微生物学通报
Microbiology
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SEP 20, 2008, 35(9): 1385~1392

研究报告

Effects of Oxidative Challenge on Defensive Enzyme and Cofactors Level in *Alkalibacterium* sp. F26

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Abstract: In this paper, a slight halophilic alkaliphile strain, *Alkalibacterium* sp. F26, which produced high level of intracellular CAT observed in previous research, was selected as a model microbial to explain the responses of this bacterium to oxidative stress. The results indicated that *Alkalibacterium* sp. F26 had obvious responses to higher concentration (>1 mmol/L) of H_2O_2 than that to lower H_2O_2 (<1 mmol/L) challenge from the aspects of defensive enzyme synthesis and cofactors level variation. As for catalase production, the activity increased up to 106.54 U/mg protein which was 1.76 fold of the control when cells were challenged by 3 mmol/L H_2O_2, but its activity only was 1.13 fold when H_2O_2 was 100 μmol/L. As far as energy state was concerned, ATP production and NAD^+ generation were significantly inhibited from 20.55 μmol/L to 17.80 μmol/L and 69.89 μmol/L to 31.77 μmol/L, respectively, leading to the drop of energy charge from 0.77 to 0.68 and the increase of the portion of NADH/NAD^+ from 0.08 to 0.41 in the former case. However, these effects were less distinct under lower concentration of H_2O_2. Except of the condition of 100 μmol/L H_2O_2, under which the activation of defensive mechanism resulted in an increase of ATP, the level of ATP dropped from 22.69 μmol/L of the control to 22.38 μmol/L and 13.70 μmol/L when challenged by 50 μmol/L and 500 μmol/L H_2O_2. Besides, the concentration of NADH fluctuated and the NAD^+ gradually reduced when H_2O_2 below 1 mmol/L.
Keywords: Reactive oxygen species stress, Catalase (CAT), ATP, NADH, NAD^+

氧化胁迫对 *Alkalibacterium* sp. F26 防御酶和辅因子的影响

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摘 要: 将一株能够高产过氧化氢酶的低度嗜盐嗜碱菌 *Alkalibacterium* sp. F26 作为模式微生物, 采用高效液相色谱技术测定胞内代谢物浓度, 研究氧化胁迫对其防御酶活性和辅因子的影响。研究结果表明: 相比低浓度 H_2O_2 (<1 mmol/L)胁迫, 此菌株在高浓度 H_2O_2(>1 mmol/L)胁迫下的应答表现更为明显: 经 3 mmol/L H_2O_2 胁迫后胞内 CAT 酶活为 106.54 U/mg protein, 是对照产量的 1.76 倍; ATP 浓度则从对照浓度 20.55 μmol/L 下降到 17.80 μmol/L; NAD^+浓度自对照样品的 69.89 μmol/L 减少至 31.77 μmol/L。由于 ATP 和 NAD^+浓度的减少, 相比未经过 H_2O_2 胁迫菌体, 细胞能荷值 EC 从 0.77 降低至 0.68, NADH/NAD^+则从 0.08 增加至 0.41。然而, 这种应答机制在细胞受到低浓度 H_2O_2 的胁迫后并不明显: 除发现 100 μmol/L H_2O_2 能够导致细胞防御机制的激活而使胞内 ATP 浓度相比

Foundation item: This work was supported by the Key Project of China National Programs for Fundamental Research and Development (No. 2007CB714306); National Natural Science Foundation of China (No. 20676056)
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Received: January 17, 2008; **Accepted:** April 10, 2008

对照有所增加的情况外，经 50 μmol/L 和 500 μmol/L H_2O_2 胁迫后胞内 ATP 水平从对照的 22.69 μmol/L 只下降到 22.38 μmol/L 和 13.70 μmol/L；并且此种胁迫条件下 NADH 浓度变化也不显著。

关键词：活性氧胁迫，过氧化氢酶(CAT), ATP, NADH, NAD^+

1 Introduction

Reactive oxygen species (ROS) such as superoxide radical anion (O_2^-), hydroxyl radical (·OH), and hydrogen peroxide (H_2O_2), are generated through the stepwise reduction of O_2 to H_2O_2. Among of them, hydrogen peroxide (H_2O_2) is a highly reactive molecule, which is used as a challenge paradigm for studying cells' responsive and defensive strategies to ROS.

