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Effects of bmim[PF6] treatments with different concentrations on microbial activity of *Saccharomyces cerevisiae*

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Abstract−To study the role of ionic liquid as a solvent in whole cell biocatalyst, it is necessary to probe the effects of ionic liquid treatments on microbial activity. In this paper, *Saccharomyces cerevisiae* was selected as a test bacterium to study the effects of bmim[PF6] ionic liquid in different concentration on yeast activity through determining the growth curve, sugar degradation curve, microbial activity, colonial morphology and cell morphology. The results revealed that the growth of yeast is inhibited strongly in low concentration ionic liquid, while the lethal effect is feeble in high concentration ionic liquid. This result was totally different from that of supercritical CO_2 treatment or high concentration benzene methanol treatment which would lead most yeast to death.

Key words: Biocatalysis, Ionic Liquid, Yeast, Microbial Activity

INTRODUCTION

Biocatalysis is a chemical reaction process using biological catalysts (enzyme or microbial cell), which has many advantages such as mild reaction conditions, no environmental pollution, fast reaction speed and high selectivity. At present, biocatalysis is widely applied in many areas such as chiral synthesis and resolution, drug intermediates, food additives and bioremediation [1-3]. But most organic syntheses must be performed in organic solvents, because the toxicity of organic solvents to biocatalyst their real application is limited. Meanwhile, volatility of organic solvents may lead to environmental pollution. Hence the searching for alternative green solvents has been a hot issue.

Ionic liquid is ionic compound in liquid style at room temperature or near room temperature consisting of organic cations and inorganic or organic anions. It's also known as room-temperature molten salt. With numerous fascinating properties such as non-volatility, non-flammable, low melting points, high thermal stability, adjustable physico-chemical property and good solubility for many organic and inorganic compounds [4], ionic liquid is showing great potential in organic synthesis [5], biocatalyst [6,7], extraction [8,9], electrochemical [10] and many other fields. Along with supercritical CO_2 and biphasic aqueous-organic system as the three green solvents, ionic liquids have a promising future in its application [11].

Compared with conventional organic solvents, ionic liquids have shown great advantages as biocatalytic reaction medium. A large number of biocatalysts in ionic liquids can maintain and even enhance their catalytic activity, operational stability and enantioselectivity. Currently, most applications of ionic liquids are mainly located in enzyme catalyzed reactions, especially in lipase. Most of those researches were about the relationship between ionic liquids and enzyme activity [12-15] and optimization of their catalysis [16-18]. Compared with the enzyme catalysis, whole cell biocatalysis has the prominent advantage of avoiding separation and purification of enzyme. For some synthesis reactions where coenzyme needs to take part, microbial cells can make use of its own multiple-enzyme system to produce suitable coenzyme through metabolism so that the catalytic reaction can be completed successfully. In recent years, the research of whole cell biocatalysis in ionic liquids has aroused more and more attention. Especially in the preparation of important drugs and chiral aromatic alcohol compounds used as chemical intermediates, the asymmetric reaction of prochiral carbonyl is usually completed by whole cell biocatalysis [19-21]. Acting as substrate and product reservoir in such biocatalysis ionic liquids can reduce the inhibition of reaction , control the target reaction accurately and separate the product easily. However, research shows that the same kinds of microorganisms in different ionic liquids have different catalytic activity and some even could not show catalytic activity [19]. Therefore, it is very important to study the microbial activity in ionic liquids. But there are few researches and reports in this respect at present, especially the researches on microbial growth, metabolism and physiological activity in ionic liquids.

This is because bmim[PF6] ionic liquid is often used as reaction medium in whole cell biocatalysis [21]. In this paper, *Saccharomyces cerevisiae* was selected as test bacterium to study the effects of bmim[PF6] ionic liquid on microbial growth, glucose metabolism and physiological activity from both macro and micro perspectives.

MATERIAL AND METHODS

1. Microorganism and Ionic Liquid

Saccharomyces cerevisiae was provided by the microbiology laboratory of the Food College in South China Agriculture University. The cells were cultured in YEPD medium which consisted of 20 g/L glucose, 20 g/L peptone, 10 g/L yeast extract, with pH 6.0. 1-buty l-3-methy limidazolium hexafluorophosphate (bmim[PF6]) (97%) provided by Shanghai Chengjie Chemistry Company Lim-

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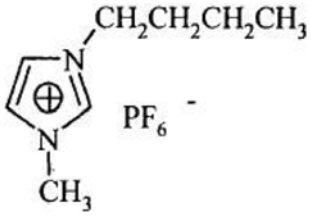

Fig. 1. The structure of ionic liquid bmim[PF6].

ited. The structure of ionic liquid is shown in Fig 1.

