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Synergistic reduction of toluylene blue induced by acetaldehyde and menadione in yeast cell suspension: Application to determination of yeast cell activity

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ABSTRACT

Membrane permeant acetaldehyde and menadione induced the synergistic reduction of toluylene blue (TB) acting as non-membrane permeant redox indicator in yeast cell suspension. NADH and acetaldehyde also induced the synergistic TB reduction in permeabilized yeast cells and phosphate buffer, but menadione had no ability to promote TB reduction. The pre-incubation of acetaldehyde inhibited the above synergistic reduction of TB in intact and permeabilized yeast cell suspension. The pre-incubation of acetaldehyde might promote NADH oxidation by alcohol dehydrogenase, because acetaldehyde decreased the intracellular NAD(P)H concentration. The above facts indicate that the synergistic reduction of TB is controlled by the order of addition of menadione and acetaldehyde. The synergistic reduction of TB by menadione and acetaldehyde was proportional to viable yeast cell number from 10^4 to 2×10^6 cells/ml, and this assay was applicable to cytotoxicity test. The time required for the above assay was only 2 min.

1. Introduction

The various assays of cell activity have been developed for the evaluation of biological, chemical, and physical effects on cell growth or viability. For example, antimicrobial susceptibility test [1] and drug discovery screens [2] are performed by the determination of viability of bacteria, fungi or animal cells exposed to antibiotics or drugs. The physical growth conditions including temperature, aeration, and agitation are also investigated by the various assays.

The determination of yeast cell activity has been performed with flow cytometric methods [3,4], fluorescent assays [5,6], biological [7] or chemical [8–10] luminescent assays, colorimetric assays [11,12], and electrochemical assays [13,14]. In general, the colorimetric WST-1 (Water Soluble Tetrazolium) assay [15] and MTT (3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide) assay [16] have been used as the conventional methods among the above assays.

WST-1 is reduced by NAD(P)H in viable cells through redox mediator such as 1-methoxy PMS (1-Methoxy-5-methylphenazinium methylsulfate) and is changed into water-soluble colored formazan. MTT reduced by various dehydrogenases in cells also changed into water-insoluble colored formazan. However, these colorimetric assays are inferior to biological or chemical luminescent assays in the measuring time or the detection limit of viable cell activity. Quinone-mediated WST-1 assay [17] was improved in order to shorten the amount of measuring time for the determination of viable yeast cell activity. However, this assay required at least 10 min and the additional treatment such as the filtration with membrane filter and the neutralization of filtrate.

This study proposed the simple, rapid, and sensitive assay for determination of yeast cell activity compared to the above colorimetric assays. The colorimetric assay developed by this study was dependent on the synergistic reduction of toluylene blue (TB) by the combination of menadione and acetaldehyde. The mechanism about the synergistic reduction of TB was discussed, and the application to the assay of yeast cell activity was demonstrated in this study.

2. Materials and methods

2.1. Yeast strain and growth conditions

Baker yeast cells *Saccharomyces cerevisiae* IFO2044 were supplied from National Institute of Technology and Evaluation in Japan. The cells were grown in test tube filled with YPD medium (2% glucose, 1% peptone, and 0.5% yeast extract) at 30 °C for 18 h. The test tube was degassed and sealed during the cultivation. The cells were washed two times by centrifugation, and the cell density was adjusted to the desirable density with 0.1 M potassium phosphate buffer (pH 7.0) on the basis of the analytical curve between the turbidity (absorbance at

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600 nm) and CFU (colony forming unit) of yeast cell suspension.

CFU was counted after incubating the portion of yeast cell suspension on agar plate at 30 °C for 2 days. Agar plate was composed of 2% glucose, 1% peptone, 0.5% yeast extract, and 1.4% agar.

2.2. Permeabilization of yeast cells [18]

Yeast cells were collected by centrifugation, and 1g of pellet was suspended in 4 ml of permeabilization buffer (0.4 M sorbitol/0.1 M potassium phosphate buffer; pH 7.0). The suspension was mixed with 9 ml of 99.5% toluene and agitated at 42 °C for 5 min. The mixture was cooled in ice and washed two times by centrifugation at 10,000g for 10 min. The pellet was suspended in the above permeabilization buffer and adjusted to the desirable cell density.

