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# Investigation of the toxicity of the ionic liquid 1-butyl-3-methylimidazolium chloride to *Saccharomyces cerevisiae* AY93161 for lignocellulosic ethanol production

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Ionic liquid (IL) pretreatment of lignocellulosic materials has provided a new technical tool to improve lignocellulosic ethanol production. To evaluate the influence of the residual IL in the fermentable sugars from enzymatic hydrolysis of IL pretreatment of lignocellulosic materials on the subsequent ethanol fermentation, the toxicity of the IL 1-butyl-3-methylimidazolium chloride ([BMIM]Cl) to *Saccharomyces cerevisiae* AY93161 was investigated. Firstly, the morphological structure, budding and metabolic activity of *Saccharomyces cerevisiae* AY93161 at different [BMIM]Cl concentrations were observed under an optical microscope. The results show that its single cell morphology remained unchanged at all [BMIM]Cl concentrations, but its reproduction rate by budding and its metabolic activity decreased with the [BMIM]Cl concentration increasing. The half effective concentration (EC50) and the half inhibition concentration (IC50) of [BMIM]Cl to *Saccharomyces cerevisiae* AY93161 were then measured using solid and liquid suspension culture and their value were 0.53 and 0.39 $g \cdot L^{-1}$ respectively. Finally, the influence of [BMIM]Cl on ethanol production was investigated. The results indicate that the [BMIM]Cl inhibited the growth and ethanol production of *Saccharomyces cerevisiae* AY93161. This toxicity study provides useful basic data for further development in lignocellulosic ethanol production by using IL technology and it also enriches the IL toxicity data.

**Keywords:** Ionic liquid, [BMIM]Cl, toxicity, *Saccharomyces cerevisiae* AY93161, lignocellulosic ethanol production.

## INTRODUCTION

Ionic liquids (ILs) are compounds, composed only of ions and are liquid at room temperature. Thus, it is normally named room temperature IL[1, 2]. Interest in ILs has grown steadily in recent years because their non-detectable vapor pressure, non-flammability, high thermo-stability, unique solvent properties and close to infinite structural variation provide a possibility for clean manufacturing in the chemical and energy related industry, including the lignocellulosic ethanol production[3, 4]. Several studies have documented that IL pretreatment of lignocellulosic materials can significantly improve their enzymatic hydrolysis efficiency by increasing their hydrolysis rate and fermentable sugars yield[5–7]. However, it is unavoidable that some IL still remains in the obtained fermentable sugars from enzymatic hydrolysis of IL pretreatment of lignocellulosic materials. To date, researches on the influence of the residual IL on the subsequent ethanol fermentation are still scarce. Hence, it is desirable to know how the residual IL in fermentable sugars affects the subsequent ethanol fermentation process[8].

*Saccharomyces cerevisiae* is the most widely used micro-organism for ethanol fermentation. The influence of the residual IL on its growth has a close relationship with its ethanol production. The growth toxicity study on the residual IL to *Saccharomyces cerevisiae* can provide useful information for further development in lignocellulosic ethanol production by using ionic liquid technology and it also enriches the IL toxicity data. Although there are many reports on the IL toxicity to microorganism growth[9–11], very few studies have investigated the IL toxicity on the growth of *Saccharomyces cerevisiae*[12]. The aim of this work is to study the toxicity of residual IL in fermentable sugars on the growth of *Saccharomyces cerevisiae*. To simplify this study, the D-glucose supplemented with suitable amount of IL was used in place of the hydrolysates from enzymatic hydrolysis of the IL pretreated lignocellulosic materials. Because the 1- butyl-3-methylimidazolium chloride ([BMIM]Cl) is one of the most widely-used and cheapest IL in pretreatment of lignocellulosic materials for ethanol production[5–7], it was chosen as a model IL. In this work, the toxicity of [BMIM]Cl in the medium at different concentrations to the growth of *Saccharomyces cerevisiae* AY93161 was systematically investigated.

## MATERIALS AND METHODS

All experiments were carried out three times, and the given numbers are the mean values with relative error within $\pm$ 5%.

### Chemicals

The [BMIM]Cl used in this study was obtained from Henan Lihua Pharmaceutical Co. Ltd., China. Its purity was 98.5% up and its water content was less than 0.5%. All other chemicals employed in this study were of reagent grade and purchased from Wuhan Chemicals & Reagent Corp., China.

