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Short communication

Electrochemical insights into the ethanol tolerance of *Saccharomyces cerevisiae*

Min Wang [a], Jinsheng Zhao [b], Zhenyu Yang [c], Zhankui Du [d], Zhengyu Yang [d,*]

[a] *School of Medicine, Ehime University, 791-0295, Toon, Japan*
[b] *Department of Chemistry and Chemical Engineering, Liaocheng University, 252059, Liaocheng, PR China*
[c] *Department of Chemistry, Nanchang University, 330047, Nanchang, PR China*
[d] *Technical Institute of Physics and Chemistry, Chinese Academy of Sciences, 100101, Beijing, PR China*

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Abstract

It is expected that intracellular redox activity may closely related to catabolic states of living cells, based on which a mediated electrochemical method has been proposed to measure the ethanol tolerance of the yeast *Saccharomyces cerevisiae* AS 3800. The couple menadione/ferricyanide was employed as a carrier mediator system, sensing intracellular redox activity. Microelectrode voltammetric method was introduced to assay the ferrocyanide accumulations arising from menadione mediated reduction of ferricyanide by the yeast. The mediated electrochemical study show that the maximal ethanol tolerance limit of *S. cerevisiae* is about 25% (v/v) ethanol, which is consistent with the result obtained by the conventional fermentative ability measurement. Moreover, the electrochemical method for the first time confirmed that the specific activities of the glycolytic and alcohologenic enzymes within intact living cells remained high by the presence of sublethal ethanol, which was only predicted by *in vitro* enzymatic assay and cannot be measured by conventional method. The new method can be used as an easy and rapid method to determine the maximal ethanol tolerance of yeast cells.
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1. Introduction

One of the fundamental difficulties in assessing ethanol tolerance in various yeasts is that there is no universally accepted technique of measurement or definition of ethanol tolerance. Ethanol has three major effects on yeast cells. It inhibits cell growth [1], cell viability and fermentation ability [2,3], although to different extents. Thus, the definition of ethanol tolerance depends on which parameter one is referring to. It has been proposed that inhibition of fermentative ability is the best indicator of ethanol tolerance [4]. The rate of fermentative ability was usually estimated by measuring carbon dioxide evolution rate, which was taken as equivalent to glycolytic flux, assuming that per molecular glucose produced two molecular of ethanol and two molecular of carbon dioxide under fermentation conditions.

Mediated electrochemical method has been employed in antibiotic susceptibility tests and in viable cell population determinations *etc*., which might be also applicable to the evaluation of ethanol tolerance of yeast cells [5,6]. The mediated electrochemical method is based on the utilization of some of the redox mediators. The appropriate redox mediators or their combinations are capable of exchanging electrons between intracellular redox enzymes and extracellular acceptors due to their ability to permeate across the cell membrane. The double mediator system, containing 2-methyl-1,4-naphthalenedione(menadione) and ferricyanide was shown to be efficient for probing the intracellular redox activity in intact cells. The function of the lipophilic mediator menadione was to shuttle electrons from intracellular redox sites to the cell surface for reaction with the hydrophilic mediator ferricyanide (Fig. 1). In eukaryotic cells, plasma membrane redox system or mitochondrial electron transport enzymes or both of them have been proposed responsible for the intracellular reduction of menadione and, with both NADH and NADPH as electron

* Corresponding author. Tel.: +86 10 82543565; fax: +86 10 64879375.
E-mail address: zhengyuyang@vip.sina.com (Z. Yang).