2.3. TB reduction mediated by menadione and acetaldehyde in yeast cell suspension

The decrease in the absorbance at 645 nm due to TB reduction was followed after the addition of TB, menadione, and/or acetaldehyde to yeast cell suspension (4×10^6 cells/ml). The final concentration of TB, menadione, and acetaldehyde was 200 μM, 50 μM, and 50 mM, respectively. The final concentration of ethanol used as solvent of menadione was 70 mM and had little effect on TB reduction due to yeast cell activity. The reaction temperature was 25 °C.

2.4. TB reduction mediated by menadione and acetaldehyde in permeabilized yeast cell suspension

The decrease in the absorbance at 645 nm was followed after the addition of TB, NADH, menadione, and/or acetaldehyde to permeabilized yeast cell suspension (7.5×10^5 cells/ml). The final concentration of TB, NADH, menadione, and acetaldehyde was 200 μM, 500 μM, 50 μM and 50 mM, respectively. The final concentration of ethanol used as solvent of menadione was 70 mM. The reaction temperature was 25 °C.

2.5. TB reduction mediated by menadione and acetaldehyde in phosphate buffer

The decrease in the absorbance at 645 nm due to TB reduction was followed in the absence of permeabilized yeast cells under the conditions as described in Section 2.4.

2.6. Determination of electron flow from yeast cells to ferricyanide

Disposition of reaction container, electrode, salt bridge, and amperemeter was the same as described in the previous paper [19]. The cathodic reaction mixture was composed of 0.1 M potassium phosphate buffer (pH 7.0) and 1 mM potassium ferricyanide, and the anodic reaction mixture was composed of 0.1 M potassium phosphate buffer (pH 7.0), 200 μM TB, and yeast cells (4×10^6 cells/ml). The volume of each reaction mixture was 5 ml, and the both mixtures were shaken at 25 °C. The change in current was followed after the addition of menadione and/or acetaldehyde to the anodic reaction mixture. The final concentration of menadione and acetaldehyde was 50 μM and 50 mM, respectively. The final concentration of ethanol used as solvent of menadione was 70 mM.

2.7. NAD(P)H oxidation by menadione and acetaldehyde in permeabilized yeast cells suspension

The change in the absorbance at 340 nm was followed after the addition of NAD(P)H, menadione, and/or acetaldehyde to permeabilized yeast cell suspension (7.5×10^5 cells/ml). The final concentration of NAD(P)H, menadione, and acetaldehyde was 250 μM, 50 μM, and 50 mM, respectively. The final concentration of ethanol used as solvent of menadione was 70 mM. The oxidation rate of NAD(P)H was calculated on the basis of molar extinction coefficient of 6270 at 340 nm. The reaction temperature was 25 °C.

2.8. Fluorescence intensity due to NAD(P)H in yeast cell suspension

The concentration of NAD(P)H in yeast cells was determined by fluorescence (ex. 365 nm, em. > 430 nm). The change in fluorescence intensity was followed after the addition of menadione and/or acetaldehyde to yeast cell suspension (7.5×10^6 cells/ml). The final concentration of menadione, acetaldehyde, and ethanol was 50 μM, 50 mM, and 70 mM, respectively. In the case of the excessive addition of menadione solution, the final concentration of menadione and ethanol was 300 μM and 420 mM, respectively.

2.9. Determination of viable yeast cell number by TB reduction mediated by menadione and acetaldehyde

The yeast cell suspension was diluted to the desirable cell density with 0.1 M phosphate buffer (pH 7.0). The cell density was determined on the basis of CFU and the absorbance at 600 nm due to yeast cell suspension described in Section 2.1. The decrease in the absorbance at 645 nm due to TB reduction was determined after the addition of menadione for 1 min and then after the subsequent addition of acetaldehyde for 1 min at 25 °C. The final concentration of TB, menadione, acetaldehyde, and ethanol was the same as described in Section 2.3. The initial reduction rate of TB was calculated on the basis of the difference between the absorbance before and after the addition of menadione and/or acetaldehyde.

