Ann Microbiol (2013) 63:495–502
DOI 10.1007/s13213-012-0494-8

ORIGINAL ARTICLE

Fungicidal mechanism of chlorine dioxide on *Saccharomyces cerevisiae*

Chuanhe Zhu · Zhao Chen · Guoyong Yu

Received: 17 October 2011 / Accepted: 28 May 2012 / Published online: 14 June 2012

Abstract The fungicidal mechanism of chlorine dioxide on *Saccharomyces cerevisiae* was investigated. During *S. cerevisiae* inactivation by ClO_2, protein, DNA, and ion leakage, enzyme activity, genomic DNA structure, and cell ultrastructure were examined. Protein and DNA leakages were not observed, while ion leakages of K^+, Ca^{2+}, and Mg^{2+} were detected and were related to the inactivation rate. The glucose-6-phosphate dehydrogenase, citrate synthase, and phosphofructokinase activities were inhibited and were also correlated with the inactivation rate. Genomic DNA structure was not damaged except for an extremely high ClO_2 concentration (100 mg L^{-1}). Electron micrographs showed that cell surface damage was pronounced and disruption in inner cell components was also apparent. The ion leakage, the inhibition of key enzyme activities of metabolic pathway, and the alteration of cell structure were critical events in *S. cerevisiae* inactivation by ClO_2.

Keywords Chlorine dioxide · Fungicidal · Mechanism · *Saccharomyces cerevisiae* · Inactivation

C. Zhu (✉) · G. Yu
College of Food Science and Engineering,
Shandong Agricultural University,
Daizong Street 61,
Taian, Shandong 271018, People's Republic of China
e-mail: chhzhu@sdau.edu.cn

Z. Chen
College of Life Sciences, Shandong Agricultural University,
Daizong Street 61,
Taian, Shandong 271018, People's Republic of China

Introduction

Chlorine dioxide (ClO_2) is a powerful sanitizing agent that has broad and high germicidal activity. It is more stable and has a higher oxidizing capability than chlorine. (Chen et al. 2010; Li and Xia 2006). ClO_2 has fungicidal, bactericidal and viricidal properties. Numerous studies have been conducted to prove the highly effective inactivation of a wide variety of microorganisms by ClO_2 (Gómez-López et al. 2009). ClO_2 has broadly been applied in the fields of health and quarantine, medical sterilization, agricultural and industrial sterilization, food preservation, etc. It is legally permitted in China and USA for sanitizing fruit and vegetables in water and recommended by both the World Health Organization. (Ministry of Health of the People's Republic of China 2008; USFDA 2010).The relative reports indicated that the resistance of the different groups of microorganisms towards ClO_2 generally increased in the order Gram-negative bacteria, Gram-positive bacteria, yeasts and mould spores and *Bacillus cereus* spores (Vandekinderen et al 2009). *Salmonella* spp., *Escherichia coli* O157:H7 and *Listeria monocytogenes* inoculated onto cabbage, carrot, lettuce, strawberry and melon were effectively reduced using the 4-5 mg L^{-1} chlorine dioxide (Tomás-Callejas et al 2012). The treatment of 8.0 mg L^{-1} ClO_2 for 120 min were effective to reduce yeast and mould populations of 2.06-2.32, 4.07-4.16 and 2.56 log CFU g^{-1} for blueberries, strawberries and raspberries were found, respectively (Gómez-López et al. 2009). Currently, it is internationally accepted as a disinfectant and food preservative with a high level of performance and safety. However, the uncertainty of the germicidal mechanism of chlorine dioxide has restricted its application in a more extensive field.

Some authors have attempted to elucidate the biocidal mechanisms of ClO_2 and have concluded that ClO_2 could cause series of damage, such as content leakage (Zhang et al. 2007; Wei et al. 2008), protein and nucleic acid denaturation (Hauchman et al. 1986; Noss et al. 1986; Li et al. 2004; Cho et al. 2010; Simonet and Gantzer 2006), and morphological alteration (Chen et al. 2002; Wang et al. 2010). However, there is still a lack of understanding on the major target site of ClO_2 interaction with microorganisms. Moreover, most of the published studies deal with inactivation mechanisms of ClO_2 on bacteria and viruses. The inactivation mechanisms would strongly depend on the type of microorganisms (Min et al 2010). *Saccharomyces cerevisiae* was used as a representative microorganism. It is associated with a variety of food decaying and is being applied in industry units in which ethanol is produced. The objective of this research was thus to investigate the fungicidal mechanism of ClO_2 on *Saccharomyces cerevisiae*. Comprehensive examinations, including protein, DNA, and ion leakage, key enzyme activity of metabolic pathway, genomic DNA structure, and cell ultrastructure, were performed to study whether they were correlated with the inactivation rate.

Materials and methods

Strain and growth condition

Saccharomyces cerevisiae 31482 was obtained from China Center of Industrial Culture Collection. The strain was cultured in yeast extract peptone dextrose (YPD) broth at 30 °C for 14 h (exponential phase) and harvested by centrifugation at 3000×*g* for 10 min at 4 °C. The pellets were then washed three times and resuspended in phosphate buffered saline (50 mmol L^{-1}, pH 6.0) and adjusted to 1×10^8 CFU mL^{-1}

Reagent preparation

A commercially available brand of stabilized ClO_2 powder (Charmstar, Tianjin Charmstar Technology Development Co., Ltd., Tianjin, PR China) was dissolved in deionized water to obtain ClO_2 solutions. The ClO_2 concentration was further measured by a standard method using iodimetry immediately before use (APHA 1998). $Na_2S_2O_3$ solution (5 g L^{-1}) was used as a neutralizing solution to end the sterilization reaction.