2. Preparation of Standard Yeast

Place some seeds into several cuvettes with 5 mL solid medium in each, and then incubate them at 30 °C for 48 h to activate the yeast. Transfer the activated yeast to fresh medium in culture bottles and cultivate them at 30 °C, 180 rpm for 24 h to prepare the standard yeast.

3. Low Concentration and Long Time Treatment

The first time cultivation: take 1 mL inoculum from standard yeast to fresh medium in six culture bottles and add in ionic liquid and sterilized water to make the concentration (v/v) of ionic liquid in corresponding culture bottles 0.0%, 0.3%, 0.5%, 0.7%, 0.9% and 1.1%. The culture bottles were airtight and incubated at 30 °C, 180 rpm. The blank control (0.0%) lasts for 11 h while the others 33 h. Check the growth yield and residual sugar content in optical density 560 nm and 540 nm every 1 h for blank control and 3 h for other samples during the culture. By measuring the curve between optical density and dry cell weight curve of 20 ml bacterial suspension, the absorbance at 560 nm could be converted to dry cell weight of 20 ml.

Test yield of ester favor, the ability of tolerant pH, alcohol and sodium chloride of yeast after culture in different concentration ionic liquids [22].

The second time cultivation: transfer an inoculum of 1 mL from the first time cultivation culture to fresh medium, and then incubate them at 30 °C, 180 rpm for 26 h. Check the growth yield at 0 h and 26 h, and check the residual sugar content at 26 h.

4. High Concentration and Short Time Treatment

Inoculate 1 mL standard yeast to culture bottles with fresh medium, then incubate them at 30 °C, 180 rpm for 12 h. Transfer the culture into sterilized centrifuge tube. Discard the supernatant by centrifugation.

Ionic liquid bmim[PF6]/benzyl alcohol treatment: Add 1 ml times 4 ionic liquid/benzyl alcohol and 24 ml times 4 sterilized buffer solution in four corresponding centrifuge tubes, three with ionic and one with benzyl alcohol. Make corresponding concentration (v/v) of ionic liquid in the tubes 0%, 20%, 40%, and the concentration of benzyl alcohol is 20%. Transfer them in new culture bottles, then incubate them at 30 °C, 180 rpm for 2.5 h. After that, centrifuge the culture again and remove all the ionic liquid and benzyl alcohol, then add buffer to 25 mL and shake up.

Supercritical CO_2 treatment: Add buffer to 10 mL in the centrifuge tube and shake up, then put it in 14 MPa supercritical CO_2 at 33 °C for 1.5 h.

After culture, do the pretreatment of ultrathin section immediately, and then observe the shape of cell walls of different samples in TEM (analytical transmission electron microscope).

5. High Concentration and Long Lime Treatment

Inoculate 1 mL standard culture to a culture bottle with 24 ml fresh medium, and then incubate it at 30 °C and 180 pm for 12 h. Transfer the culture into sterilized centrifuge tube. The supernatant is discarded after centrifugation. Add 1.5 mL ionic liquid and 13.5 mL sterilized buffer solution and then shake up. Transfer them into the culture bottle and incubate at 30 °C, 180 rpm for 2.5 h. After the treatment transfer the culture in sterilized cuvette, stratify, remove buffer and then cultivate it at room temperature for 3 months. Inoculate 4 drops sample to fresh plan media after 3 months, and incubate it for 2 days. Then observe their colonial morphology.

6. Analysis Methods

Optical density at 560 nm and 540 nm was determined by using a spectrophotometer (752, Shanghai Jingmi Scientific Instrument Company Limited, China). Microbial growth curve was expressed through dry cell weight. EC_{50} was calculated by the least square method. Residual sugar content was measured by DNS method. Ultrathin section of the culture cell was observed from the TEM (TECNAI 12, FEI, Holland). The ability of production ester and the ability of tolerant different pH, alcohol and sodium chloride for yeast were determined following the method of Zhuge [23], adopting sensory evaluation methods.