2.10. Cytotoxic effect of triton X-100

Yeast cells (4×10^6 cells/ml) was incubated with Triton X-100 at 25 °C for 90 min, and the initial reduction rate of TB was determined under the conditions described in 2.9. Yeast cells (2.5×10^5 cells/ml) was incubated in the YPB broth (2% glucose, 1% peptone, and 0.5% yeast extract) including Triton X-100 at 30 °C for 15 h. The absorbance due to the turbidity of yeast cell suspension was determined at 600 nm as described in Section 2.1.

2.11. Chemicals

Chemicals were obtained from Wako Pure Chemical Industries, Ltd.

2.12. Statistical analysis

The mean and the standard deviation were calculated by using Office Excel.

3. Results

3.1. TB reduction by menadione and acetaldehyde in yeast cell suspension

TB reduction in yeast cell suspension was promoted by the addition of menadione (•, ▲ at 2 min in Fig. 1), and TB reduction promoted by menadione was faster than that by acetaldehyde (■ at 2 min in Fig. 1). The combination of menadione and acetaldehyde (▲, ■ at 4 min in Fig. 1) promoted TB reduction faster than menadione-mediated TB reduction (• at 2 min in Fig. 1) and induced the synergistic reduction of TB. The addition of acetaldehyde to menadione-mediated TB reduction (▲ at 4 min in Fig. 1) induced greater synergistic TB reduction than the addition of menadione to acetaldehyde-mediated TB reduction (■ at 4 min Fig. 1). The above facts indicate that the order of addition of acetaldehyde and menadione influences the synergistic reduction of TB.

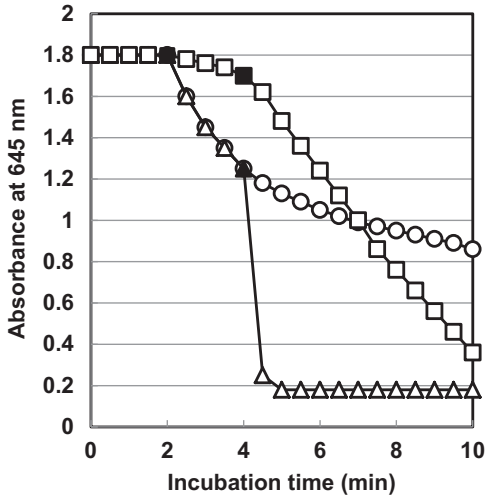

Fig. 1. Effects of menadione and acetaldehyde on TB reduction in yeast cell suspension. Symbols □ show TB reduction, after the addition of acetaldehyde (■ at 2 min), and after the subsequent addition of menadione (■ at 4 min). Symbols ○ show TB reduction and after the addition of menadione (• at 2 min). Symbols △ show TB reduction, after the addition of menadione (▲ at 2 min), and after the subsequent addition of acetaldehyde (▲ at 4 min). Each symbol represents the mean of three different determinations, and the standard deviation was less than 5% of the mean.

3.2. TB reduction by menadione and acetaldehyde in permeabilized yeast cell suspension

TB was not reduced by permeabilized yeast cells, but the addition of acetaldehyde promoted TB reduction in the absence of NADH (■ at 2.5 min in Fig. 2). Though the cell density of permeabilized yeast cell suspension was lower approximately 5-fold than that of yeast cell suspension, the reduction rate of TB by acetaldehyde in permeabilized yeast cell suspension (■ at 2.5 min in Fig. 2) was faster than that in yeast cell suspension (■ at 2 min in Fig. 1), because the decrease in absorbance due to TB reduction in yeast and permeabilized yeast cell suspension was 0.1 and 0.4 per 2 min, respectively. This fact suggests that TB reduction by acetaldehyde is promoted by the enzymes in the absence of NADH.