ClO_2 treatment

In concentration-dependent inactivation, 5 mL of cell suspensions was treated with equal volumes of ClO_2 with final concentration of 1, 2, 4, 5, 10, 20, 50, and 100 mg L^{-1} ClO_2 for 1 min; in time-dependent inactivation, 5 mL of cell suspensions was treated with equal volumes of ClO_2 with final concentration of 4 mg L^{-1} for 1, 5, 10, 15, 20, 25, 30, and 60 min. Our preliminary experiment proved that the ClO_2 concentration were constant during all treatments. Immediately after treatments, 5 mL of cell suspensions was transferred to a 1 mL of $Na_2S_2O_3$ solution to instantaneously quench the residual ClO_2. These solutions were used for subsequent assays. The sterile deionized water-treated sample was used as the control.

Determination of inactivation rate

Suspensions were spread on malt-extract agar (MEA) to determine the inactivation rates. Colonies were counted and results expressed as log CFU mL^{-1} after incubation at 30 °C for 3 d. The minimal fungicidal ClO_2 concentration and treatment time were defined as the minimal fungicidal ClO_2 concentration and treatment time used to produce 99.9 % inactivation rate, respectively.

Measurement of ClO_2 on content leakage

The samples were extracted and centrifuged at 3000×*g* for 10 min at 4 °C. The supernatants were collected for determination of content leakage. The protein content was measured at 595 nm by the Bradford method using bovine serum albumin as the standard (Bradford 1976) and DNA at 280 nm using bovine thymus DNA as the standard. The ion (K^+, Ca^{2+}, and Mg^{2+}) contents were detected by ICP-MS (VISTA-PRO, Varian, Varian Medical Systems, Inc., Palo Alto, CA, USA).

Measurement of ClO_2 on enzyme activity

The samples were centrifuged at 3000×*g* for 10 min at 4 °C and the pellets suspended in 5 mL of 0.05 mol L^{-1} Tris-HCl buffer (pH 7.5). Suspended cells were then disrupted by submitting them to a vortex for 5 min, in the presence of 5 g of glass beads (0.5 mm diameter). The disruption period was 1 min separated by 30 s interval in an ice bath. Cell debris and glass beads were then removed by centrifugation for 10 min (3000×*g*, 4 °C) and the supernatant was used for determinations of enzyme activity.

The glucose-6-phosphate dehydrogenase (G6PD) activity was determined by spectrophotometry at 30 °C and 340 nm using $NADP^+$ as cofactor (Gurpilhares et al. 2006). G6PD activity was determined in a medium composed by: 500 μL Tris-HCl buffer (0.05 mol L^{-1}, pH 7.5), 100 μL $MgCl_2$ (0.035 mol L^{-1}), 5 μl $NADP^+$ (0.131 mol L^{-1}), 10 μL G6P (0.5 mol L^{-1}) and 100 μL of sample. The citrate synthase (CS) activity was determined at 30 °C and 412 nm using 520 μL buffer (50 mmol L^{-1} Tris-HCl containing 100

mmol L^{-1} KCl and 1 mmol L^{-1} EDTA, pH 7.5), 20 μL DTNB, 20 μL acetyl coenzyme A (2.5 mmol L^{-1} in distilled water), 20 μL oxaloacetate (5.0 mmol L^{-1} in distilled water), and 20 μL of the sample (Lemos et al. 2003). The phosphofructokinase (PFK) activity was assayed spectrophotometrically by monitoring the changes in a cuvette compartment controlled at 30 °C and 340 nm (Gancedo and Gancedo 1971). The assay was carried out in 1 mL mixture of the following composition: 0.05 mol L^{-1} Tris-HCl buffer, pH 7.5, 0.05 mmol L^{-1} ATP, 5 mmol L^{-1} $MgCl_2$, 0.125 mmol L^{-1} NADH, 0.25 mmol L^{-1} F6P, 0.5 units aldolase, glycerophosphate dehydrogenase, and triosephosphate isomerase. One unit of each enzyme activity (U) was defined as the quantity of enzyme catalyzing the conversion of 1 μmol of substrate per minute under the assay condition used. Specific activity was expressed as units per milligram of total protein. Total protein concentration was determined by Bradford method using bovine serum albumin as the standard.

Analysis of ClO_2 on genomic DNA structure

Genomic DNA structures of untreated control, samples treated with 1, 10, and 100 mg L^{-1} ClO_2 for 1 min, and samples treated with 4 mg L^{-1} ClO_2 for 1, 20, and 60 min were examined. Genomic DNA of *S. cerevisiae* was isolated as previously described by Clemons et al. (2010). *S. cerevisiae* cells were collected by centrifugation for 10 min (3000×*g*, 4 °C), washed in 1 mol L^{-1} sorbitol, and protoplasts generated using yeast lytic enzyme. Protoplasts were washed by centrifugation and lysed using 10 % sodium dodecyl sulfate. DNA was recovered by sequential precipitation with 95 % ethanol and 70 % isopropanol. RNA was removed by adding RNase. Pellets of genomic DNA were solubilized in TE (10 mmol L^{-1} Tris, 1 mmol L^{-1} EDTA, pH 7.4). Genomic DNA was separated by electrophoresis through a 0.8 % agarose gel and stained with ethidium bromide. DNA band was then visualized by UV transillumination.

