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Electrochemical Evaluation of the Inhibitory Effects of Weak Acids on *Zygosaccharomyces bailii*

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The changes in intracellular redox activity or in mitochondrial electron transport could be taken as indications of the changes in the physiological state of living cells, based on which a mediated electrochemical method was purposed to evaluate the inhibitory effects of weak acids on *Zygosaccharomyces bailii*, a known food spoilage yeast. The dual mediator systems menadione/ferricyanide and 2,6-dichlorophenolindophenol/ferricyanide were employed as probes to detect the variance in intracellular redox activity and in mitochondrial electron flux, respectively. Measurements were made with a microelectrode voltammetric method to assay the ferrocyanide accumulations arising from menadione- or 2,6-dichlorophenolindophenol-mediated reduction of ferricyanide by *Z. bailii* suspensions by the presence or absence of increasing concentrations of weak acids. The results obtained from 2 h of incubation showed that the variance in electrochemical response revealed some physiological information underlying the inhibitory effects of weak acid on the yeast. For the first time, it was shown that the mediated electrochemical method provides an adjunct to the conventional method based on respiration inhibition for establishing levels for the utilization of preservatives in the food industry.

KEYWORDS: Electrochemical; weak acid; toxicity; mediator; spoilage yeast

INTRODUCTION

Weak carboxylic acids were used as food preservatives to inhibit microbial activity. Most references on this subject referred to their toxic effects on growth or on respiration rate (*1*−*4*). However, cellular catabolic activity may continue under conditions at which growth is fully arrested, causing alteration to foods and drinks (*5*, *6*). In fact, food spoilage is frequently due to CO_2 evolution during fermentation without any measurable microbial growth, based on which the measure of respiration inhibition has been proposed as a reasonable approach for establishing levels for the utilization of preservatives in the food industry (*3*, *4*). However, the results from respiration inhibition reflect only the activities in the mitochondrion and have been considered to be of no significance in terms of the elucidation of physiological mechanisms underlying the inhibitory effects of weak acids.

All of the major metabolic pathways in cells involve redox reactions and specific redox couples that cycle between oxidized and reduced states. It has been shown that redox activity cannot be viewed as an isolated process; its regulation is closely connected to central and peripheral reactions in carbon and nitrogen metabolism. It is expected that the intracellular redox activity may change selectively with changes in the cellular environment, such as those due to the introduction of toxic substances, growth factors, and nutrients (*8*). Changes in the mitochondrial electron transport have also been used as the indication of the changes in the physiological state of living cells (*9*). Perhaps we could indirectly evaluate the cytotoxic effects of weak acids on microorganisms by referring to intracellular redox activity inhibition or electron transport inhibition. Mediated electrochemical measurements have been proved to be potent in evaluating intracellular redox activity or mitochondrial electron transport of living cells with the aid of appropriate mediator systems (*8*, *10*).

The double-mediator system, containing menadione/ferrocyanide, has been used to detect intracellular redox activity of eukaryotic cells. Menadione mediates the transfer of electrons from the insides of cells to ferricyanide, which cannot permeate into the membrane of eukaryotic cells (**Figure 1**). The detection of intracellular redox activity using menadione as electron-transfer mediator was based on the fact that menadione is lipophilic and can diffuse through the cell membrane and then be reduced by the cytosolic and mitochondrial enzymes catalyzing electron transfer from NAD(P)H to quinone substrates (*11*). The 2,6-dichlorophenolindophenol/ferricyanide system has been frequently used to detect cellular respiration activity in living cells (*12*, *13*), because 2,6-dichlorophenolindophenol (DCPIP) has been considered directly accept electrons from the mitochondrial electron transport chain of eukaryotic cells (*14*−*16*).