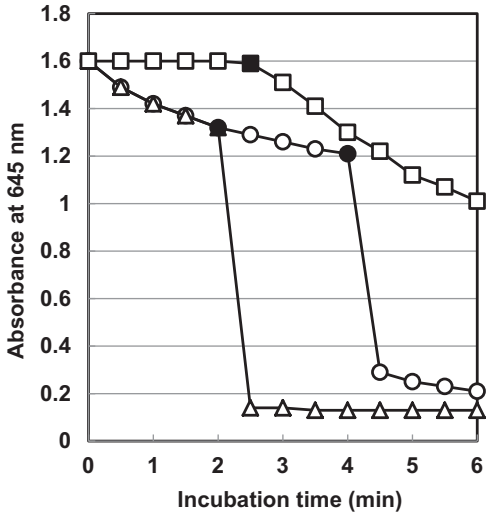

Fig. 2. Effects of menadione and acetaldehyde on TB reduction in permeabilized yeast cell suspension. Symbols □ show TB reduction in the absence of NADH and after the addition of acetaldehyde (■ at 2.5 min). Symbols ○ show TB reduction by NADH, after the addition of menadione (• at 2 min), and the subsequent addition of acetaldehyde (• at 4 min). Symbols △ show TB reduction by NADH and after the addition of acetaldehyde (▲ at 2 min). Each symbol represents the mean of three different determinations, and the standard deviation was less than 6% of the mean.

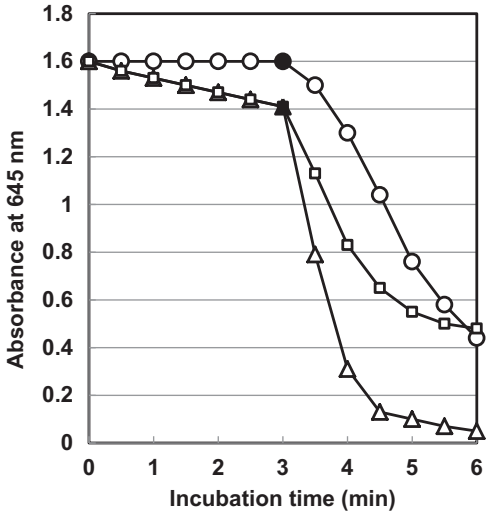

Fig. 3. Effects of NADH, acetaldehyde and alcohol dehydrogenase on TB reduction in phosphate buffer. Symbols ○ show TB reduction by acetaldehyde and after the addition of NADH (• at 3 min). Symbols △ show TB reduction by NADH and after the addition of acetaldehyde (▲ at 3 min). Symbols □ show TB reduction by NADH and after the addition of acetaldehyde and alcohol dehydrogenase (10U) (■ at 3 min). Each symbol represents the mean of three different determinations, and the standard deviation was less than 4% of the mean.

TB reduction was promoted by the addition of NADH (○, △ in Fig. 2), and the subsequent addition of acetaldehyde induced the rapid TB reduction (▲ at 2 min and • at 4 min in Fig. 2). As the rate of TB reduction by the combination of NADH and acetaldehyde was much faster than the sum of each TB reduction rate by NADH or acetaldehyde, the combination of NADH and acetaldehyde was demonstrated to induce synergistic reduction of TB.

The addition of menadione (• at 2 min in Fig. 2) had little effect on TB reduction by NADH in permeabilized yeast cell suspension, suggesting the important role of plasma membrane in menadione-mediated TB reduction.

3.3. TB reduction by NADH and acetaldehyde in phosphate buffer

TB reduction was not promoted by the addition of acetaldehyde in phosphate buffer (○ in Fig. 3) but by the subsequent addition of NADH (• at 3 min in Fig. 3), suggesting that acetaldehyde mediates the electron flow from NADH to TB. TB reduction was promoted by NADH (□, △ in Fig. 3), and the addition of acetaldehyde (■, ▲ at 3 min in Fig. 3) induced the synergistic reduction of TB. As the rate of TB reduction by the combination of NADH and acetaldehyde was promoted by pre-addition of NADH (▲ after 3 min in Fig. 3) rather than the pre-addition of acetaldehyde (● after 3 min in Fig. 3), the complex of acetaldehyde and TB might be formed after pre-incubation of acetaldehyde and inhibit TB reduction by NADH.

The synergistic reduction of TB by the combination of NADH and acetaldehyde in phosphate buffer was slower than that in permeabilized yeast cells (Figs. 2 and 3). This fact also suggests that NADH and acetaldehyde promote TB reduction with enzymes present in permeabilized yeast cells.

The synergistic reduction of TB induced by NADH and acetaldehyde was inhibited in the presence of alcohol dehydrogenase (EC.1.2.1.3) which oxidizes NADH by reducing acetaldehyde to ethanol [20,21] (■ at 3 min in Fig. 3). This fact suggests that the pre-incubation of acetaldehyde promotes oxidation of NADH by alcohol dehydrogenase to decrease NADH-dependent TB reduction in yeast cell suspension as shown in Fig. 1.