Microscopic analysis of ClO_2 on cell ultrastructure

Cell ultrastructures of control, samples treated with 10 mg L^{-1} ClO_2 for 1 min, and samples treated with 4 mg L^{-1} ClO_2 for 20 min were examined and photographed using a transmission electron microscope (JEM-1200EX, JEOL, JEOL Ltd., Tokyo, Japan).

Statistical analysis

All experiments were carried out in triplicate. SigmaPlot 11.1 (Systat Software Inc., San Jose, CA, USA) were used for data analysis. Analysis of variance (ANOVA) was carried out to determine whether significant differences ($P<0.05$) existed.

Results

Inactivation rate

According to Fig. 1, the inactivation rate of *S. cerevisiae* increased gradually at the range of 0 to 10 mg L^{-1} ClO_2 and 0 to 20 min. And when ClO_2 concentration reached 10 mg L^{-1} and treatment time extended to 20 min, the rate was 99.9 %. Thereafter, the inactivation rate remained at 100 %. The minimal fungicidal ClO_2 concentration and treatment time were 10 mg L^{-1} and 20 min, respectively.

The influence of ClO_2 on content leakage

Protein and DNA leakages were not observed in our study. Therefore, no detectable protein or DNA leaked out, irrespective of ClO_2 concentration and treatment time. However, ion leakages, though at very low levels, were detected

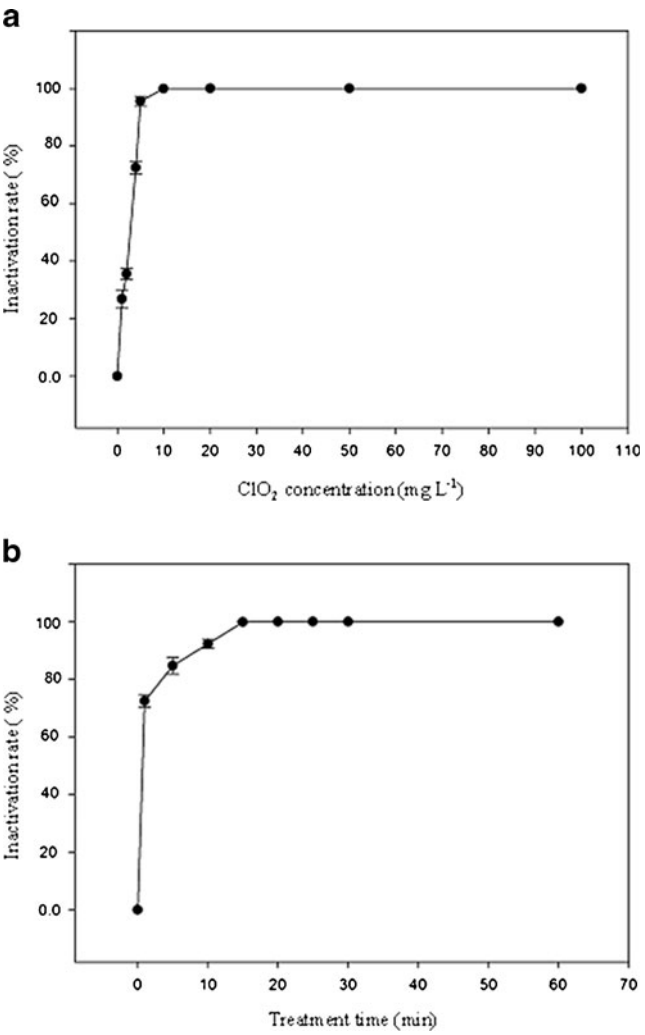

Fig. 1 The inactivation rate of ClO_2 with different concentration (**a**) and treatment time (**b**) on *S. cerevisiae*

(Fig. 2). The leakages of K^+, Ca^{2+}, and Mg^{2+} showed gradual increases before 99.9 % of the cells were killed. Thereafter, even though ClO_2 concentration and treatment time continued to increase, no significant increases in ion leakage could be measured. The analysis of correlation indicated the inactivation rate of *S. cerevisae* treated by chlorine dioxide with different concentrations had positive correlation with K^+ ion leakages (R^2=0.893), Ca^{2+} ion leakages (R^2=0.887), and Mg^{2+}ion leakages (R^2=0.951). The inactivation rate of *S. cerevisae* treated by chlorine dioxide with different treatment time showed positive correlations with K^+ ion leakages (R^2 =0.870), Ca^{2+} ion leakages (R^2=0.933), and Mg^{2+}ion leakages (R^2=0.845).

The influence of ClO_2 on enzyme activity

As are shown in Fig. 3, ClO_2 effectively inhibited G6PD, CS, and PFK activities of *S. cerevisiae*. Initially, increases in the