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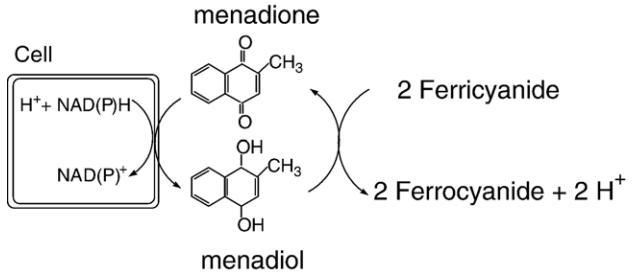

Fig. 1. Detection of intracellular redox activities with use of menadione to link intra- and extracellular redox pairs. Hypothetical menadione reaction cycle. Menadione diffuses into a yeast cell, where it is reduced to menadiol, an then diffuses out of the cell and reacts with ferricyanide, converting it into ferrocyanide, and regenerating menadione to repeat the cycle.

donors. With respect to *S. cerevisiae* cells, it has been reported that menadione accepted protons and electrons from the plasma membrane redox system and released protons by reducing ferricyanide to ferrocyanide [7]. As mentioned above, the electron donors for menadione mediated reduction of ferricyanide are NADH and NADPH, which are produced during the glucose catabolism in *S. cerevisiae* cells. In this case, menadione mediated reduction of ferricyanide may indicate the catabolic states of yeast cells, which forms the basis for the present study to evaluate the ethanol tolerance of *S. cerevisiae* electrochemically [8].

In this communication, a mediated electrochemical method was proposed for determination of the maximal ethanol tolerance of the yeast *S. cerevisiae* AS 3800, and the electrochemical results were agree with the previous studies based on measurements of fermentation ability. Moreover, it is most worthy of to be stated that the curve shape of the electrochemical response under ethanol stress for the first time indicates that the activities of glycolytic and alcohogenic enzymes measured *in vivo* remained high and did not appear limiting, which was only measured *in vitro* by now.

2. Materials and methods

2.1. Solution preparations

Phosphate-buffered saline (pH 6.0) was prepared from 0.1 M KH_2PO_4, 0.08 M Na_2HPO_4, and 0.1 M KCl. Potassium ferricyanide solutions were prepared in phosphate buffered saline to give a concentration of 0.30 M. Menadione was dissolved in 96% ethanol to give a 20 mM solution, filter-sterilised, and stored in a light-proof container at 4 °C. Glucose, 1 M in distilled water was sterilized at 121 °C for 15 min and stored at 4 °C.

2.2. Microorganism cultivation and preparation

S. cerevisiae AS 3800 was obtained from the strain collection center of the institute of Microbiology, Chinese Academy of Science (Beijing, China) The stain was maintained on YPD agar (0.5% w/v yeast extract, 1% w/v peptone, 2% w/v glucose, and 2% w/v agar) slants at 4 °C and fresh cultures (24–48 h) on YPD were used as inoculums. The yeasts were cultured in 250 ml shake flasks rotated at 200 rpm at 30 °C and using the growth medium and fermentation conditions as previously described [9]. Growth proceeded overnight for 12 h to allow cell growth to stationary phase, after which the broth was centrifuged at 10 000 ×*g* for 20 min and the cell pellet was washed twice with distilled water and suspended in phosphate buffered saline solution. Cells density was adjusted to an optical density at 600 nm (OD_{600}) of 9.0 using a spectrophotometer. The yeast cell suspensions were then subjected to the following experiments.

2.3. Incubation of cells with mediator and substrate

A total volume of 20 ml of incubation suspension was prepared for each trial. The standard incubation suspension comprised: 10 ml of cell suspension, 3.0 ml of ferricyanide solution, 100 μl of menadione solution, 150 μl of glucose solution, and 6.75 ml of phosphate-buffered saline. In the trails aimed to evaluate the ethanol tolerance of the yeast, 6.75 ml of phosphate-buffered saline in the standard incubation suspension was replaced by 6.75 ml of the mixture of required amounts of ethanol and appropriate amounts of phosphate-buffered saline. The reconstituted cell suspensions were incubated in 50 ml flasks rotated at 150 rpm at 30 °C for 2 h and conducted under nitrogen sparging or under exposure to air as stated in the text. At the completion of incubation, the cells were pelleted by centrifugation (10 000 rpm, 4 °C, and 15 min) and the supernatant removed for analysis. Unless stated otherwise, all trials were performed in triplicate.