There is a large amount of research work concentrated on the direct defensive mechanisms of microbiology to ROS. With the oxidative damage by menadione, more catalase (CAT), and superoxide dismutase (SOD) were produced in *Alkalibacterium* sp. F26[1,2]. On the other hand, the influences of ROS on the injury mechanism to cells, such as cellular protein, DNA, lipid, et al. were also careful investigated[3,4]. Furthermore, John D Helmann et al[5] used global transcriptional profiling to monitor the magnitude and kinetics of changes in *Bacillus subtilis* mRNA population after expose to hydrogen peroxide, and exhibited the induction of genes under the control of three regulons, such as PerR, σ^B and OhrR. In fact, the forms and level of cofactors, such as ATP, ADP, AMP, NADH, NAD^+, NADPH, $NADP^+$, are key physiological parameters. Cells use cofactors to regulate flux through various metabolic pathways to protect themselves from different stress. However, few reports are available on the effects of oxidative stress on the forms and level of cofactors.

The aim of the present study was to investigate if H_2O_2 stress can manipulate the intracellular cofactors level. For this, the effects of different H_2O_2 concentration on the catalase production and the cofactors level of *Alkalibacterium* sp. F26 were careful investigated. The results indicated that *Alkalibacterium* sp. F26 had obvious responses to higher concentration (>1 mmol/L) of H_2O_2 than that to lower H_2O_2 (<1 mmol/L) challenge from the aspects of defensive enzyme synthesis and cofactors level variation.

2 Materials and methods

2.1 Microorganism

Alkalibacterium sp. F26, a slightly halophilic alkaliphile, was isolated from Haoji Soda Lake located in the Hulunbeir area of Inner Mongolia autonomous region, China.

2.2 Media

The strain was cultivated at 37°C on 2.0% agar slants containing (g/L): glucose 10, polypeptone 5, yeast extract 5, KH_2PO_4 1, $MgCl_2$ 0.2, NaCl 50 and Na_2CO_3 10.

The seed and fermentation medium contained (g/L): glucose, 10; beef extract, 10; corn liquid, 10 and yeast extract, 5. The composition and concentration of inorganic salt were the same as the slant medium mentioned above.

The initial pH of all media was adjusted to 9.20.

2.3 Cultivation

The seed culture inoculated from a slant was cultivated in a 500 mL flask containing 50 mL seed medium on a reciprocal shaker for 20 h. Fermentations was carried out in a 500 mL flasks containing 50 mL fermentation medium. The inoculum's size was 5% (*V*/*V*). The flask cultures were grown for 18 h and the rotation rate was controlled at 200 r/min. All experiments were done in triplicate. All cultivations were carried out at 37°C.

2.4 Challenging cells by different concentration of H_2O_2

The cells of *Alkalibacterium* sp. F26 were cultured for 18 h (exponential phase). A solution of H_2O_2 was added to the culture (the culture contained l mmol/L, 2 mmol/L, 3 mmol/L, 50 μmol/L, 100 μmol/L, 500 μmol/L H_2O_2 after oxidant addition), while the culture without oxidant was set as control.

2.5 Analytic methods

Dry cell weight (DCW) was determined after

drying the cells at 105°C to a constant weight. Glucose was measured by dinitrosalicylic acid method. Catalase activity was determined spectrophotometrically by monitoring the decrease in absorption at 240 nm caused by the disappearance of H_2O_2 at 30°C[6]. The R^2 of the calibration curve in the calculation of CAT activity is 0.998. One unit of enzyme activity is defined as the amount of catalase decomposing 1 μmol H_2O_2 per min. All the data are expressed as the means of three independent experiments. The confidence interval was calculated by a given confidence coefficient 95%. The amount of protein in the cell extract was measured using the Coomassie blue method with Bovine serum albumin as standard [7].