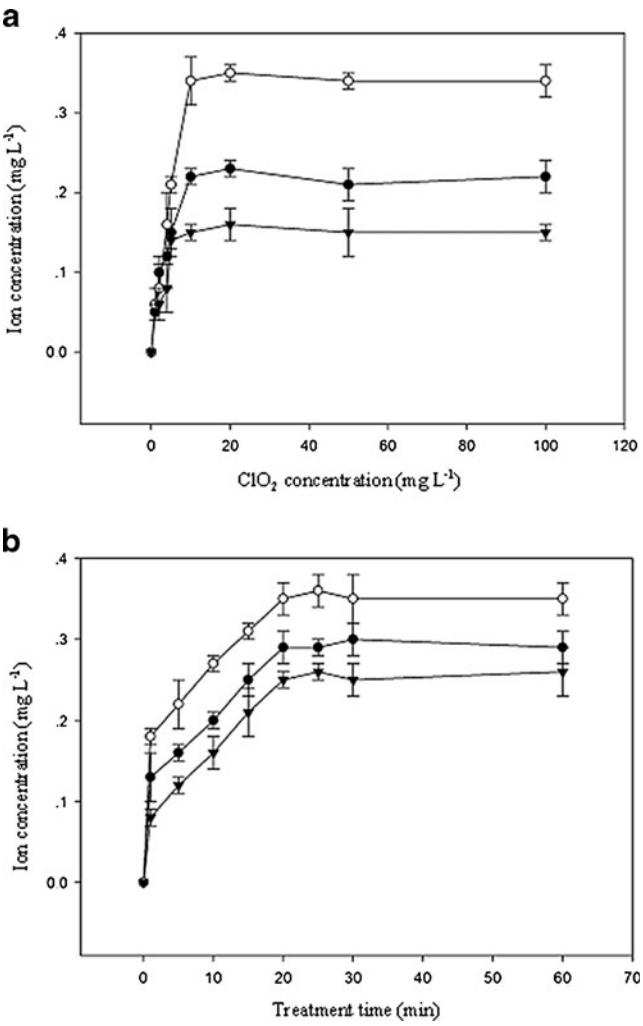

Fig. 2 The influence of ClO_2 with different concentration (**a**) and treatment time (**b**) ion leakage of *S. cerevisiae*. (▼), K^+; (○), Ca^{2+}; (●), Mg^{2+}

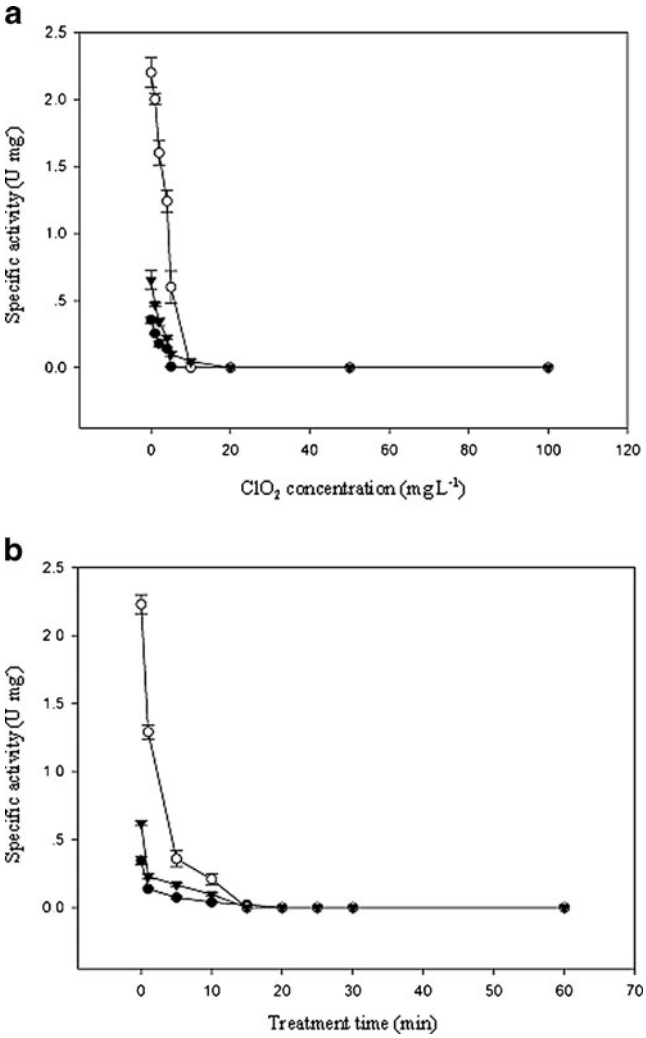

Fig. 3 The influence of ClO_2 with different concentration (**a**) and treatment time (**b**) on G6PD (●), CS (○), and PFK (▼) activity of *S. cerevisiae*

ClO_2 concentration and treatment time produced a more dramatic decrease in enzyme activity ($P<0.05$). The G6PD, CS, and PFK activities were completely inhibited when 99.9 % inactivation rate was obtained. After enzyme activities decreased to unmeasurable levels, they were kept being inhibited. The analysis of correlation indicated the inactivation rate of *S. cerevisae* treated by chlorine dioxide with different concentrations had positive correlation with the G6PD, CS, and PFK activities, the coefficient of correlation was 0.971, 0.958, 0.911 respectively. The inactivation rate of *S. cerevisae* treated by chlorine dioxide with different treatment time showed significant positive correlations with the activities of G6PD (R^2 =0.983), CS (R^2=0.990), and PFK (R^2=0.961).