RESULTS AND ANALYSIS

1. Effect of Low Concentration Ionic Liquid on Yeast

To know the effect of low concentration bmim[PF6] ionic liquid in long time on the yeast activity, many microbial indexes were detected, such as growth curve, sugar degradation curve and other microbial activity.

1-1. Growth Rate and Metabolic Ability of Yeast in the First Time Cultivation

As shown in Fig. 2, as the concentration of ionic liquid bmim[PF6] increases, the dry cell weight of 20 ml bacterial suspension becomes increasingly lower. When the concentration of ionic liquid reaches 0.7% or above, the yeast stops growing significantly. The growth curves of higher concentration ionic liquid are very flat compared to that of the lower ones, which implies that the low concentration

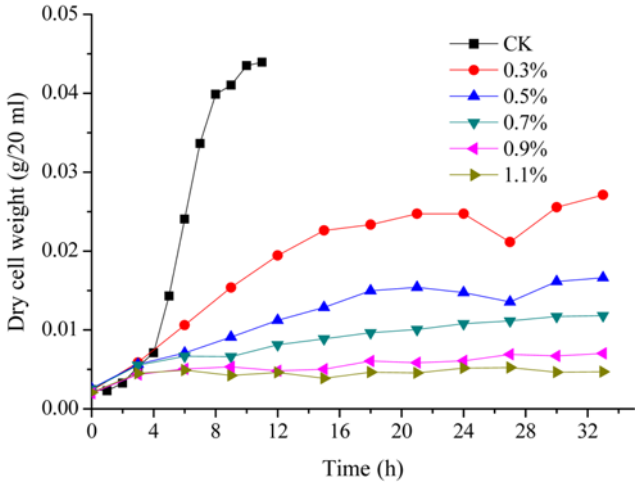

Fig. 2. Growth curve of yeast in low concentration ionic liquid bmim[PF6].

Table 1. The results of Ils toxicity to yeast after 33 hours at 30 °C

Ils concentration (v/v) %	0	0.3	0.5	0.7	0.9	1.1
Dry cell weight (mg/20 ml)	43.951	27.11	16.604	11.815	7.025	4.708
Inhibition (%)		38.318	62.222	73.118	84.016	89.288
Concentration Log (x)		−0.523	−0.301	−0.155	−0.046	0.041
Inhibition probability value (y)		4.703	5.311	5.616	5.995	6.242
Theoretical probability value of inhibition (y′)		4.693	5.292	5.686	5.981	6.216
Theory of inhibitionp (%)		38	62	75	84	89
Regression equation			y=2.701x+6.105 R2=0.9957			
EC_{50} (v/v) %			0.390			

ionic liquid will inhibit the growth of yeast by its special properties.

Result of Table 1 shows that the inhibition of yeast by ionic liquids is increased with the increasing concentration of ionic liquid. When the Ils concentration reaches 1.1%, the inhibition rate reaches 89.288%.

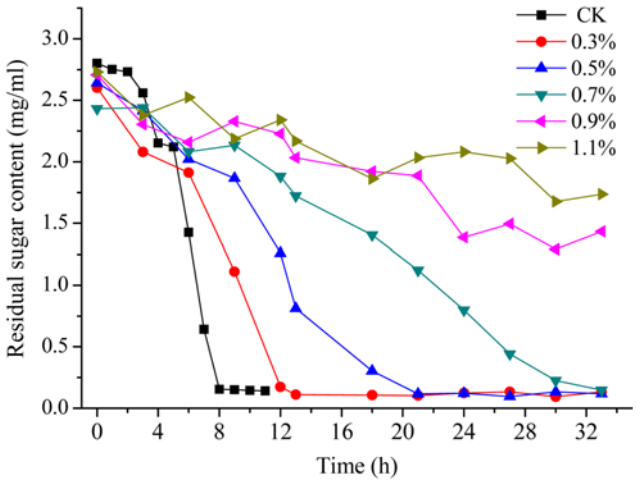

Fig. 3. Residual sugar curve of yeast in low concentration ionic liquid bmim[PF6].