### Preparation of inoculum

The yeast *Saccharomyces cerevisiae* AY93161 was used throughout this study. The stock cultures were maintained on YPD agar plates at 4°C and transferred to

fresh plates every 4 weeks to avoid the micro-organism degradation. The inoculum preparation was carried out by means of micro-organism transfer from stock cultures to a fresh YPD agar plate and grew for 48 h at 30°C. Following this period, single colonies were transferred to a 250 mL flask with 100 mL YPD medium. The flask was placed on a orbital shaker with a shaking diameter 5 cm and a shaking frequency 200 rpm and incubated at 30°C for 24 h. This was used as the inoculum for the following solid and liquid suspension culture. The compositions of culture medium used in preparation of inoculum were shown in Table 1.

### Growth of *Saccharomyces cerevisiae* AY93161 in the presence of [BMIM]Cl in solid culture

A series of 10–fold dilution for the inoculum was prepared starting with 9 ml of sterilized phosphate buffered saline added to 1.0 ml inoculum. The flask was then closed and the contents were stirred for 30 min; and 1.0 ml diluted inoculum was added to 9.0 ml of sterilized phosphate buffered saline. The dilutions were repeated to 5 continuous dilutions. After 5 series of 10-fold dilution, 100 μl inoculum of *Saccharomyces cerevisiae* AY93161 were spread over the YPD agar plates with different [BMIM]Cl concentrations. The [BMIM]Cl concentration in the YPD agar plates were 50, 10, 1, $10^{-1}$, $10^{-2}$, $10^{-3}$, $10^{-4}$, $10^{-5}$ and $10^{-6}$ g·L$^{-1}$ respectively. All plates were incubated for 48 h at 30°C until colonies appeared and the colony forming units (CFU) were counted.

### Growth of *Saccharomyces cerevisiae* AY93161 in the presence of [BMIM]Cl in liquid suspension culture

The liquid suspension culture of *Saccharomyces cerevisiae* AY93161 was carried out in a 250 mL flask with 99 mL YPD medium having different [BMIM]Cl concentrations and 1 mL inoculum at 30°C and 200 rpm for 32 h. The [BMIM]Cl concentration in the YPD medium were 5, 1, $10^{-1}$, $10^{-2}$, $10^{-3}$, $10^{-4}$, $10^{-5}$ and $10^{-6}$ g·L$^{-1}$ respectively. At 12 and 24 h, the samples were taken for the observation of the yeast morphological structure, budding and metabolic activity. During the culture process, small samples were taken at regular intervals for yeast concentration analysis. When the culture process finished, the final concentration of yeast, ethanol and the fermentable sugars was determined.

### Analytical methods

The yeast morphological structure, budding and metabolic activity were observed using an OLYMPUS CX41 microscope (Olympus Corporation, Tokyo, Japan). Among them, the yeast metabolic activity was observed by methylene blue staining method[13]. Yeast concentration was determined by the dry weight method[14]. Ethanol content was determined by gas chromatography[15] and the fermentable sugars concentration was estimated using the 3,5-dinitrosalicylic acid method[16]. The half effective concentration (EC50) was calculated by the least square regression method based on the CFU number at different [BMIM]Cl concentrations[17]. The half inhibition concentration (IC50) was also calculated by the least square regression method, but it based on the specific growth rate value at different [BMIM]Cl concentrations which came from the yeast growth process data[17].

## RESULTS AND DISCUSSION

### Effect of [BMIM]Cl on the morphological structure and metabolic activity of the yeast *Saccharomyces cerevisiae* AY93161