The influence of ClO_2 on genomic DNA structure

Figure 4 showed there were no significant differences between genomic DNA extracted from *S. cerevisae* treated

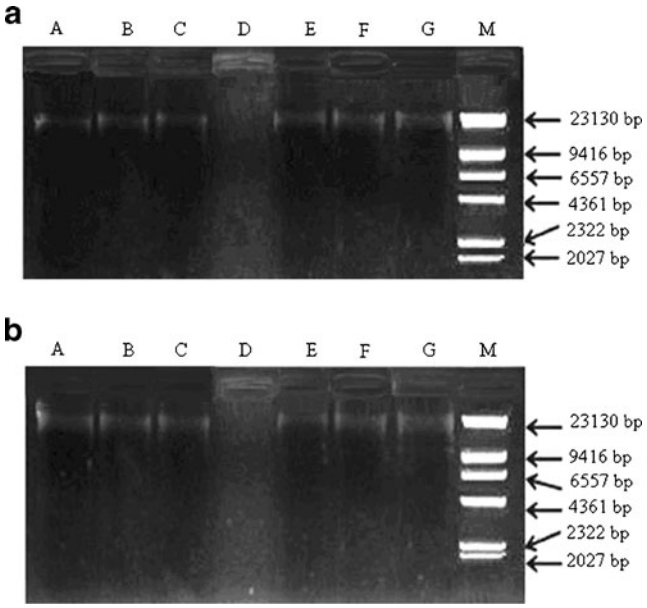

Fig. 4 The influence of ClO_2 with different concentration and treatment time on genomic DNA of *S. cerevisiae*. **a**: Genomic DNA of *S. cerevisae* directly treated by chlorine dioxide; **b**: Genomic DNA extracted from *S. cerevisae* treated by chlorine dioxide. A: untreated control; B: 1 mg L^{-1} ClO_2 for 1 min; C: 10 mg L^{-1} ClO_2 for 1 min; D: 100 mg L^{-1} ClO_2 for 1 min; E: 4 mg L^{-1} ClO_2 for 1 min; F: 4 mg L^{-1} ClO_2 for 15 min; G: 4 mg L^{-1} ClO_2 for 60 min; M: DNA marker

by chlorine dioxide genomic DNA of *F. tricinctum* spores directly untreated by chlorine dioxide, irrespective of ClO_2 concentration and treatment time. The entire genomic DNA of *S. cerevisiae* exposed to ClO_2 treatments showed similar band patterns with the untreated control, except for the samples treated with 100 mg L^{-1} ClO_2 for 1 min, showing no measurable bands (Fig. 4a). Obviously, the band was not absent even when the inactivation rate reached 99.9 %. Only when the ClO_2 concentration was extremely high (100 mg L^{-1}), the treatment could cause damage to the genomic DNA structure. And similarly, the extension of treatment time did not significantly influence the genomic DNA.

The influence of ClO_2 on cell ultrastructure

The normal *S. cerevisiae* cell showed a very typical fungal structure (Fig. 5a), which had a clear observation of plasma membrane and intracellular cytoarchitecture. With time and concentration respectively, the membrane structure became gradually dissolved, cytoplasm was more pycnotic, and organelles (eg. endoplasmic reticulum, nucleus) could gradually not be identified (Fig. 5 and Fig. 6). After the treatments of 10 mg L^{-1} ClO_2 for

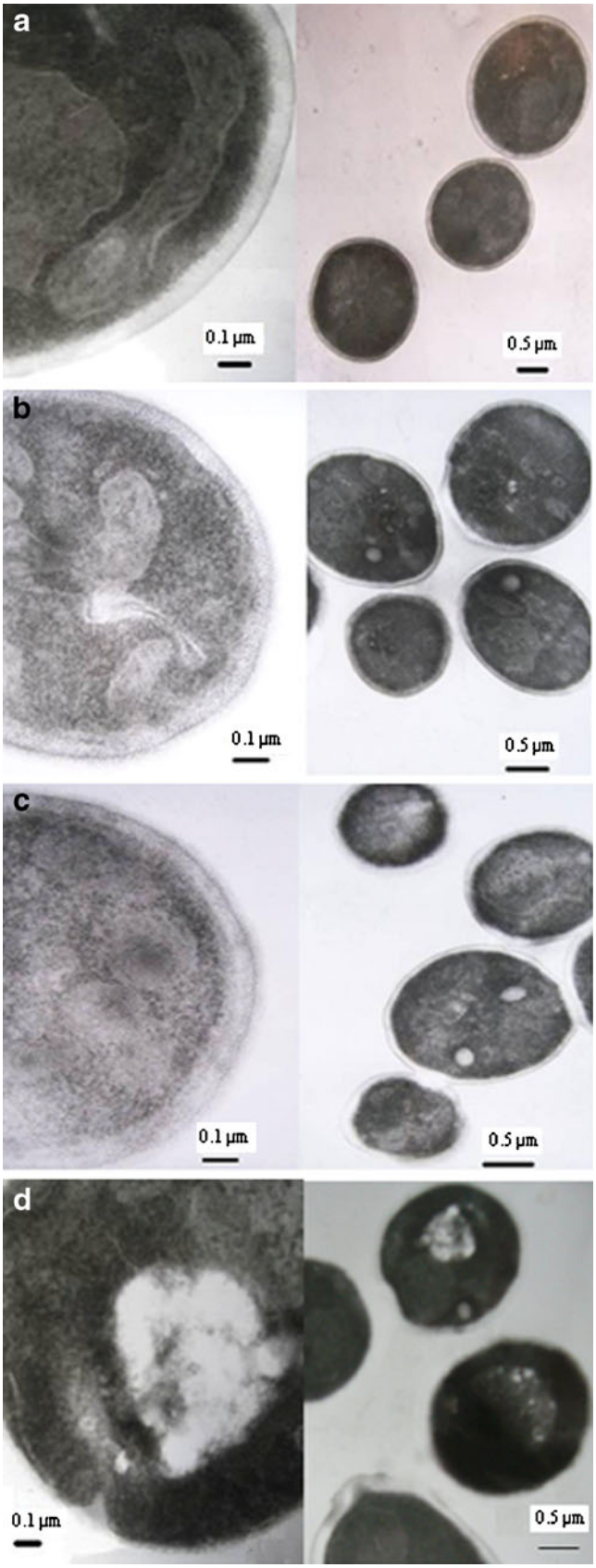

Fig. 5 The influence of ClO_2 with different treatment concentration on cell structure of *S. cerevisiae.* **a** Normal cell of *S. cerevisae* **b**: 1 mg L^{-1} chlorine dioxide for 1 min; **c**: 5 mg L^{-1} chlorine dioxide for 1 min; **d**: 10 mg L^{-1} chlorine dioxide for 1 min