2.6 Estimation of intracellular metabolites concentration

Intracellular metabolite concentrations were measured by using an in vitro procedure based on inactivation of metabolism followed by metabolite extraction directly in the cell sample.

80 mL (for ATP detection), 20 mL (for NADH detection), and 40 mL (for NAD^+ detection) cell samples were removed from the culture, frozen immediately in liquid nitrogen for 60 s, and stored at −20°C for following tests.

The cells for ATP detection were harvested by centrifugation at 2000×g for 10 min, then, were washed twice with distilled water. For extracting ATP, 10 mL of 0.6 mol/L $HClO_4$ was added to the cell pellets and mixed thoroughly with a magnetic stirrer for 10 min. The mixture was centrifuged for 10 min at 10000×g to collect the supernatant. Another 10 mL of 0.6 mol/L $HClO_4$ was added to the pellets, mixed thoroughly for 10 min, and the supernatant was collected after centrifugation. Two portions of the supernatant were combined in a 25 mL volumetric flask and made up to 25 mL with 0.6 mol/L $HClO_4$. Ten milliliter of the prepared solution was taken and the pH adjusted to 7.0 with 0.8 mol/L potassium hydroxide. After being kept at 4°C for 30 min, crystal $KClO_4$ was removed from the solution by filtration (pore size = 0.22 μm) and then diluted to 25 mL with phosphate buffer (pH 7.0) before HPLC column application. The injection volume for the HPLC analysis was 10 μL.

NADH and NAD^+ are acid and alkali labile, respectively. Therefore, measurement of both oxidized and reduced forms requires that extraction be carried out in separate samples[8].

For extracting the NADH, 20 mL cell sample were freeze-dried for 24 h and transferred to 20 mL solution containing 50 mmol/L KOH, 30% ethanol and 22 mmol/L borate (pH 10). Extracted samples were left on ice for 30 min before pH being adjusted to 9.0~9.4 with 3 mol/L HCl under vigorous agitation. After centrifugation at 4°C for 10 min at 10000×g, the supernatant was immediately tested for NADH without neutralizing to avoid destruction of NADH by locally too high concentrations of acid.

For extracting the NAD^+, 20 mL cell sample were freeze-dried for 24 h and transferred to 20 mL 70% (*V*/*V*) cold ethanol containing 10 mmol/L potassium phosphate buffer, pH 6.5, and extracted for 30 min at room temperature (25°C). During the extraction procedure, the pH was kept below 7.0 with 0.5 mol/L HCl under vigorous agitation to avoid NAD^+ degradation. After centrifugation for 10 min (4°C and 10000×g), the supernatant was immediately used for metabolite concentration measurements.

The concentrations of the intracellular metabolites were measured by HPLC (Agilent 1100 series, column stable-C18, Thermo electron corporation, MA, USA)[9]. Briefly, separation of different metabolites was obtained by forming a step gradient (adapted to the column length increase with respect to the original method) with two buffers of the following composition: buffer A, 100% methanol; buffer B, 0.1 mmol/L KH_2PO_4, pH 7.0. The gradient was: 10 min 100% buffer A; 3 min 90% buffer A; 10 min 70% buffer A; 12 min 55% buffer A; 15 min 45% buffer A; 10 min 25% buffer A; 5 min 0% buffer A. The flow rate throughout chromatographic runs was 1.0 ml/min, the column temperature was kept at a constant 35°C.

All values of metabolites concentrations were mean values of at least three independent extraction procedures.

3 Results

3.1 Responses of *Alkalibacterium* sp. F26 to high concentration of H_2O_2

3.1.1 Induction of CAT synthesis and the effects on cellular growth under high concentration of H_2O_2: To investigate whether *Alkalibacterium* sp. F26 can adapt to peroxides stress, the exponentially grown cells (18 h) were treated with 1 mmol/L, 2 mmol/L, 3 mmol/L H_2O_2. Previous work showed that such levels of H_2O_2 could greatly inspire the responses of *Alkalibacterium* sp. F26 to oxidative stress.