Regression line equation for the virulence was calculated by least square, y=2.701x+6.10, R2=0.9957>0.99, which shows a good linear relationship. So this regression line equation for the virulence is usable. From the regression equation, EC_{50} is 0.39%. Lee [24] determined the EC_{50} value of [Bmim][PF_6] to Escherichia coli: 0.29%. This suggests that the toxicity of [Bmim][PF_6] to yeast than to *Escherichia coli* is lower.

Fig. 3 shows that when the ionic liquid bmim[PF6] concentration is lower than 0.9%, the residual sugar reaches the lowest point, and their curves are prone to be horizontal, yet the equilibration time expands as the concentration grows. The shapes of curves of samples in 0.9% and 1.1% ionic liquid are different from others. They are waveforms, and both of their residual sugars are at high level, the trend of which, however, is going down as the culture time prolongs. There is a possibility that the sugar residual yield will continue decreasing.

From the result of Table 2, we can see the general trend of yeast activity in many indexes, including yield of ester favor, ability of tolerant different pH, alcohol and sodium chloride. In short, the concentration of bmim[PF6] and every microbial activity index is in inverse relation.

1-2. Growth Rate and Metabolic Ability in Second Time Cultivation

As shown in Fig. 4, on the condition of the same inoculum con-

Table 2. Other microbial activity index of yeast after long time treatment in low concentration ionic liquid bmim[PF6]

Concentration of bmim[PF6] (v/v)		CK	0.3%	0.5%	0.7%	0.9%	1.1%
Ability of production ester	Single cultivation	+++	++	+	-	-	-
	Second cultivation	+++	++	+	+	+	+
Ability of tolerant different pH	pH 3	++	++	++	+	+	-
	pH 5	+++	++	++	+	+	-
	pH 9	+++	++	+	-	-	-
Ability of tolerant alcohol	11%	+++	+	+	-	-	-
	13%	+++	+	-	-	-	-
	17%	+++	+	-	-	-	-
Ability of tolerant sodium chloride	1%	++++	++++	++	++	+	-
	5%	++	+	-	-	-	-
	10%	-	-	-	-	-	-

Note: microbial activity index is the ability of production ester and the ability of tolerant different pH, alcohol and sodium chloride of yeast after long time treatment in low concentration ionic liquid bmim[PF6]. For the ability of production ester, "-" means no ester aroma, "+" means having ester aroma, one more "+" means having stronger ester aroma. For the ability of tolerant different pH, alcohol and sodium chloride, "-" means no microbial growth, "+" means having small amount of microbial growth, one more "+" means having more microbial growth, and so on

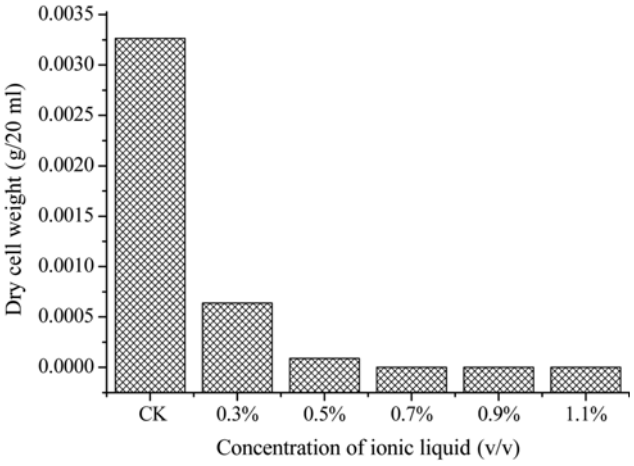

Fig. 4. Dry cell weight of yeast at 0 h in second cultivation.

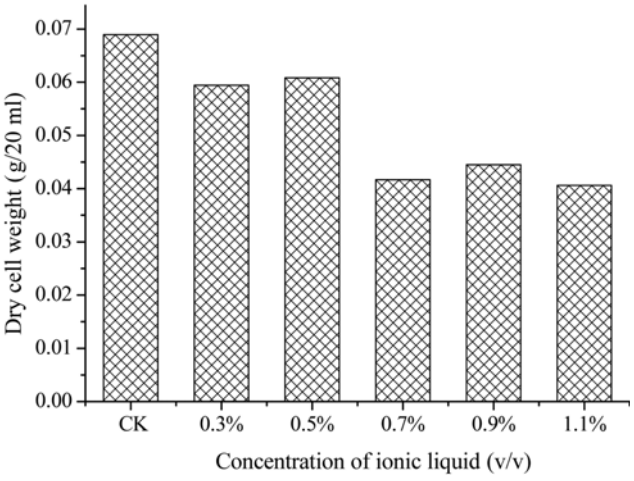

Fig. 5. Dry cell weight of yeast at 26 h in second cultivation.