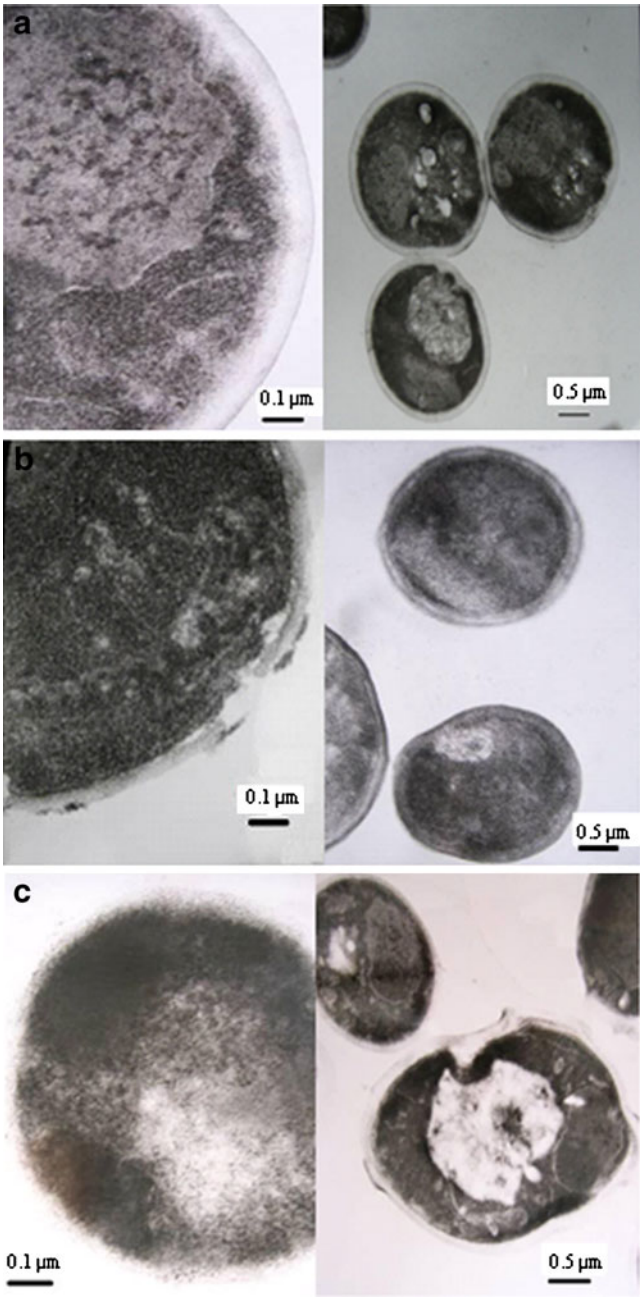

Fig. 6 The influence of ClO_2 with different treatment concentration on cell structure of *S. cerevisiae.* **a**: 4 mg L^{-1} chlorine dioxide for 1 min; **b**: 4 mg L^{-1} chlorine dioxide for 5 min; **c**: 4 mg L^{-1} chlorine dioxide for 15 min

1 min and 4 mg L^{-1} ClO_2 for 20 min (99.9 % of the cells were inactivated), the membrane structure became unclear, the cell wall be destroyed, cytoplasm was turbid and appeared agglutinate phenomenon, and it was difficult to identify organelles (eg. endoplasmic reticulum, nucleus) (Fig. 5 and Fig. 6). From the Fig. 5 and Fig. 6, serious damages on inner cell components, such as visible holes in the cytoplasm, were also observed.

Discussion

In this study, no protein or DNA leakages were detected, irrespective of ClO_2 concentration (1 to 100 mg L^{-1}) and treatment time (1 to 60 min). Even though the integrity of plasma membrane of *S. cerevisiae* was, to some extent, damaged, considering that protein and DNA are all macromolecules with large size, they might not leak out of the cell easily. In despite of no detectable protein or DNA leakage, we could not immediately conclude that cell membranes were not permeated by ClO_2, since ions, including K^+, Ca^{2+}, and Mg^{2+}, showed different levels of leakage. The levels of ion leakage stabilized after inactivation rate reached 99.9 %, showing that the ion leakage was at its maximum, which was consistent with the results of Wei et al. (2008) and Wang et al. (2010). Noticeably, K^+, Ca^{2+}, and Mg^{2+} are stabilizers of the cell wall and the cell membrane, and K^+ is even essential for cell membrane permeability (Talaro and Talaro 2002). The leakage of these ions might thus result in more severe membrane disruption and more ion leakage. Wang et al. (2010) found that the content of K^+, Ca^{2+}, and Mg^{2+} was lost immediately under the treatment of ClO_2 at 50 mg L^{-1} for 1 min. However, they also indicated that a large amount of cell protein and DNA of the spores leaked out in a short time under the treatment of 50 mg mLL^{-1} ClO_2. This difference may attribute to the different biological responses of plasma membrane to ClO_2 treatment depending on the type of organism. Therefore, it is rather necessary to make investigation on biocidal mechanisms of ClO_2 on various species of organisms.