Fig. 1 depicted the influences of high concentration of H_2O_2 on the cell growth and CAT production. The CAT activity increased rapidly as expected due to the elevated concentration of H_2O_2, but the cellular growth was seriously affected under such conditions. The largest effects were seen after 3 mmol/L H_2O_2, the activity of CAT increased from 60.60 U/mg protein to 106.54 U/mg protein and the DCW reduction to 3.31 g/L occurred with this provision. The obvious increase of CAT activity and decrease of DCW indicated the most obvious extent of oxidative stress was achieved compared to other experimental conditions.

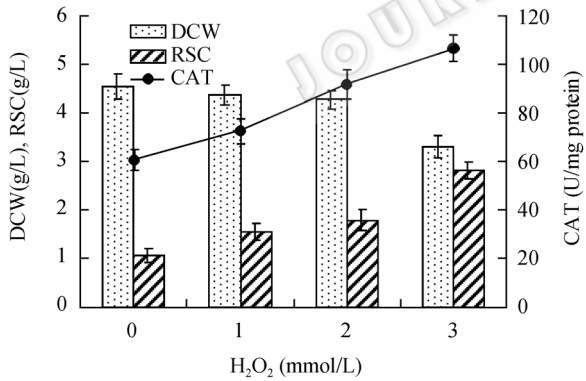

Fig. 1 Effects of high concentration of H_2O_2 on the cell growth and CAT production

3.1.2 Variations of energy metabolism under high concentration of H_2O_2: A similar physiological change was also observed in the case of intracellular ATP, ADP and ATP/ADP. As illustrated in Fig. 2, ATP was also significantly reduced after H_2O_2 challenge in a dose-dependent manner. The largest ATP-depletion was detectable after treatment with 3 mmol/L H_2O_2, and ADP, the degradation product of ATP, increased from 18.73 μmol/L in untreated cells to 33.24 μmol/L under such conditions.

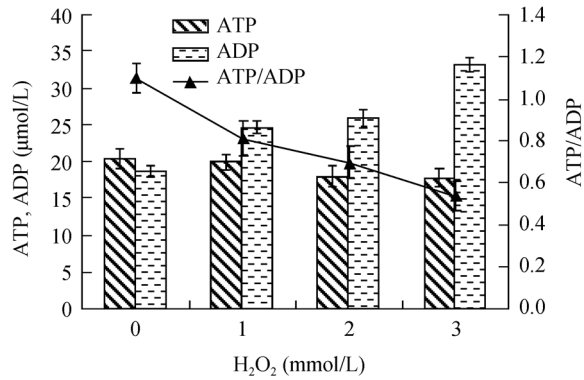

Fig. 2 Effects of high concentration of H_2O_2 on the cell energy metabolism

Energy charge (EC), the extent of phosphorylation and/or dephosphorylation, may be calculated by the formula: [(ATP + 0.5 ADP)/(ATP + ADP + AMP)][10]. In our study, H_2O_2 concentration of 1 mmol/L led to a decreased EC level from 0.77 of the control condition to 0.73, 2 mmol/L H_2O_2 to 0.71 and 3 mmol/L H_2O_2 to 0.68 (Fig. 3).

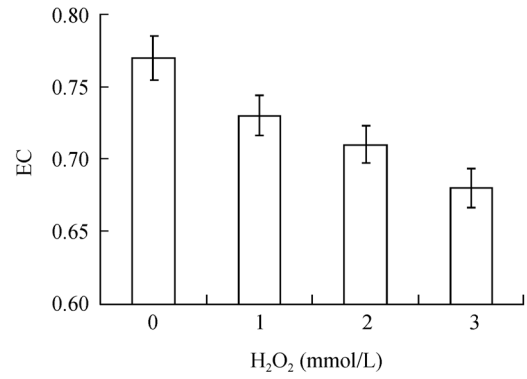

Fig. 3 Effects of high concentration of H_2O_2 on EC

3.1.3 Variations of coenzyme I under high concentration H_2O_2: Mitochondria contain two central water-soluble electron carrier systems, NADH/NAD^+ and NADPH/$NADP^+$, which transfer electrons from intermediary metabolites to electron transfer chain[11]. NAD^+ is one of the most used coenzymes in cellular metabolism, and NADH is its reduced form, which is a key member of the electron transfer chain in mito-