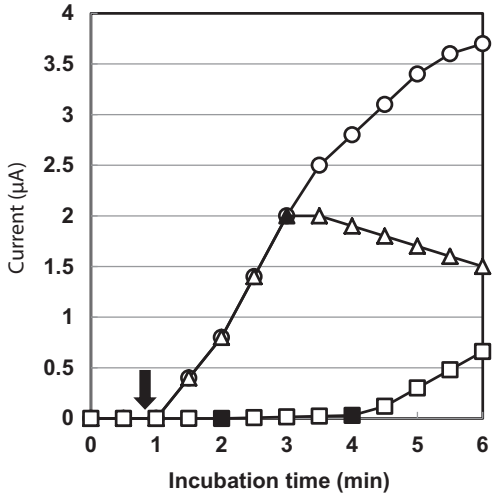

Fig. 4. Effects of TB, menadione and acetaldehyde on electron flow from yeast cells to ferricyanide. Symbols ○ show the current in the presence of TB and after the addition of menadione (black arrow at 1 min). Symbols △ show the current in the presence of TB, after the addition of menadione (black arrow at 1 min), and after the subsequent addition of acetaldehyde (▲ at 3 min). Symbols □ show the current in the presence of TB, after the addition of acetaldehyde (■ at 2 min), and after the subsequent addition of menadione (■ at 4 min). Each symbol represents the mean of three different determinations, and the standard deviation was less than 6% of the mean.

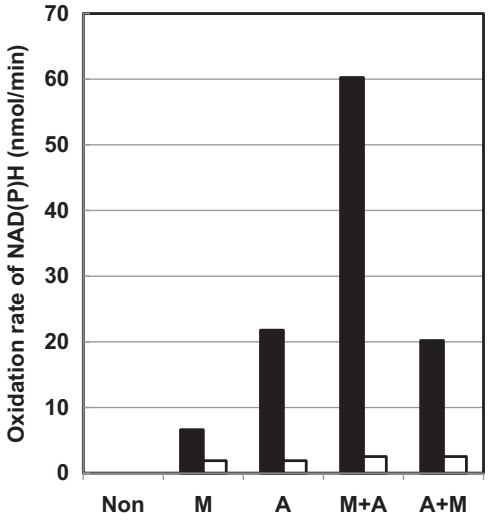

Fig. 5. Effects of menadione and acetaldehyde on NAD(P)H oxidation in permeabilized yeast cell suspension. Black and white bars show oxidation rate of NADH and NADPH, respectively. Non, M and A mean non-addition, the addition of menadione and acetaldehyde, respectively. M+A means the addition of acetaldehyde after 1 min-incubation with menadione. A+M means the addition of menadione after 1 min-incubation with acetaldehyde. Each bar represents the mean of three different determinations, and the standard deviation was less than 6% of the mean.

3.4. Menadione-mediated electron flow from yeast cells to anode

Fig. 4 shows the current depending on the electron flow from yeast cells to ferricyanide via anode and cathode. Yeast cells could not transport electron directly to anode in the presence of TB, but electron flow from yeast cells to anode was observed by the addition of menadione (arrow at 1 min in Fig. 4). Menadione-mediated electron flow was inhibited by pre- or post-addition of acetaldehyde (■ at 2 min and ▲ at 3 min in Fig. 4), suggesting that acetaldehyde is involved in NADH oxidation by alcohol dehydrogenase to inhibit reduction of menadione by NAD(P)H: menadione reductase (called DT-diaphorase, EC.1.6.99.2). On the other hand, acetaldehyde induced the synergistic reduction of TB with menadione. This discrepancy suggests that acetaldehyde inhibits reduction of menadione by NADH:menadione reductase by promoting oxidation of NADH by alcohol dehydrogenase and that menadiol produced by NADH:menadione reductase is rapidly oxidized by TB or dissolved oxygen before menadiol transports electron to anode.