2.4. Respirometry measurements

The rate of the fermentation ability was estimated by measuring carbon dioxide evolution with a Gilson differential respirometer (Gilson, Middleton, Wis)[10]. Samples (4 ml) were withdraw from the fermentation medium after 12 h incubation, harvested, and suspended in 4 ml fresh medium containing various concentrations of ethanol. The measurement of the fermentative ability of the yeast cells was conducted at 30 °C, and continued for 2 h. Fermentation rates were calculated by the least squares fitting method, and expressed in μl CO_2 min^{-1} mg dry $weight^{-1}$. Dry weight was determined after incubating membrane (0.45 μm pore size) filtered cell suspensions at 80 °C for 24 h.

2.5. Analytical methods

Steady-state voltammetry was conducted using an electrochemical working station (CHI 660, USA) controlled by CHI 660 software. Steady-state voltammograms were obtained at a scan rate of 10 mV s^{-1} scanning from 100 to 500 E/mV (*vs.* saturated Ag/AgCl). A 15 μm diameter Pt disk electrode was used as the working electrode, and a Pt gauze auxiliary electrode was used to complete the three-electrode electrochemical cell. The microelectrode was polished with 0.05 μm alumina/water slurry on a flocked twill polishing cloth. All

measurements were performed in triplicate, and the steady-state currents at 500 mV were measured and taken as the oxidative limiting current of microbially produced ferrocyanide. The data are the mean ± standard deviation (SD) from triplicate analyses in duplicate experiments.

2.6. Effects of added ethanol on ΔpH

ΔpH represents the difference between intracellular pH and external pH. The detection of external pH was simple and straightforward. Since determination of intracellular pH by distribution of weak acid requires the assumption of impermeability of the anion, intracellular pH was estimated by the freeze-thaw method [11]. Samples (20 ml) were removed from 12 h batch fermentation, harvested, and suspended in 20 ml fresh medium containing various concentrations of ethanol. The reconstituted cell suspensions were incubated in 50 ml flasks rotated at 150 rpm at 30 °C. After 2 h incubation, the ethanol-stressed yeast cells were harvested and, the intracellular pHs were measured according to the following procedures. Cells (250 mg [wet weight] were rapidly filtered on Nuclepore membranes and scraped off into a polythene cap (9 mm [inner diameter]) and frozen in liquid N_2. The caps were then thawed in air or in hexane and immediately refrozen. After six cycles, the temperature was quickly raised to 25 °C and the pH was determined by using a flat, combination electrode.

2.7. Glucose and ethanol analysis

Glucose concentration was measured by HPLC using a Hewlett-Packard 1050 equipped with a refractive index detector. HPLC analyses were carried out using an AMINEX HPX-87P carbohydrate analysis column operating at 85 °C with deionized water mobile-phase at a flow of 1 ml/min. Ethanol was determined by gas chromatography using a Packard chromatograph equipped with a chromosorb 101 column.

3. Results and discussion

3.1. Voltammetric measurement of menadione mediated reduction of ferricyanide

The use of voltammetric microelectrodes has been shown to be a simple, reliable, and rapid method for spelling the concentration of electroactive species such as ferrocyanide, diffusion limiting currents can be established in the second time scale with virtually zero destruction of analyte [12]. Fig. 2 shows the steady-state voltammogram of the supernatant before and after 2.5 h incubation with yeast suspensions. The steady-state anodic plateau currents measured at $E = 500$ mV are taken as the oxidative limiting current of microbially produced ferrocyanide, and can be quantitatively related to the concentration of microbially produced ferrocyanide *via* the following equation [10].