In the present paper, dual mediator systems menadione/ferricyanide and DCPIP/ferricyanide were employed as probes to detect the inhibitory effects of weak acids on *Zygosacchromyces*

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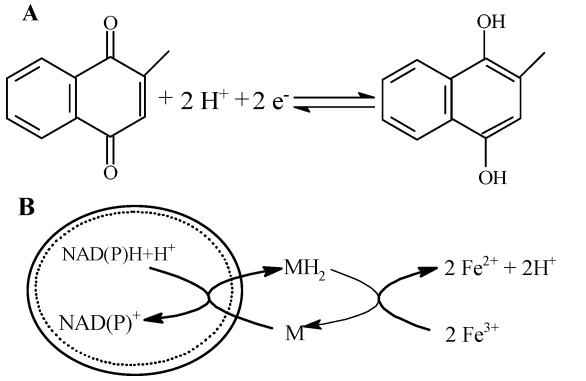

Figure 1. Detection of intracellular redox activities with use of menadione to link intra- and extracellular redox pairs: (**A**) two-electron reduction of menadione to menadiol; (**B**) hypothetical menadione reaction cycle. Menadione (M) diffuses into a yeast cell, where it is reduced to menadiol (MH_2), which then diffuses out of the cell and reacts with ferricyanide, labeled for simplicity Fe^{3+}, converting it into ferrocyanide, labeled Fe^{2+}, and regenerating menadione (M) to repeat the cycle. Ferrocyanide accumulations arising from the above reaction cycle was assayed by microelectrode voltammetric measurements

bailii, a known food spoilage yeast (*17*). Measurements were undertaken with a microelectrode voltammetric method to assay ferrocyanide accumulations arising from menadione- or DCPIP-mediated reduction of ferricyanide by cell suspensions in the absence or presence of increasing concentrations of weak acids (*18*). The results of this study suggested that the mediated electrochemical method appears to be an adjunct method for establishing levels for the utilization of preservatives in the food industry.

MATERIALS AND METHODS

Reagents. Potassium hexacyanoferrate(III), D-(+)-glucose, sodium chloride, yeast extract power, potassium dihydrogen phosphate, disodium hydrogen phosphate trihydrate, potassium chloride, peptone, 96% ethanol, acetic acid, benzoic acid, and 37% hydrochloric acid were obtained from Shanghai Reagent Corp. (Shanghai, China). Menadione and DCPIP were purchased from Merck (Darmstadt, Germany). All chemicals used in this study were of analytical reagent grade, and all solutions were prepared using distilled and deionized water.

Microorganism. *Z. bailii* AS 2.312 was obtained from the strain collection center of the Institute of Microbiology, Chinese Academy of Science (Beijing, China). The strains were maintained on agar slants containing (g/L): glucose, 30 g/L; yeast extracts, 3 g/L; peptone, 5 g/L; and agar, 15 g/L; pH 6.0 at 4 °C.

Solution Preparations. Phosphate buffer (pH 6.0), containing 0.1 M KH_2PO_4 and 0.08 M Na_2HPO_4, was used for washing cell pellets. 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. Glucose solution was prepared in phosphate-buffered saline to give a concentration of 15.0 g/L glucose. Menadione was dissolved in 96% ethanol to give a 20 mM solution, filter-sterilized, and stored in a light-proof container at 4 °C. DCPIP was dissolved in 96% ethanol to give a 20 mM solution, filter-sterilized, and stored at 4 °C.

Cultivation Conditions. *Z. bailii* was prepared in a medium containing the following compounds: D-glucose, 21.5 g/L; yeast extracts, 3 g/L; peptone, 5 g/L; KH_2PO_4, 4 g/L; Na_2HPO_4, 4 g/L; pH 5.5. The media were sterilized by autoclaving at 120 °C for 15 min, and glucose was autoclaved separately at 110 °C for 10 min. After sterilization and cooling, solutions were mixed to form a complete medium prior to inoculation. A 250 mL Erlenmeyer flask containing 50 mL of medium was inoculated from a fresh agar plant and inoculated at 30 °C on a rotary shaker at 250 rpm. Growth proceeded overnight for a minimum of 9 h and a maximum of 13 h to allow cell growth to stationary phase, after which the broth was centrifuged at 10000*g* for 10 min and the cell pellet was washed twice with phosphate buffer and resuspended in phosphate-buffered saline solution. The cells were adjusted to the desired optical density measured at 600 nm (OD_{600} = 9.0).