G6PD catalyzes the oxidation of glucose-6-phosphate to form 6-phosphoglucono-δ-lactone in the pentose phosphate pathway (PPP), which produces NADPH. If G6PD was inhibited, PPP could not supply reducing equivalents for NADPH synthesis and pentoses for DNA and RNA synthesis. The first reaction of tricarboxylic acid (TCA) cycle is the condensation of acetyl-CoA with oxaloacetate to form citrate catalyzed by CS. If CS was inhibited, cell could not burn the acetyl-CoA made from fat, glucose, or protein to provide ATP in cooperation with oxidative phosphorylation. PFK catalyzes the transfer of a phosphoryl group from ATP to fructose-6-phosphate to yield fructose-1,6-bisphosphate. If PFK was inhibited, cell could not convert glucose to ATP and pyruvate (pyruvate can be burned for energy in TCA or converted to fat in fatty acid synthesis) (Nelson and Cox 2004). In this study, G6PD, CS, and PFK activities of *S. cerevisiae* were all significantly inhibited by ClO_2. Hence, all related biosynthetic reactions would be blocked. Moreover, the activities decreased more rapidly, as within a certain range ClO_2 concentration increased and treatment time prolonged. Cho et al. (2010) reported that β-$_D$-galactosidase in *Escherichia coli* could be degraded by ClO_2 and the reaction between ClO_2 and β-$_D$-galactosidase was nearly

instantaneous. And in the study of Wei et al. (2008), Na^+-K^+-ATPase or Ca^{2+}-Mg^{2+}-ATPase activities were significantly decreased. They indicated that as ClO_2 concentration increased, the ATPase activity decreased more rapidly, which was consistent with our results. Based on what has been reported by Navalon et al. (2009), some amino acids could be degraded to simple, small carbon chain products by ClO_2. Accordingly, considering that enzyme (as a protein) did not leak out of the cells, ClO_2 may penetrate into the cells and attack functional amino acid residues on an enzyme that are essential for the enzyme's activity and trigger a change in its structure. If an enzyme undergoes changes in structures, its catalytic activity will be destroyed (Nelson and Cox 2004). Hence, it is possible to consider that ClO_2 has the ability to exhibit inhibition effects on enzyme activity. Noticeably, the inhibition of enzyme activity by ClO_2 was related to the inactivation rate. When nearly all of the *S. cerevisiae* cells were dead, the G6PD, CS, and PFK were simultaneously inactivated. Therefore, we can speculate that ClO_2 penetrated into cells and broke down the structures of several enzymes, which had deleterious effects on some critical microbial metabolic processes. And interestingly, the inhibition of enzyme activity seemed to be related to the metal ion loss. It is well known that some enzymes require an additional chemical component such as metal ion called a cofactor for activity. For example, Mg^{2+} is the cofactor of both G6PD and PFK. If the metal ion that is tightly or even covalently bound to the enzyme protein is disengaged, the enzyme activity can be largely influenced. However, in the metabolic reaction catalyzed by CS, there is no participation of metal ion. Thus, there was only a connection between the leakage of metal ion and the inhibition of some enzymes' activities, but it should not be concluded that the ion loss can cause more severe inhibition. These two biological disorders in the ClO_2-treated *S. cerevisiae* cells occurred in two individual pathways, although some possible synergistic effects might exist between them.

Damage of genomic DNA by ClO_2 was initially sluggish, happening only when ClO_2 concentration reached 100 mg L^{-1}. Moreover, prolonging the treatment time did not have any marked influences on the genomic DNA structure. And most importantly, the damage caused by ClO_2 was not correlated with the inactivation rate. The fact that only extremely high ClO_2 concentration gave rise to the disintegration of genomic DNA indicated that this might be the result of damage accumulation. Thus, damage of genomic DNA could not be considered as a critical factor for *S. cerevisiae* inactivation. Our results were in consistency with Zhang et al. (2007), who reported that the ClO_2 treatment could not damage the genomic DNA of *Escherichia coli* when ClO_2 concentration were lower than 100 mg L^{-1}. The degradation of DNA occurred only when ClO_2 concentration reached 500 mg L^{-1}. They concluded that this concentration was much higher compared with the minimal concentration needed to inactivate the cells and was thus beyond the scope of mechanism study.

According to transmission electron microscopy, ClO_2 could not only destroy the cell surface but also diffused into *S. cerevisiae* cells to damage the intracellular structures when 99.9 % of the cells were inactivated. The results obtained in this study suggest that as a sort of strong oxidant, ClO_2 could still penetrate into the cells to act upon various inner components after damaging certain cell surface structures. And our assay on the activities of intracellular enzymes, which was largely inhibited by ClO_2, also proved this point. According to some literatures, ClO_2 gave rise to various levels of morphological alteration for different species. Our observations were in contrast with Wei et al. (2008) when making study on inactivation mechanism of ClO_2 on *Candida albicans*. They observed no obvious damages on the plasma membranes and inner structures, and the cell wall was intact, although nearly all the cells were inactivated after treatment with 15 mmol L^{-1} of ClO_2 for 10 min. Nonetheless, they reported that the cytoplasm appeared a little 'rougher' and the cell wall became less dense. Our results agreed with Cho et al. (2010) who found that the *Escherichia coli* cells inactivated by ClO_2 showed partial damages both in cell surfaces and inner components. The differences among reports may also be explained by the different responses of microbial cells to ClO_2 treatment.