chondria. The effects of H_2O_2 concentration on the NADH and NAD^+ level were also investigated and the results were listed in Fig. 4. As shown in Fig. 4, the intracellular NAD^+ level decreased but the NADH concentration increased when *Alkalibacterium* sp. F26 was exposed to hydrogen peroxide. At the range of 1 mmol/L~3 mmol/L, the change of intracellular NADH and NAD^+ was also dose-dependent. The increasing NADH but decreasing NAD^+ resulted in the ratio of NADH/NAD^+ raised from 0.08 (without H_2O_2 addition) to 0.41 after treated with 3 mmol/L H_2O_2.

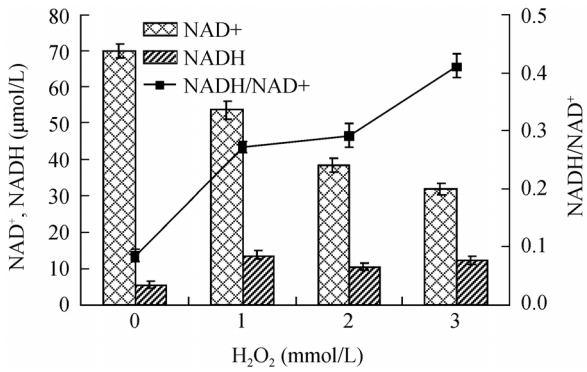

Fig. 4 Effects of high concentration of H_2O_2 on NADH and NAD^+

3.2 Responses of *Alkalibacterium* sp. F26 to low concentration of H_2O_2

3.2.1 Induction of CAT synthesis and the effects on cellular growth under low concentration of H_2O_2: Some researches showed that cells often had different responses to different extent of stress[12,13]. In the case of *Alkalibacterium* sp. F26, low-concentration of H_2O_2 didn't have such obvious inhibitive effects on the growth and the CAT production compared with the results of high challenge of H_2O_2. As shown in Fig. 5, the cellular growth was not significantly changed, and DCW only dropped from 4.85 g/L of the control to 4.13 g/L under the stress concentration of 500 μmol/L H_2O_2. Moreover, the remaining sugar concentration in fermentation solution was higher than control, which meant that low oxidative stress could weaken the rate of glucose consumption.

3.2.2 Variations of energy metabolism under low concentration of H_2O_2: Interestingly, as shown in Fig. 6, the intracellular ATP level increased from 22.69 μmol/L to 35.25 μmol/L, and the greatest ratio of ATP/ADP from 1.00 to 1.26, respectively, when low concentration (100 μmol/L H_2O_2) added to the fermentation broth. To explain this phenomenon, some researchers speculated that a disturbed energy metabolism rendered cells more susceptible to H_2O_2-induced oxidative stress[14]. From this point of view, the elevated ATP level after treatment with 100 μmol/L H_2O_2 might reflect an initial activation of intracellular, energy-dependent antioxidative pathway. This viewpoint could also explain the fact that the energy charge (EC) increased from the control level of 0.74 to 0.77 (Fig. 7), which reflected the degree of oxidative stress didn't have serious effects on the structure and the function of the respiration chain, and oxidative phosphorylation function was normal.

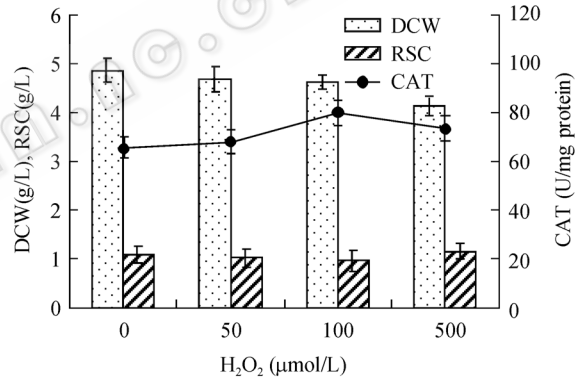

Fig. 5 Effects of low concentration of H_2O_2 on the cell growth and CAT production