Determination of Cell Optical Density. All spectrophotometric measurements were performed using a Shimadzu UV-1601 UV−bis spectrophotometer controlled by UV-1601 PC V.3 software (Shimadzu Corp., Kyoto, Japan). A measured aliquot of the cell suspension was centrifuged at 10000 rpm for 10 min. The supernatant was removed, and the pellet was reconstituted by the addition of 1.5 mL of distilled water. Sample dilution factors were chosen such that the measured optical density (600 nm) was between 0.1 and 0.6 unit, using water as a reference.

Incubation of Cells with Mediator(s) and Substrates. A total volume of 20.0 mL of incubation suspension was prepared for each trial. The standard incubation suspension comprised 12 mL of cell suspension (final OD_{600} = 6.0), 3.0 mL of ferricyanide solution (final concentration = 45 mM), 4.0 mL of glucose solution (final concentration = 3.0 g/L), and 100 μL of menadione or DCPIP solution (final concentration = 100 μM), and required amounts of acetic or benzoic acid were then added to the cell suspensions. Cell suspensions were adjusted to pH 4.5 with 2 mol/L HCl, and the final volume of cell suspensions was adjusted to 20 mL with sterile distilled water. Incubation of cells with mediators and substrate was for 2 h at 30 °C on a rotary shaker under oxygen-free nitrogen sparging. At the completion of incubation, the cells were pelleted by centrifugation (10000 rpm, 4 °C, 15 min), and the supernatants were adjusted to pH 7.0 with 2 M NaOH and then taken for analysis. Unless stated otherwise, all trials were performed in duplicate.

Analytical Methods. Steady-state voltammetry was conducted using an electrochemical working station (CHI 900) (CHI Corp., Austin, TX) controlled by CHI 900 software. Steady-state voltammograms were obtained at a scan rate of 10 mV s^{-1} scanning from 500 to 100 mV versus Ag/AgCl reference electrode. A 15 μm diameter Pt disk working electrode (CHI Corp.) was used as the working electrode, and a Pt gauze auxiliary electrode was used to complete the three-electrode electrochemical cell. The microelectrode was pretreated by polishing with a 0.05 μm alumina/water slurry on a flocked twill polishing cloth (Leco Corp., St. Joseph, MI) and then cleaned with pure water in an ultrasonic bath. 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. The standard deviations are shown as error bars on the plotted data.

RESULTS AND DISCUSSION

Voltammetric Measurements of Menadione- or DCPIP-Mediated Reduction of Ferricyanide. The use of voltammetric microelectrodes has been shown to be a simple, reliable, and rapid method for determining the concentration of electroactive species such as ferrocyanide (*19*); diffusion limiting currents (quantitatively related to concentration) can be established in the second time scale with virtually zero destruction of analyte. In this work, ferrocyanide accumulations arising from menadione- and DCPIP-mediated reduction of ferricyanide indicate the intracellular redox activity and the electron flux through the electron transport chain, respectively, within *Z. bailii* cells. Before incubation, the mediators were fully in their oxidized form; voltammogram measurements showed only cathodic currents due to reduction of ferricyanide (**Figure 2**). After incubation for 2 h with cell suspensions, the voltammogram has shifted down the current axis and there is both cathodic current (arising from the reduction of ferricyanide) and anodic current (arising from the oxidation of ferrocyanide) (**Figure 2**). The cathodic current in the 100−200 mV region decreased as some of the ferricyanide had been microbially converted to ferrocyanide. At the same time, the anodic current in the 400−

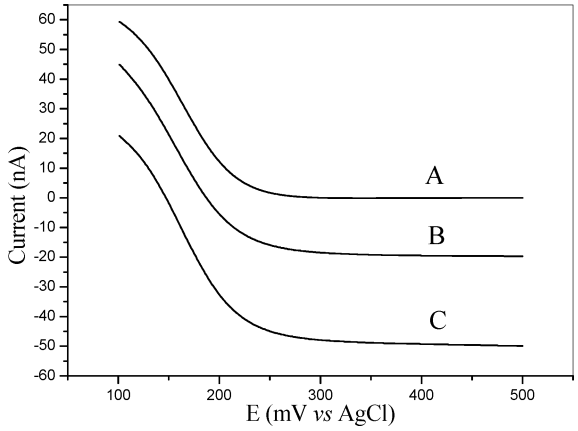

Figure 2. Steady-state voltammograms of 45 mM ferricyanide, 3 g/L glucose, and 100 μM menadione or 100 μM DCPIP in phosphate-buffered saline (curve **A**) and of the solutions after a 2 h incubation of *Z. bailii* suspensions with 100 μM menadione (curve **C**) or 100 μM DCPIP (curve **B**), 45 mM ferricyanide, and 3 g/L glucose in phosphate-buffered saline.