3.5. Effects of menadione and acetaldehyde on oxidation of NAD(P)H in permeabilized or intact yeast cells

Acetaldehyde or menadione promoted oxidation of NADH rather than NADPH in permeabilized yeast cells, and the rate of acetaldehyde-mediated NADH oxidation was faster than that of menadione-mediated NADH oxidation as shown in Fig. 5. This fact indicates that NADH oxidation by alcohol dehydrogenase is faster than that by NADH:menadione reductase. Though acetaldehyde-mediated NADH oxidation was little affected by the addition of menadione (A, A+M in Fig. 5), menadione-mediated NADH oxidation was promoted by the addition of acetaldehyde (M, M+A in Fig. 5), and NADH oxidation by the combination of menadione and acetaldehyde (M+A in Fig. 5) was faster than the sum of menadione- and acetaldehyde-mediated NADH oxidation (M, A in Fig. 5), indicating the synergistic NADH oxidation. The above facts indicate that synergistic NADH oxidation is controlled by the order of addition of menadione and acetaldehyde and that synergistic NADH oxidation is involved in the synergistic TB reduction.

Fig. 6 shows the effects of menadione, acetaldehyde and ethanol on the fluorescence intensity due to NAD(P)H concentration in yeast cells.

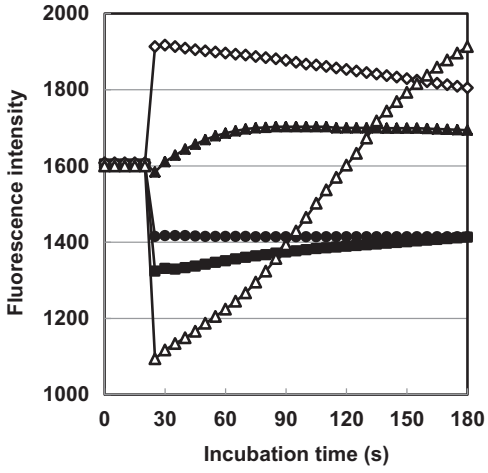

Fig. 6. Effects of menadione, acetaldehyde and ethanol on the fluorescence intensity in yeast cell suspension. Menadione, acetaldehyde and ethanol were added to yeast cell suspension after 20 s, and the change in fluorescence intensity was followed. Each symbol was as follows: ◇, ethanol; ▲, menadione;•, acetaldehyde; ■, menadione and acetaldehyde; △, the addition of 6-fold menadione and ethanol. Each symbol represents the mean of three different determinations, and the standard deviation was less than 5% of the mean.

A small decrease in the fluorescence intensity was observed just after the addition of menadione solution (50 μM menadione and 70 mM ethanol), and the fluorescence intensity increased with incubation time (▲ in Fig. 6). Ethanol used as the solvent of menadione induced the increase in the fluorescence intensity (◇ in Fig. 6), indicating the production of NADH by alcohol dehydrogenase. When 300 μM menadione and 420 mM ethanol were added to yeast cell suspension, the great decrease in fluorescence intensity due to NAD(P)H:menadione reductase oxidizing NAD(P)H was observed, and the increase in fluorescence intensity due to alcohol dehydrogenase reducing NAD(P) was continuing (△ in Fig. 6).

Acetaldehyde induced the rapid decrease in fluorescence intensity, and the constant decrease was kept (• in Fig. 6). The decrease in fluorescence intensity by the combination of menadione and acetaldehyde (■ in Fig. 6) was larger than the sum of the decrease in

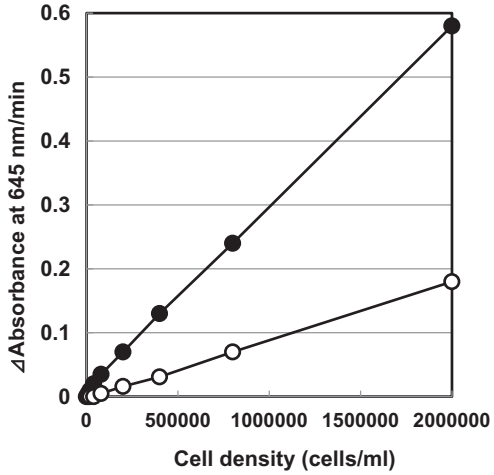

Fig. 7. Proportionality relation between viable yeast cell number and TB reduction. Symbols • show the initial TB reduction mediated by both menadione and acetaldehyde for 1 min after 1 min-incubation with menadione. Symbols ○ show the initial TB reduction mediated by menadione for 1 min. Each symbol represents the mean of three different determinations, and the standard deviation was less than 7% of the mean.