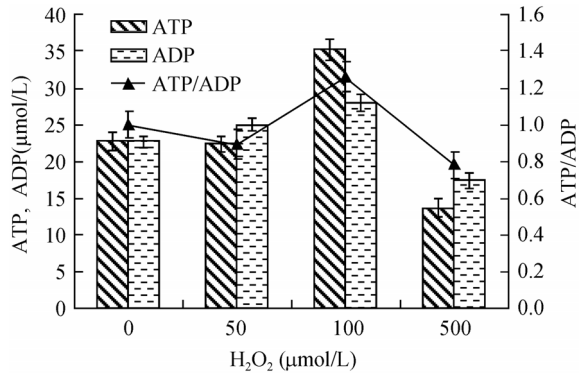

Fig. 6 Effects of low concentration of H_2O_2 on the cell energy metabolism

3.2.3 Variations of coenzyme I under low concentration of H_2O_2: It is well known that cells can syn-

thesis CAT to catalyze the conversion of H_2O_2 to O_2 and H_2O, and CAT production plays an important role in maintaining ROS at a normal level. If the amount of CAT synthesized by bacteria themselves was enough for the degradation of trace H_2O_2, cellular metabolism will not be disturbed. As far as *Alkalibacterium* sp. F26 is concerned, the level of 50 μmol/L H_2O_2 is far more below the extent of disturbing its metabolism. Therefore, the cellular coenzyme level was not significantly different just like the results of high stress: NADH production decreased with the increasing stress, and NAD^+ level didn't show a marked ascending trend and was in a state of fluctuation.

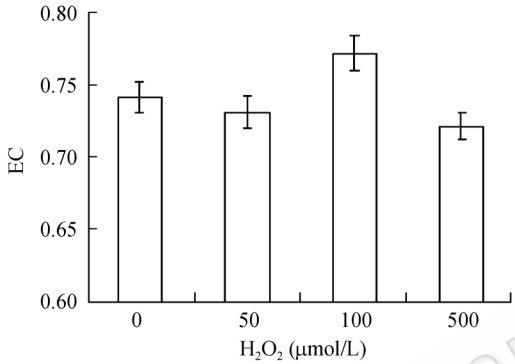

Fig. 7 Effects of low concentration of H_2O_2 on EC

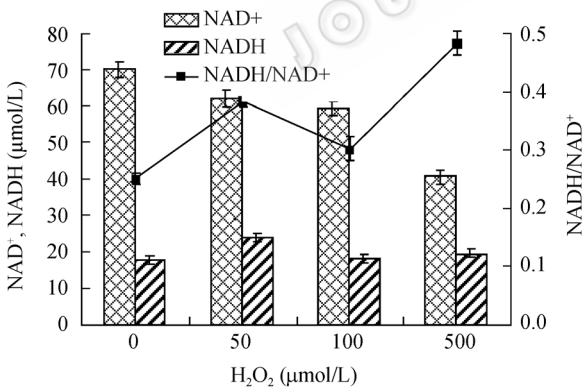

Fig. 8 Effects of low concentration of H_2O_2 on NADH and NAD^+

4 Discussion

Cellular overload with H_2O_2 induces oxidative stress, accompanied by lipid peroxidation, DNA and protein damage and some other adverse effects. The aim of the present study was to illustrate the variation of defensive enzyme production and cofactors level under H_2O_2 challenge. Taken together, when high H_2O_2 concentration was fed to the culture broth, a high catalase production, and high intracellular ADP and NADH concentration were detected, while the effects were not so obvious when exposed to low stress condition.