500 mV regions increased, representing the diffusion limiting current for the electrochemical oxidation of microbially produced ferrocyanide. The steady-state anodic plateau current measured at $E = 500$ mV was used as a relative measure of the amount of ferrocyanide produced. The lipophilic mediators menadione and DCPIP have $E°$ values (−81 and 5 mV versus Ag/AgCl, respectively) significantly more negative than that of ferricyanide (113 mV versus Ag/AgCl) and hence will not interfere with the ferricyanide voltammogram. In fact, at the concentrations used in this work, meaningful voltammograms could not be obtained for either menadione or DCPIP. **Figure 2** shows the steady-state voltammograms of the supernatant obtained after a 2 h incubation of *Z. bailii* suspensions with menadione/ferricyanide mediator system or DCPIP/ferricyanide mediator system. The limiting oxidative currents of ferrocyanide arising from menadione- and DCPIP-mediated reduction of ferricyanide were 49.9 ± 3.49 and 19.7 ± 0.98 nA, respectively.

Effect of Menadione and DCPIP Concentrations on the Signal Detected in the Double-Mediator System. Steady-state voltammetry of the supernatant after incubation indicates the amounts of reduced and oxidized ferricyanide. Thus, monitoring the concentration of this mediator to ensure oversupply is straightforward. However, the optimal concentration of the lipophilic mediator can be determined only indirectly. Fresh *Z. bailii* cells were incubated with several concentrations of menadione in the presence of excess (45 mM) ferricyanide, and the results are presented in **Figure 3A**. The limiting current values were independent of menadione concentration above 70 μM but decreased (as expected) as the menadione concentration decreased. **Figure 3B** shows how the limiting current response varied with DCPIP concentrations in the presence of 45 mM ferricyanide. The limiting current increased linearly at lower DCPIP concentrations before leveling off at ~90 μM DCPIP. The concentrations selected for both menadione and DCPIP were 100 μM, and the concentration selected for ferricyanide is 45 mM in the following experiments.