$$i_{\mathrm{Lim}} = 4nFDrC$$

Where i_{Lim} is the experimentally measured diffusion limiting current, n is the number of electrons transferred per mol of electroactive species ($n = 1$ in this case), F is the Faraday constant, D is the diffusion coefficient, r is the radius of microelectrode, and C is the concentration of the electroactive species. In this work, ferricyanide is converted to ferrocyanide by yeast catabolism. Fig. 2 (curve a) shows that before incubation, the mediator is fully in the oxidized form, and the steady-state anodic plateau current measured at $E = 500$ mV was about zero. After incubation for 2.5 h with yeast, glucose, menadione/ferricyanide dual mediator system, and under oxygen-free nitrogen sparging, the steady-state anodic plateau current of the obtained supernatant was 72.0 nA. However, the corresponding date for the same incubation conducted by exposure to air during the 2.5 h incubation was only 12.4 nA. Therefore, the presence or absence of oxygen in the menadione/ferricyanide mediator system influenced the responses to glucose greatly, which might result from the switchover in catabolic modes of *S. cerevisiae* from fermentative-predominant mode to respiratory-predominant mode when the inoculation conditions were changed from the presence of oxygen to the absence of oxygen. The above assumption was confirmed by the following glucose fermentation experiments, which showed that after incubation for 2.5 h with yeast, 7.5 mM glucose, menadione/ferricyanide, the ethanol yield for the incubation conducted under exposure to air was 0.45 g ethanol per gram of glucose (88.2% of theoretical maximum yield) and, the ethanol yield for the incubation conducted under oxygen-free nitrogen sparging was only 0.09 g ethanol per gram of glucose consumed (17.6% of theoretical maximum yield).

3.2. Measurement of inhibitory effect of ethanol on S. cerevisiae by conventional respirometry measurements method

In order to investigate the inhibition of fermentation ability by added ethanol on *S. cerevisiae*, cells were harvested and suspended in fresh medium containing various concentrations

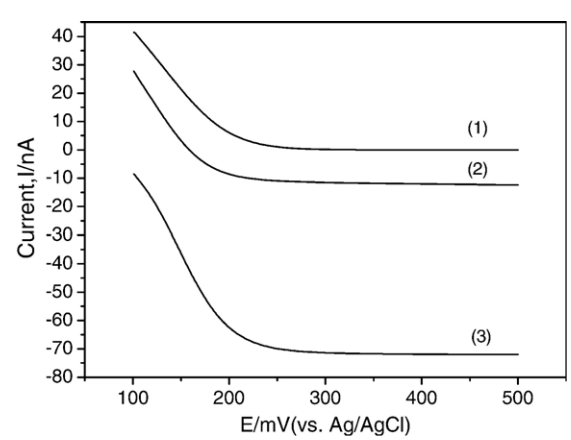

Fig. 2. Steady-state voltammograms recorded at a 15 μm Pt microelectrode before (1) and after a 2.5 h incubation of *S. cerevisiae* AS 3800 suspension under air (2) or under oxygen-free nitrogen sparging (3). Yeast absorbance (OD_{600}), 4.5; ferricyanide concentration, 45 mM; menadione concentration, 100 μM; glucose concentration, 7.5 mM; initial pH, 6.0; Scan rate, 10 mV s^{-1}.

of ethanol. Fig. 3 shows that ethanol caused a progressive, dose-dependent inhibition of fermentation ability on *S. cerevisiae* and with only 20.0% of the original activity remaining by the presence of 25.0% (v/v) ethanol, which indicates that the maximal ethanol tolerance of *S. cerevisiae* is around 25.0%. The basis for the decline in fermentation rate is not fully understood. According to the previous studies, plasma membrane and some of the catabolic enzymes represented the most possible target sites of ethanol in yeast cells. However, the specific target sites which are mainly responsible for the decline in the fermentation abilities have not yet been identified.

3.3. Electrochemical measurement of the inhibitory effect of ethanol on S. cerevisiae

The ethanol tolerance of *S. cerevisiae* was assayed through the variance in relative ferrocyanide accumulations by the presence or absence of increasing concentrations of ethanol present in cell suspensions. It has to be noted that the D values of ferrocyanide in phosphate-buffered saline are greatly affected by the presence of ethanol, so that D is a function of ethanol concentrations, and should be determined beforehand. By measuring the steady-state diffusion controlled limiting current of 1 mM ferrocyanide in phosphate-buffered saline by the presence of 0%–30% (v/v) ethanol, the D values of ferrocyanide under various ethanol concentrations can be obtained by the above equation. The list of D values under different ethanol concentrations are shown in Table 1. Having measured the limiting oxidative currents of the ferrocyanide accumulations arising form menadione mediated reduction of ferricyanide in a 2.5 h incubation, it is trivial to determine the ferrocyanide accumulations.