The morphological structure is one of the most important physiological properties for a micro-organism. Observation of the morphological structure changes of the yeast *Saccharomyces cerevisiae* AY93161 in the medium containing [BMIM]Cl under a microscope can provide useful information on the toxicity of [BMIM]Cl to its growth. The morphological structures of the yeast *Saccharomyces cerevisiae* AY93161 at different [BMIM]Cl concentrations in the medium during the liquid suspension culture were observed at log phase and stationary phase using an OLYMPUS CX41 microscope (Olympus Corporation, Tokyo, Japan) and Figures 1 and 2 showed these morphological images when culture time was 12 and 24 h respectively. As indicated in Figures 1 and 2, the single cellar morphology of the yeast *Saccharomyces cerevisiae* AY93161 kept almost unchanged at all [BMIM]Cl concentrations in comparison with the control whenever the culture was during log phase or stationary phase, but Figure 1 demonstrated that its reproduction rate by budding decreased with the increase of [BMIM]Cl concentration from $10^{-6}$ to 1 g·L$^{-1}$ during log phase although its budding rate had no obvious difference between the control and the [BMIM]Cl concentration less than $10^{-3}$ g·L$^{-1}$.

The metabolic activity is another important physiological property for a micro-organism. In order to evaluate the effect of [BMIM]Cl on its metabolic activity, the yeast *Saccharomyces cerevisiae* AY93161 at different [BMIM]Cl concentrations in the medium during the liquid suspension culture was observed at log phase and stationary phase using an OLYMPUS CX41 microscope (Olympus Corporation, Tokyo, Japan) by methylene blue staining method. Figures 3 and 4 showed these morphological images when culture time was 12 and 24 h respectively. As shown in Figures 3 and 4, its metabolic activity decreased with the increase of [BMIM]Cl concentration from $10^{-6}$ to 1 g·L$^{-1}$ whenever the culture was during the log phase or stationary phase, but its metabolic activity during the stationary phase decreased more significantly than that during the log phase. This is because the metabolic activity of the yeast *Saccharomyces cerevisiae* AY93161 during the log phase was much stronger than that during the stationary phase.

**Table 1.** Compositions of culture medium used in preparation of inoculum

| Medium Type | Composition (g.L$^{-1}$) | | | |
|---|---|---|---|---|
| | D-glucose | peptone | yeast extract | agar |
| The YPD agar medium | 20 | 20 | 10 | 15 |
| The YPD agar medium | 20 | 20 | 10 | 0 |

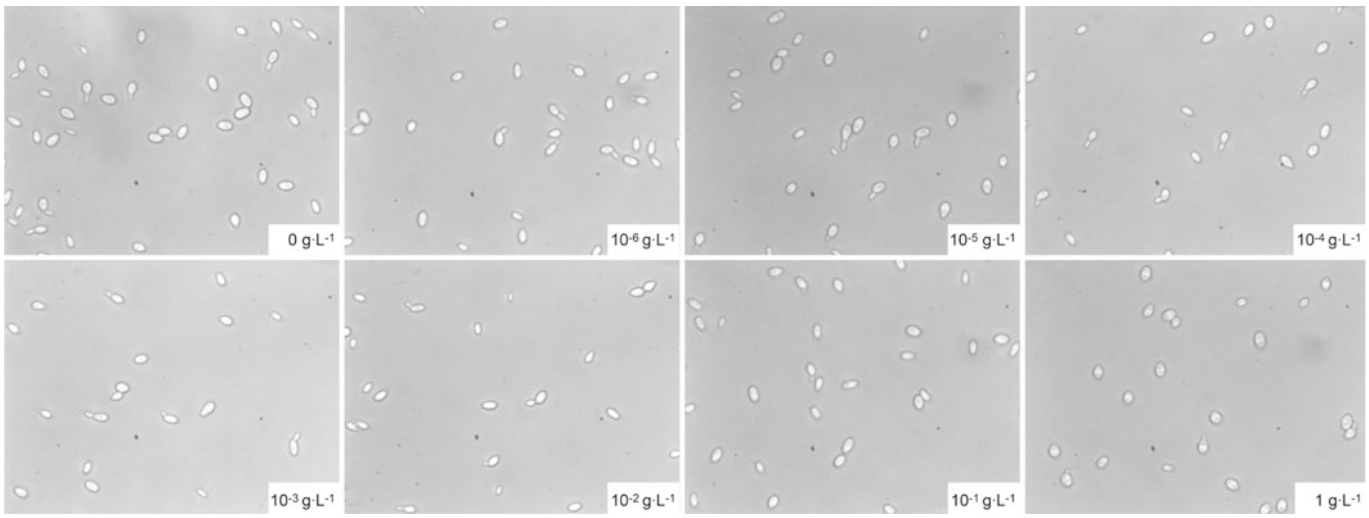


**Figure 1.** Morphological structure of the yeast *Saccharomyeas cerevisiae* AY93161 at different [BMIM]Cl concentrations during log phase (culture time was 12 h)