The CAT level increased from 60.6 U/mg protein (the control) to 106.5 U/mg protein with the H_2O_2 concentration rose from 0 mmol/L to 3 mmol/L in the culture broth. In the past researches, it has detected that superoxide radical (O_2^-), hydroxyl radical (·OH) and hydrogen peroxide (H_2O_2) were produced, when the stress chemicals, such as H_2O_2 was supplemented to the microbiology culture broth. The production of ROS induced more CAT synthesis to alleviate the toxicity of ROS[15]. For *E. coli* case, the resistance to oxidative stress was largely coordinated by OxyR and SoxRS, the former activated genes in response to either peroxide stress or change in the thiol-disulfide status. The later controlled the gene expression which in response to reactive free radicals, such as superoxide anion and nitric oxide[16,17]. Similarly, the catalase in *Bacillus subtilis* was also strongly induced by peroxides[18]. Therefore, as far as *Alkalibacterium* sp. F26 was concerned, for the existence and expression of relative genes, enhanced CAT-level exhibited under the induction of hydrogen peroxide.

The intracellular ATP and NAD^+ decreased 22% and 55%, respectively, under high H_2O_2 concentration challenge. There were two different reasons related to the ATP reduction, the first reason was that H_2O_2 destroyed the structure and the function of mitochondria, and impaired the F_0F_1-ATPase activity[18,19]. The second reason was that the microbial cells required a large amount of ATP to synthesis CAT, SOD, and some other antioxidants to overcome the bad effects caused by high ROS.

Nicotinamide adenine nucleotide (NAD^+) was one of the key cofactors in the metabolic networks, functions as a cofactor in over 300 oxidation-reduction reactions[20]. There were also two different mechanism involved in the reduction of NAD^+ and the accumula-

tion of NADH. The first mechanism was that H_2O_2 inhibited the NADH oxidation to NAD^+ through the electron transfer chain (ETC) by damaging the ETC activity. The other mechanism was that H_2O_2 could inhibit the specific activity of some key enzymes, such as glyceraldehydes-3-phosphate dehydrogenase (GADPH), aconitase, α-Ketoglutarate dehydrogenase (α-KGDH) and succinate dehydrogenase (SDH)[21,22]. Among of them, GADPH and α-KGDH were involved in both NADH and NAD^+ metabolism. Therefore, NADH couldn't be converted to NAD^+ and then brought the reduction of NAD^+. However, as we known, NADH and NADPH, the intracecellular reductants, play a key role in providing reducing equivalents in living cells to protect from oxidative damage[20]. From this point of view, the more NADH production might be benefit for cells to avoid bad influences of stress.

In this work, we had clarified that the changes in the enzyme synthesis and cofactors level (ATP, ADP, NADH, NAD^+) with H_2O_2 challenges. The results could increase our understanding to the oxidative stress on the bacteria physiology, especially on cellular cofactors level. Moreover, the results presented here are also of industrial importance, especially in the case that H_2O_2 stress could enhance the production catalase, an important industrial enzyme.

Acknowledgements

This work was supported by a grant from the Major State Basic Research Development Program of China (973 Program) (No. 2007CB714306) and the National Natural Science Foundation of China (No. 20676056). We also would like to thank Miss Jin Gao for her kind help in this study.

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征 稿 简 则

3.4 摘要写作注意事项

3.4.1 英文摘要:

1) 建议使用第一人称, 以此可区分研究结果是引用文献的还是作者的; 2) 建议用主动语态, 被动语态表达拖拉模糊, 尽量不用, 这样可以避免好多长句, 以求简单清晰; 3) 建议使用过去时态, 要求语法正确, 句子通顺; 4) 英文摘要的内容应与中文摘要一致, 但可比中文摘要更详尽, 写完后务必请英文较好、且专业知识强的专家审阅定稿后再返回编辑部。 5) 摘要中不要使用缩写语, 除非是人人皆知的, 如：DNA, ATP 等; 6) 在英文摘要中, 不要使用中文字体标点符号。

3.4.2 关键词：应明确、具体, 一些模糊、笼统的词语最好不用, 如基因、表达……

4 特别说明

4.1 关于测序类论文

凡涉及测定 DNA、RNA 或蛋白质序列的论文, 请先通过国际基因库 EMBL (欧洲)或 GenBank (美国)或 DDBJ(日本), 申请得到国际基因库登录号 (Accession No.)后再投来。

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