Inhibitory Effects of Weak Acids on *Z. bailii*. In this paper, the inhibitory effects of weak acids on *Z. bailii* were assessed indirectly through the intracellular redox activity or the mitochondrial electron transport inhibition by the presence or absence of increasing concentrations of acetic acid or benzoic acid in *Z. bailii* suspensions. The oxidative limiting currents of the ferrocyanide accumulations arising from menadione-mediated reduction of ferricyanide were designated R_M, and the oxidative limiting currents of ferrocyanide accumulations arising from DCPIP-mediated reduction of ferricyanide were designated R_D. **Figure 4** shows a plot of relative R_M against acetic acid concentrations present in *Z. bailii* suspensions. The relative R_M values decreased steadily in the presence of increasing concentrations of acetic acid, which indicates the steadily lowered intracellular redox activity by the presence of increasing concentrations of acetic acid in *Z. bailii* suspensions. Some previous studies have reported that the activities of some glycolytic enzymes of *Saccharomyces cerevisiae* ethanol-producing yeast) was inhibited by acetic acid, and enolase was the enzyme most severely affected among the glycolytic enzymes (*20*, *21*). The inhibition on some glycolytic enzymes of *Z. bailii* might account for the decrease in intracellular redox activity, which further led to the decrease in the relative R_M values. **Figure 4** also shows how the R_D values varied with acetic acid concentrations in *Z. bailii* suspensions. The curve for the relative R_D values showed two distinct trend tendencies. Initially, the relative R_D values increased rapidly, before reaching the maximum value at ~40 mM acetic acid. This was followed by an almost linear decrease. Uncoupling theory has frequently been proposed to interpret the inhibitory effect of weak acid. According to the theory, the drop in intracellular pH resulting from inflow of weak acid is neutralized by the action of the plasma membrane ATPase, which pumps protons out of the cell at the expense of ATP hydrolysis. To maintain the intracellular pH, additional ATP must be generated, which should be achieved by increasing the respiratory activity. However, ATP production cannot be raised infinitely; at high acid concentrations the proton pumping capacity of the cell is exhausted, resulting in depletion of the proton motive force and the death of living cells (*22*, *23*). With regard to present work, at lower acetic acid concentrations (<40 mM acetic acid), the inflow of acetic acid caused a minimal drop in intracellular pH, which was tolerable for the yeast strain to maintain its normal activity, and in response to the drop in intracellular pH, the yeast would increase ATP production by enhancing respiration, which was reflected by the enhanced fluctuations in relative R_D values. Previous studies have reported that any compounds that destroy the proton motive force would stimulate respiration (*24*). The increase in respiratory activity of yeast cell upon addition of lower concentrations of weak acid has been few reported in the previous works based on respiration rate (*25*, *26*), the reason for which might be the lesser precision of the test apparatus. Further increase in acetic acid concentrations led to significant decrease in glycolytic activities and depletion of the proton motive force, and also acidification of the cytoplasm finally. At higher acetic acid concentrations (>40 mM), the values in both relative R_M and relative R_D decreased steadily and reduced by 70% in both cases by the presence of 160 mM acetic acid. Interestingly, the acetic acid concentrations responsible for 50% inhibition of both relative R_M and relative R_D ($C_{50\%}$) are comparative and comparable to the previous results obtained from respiration inhibition ($C_{50\%}$) (*25*). **Figure 5** shows the inhibitory effects of benzoic acid on the relative R_M and the relative R_D values of *Z. bailii*, which followed the similar spectra depicted in **Figure 4** except that the initial stimulation magnitude in the relative R_D values was not so evident as that in case of acetic acid. Benzoic acid was more effective than acetic aid with respect to their inhibitory effect on *Z. bailii*, which was consistent with the previous reports based on respiration inhibition (*25*). Practical operations in our laboratory have found that the lowest concentrations of acetic acid and benzoic acid to keep the apple juice intact from the spoilage of *Z. bailii* cells

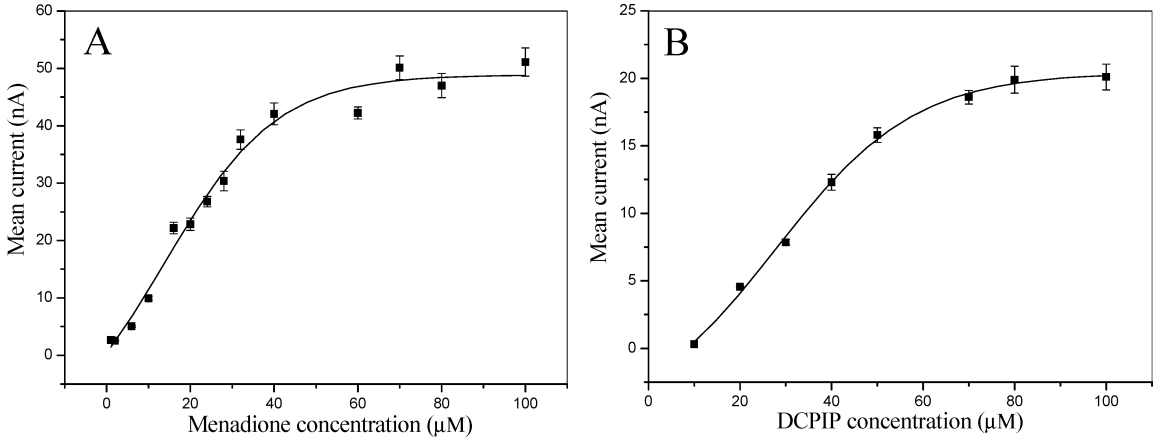

Figure 3. Effects of menadione (**A**) and DCPIP (**B**) concentrations on the electrochemical response detected after a 2 h incubation of fresh *Z. bailii* cells with 45 mM ferricyanide and 3 g/L glucose. Incubation and voltammetric measurement conditions are identical to those in **Figure 2**, except the lipophilic mediator concentrations. Each experiment was performed in duplicate, and each sample was analyzed in triplicate. The data are the mean ± SD from triplicate analyses in duplicate experiments.