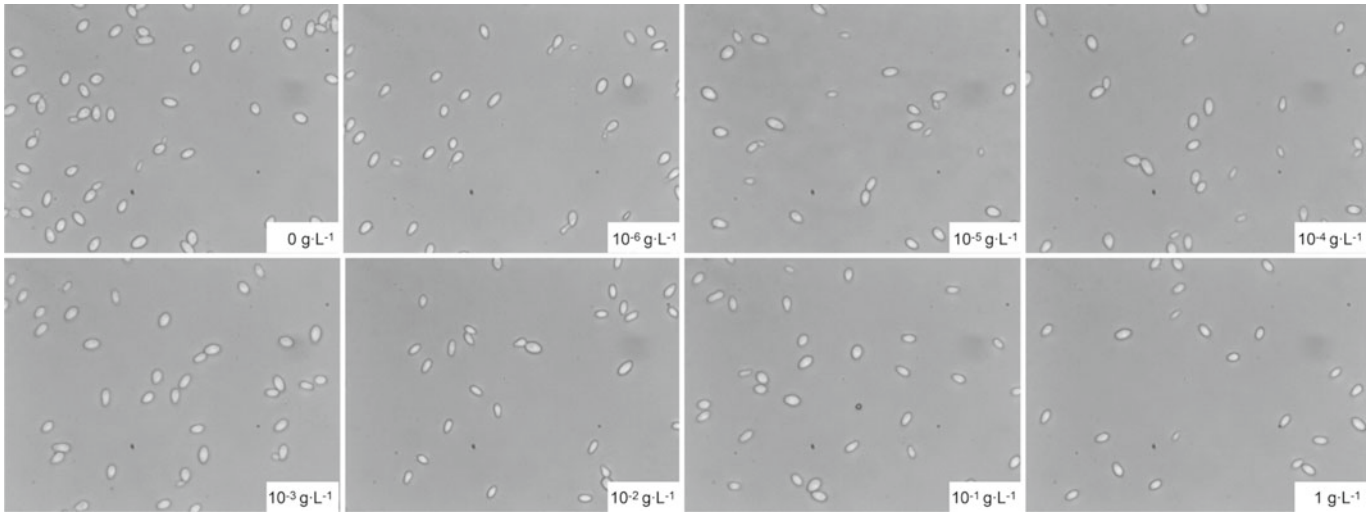


**Figure 2.** Morphological structure of the yeast *Saccharomyeas cerevisiae* AY93161 at different [BMIM]Cl concentrations during stationary phase (culture time was 24 h)