In conclusion, the protein and DNA leakage, and the damage of genomic DNA structure were not correlated with inactivation rate of *S. cerevisiae* in comparison with bacteria. our research suggest that the inactivation mechanism of *S. cerevisiae* by ClO_2 is related to the leakage of K^+, Ca^{2+}, and Mg^{2+}, the inhibition of enzyme activities of metabolic pathway, and the disruption of the cell integrity.

Acknowledgements This research was supported by Department of Science and Technology of Shandong Province, China (2007BS06022).

References

American Public Health Association (APHA) (1998) Standard methods for the examination of water and waste water. American Public Health Association, Washington, D. C

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgam quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248–254

Chen C, Li D, Liu X, Bao L, Liu Z (2002) Experimental observation on mechanism of bactericidal effect of chlorine dioxide. Chinese J Disinfect 19:137–141

Chen Z, Zhu C, Zhang Y, Niu D, Du J (2010) Effects of aqueous chlorine dioxide treatment on enzymatic browning and shelf-life of fresh-cut asparagus lettuce (Lactuca sativa L.). Postharvest Biol Technol 58:232–238

Cho M, Kim J, Kim JY, Yoon J, Kim J (2010) Mechanisms of Escherichia coli inactivation by several disinfectants. Water Res 44:3410–3418

Clemons KV, Salonen JH, Issakainen J, Nikoskelainen J, McCullough MJ, Jorge JJ, Stevens DA (2010) Molecular epidemiology of Saccharomyces cerevisiae in an immunocompromised host unit. Diagn Microbiol Infec Dis 68:220–227

Gancedo JM, Gancedo C (1971) Fructose-1,6-diphosphatase, phosphofructokinase and ghcose-6-phosphate dehydrogenase from fermenting and non fermenting yeasts. Arch Microbiol 76:132–138

Gómez-López VM, Rajkovic A, Ragaert P, Smigic N, Devlieghere F (2009) Chlorine dioxide for minimally processed produce preservation: a review. Trends Food Sci Tech 20:7–26

Gurpilhares DB, Hasmann FA, Pessoa JA, Roberto IC (2006) Optimization of glucose-6-phosphate dehydrogenase releasing from Candida guilliermondii by disruption with glass beads. Enzyme Microb Technol 39:591–595

Hauchman FS, Noss CI, Olivieri VP (1986) Chlorine dioxide reactivity with nucleic acids. Water Res 20:357–361

Lemos D, Salomon M, Gomes V, Phan VN, Buchholz F (2003) Citrate synthase and pyruvate kinase activities during early life Stages of the shrimp Farfantepenaeus paulensis (Crustacea, Decapoda, Penaeidae): effects of development and temperature. Comp Biochem Phys B 135:707–719

Li GP, Xia HL (2006) Review on the application of chlorine dioxide in food sterilization and disinfection [J]. Food Sci Technol 9:21–25

Li JW, Xin ZT, Wang XW, Zheng JL, Chao FH (2004) Mechanisms of inactivation of hepatitis a virus in water by chlorine dioxide. Water Res 38:1514–1519

Min C, Jaeeun K, Kim JY, Jeyong Y, Kim JH (2010) Mechanisms of Escherichia coli inactivation by several disinfectants [J]. Water Res 44:3410–3418

Ministry of Health of the People's Republic of China (2008) Hygienic Standards for Use of Food Additives.

Navalon S, Alvar M, Garcia H (2009) Chlorine dioxide reaction with selected amino acids in water. J Hazard Mater 164:1089–1097

Nelson DL, Cox MM (2004) Enzymes. In Lehninger Principles of Biochemistry, 4th edn. W. H. Freeman & Co, New York, pp 190–237

Noss CI, Hauchman FS, Olivieri VP (1986) Chlorine dioxide reactivity with proteins. Water Res 20:351–356

Simonet J, Gantzer C (2006) Degradation of the Poliovirus 1 genome by chlorine dioxide. J Appl Microbiol 100:862–870

Talaro KP, Talaro A (2002) From Atoms to Cells: A Chemical Connection In Foundations in Microbiology, 4th edn. McGraw-Hill Co, New York, pp 26–57

Tomás-Callejas A, López-Gálvez F, Adrian S, Artés F, Artés-Hernández F, Trevor VS (2012) Chlorine dioxide and chlorine effectiveness to prevent Escherichia coli O157:H7 and Salmonella cross-contamination on fresh-cut Red Chard [J]. Food Control 23:325–332

USFDA (United States Food and Drug Administration) (2010) Secondary direct food additives permitted in food for human consumption. 21 CFR. Sec. 173. 300 Chlorine dioxide.

Vandekinderen I, Devlieghere F, Van Camp J, Kerkaert B, Cucu T, Ragaert P, De Bruyne J, De Meulenaer B (2009) Effects of food composition on the inactivation of foodborne microorganisms by chlorine dioxide [J]. Int J Food Microbiol 131:138–144

Wang Z, Liao F, Lin J, Li W, Zhong Y, Tan P, Huang Z (2010) Inactivation and mechanisms of chlorine dioxide on Nosema bombycis. J Invertebr Pathol 104:134–139

Wei MK, Wu QP, Huang Q, Wu JL, Zhang JM (2008) Plasma membrane damage to Candida albicans caused by chlorine dioxide (ClO_2). Lett Appl Microbiol 47:67–73

Zhang X, Wu Q, Zhang J, Wu H, Que S (2007) Study on mechanism of effect of chlorine dioxide on Escherichia coli. Chinese J Disinfect 24:16–20