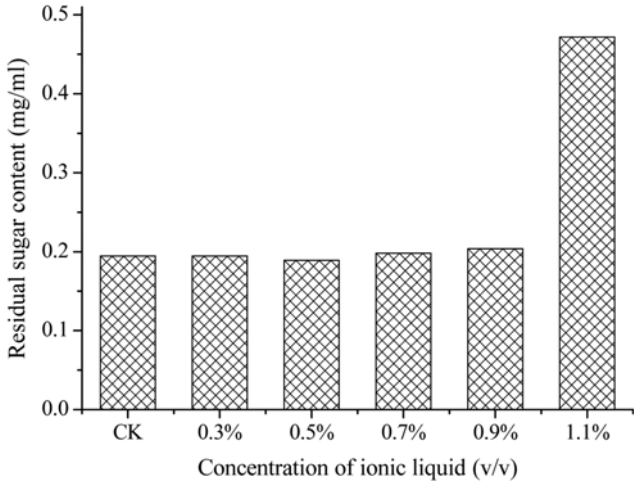

Fig. 6. Residual sugar of yeast at 26 h in second cultivation.

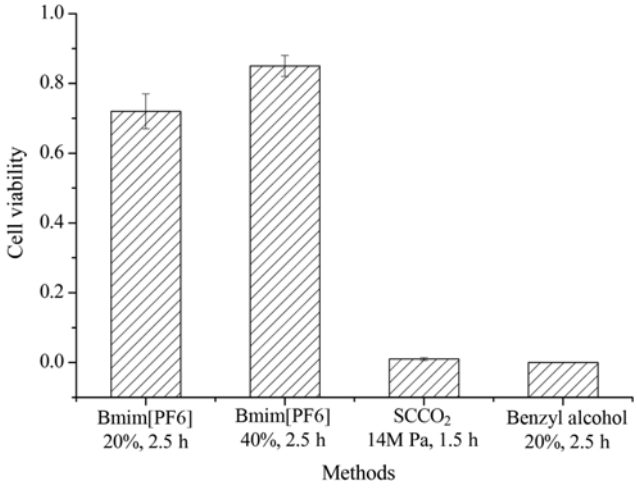

Fig. 7. Cell viability of yeast after short time treatment in 3 methods.

centrations the dry cell weight decreases as the ionic liquid concentration increases dramatically at the very beginning of the second cultivation. The dry cell weight is 0 when the concentration of ionic liquid is 0.7%, 0.9% and 1.1%. It means the samples with ionic liquid just contain very small amount of yeast after the first time cultivation.

Comparing Fig. 4 and Fig. 5, we find that the yeast in all samples increase significantly after incubating for 26 h, especially the ones in ionic liquid. We could infer that the growth of yeast in first cultivation is restrained, but when the concentration of ionic liquid decreases drastically, the growth of yeast recovers very well.

From Fig. 6, besides the sample in 1.1% ionic liquid, the residual sugar of others is almost the same, namely reaching lowest level. It's another fact to prove that the yeast really recovers after second cultivation. Combining Fig. 5 and Fig. 6, we note that the main inhibition of yeast growth by low concentration ionic liquid is in the time when they are in the same system. The effects of ionic liquid on yeast are weakened greatly after separation.

2. Effects of High Concentration Ionic Liquid on Yeast

2-1. Macro Effects of Ionic Liquid on Yeast

After short time treatments in high concentration (20% and 40%) ionic liquid bmim[PF6], the cell viability of yeast does not change much, which is very different from the samples treated in 14 MPa supercritical CO_2 or 20% benzyl alcohol (Fig. 7). The cell viability in ionic liquid is more than 0.7, yet other treatment would cause most yeast to die, from which we could deduce that high concentration ionic liquid treatment in short time does not do much harm to yeast. However, what's surprising is that the cell viability in 20% ionic liquid was lower than that in 40% ionic liquid. The same test was carried out twice, but same result came out. This result is similar to the results from Ganske et al. [25], who found that the growth of P. pastoris was not affected in 10% (v/v) [BMIM][PF6], but inhibited to a certain extent in 4%. This may be because ionic liquid bmim [PF6] is hydrophobic and its density is much bigger than H_2O. The degree of scatter in water of 20% ionic liquid is larger than the 40% one. In this way, the contact area of 20% ionic liquid with yeast is bigger than that of the 40% one, so the effects of 20% ionic liquid on yeast may also be stronger.