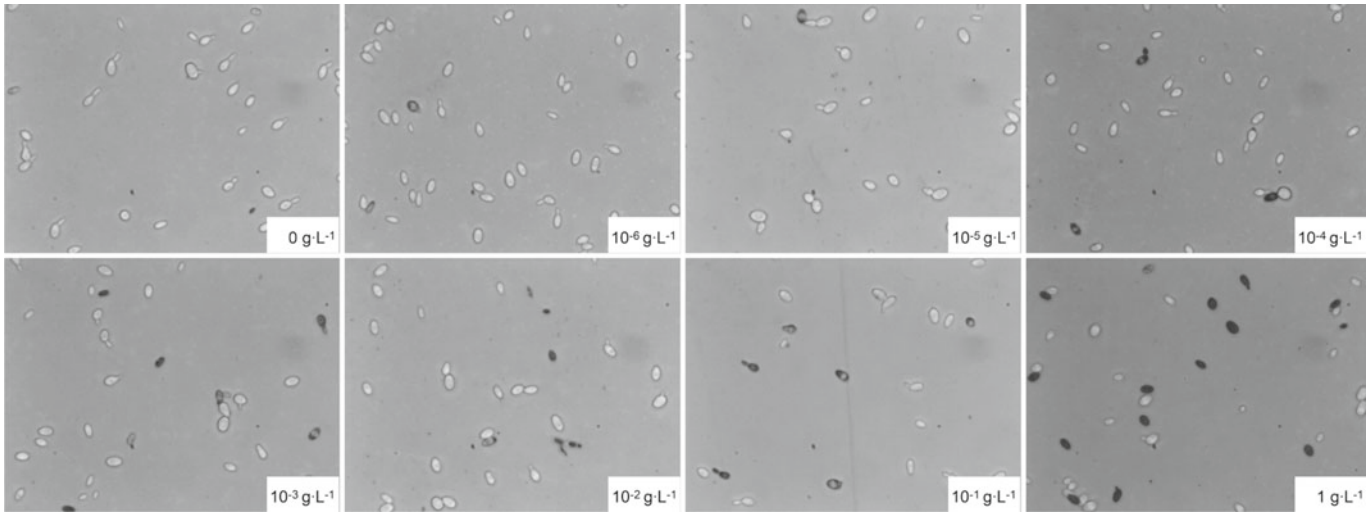


**Figure 3.** Morphological image of the yeast *Saccharomyeas cerevisiae* AY93161 at different [BMIM]Cl concentrations during log phase by methylene blue staining (culture time was 12 h)

### Effect of [BMIM]Cl on the growth of the yeast *Saccharomyces cerevisiae* AY93161 in solid culture

The effect of [BMIM]Cl on the growth of the yeast *Saccharomyces cerevisiae* AY93161 in solid culture is an important expression form of its toxicity. The CFU number with different [BMIM]Cl concentrations in solid culture is a comprehensive indicator of its toxicity to the growth of the yeast *Saccharomyces cerevisiae* AY93161. Table 2 listed the CFU number in the YPD agar plates with different [BMIM]Cl concentrations. As shown in Table 2, the yeast could not grow when the [BMIM]Cl concentration was greater than 50 $g \cdot L^{-1}$. The [BMIM]Cl had negative effect on the yeast growth when its

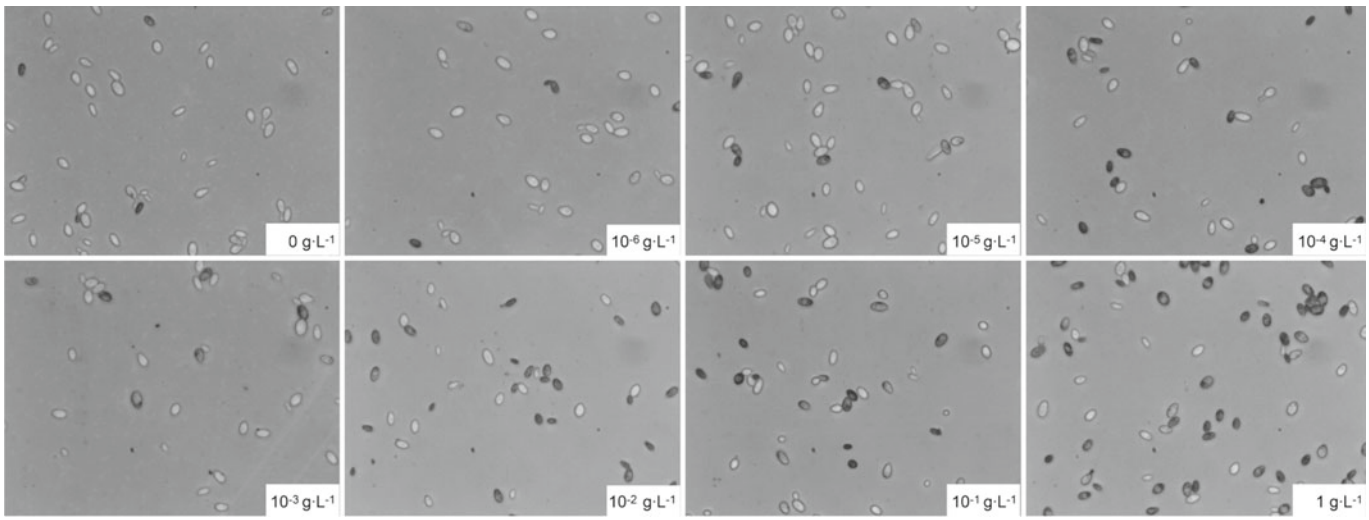


**Figure 4.** Morphological image of the yeast *Saccharomyeas cerevisiae* AY93161 at different [BMIM]Cl concentrations during stationary phase by methylene blue staining (culture time was 24 h)