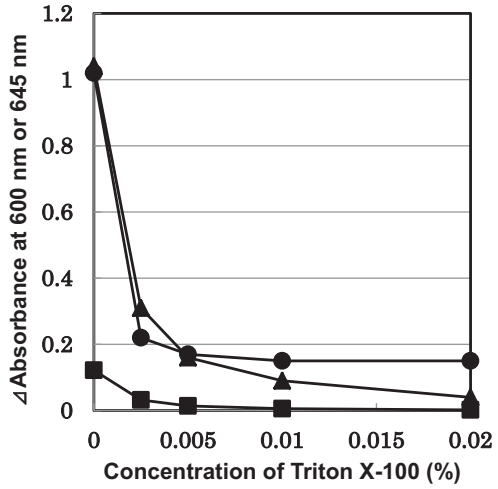

Fig. 8. Effects of Triton X-100 on viability of yeast cells. Symbols ▲ show the initial TB reduction mediated by the combination of menadione and acetaldehyde for 1 min after 1 min-incubation with menadione. Symbols ■ show the initial TB reduction mediated by menadione for 1 min. Symbols • show the turbidity (absorbance at 600 nm) of culture medium after 15-h cultivation. Each symbol represents the mean of three different determinations, and the standard deviation was less than 6% of the mean.

fluorescence intensity by the individual addition of menadione and acetaldehyde (•, ▲ in Fig. 6), indicating the synergistic oxidation of NADH. This synergistic NADH oxidation indicates that 50 mM acetaldehyde has enough ability to keep NAD(P)H oxidation in yeast cells in spite of the presence of 70 mM ethanol used as solvent of menadione. The above NAD(P)H oxidation by acetaldehyde in yeast cell suspension might be related to the inhibitory effect of pre-added acetaldehyde on both menadione-mediated TB reduction (Fig. 1) and menadione-mediated electron flow from yeast cells to anode (Fig. 4).

3.6. Assay of viable cell number and yeast cell activity by TB reduction

Fig. 7 shows the proportionality relation between viable cell number and TB reduction. The viability assay was composed of 1 min-TB reduction by the addition of menadione and the following 1 min-TB reduction by the subsequent addition of acetaldehyde. The viable cell number was determined on the basis of the relationship between CFU and the absorbance at 600 nm due to turbidity of yeast cell suspension. The linear proportionality was observed from 10,000 to 2,000,000 cells/ml (• in Fig. 7). Detection limit of viable cells determined by menadione-mediated TB reduction (○ in Fig. 7) and by the synergistic TB reduction (• in Fig. 7) was 80,000 and 10,000 cells/ml, respectively. The above facts show that TB reduction by the combination of menadione and acetaldehyde is superior to menadione-mediated TB reduction in the sensitivity to viable cell number.

Fig. 8 shows the cytotoxic effect of Triton X-100 which inhibits plasma membrane associated NADH-dependent redox activities of yeast cells [22]. TB reduction by the combination of menadione and acetaldehyde (▲ in Fig. 8) showed more obvious dose-dependent response than that by menadione-mediated TB reduction (■ in Fig. 8). The activity of yeast cells exposed to 0.01 or 0.02% Triton X-100 was not observed by menadione-catalyzed TB reduction (■ in Fig. 8) but by TB reduction by the combination of menadione and acetaldehyde. This fact shows that the synergistic reduction of TB by the combination of menadione and acetaldehyde is useful for the detection of low cell activity.

The turbidity of yeast cell suspension after 15-h cultivation (• in Fig. 8) showed less dose-dependent response than TB reduction by the combination of menadione and acetaldehyde (▲ in Fig. 8). Therefore, the synergistic TB reduction is expected to be applicable to the rapid determination of yeast cell activity or viability after the short-time incubation with toxic compounds.