Fig. 8 shows the colonial morphology of yeast which was treated in 100% ionic liquid bmim[PF6] for three months. Fig. 8(b) is the amplified photo of Fig. 8(a). Even in such high concentration ionic liquid and for such a long time, the yeast was still alive, and the co-

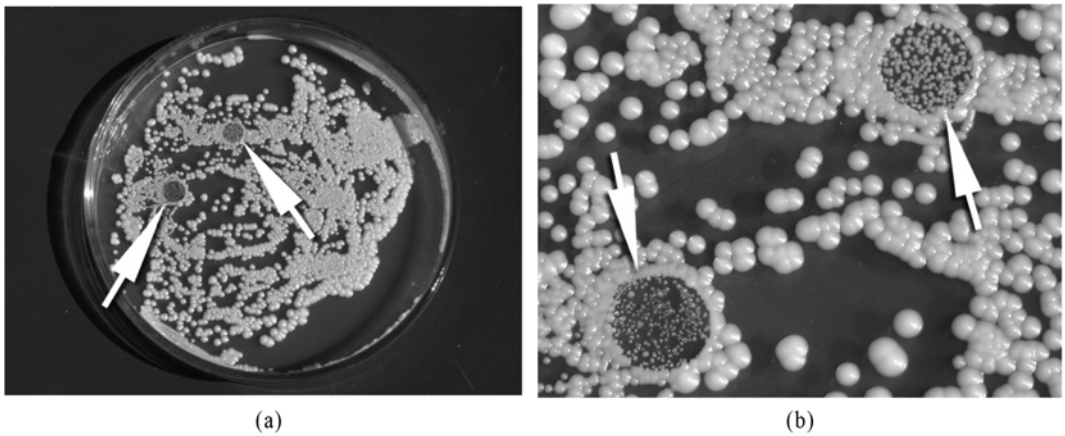

Fig. 8. Colonial morphology of yeast after long time treatment in 100% ionic liquid bmim[PF6].

lonial morphology is more or less as that of normal one. We can see the two small circles in Fig. 8(a) and 8(b). In these two circles there are a great number of very small colonies which may be caused by the restriction from ionic liquid. Yet more repeated experiments need to be done to prove this conclusion.