concentration was between $10^{-4}$ to 50 g·L$^{-1}$. The [BMIM]Cl had almost no effect on the yeast growth when its concentration was less than $10^{-4}$ g·L$^{-1}$. Based on the data in Table 2, the EC50 could be calculated by a regression method and its value was 0.53 g.L$^{-1}$. Compared with the reported EC50 in literature, it was close to those of the *Vibrio fischeri* and some soil bacteria[9, 18]. Because the EC50 was at relatively lower concentration, it indicated that the [BMIM]Cl had a relatively stronger inhibition on the yeast growth. This result demonstrated that the residual [BMIM]Cl in the fermentable sugars from enzymatic hydrolysis of [BMIM]Cl pretreatment of lignocellulosic materials was the primary source of inhibition on the yeast growth for ethanol production.

### Effect of [BMIM]Cl on the growth of the yeast *Saccharomyces cerevisiae* AY93161 in liquid suspension culture

The growth of the yeast *Saccharomyces cerevisiae* AY93161 in liquid suspension culture is closely related to its ethanol production. To evaluate the influence of [BMIM]Cl on its ethanol production, it is essential to know how [BMIM]Cl affects its growth. Figure 5 showed its growth curves at different [BMIM]Cl concentrations in liquid suspension culture. As shown in Figure 5, its growth curves at all [BMIM]Cl concentrations were fit for the typical batch bacterial growth curves and the [BMIM]Cl inhibited its growth with the increase of [BMIM]Cl concentration. When the [BMIM]Cl concentration was less than $10^{-3}$ g·L$^{-1}$, the [BMIM]Cl slightly inhibited its growth. When the [BMIM]Cl concentration was between $10^{-3}$ to 1 g·L$^{-1}$, the [BMIM]Cl moderately inhibited its growth. When the [BMIM]Cl concentration was greater than 1 g·L$^{-1}$, the [BMIM]Cl significantly inhibited its growth. When the [BMIM]Cl concentration reached 5 g·L$^{-1}$, its growth completely ceased. From its growth curves, it also could be found that the [BMIM]Cl inhibited its growth mainly by decreasing its specific growth rate**.** Its specific growth rate at different [BMIM]Cl concentrations could be obtained from Figure 5. The IC50 was then able to be calculated by a regression method and its value was 0.39 g·L$^{-1}$. Because the IC50 was at relatively lower concentration, it indicated that the [BMIM]Cl had a relatively stronger inhibition on its growth in liquid suspension culture.

Table 3 listed some important process parameters in liquid suspension culture at different [BMIM]Cl concentrations. As indicated in Table 3, when the [BMIM]Cl concentration increased, the final yeast and ethanol concentration as well as the ethanol yield

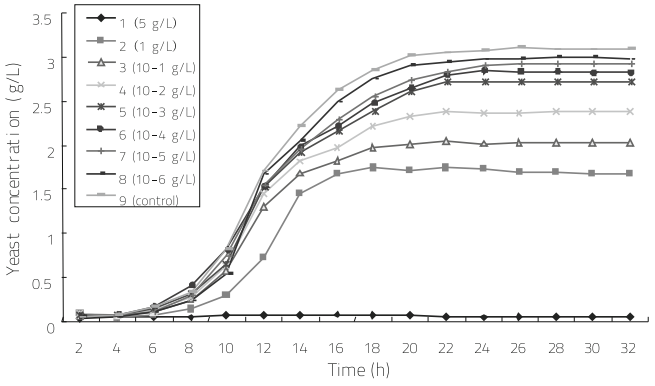


**Figure 5.** Growth curves of the yeast Saccharomyeas cerevisiae AY93161 at different [BMIM]Cl concentrations in liquid suspension culture

**Table 2.** The CFU number in the YPD agar plates with different [Bmim]Cl concentrations

| $C_i$ (g.L$^{-1}$) | 50 | 10 | 1 | $10^{-1}$ | $10^{-2}$ | $10^{-3}$ | $10^{-4}$ | $10^{-5}$ | $10^{-6}$ | 0 |
|---|---|---|---|---|---|---|---|---|---|---|
| CFU Number | 0 | 82 | 198 | 255 | 310 | 372 | 422 | 419 | 431 | 429 |