4. Discussion

4-(4,6-diamino-m-tolyl)imino-2,5-cyclohexadien-1-ylidene) dimethlyammonium chloride is called toluylene blue (TB) and has been used as a redox indicator. As TB is cationic compound, TB is estimated to be non-membrane permeant redox indicator. In fact, TB reduction in yeast cell suspension was slower than that in permeabilized yeast cell suspension (Figs. 1 and 2) and was promoted by the addition of menadione acting as membrane permeant redox mediator (Fig. 1).

In this study TB was found to be as a specific redox indicator which was reduced synergistically by menadione and acetaldehyde, because other redox indicators such as 2,6-dichlorophenol indophenol or ferricyanide showed no synergistic reduction by menadione and acetaldehyde (data not shown here).

Non-enzymatic reduction of TB by acetaldehyde was not observed in phosphate buffer, but acetaldehyde enhanced reduction of TB by NADH as shown in Fig. 3. Acetaldehyde might act as redox mediator between TB and NADH, because the redox potential of NADH, acetaldehyde, and TB is −0.32 V [23], −0.197 V [24], 0.115 V [25,26] at pH 7.0, respectively. The effect of acetaldehyde acting as redox mediator might be enhanced by enzymes in permeabilized yeast cells, because the synergistic reduction of TB in permeabilized yeast cell suspension was faster than that in phosphate buffer (Figs. 2 and 3).

On the other hand, menadione was not involved in the synergistic reduction of TB by the combination of NADH and acetaldehyde in permeabilized yeast cell suspension (Fig. 2). This fact suggests that menadione reduced by NAD(P)H:menadione reductase is rapidly oxidized by oxygen rather than TB, because menadiol was rapidly oxidized under the aerobic condition [28] and promoted the generation of the reactive oxygen species in both intact and permeabilized yeast cell suspension [29]. The destruction of plasma membrane might damage the anaerobic environment required for both the effective production of menadiol by NAD(P)H:menadione reductase and the interaction between TB and menadiol on the surface of plasma membrane because of the electrostatic action between cationic TB and negatively charged plasma membrane.

Acetaldehyde migrated into yeast cells to oxidize NADH with alcohol dehydrogenase (Fig. 6), and acetaldehyde promoted TB reduction in permeabilized yeast cells (Fig. 2). However, TB reduction by acetaldehyde in yeast cell suspension was slower than that in permeabilized yeast cell suspension. This fact suggests that cationic TB on the

surface of negatively charged plasma membrane is hard to get close to intracellular acetaldehyde and NADH. Menadione might promote the redox reaction among TB, acetaldehyde, and NADH in plasma membrane to show the synergistic TB reduction.

As the concentration of acetaldehyde was estimated to be approximately 10 mM in yeast cells [27], yeast cells were considered to metabolize acetaldehyde and to have resistance to acetaldehyde inducing stress. For example, excess acetaldehyde might be converted to ethanol by alcohol dehydrogenase. In fact, rapid NAD(P)H oxidation by addition of acetaldehyde was observed in intact and permeabilized yeast cells (Figs. 5 and 6).

The conventional WST-1 assay has been used to evaluate the viability of yeast cells or animal cells. This assay requires 1-methoxy PMS which acts as membrane permeant redox mediator, and the incubation time of yeast cells with WST-1 and 2-methoxy PMS is approximately 2 h [15]. In order to shorten the above incubation time, quinone-catalyzed WST-1 assay was developed as rapid colorimetric assay and showed the proportionality relation between WST-1 reduction and the cell density from 100,000 to 10,000,000 cells/ml [17]. The incubation time of yeast cells with quinone and WST-1 was 10 min, and then the filtration with membrane filter and the neutralization of filtrate were required before the determination of absorbance.

On the other hand, the colorimetric TB reduction assay required 2 min-incubation including 1 min-incubation of yeast cells with menadione and the following 1 min-incubation after the subsequent addition of acetaldehyde. The proportionality relation between TB reduction and viable yeast cell number was observed from 10,000 to 2,000,000 cells/ml.

As Bindschedler's green leuco base being similar to TB was reduced directly by animal cells and applied to their viability assay [30], the synergistic TB reduction by the combination of menadione and acetaldehyde is expected to be applied to the rapid screening tests for antimicrobials and new drug discovery.

Appendix A. Transparency document

Transparency document associated with this article can be found in the online version at http://dx.doi.org/10.1016/j. bbrep.2017.01.015.

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