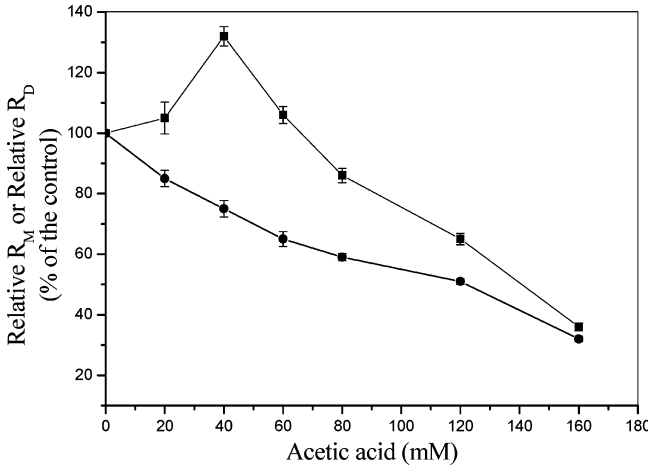

Figure 4. Toxic effects of acetic acid on the relative R_M (●) and relative R_D (■) of *Z. bailii* cells. Measurements were undertaken after a 2 h incubation by the presence or absence of increasing concentrations of acetic acid. Incubation and voltammetric measurement conditions are identical to those depicted in **Figure 2**. Each experiment was performed in duplicate, and each sample was analyzed in triplicate. The data are the mean ± SD from triplicate analyses in duplicate experiments.

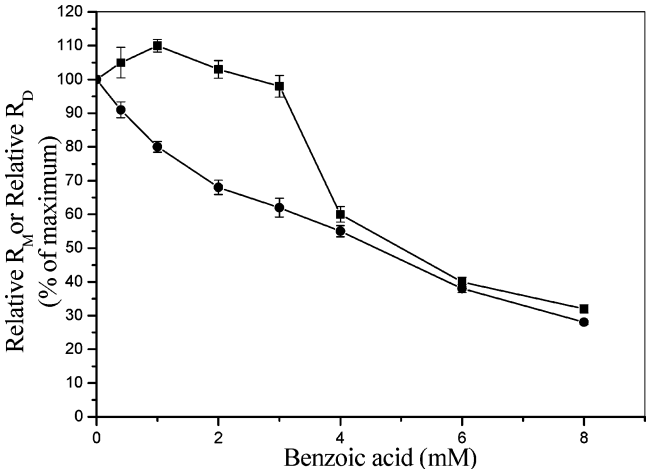

Figure 5. Toxic effect of benzoic acid on the relative R_M (●) and relative R_D (■) of *Z. bailii* cells. Measurements were undertaken after a 2 h incubation by the presence or absence of increasing concentrations of benzoic acid. Incubation and voltammetric measurement conditions are identical to those in **Figure 2**. Each experiment was performed in duplicate, and each sample was analyzed in triplicate. The data are the mean ± SD from triplicate analyses in duplicate experiments.

(under pH 4.5 and 30 °C) for a week were 200 and 10 mM, respectively. Under the above weak acid concentrations, both the relative R_M and the relative R_D values of *Z. bailii* suspensions treated as in **Figures 4** and **5** were approaching zero (data note shown). To establish levels for the utilization of preservatives in the food industry, the lowest dose levels of weak acid should be the concentration levels at which both the relative R_M and the relative R_D values approach zero. Further effort should be made to optimize the mediated electrochemical method and to compare the method with the conventional method based on respiration inhibition.

Conclusion. Coupling with the joint utilization two dual-mediator systems, menadione/ferricyanide and DCPIP/ferricyanide, an electrochemical method has been used for toxic evaluation of weak acids on *Z. bailii*. The variance in electrochemical response by the absence and presence of weak acid was used to indicate the inhibitory effects of weak acid on the yeast. The results discussed in this paper showed that the electrochemical method could reveal some intracellular response of yeast cells to weak acid inhibition, which cannot be completely accessible to the conventional method based on growth or respiration. The mediated electrochemical method provides us with an adjunct to the conventional method to establish levels for the utilization of preservatives in the food industry with the advantage of rapid and simple operation.

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