**Table 3.** Effect of [BMIM]Cl concentration on the liquid suspension culture process parameters

| $C_i$ (g·L$^{-1}$) | 5 | 1 | $10^{-1}$ | $10^{-2}$ | $10^{-3}$ | $10^{-4}$ | $10^{-5}$ | $10^{-6}$ | 0 |
|---|---|---|---|---|---|---|---|---|---|
| $C_b$ (g·L$^{-1}$) | 0.06 | 1.67 | 2.03 | 2.38 | 2.71 | 2.83 | 2.91 | 2.98 | 3.08 |
| $C_p$(g·L$^{-1}$) | * | 3.92 | 5.37 | 6.54 | 7.65 | 7.71 | 7.62 | 7.82 | 7.85 |
| $C_s$(g·L$^{-1}$) | 19.6 | 5.42 | 3.62 | 1.58 | 0.75 | 0.71 | 0.58 | 0.60 | 0.51 |
| Y | * | 0.269 | 0.328 | 0.355 | 0.397 | 0.400 | 0.392 | 0.403 | 0.403 |

$C_i$ represents the [BMIM]Cl concentration (g.L$^{-1}$), $C_b$ represents the final yeast concentration (g.L$^{-1}$), $C_p$ represents the final ethanol concentration (g.L$^{-1}$), $C_s$ represents the final fermentable sugars concentration (g.L$^{-1}$), Y represents the ethanol yield from the fermentable sugars.

from the fermentable sugars decreased, but the final remained fermentable sugars increased. It could also be found that when the [BMIM]Cl concentration was less than $10^{-3}$ g·L$^{-1}$, these important parameters, including the final ethanol concentration and its yield had only little difference in comparison with the control. It suggests that the residual [BMIM]Cl concentration in the fermentable sugars should be controlled below $10^{-3}$ g·L$^{-1}$, thus it would not affect the yeast growth and ethanol production. From a technological point of view, further studies should be carried out to improve the yeast tolerance to [BMIM]Cl and decrease the residual [BMIM]Cl in the fermentable sugars from enzymatic hydrolysis of its pretreatment of lignocellulosic materials for ethanol production.

## CONCLUSIONS

The work systematically investigated the toxicity of [BMIM]Cl in the medium at different concentrations to the growth of *Saccharomyces cerevisiae* AY93161 to provide useful information for lignocellulosic ethanol production. The main conclusions are as follows:

The single cellar morphology of the yeast *Saccharomyces cerevisiae* AY93161 remained unchanged at all [BMIM]Cl concentrations, but its reproduction rate by budding and metabolic activity decreased with the [BMIM]Cl concentration increasing.

The EC50 and IC50 of [BMIM]Cl to *Saccharomyces cerevisiae* AY93161 were measured using solid and liquid suspension culture. Their values were 0.53 and 0.39 g·L$^{-1}$, respectively. This indicated that the [BMIM]Cl had a relatively stronger inhibition on yeast growth and the residual [BMIM]Cl in the fermentable sugars from enzymatic hydrolysis of [BMIM]Cl pretreatment of lignocellulosic materials would become the primary source of inhibition on yeast growth for ethanol production.

For the liquid suspension culture process of the yeast *Saccharomyces cerevisiae* AY93161, with the [BMIM]Cl concentration increasing, the final yeast and ethanol concentration as well as the ethanol yield from the fermentable sugars decreased, but the final remained fermentable sugars increased. However, when the [BMIM]Cl concentration was less than $10^{-3}$ g·L$^{-1}$, the final ethanol concentration and its yield had only little difference in comparison with the control. It suggests that the residual [BMIM]Cl concentration in the fermentable sugars should be controlled below $10^{-3}$ g·L$^{-1}$, thus it would not affect the yeast growth and ethanol production.

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## LITERETURE CITED

1. Kokorin, A. (2011). Ionic Liquids: Applications and Perspectives. InTech. DOI: 10.5772/1782.

2. Wasserscheid, P. & Stark, A. (2010). Handbook of Green Chemistry: Green Solvents, Vol. 6-Ionic Liquid. Wiley-VCH, Germany.