The oxidative limiting currents of the ferrocyanide accumulations arising from menadione mediated reduction of ferricyanide were designated R_M. Under lower ethanol concentrations and up to 15% (v/v) added ethanol, the R_M increased steadily and reached to the maximum value of 132% of the controlled sample at 15% (v/v) ethanol, which indicated the increase in intracellular NADH levels (Fig. 3). However, the further increase in added ethanol concentrations led to the decrease in the R_M and, the decrease in the R_M underwent two different stages. Above 15.0% and up to 20.0% added ethanol, the decrease in the R_M was moderate. Nevertheless, above 20.0% and up to 25% added ethanol, the R_M decreased abruptly and be lowered to 23.0% of the control sample by the presence of 25% ethanol, which indicates that the maximal ethanol tolerance of *S. cerevisiae* is around 25%. Fig. 3 also illustrates the changes in the internal pH of cells by the presence of increasing concentrations of added ethanol. The internal pH values of the yeast cells remained unchanged by the presence of increasing concentrations of ethanol up to 20%, and then decreased drastically with the further increase in ethanol concentrations, and nearly collapsed by the presence of about 27.0% ethanol. The maintenance of a nearly neutral internal pH was crucial for the yeast to sustain its normal metabolic activities, and the internal pH collapses will inevitably lead to the death of yeast cells. The abrupt decrease in both internal pH and the relative R_M (% of the R_M (control)) happened simultaneously, which most probably indicates the sudden and complete disruption of the cell membrane by the presence of around 25.0% added ethanol since the maintenance of the internal pH and the redox activities are both dependant on the integrity of cell membrane. Increasing evidence has suggested that cell membrane was the primary target site of ethanol in yeast cells. Damage to the cell membrane by added ethanol will result in altered membrane organization and permeability, inhibition of nutrition transport, accelerated passive re-entry of protons in a manner resembling the action of an uncoupler, and the enhancement of thermal death and 'petite' mutation in yeast, which should be accounting for the decrease in all of the three parameters including the fermentative activity, the redox activity and the internal pH [13].

The curve shape of the relative R_M was very different from that of the fermentative ability. However, the mediated electrochemical method might be more appropriate in evaluating the inhibitory effects of ethanol on the yeast, which could be explained from the following reasons. First, the continuous increase in the relative R_M *versus* the continuous decrease in fermentative ability by the presence of increasing concentrations of ethanol represents the main difference between the mediated electrochemical method and the conventional fermentation ability method. The continuous increase in the

Table 1
The D values of ferrocyanide in phosphate-buffered saline with different ethanol concentrations

Ethanol (%, v/v)	D (10^{-10} m^2/s)	Ethanol (%, v/v)	D (10^{-10} m^2/s)
0	7.01	16	5.29
2	6.90	18	5.10
4	6.72	20	4.88
6	6.59	22	4.54
8	6.43	24	4.34
10	6.11	26	4.19
12	5.88	28	4.10
14	5.49	30	4.02

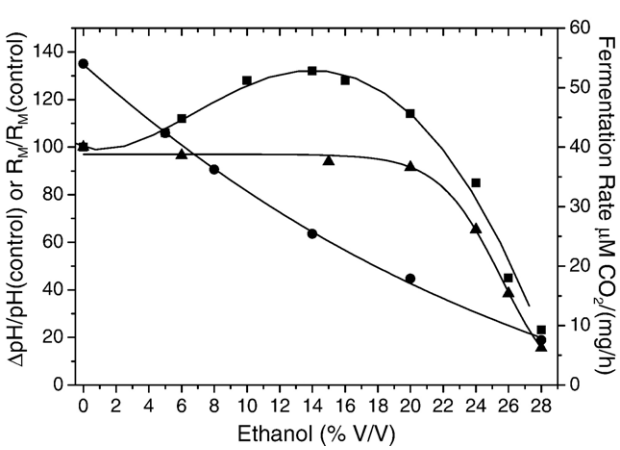

Fig. 3. Effects of ethanol exposure on the relative R_M (■), relative ΔpH (▲) and fermentative abilities (●) of yeast cells. Fermentative ability is expressed as micromoles of evolved carbon dioxide per hour milligram of cell protein. The test conditions are stated in the text.