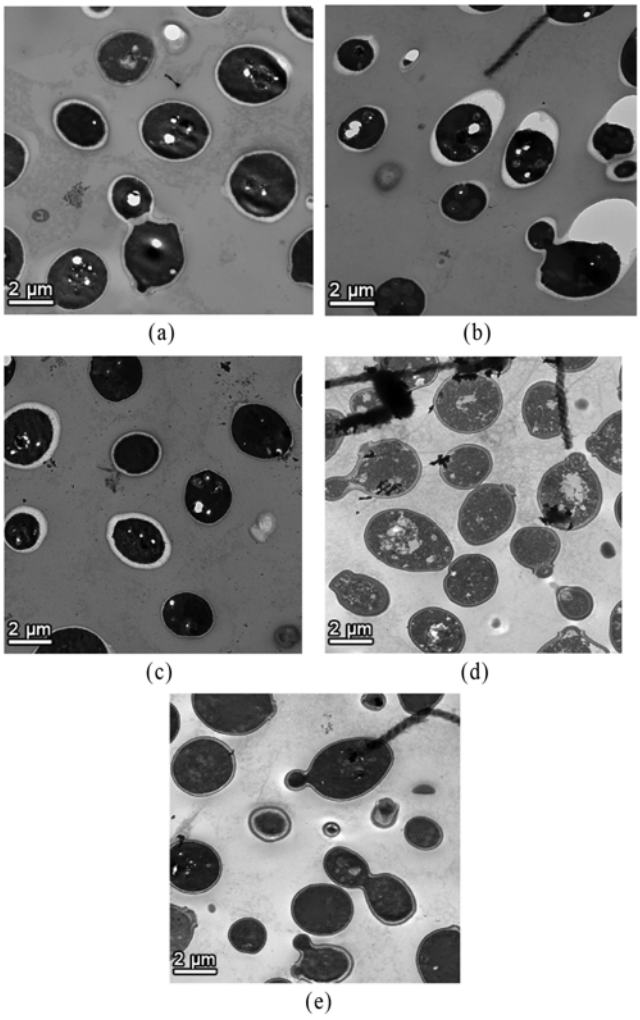

Fig. 9. Transmission electron microscope photos of yeast in different treated methods. (a) blank control; (b) 20% ionic liquid; (c) 40% ionic liquid; (d) 20% benzyl alcohol; (e) 14 MPa supercritical CO_2.

2-2. Micro Effects of Ionic Liquid on Yeast

Transmission electron microscope photos of yeast in different treated methods are shown in Fig. 9. The cell walls in Fig. 9(a) are uniform and their thickness is about 20 nm. The cell walls in Fig. 9(b) are significantly uneven, either in different cells or in the same cell. The cells seem to be stretched or squeezed in one direction, and some cell walls are even broken. The shapes of cells in Fig. 9(c) are like that of the ones in Fig. 9(a). Comparing different sections of yeast, we find the difference among the thicknesses of their cell walls. The cells in Fig. 9(d) and Fig. 9(e) are totally different from the normal one. There is some black substance out of the transparent cell walls, which forms by the denaturation of some material on cell walls. Such kind of cell wall indicates the yeast were dead. It is no doubt that their corresponding cell viabilities were at very low levels, as shown in Fig. 7.

DISCUSSION

From the result of the high concentration ionic liquid in short time treatment, we find that the cell viability of yeast in 20% ionic liquid bmim[PF6] is lower than that in 40% ionic liquid. The concentrations of ionic liquid bmim[PF6] and its effect on yeast are not in direct ratio for the first time. It's necessary to do widespread tests to confirm which concentration level of bmim[PF6] is the most harmful to yeast, and that concentration is probably not 100%.

The test methods between ionic liquid and yeast should be proper because the characters in different ionic liquids may be completely different. For example, ionic liquid bmim[PF6] is neutral and hydrophobic and its density is 1.363 kg/m^3 (298 K). So it's necessary to shake or stir during the cultivation to enlarge the interface between ionic liquid and yeast in order to present the proper effects of them.

Ionic liquid and supercritical CO_2 are called the new green solvents in this century, and the comparison between them and conventional organic solvent is always a hotspot worldwide. Yet till now, there is suchlike thesis that interprets the effect between ionic liquid and supercritical CO_2. In this paper, through macro cell viability and micro TEM observation, we compared the effects on yeast

among high concentration ionic liquid, high concentration benzyl alcohol and 14 MPa supercritical CO_2. The result showed the fatal rate of ionic liquid treatment is much lower than the others. The effects of 14 MPa supercritical CO_2 and high concentration benzyl alcohol are very familiar. It's just a brand new try among them so more deep research should be continued to confirm or find more relationships between them.

CONCLUSION

Long time incubation in low concentration ionic liquid generates inhibitory effect on the growth of yeast. As the concentration gets higher, the maximum growth rate of yeast becomes lower, the time it takes to reach the lowest residual sugar level gets longer, and microbial activity of yeast gets weaker. Still, after the separation in a second culture, all the activity of yeast is recovered very well.

Ionic liquid bmim[PF6] is not lethal to saccharomyces cerevisiae. From the macroscopic perspective, the cell viability of yeast in high concentration ionic treatment is far higher than that in 14 MPa supercritical CO_2 or high concentration benzyl alcohol treatment. From the microscopic perspective, though the teratogenesis of yeast in 20% ionic liquid is higher than that in 40% ionic liquid; neither has a lethal effect like 14 MPa supercritical CO_2 and high concentration benzyl alcohol treatment would.

Last but not least is another convincing result to show that ionic liquid bmim[PF6] does not have great harmful effects on yeast: yeasts remain alive with colonial morphology no difference from normal yeast after three months of incubation in 100% ionic liquid bmim [PF6].

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