3. Zhu, S. (2008). Use of ionic liquids for the efficient utilization of lignocel-lulosic materials. J. Chem. Technol. Biotechnol. 83, 777–779, DOI: 10.1002/jctb.1884.

4. Wang, Q., Wu, Y. & Zhu, S. (2011). Use of ionic liquids for improvement of cellulosic ethanol production. Bioresources. 6 (1), 1–2.

5. Mora-Pale, M., Meli, L., Doherty, T.V., Linhardt, R.J. & Dordick, J.S. (2011). Room temperature ionic liquids as emerging solvents for the pretreatment of lignocellulosic biomass. Biotechnol. Bioeng. 108 (6): 1229–1245, DOI: 10.1002/bit.23108.

6. Simmons, B.A., Singh, S., Holmes, B.M. & Blanch, H.W. (2010). Ionic liquid pretreatment. Chem. Eng. Prog., 116 (3), 50–55.

7. Tadesse, H. & Luque, R. (2011). Advances on biomass pretreatment using ionic liquid: an overview. Energy Environ. Sci., 4, 3913–3929, DOI: 10.1039/C0EE00667J.

8. Zhu, S., Yu, P., Tong, Y., Chen, R., Lv, Y., Zhang, R., Lei, M., Ji, J., Chen, Q. & Wu, Y. (2012). Effects of the ionic liquid 1-butyl-3-methylimidazolium chloride on the growth and ethanol fermentation of *Saccharomyces cerevisiae* AY92022. Chem. Biochem. Eng. Q., 26, 105–109.

9. Pham, T.P.T., Cho, C.W. & Yun, Y.S. (2010). Environmental fate and toxicity of ionic liquids: a review. Water Res., 44, 352–372, DOI: 10.1016/j.watres.2009.09.030.

10. Zhu, S., Wu, Y., Chen, Q., Chi, R., Shen, X. & Yu, Z. (2009). A mini-review on greenness of ionic liquids. Chem. Biochem. Eng. Q., 23, 207-211.

11. Zhao, D., Liao, Y. & Zhang, Z. (2007). Toxicity of ionic liquids. Clean-Soil, Air, Water, 35, 42–48, DOI: 10.1002/clen.200600015.

12. Docherty, K.M. & Kulpa, C.F. (2005). Toxicity and antimicrobial activity of imidazolium and pyridinium ionic liquids. Green Chem., 7, 185–189, DOI: 10.1039/B419172B.

13. Painting, K. & Kirsop, B. (1990). A quick method for estimating the percentage of viable cells in a yeast population, using methylene blue staining. World J. Microbiol. Biotechnol., 6 (3), 346–347, DOI: 10.1007/BF01201311.

14. Atala, D.I.P., Costa, A.C., Filho, M.R. & Maugeri, F. (2001). Kinetics of ethanol fermentation with high biomass concentration considering the effect of temperature. Appl. Biochem. Biotechnol., 91–93, 353–365, DOI: 10.1385/ABAB:91-93:1-9:353.

15. Zhu, S., Wu, Y., Yu, Z., Zhang, X., Wang, C., Yu, F., Jin, S., Zhao, Y., Tu, S. & Xue, Y. (2005). Simultaneous saccharification and fermentation of microwave/alkali pretreated rice straw to ethanol. Biosys. Eng., 92 (2), 229–235, DOI: 10.1016/j.biosystemseng.2005.06.012.

16. Miller, G.L. (1959). Use of dinitrosalicylic acid reagent for determination of reducing sugar. Anal. Chem., 31, 426–428.

17. Lee, S.M., Chang, W.J., Choi, A.R. & Koo, Y.M. (2005). Influence of ionic liquids on the growth of *Escherichia coli*. Korean J. Chem. Eng., 22, 687–690, DOI: 10.1007/BF02705783.

18. Tong, Y., Wang, Q., Bi, Y., Lei, M., Lv, Y., Liu, Y., Liu, J., Lu, L., Ma, Y., Wu, Y. & Zhu, S. (2012). Influence of ionic liquid 1- butyl-3-methylimidazolium chloride on the soil micro-ecological system. Open Biotechnol. J., 6, 1–4.