relative R_M indicated the continuous increase in intracellular NADH level by the presence of up to 15% ethanol. As discussed above, under oxygen-free nitrogen sparging condition, the mediated electrochemical measurement enable *S. cerevisiae* cells to operate under respiration-predominant mode and, ethanol-stressed yeast cells were able to completely oxidized ethanol and produced excess NADH, which directly led to the increase in the relative R_M. According to the previous study [14], the intracellular ethanol concentrations of ethanol-stressed cells were similar with the extracellular ethanol concentrations added to the cells suspensions, thus the progressive increase in intracellular ethanol availability made the continuous increase in the relative R_M of the ethanol-stressed yeast cells. Second, the continuous increase in the relative R_M also indicate that the intracellular metabolic enzymes were not inhibited by the presence of up to 15% added ethanol, which was consistent with the previous reports showing that during batch fermentation, the viability of the yeast cells remained at or above 90%, internal pH remained near neutrality, and the specific activities of the glycolytic and alcohogenic enzymes (measured *in vitro*) remained high throughout batch fermentation [10]. However, the nearly untouched intracellular activities just mention above could not explain the continuous decrease in the fermentation ability as depicted in Fig. 3.

Third, now that the intracellular ethanol accumulations up to 20% (v/v) have no inhibitory effects on the intracellular metabolic enzymes, the only potential cause for the continuous decrease in the fermentative ability in ethanol-stressed yeast cells might be the destructive effect of ethanol on the cell membrane of the yeast, which led to the inhibition of nutrient transport, altered membrane organization and permeability and accelerated passive re-entry of protons, *etc.* Previous studies have shown that nutrient limitation, not increased intracellular accumulation of ethanol, was responsible for the decreases in growth and fermentation abilities of yeast cells [15]. Under fermentation conditions, the inhibition on glucose transport by added ethanol resulted in nutrient limitation for the catabolic activities of yeast cells, which directly lead to the continuous decrease in fermentation ability. Moreover, the ethanol-stressed yeast cells under fermentation condition cannot oxidize the intracellular ethanol accumulations, which might be another reason accounting for the continuous decrease in fermentation ability. Finally, the slow decrease in the relative R_M by the presence of 15% and up to 20% ethanol was probably due to the leakage of essential cofactors and coenzymes from the yeast [16], and the abrupt decrease in the relative R_M by the presence of ethanol concentrations higher than 20% and up to 25% ethanol was most probably due to the sudden and complete collapse of the plasma membrane of the yeast. Increasing proofs have proven that the plasma membrane is the prime target of ethanol action on yeasts [17,18].

In conclusion, the results presented in this communication indicate that the mediated electrochemical method is an alternative method for analysis of the maximal ethanol tolerance of yeast cells. The results from the electrochemical method were consistent with the results obtained from the conventional fermentation ability tests. In addition, the electrochemical method for the first time confirmed that the specific activities of the glycolytic and alcohologenic enzymes within intact living cells remained high by the presence of sublethal ethanol concentrations, which was only predicted by *in vitro* enzymatic assay and cannot be measured by conventional method. The plasma membrane of the yeast may be the most possible and the primary target site for the inhibitory effects of ethanol on yeast cell, and nutrient limitation may be responsible for the decreases in growth and fermentation activities of yeast cells at higher ethanol concentrations. Furthermore, since the mediated electrochemical technique is rapid and straightforward, resistance to the decrease in the relative R_M may be used for screening ethanol tolerant yeasts. Besides these, the introduction of microelectrode voltammetric method for ferrocyanide quantification greatly expedites the measurement process.

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