{600} value. In other words, these results, however, indicate that even a high concentration of $[C_2mim]OAc$ is applicable under specific conditions. We believe this finding is important because $[C_2mim]OAc$ has a high biomass processing ability and is currently the most popular ionic liquid, but its toxicity is a problem.[11–13,24,40,41]

When $[C_2mim]OAc$ was used, the ethanol concentration increased slightly below 0.1 M, compared to the case of 0 M. This phenomenon is known as hormesis and is commonly observed when antibiotics are added below the minimal inhibitory concentration.[42,43]

Inhibition by L-carnitine was comparable to that by OE_2imC_3C, but fermentation was less inhibited by TMG than OE_2imC_3C. In particular, almost no inhibition was observed at 0.5 M TMG. Although the trends in fermentation differed from those in proliferation, TMG had the lowest toxicity in both assays.

The temperature-dependence was investigated. At 40 °C or higher, no clear fermentation inhibition was observed with 0.5 M OE_2imC_3C, and OE_2imC_3C resistance was improved compared to that at 30 °C. Similar results were obtained for the other zwitterions. On the other hand, there are some exceptions. Focusing on 40 °C and 1 M, fermentation inhibition by OE_2imC_3C was comparable to that at 30 °C, but the inhibitory effect of $[C_2mim]OAc$ was weaker than that at 30 °C, and the strength of toxicity was reversed (described later).

55 °C was the highest temperature adequate for fermentation (Fig. 10). In the 0.5 and 1.0 M OE_2imC_3C groups, ethanol production was suppressed by 12% and 41%, respectively, compared with that in the 0 M solution. The toxicity seems less effective at 55 °C in the high concentration range. Fermentation was less inhibited by TMG than OE_2imC_3C, and L-carnitine inhibited fermentation to the same extent as OE_2imC_3C.

To clarify the relationship between the temperature and ion concentration during fermentation inhibition, the ethanol concentration in the 1.0 M solutions at each temperature is plotted in Fig. 11. When OE_2imC_3C was added, higher ethanol concentrations were obtained at 40 °C than at 30 °C, and even higher ethanol concentrations were obtained at 45–55 °C. This tendency was not much different for TMG and L-carnitine. In contrast, $[C_2mim]OAc$ exhibited a different trend. The ethanol concentration increased at 40 °C, compared to 30 °C, and decreased at higher temperatures. The increase in ethanol concentration at 40 °C may be due to hormesis. Hormesis was observed during growth and fermentation when low concentrations of $[C_2mim]OAc$ were used. A similar phenomenon was observed in some points in the zwitterionic solutions at higher concentrations. There are some points still unknown, but this experimental method is developed for rapid screening. To study the inhibition mechanisms in detail, we need to conduct time-consuming experiments, which will be conducted in the future.

Inhibition against a different yeast: *S. cerevisiae*. A common yeast *S. cerevisiae* (BY4741) was used for fermentation at the optimal temperature of 30 °C (Fig. 12). While the fermentation by *K. marxianus* DMKU3-1042 (at 40 °C) was inhibited in 1 M OE_2imC_3C solution, that of *S. cerevisiae* was only minimally inhibited in the same solution. This suggests that *S. cerevisiae* is more resistant to OE_2imC_3C during fermentation than *K. marxianus*. Because there was no significant difference in growth tolerance between the two species, tolerance to fermentation and growth were assumed to be independent. This could be attributed to the differences in metabolic pathways, as aerobic culturing was used for growth, and anaerobic culturing was used for fermentation. Notably, $[C_2mim]OAc$ inhibited fermentation more strongly than OE_2imC_3C, reducing the ethanol concentration by less than half when 1.0 M was added.

Conclusions

A rapid screening method was established for growth inhibition of *K. marxianus* applicable to 30–50 °C. Using 96-well plates enabled direct measurement of OD_{600} with a 100 μL sample volume. Although the evaporation of culture media was a problematic issue especially at 50 °C, it was avoided by covering with sheets, putting wet towels and so on. OE_2imC_3C was less toxic for yeast growth than $[C_2mim]OAc$. Growth inhibition was increased at 50 °C, compared to the optimal growth temperature of 40 °C. Natural zwitterions, TMG and L-carnitine were further less toxic. These trends were similar in two *K. marxianus* strains and *S. cerevisiae*.

A rapid screening method for fermentation inhibition was also established at 30–55 °C. Using pressure-durable cryotubes enabled downscaling the sample volume to 1 mL, simultaneous testing over 18 samples, preventing the evaporation of media and ethanol. Using small-dead-volume syringe filters and glass inserts was also important for the downscaling. The trends in fermentation inhibition by zwitterions/ionic liquid were

somewhat complicated. A possible reason for the complication is the very rapid assay completion within 1 h and there is room to improve. The rough toxicity trend was TMG < L-carnitine < OE_2imC_3C < [C_2mim]OAc but the order changed depending on the concentration/temperature. *S. cerevisiae* was more resistant to OE_2imC_3C during fermentation than *K. marxianus*.

Data availability

All data are available in the main text or the ESI.†

Conflicts of interest

There are no conflicts to declare.

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