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ORIGINAL RESEARCH PAPER

EFFECTS OF CARBON SOURCES ON THE VALUES OF ANTIOXIDANT INDEXES AND INTERMEDIATES METABOLITES FROM *SERRATIA MARCESCES* SYBC08

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Abstract: The effects of two different carbon sources on changes of catalase (CAT) yield, the values of antioxidant indexes and intermediate metabolites from *Serratia marcescens* SYBC08 were investigated at different time points (4, 8, 12, 16, 20 and 24 h). The highest CAT yield (20008 $U \cdot mL^{-1}$) in presence of citric acid was approximately 1.46-fold higher than that in presence of glucose (13680 $U \cdot mL^{-1}$). The maximum superoxide dismutase activity (21.53 $U \cdot mL^{-1}$) was achieved in presence of citric acid, significantly higher than that in presence of glucose (15.20 $U \cdot mg^{-1}$protein). Intracellular alkyl hydroperoxide reductase activity in presence of citric acid or glucose was sharply increased during culture period and reached the maximum value at 24 h. Additionally, the NADH oxidase activities, total antioxidant capacities, intracellular and extracellular ascorbic acid contents, intracellular H_2O_2 level, malondialdehyde contents and intracellular contents of pyruvate, succinic acid and citric acid in presence of citric acid or glucose were also evaluated.

Keywords: *antioxidant indexes, carbon sources, intermediate metabolites, Serratia marcescens*

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INTRODUCTION

Serratia marcescens, which are ubiquitous in the environment found in soil and water as well as associated with plants, insects, humans, and other animals [1], is a species of gram-negative, oxidase-negative, facultatively anaerobic, indole and citrate-positive, rod-shaped mobile bacterium of the family *Enterobacteriaceae* [2, 3]. *S. marcescens* is regard as an important industrial strain which can use different carbon sources for growth and producing several kind of enzymes, such as catalase (CAT), lipase, L-asparaginase, chitinase, and so on [4 – 7]. In our previous studies, we found that *S. marcescens* SY08 isolated from sludge containing H_2O_2 produced high CAT yield cultured in presence of citric acid as sole carbon source [8, 9]. Under oxidative stress, CAT is one of important antioxidant substances that prevent the formation of highly reactive hydroxyl radical by catalyzing the decomposition of hydrogen peroxide (H_2O_2) into water and dioxygen by two-electron transfer. However, the change of other antioxidant substances with the variety of CAT yield was still not investigated in previous studies.

When microorganism use glucose as sole carbon source for growth under aerobic condition, glucose is converted into pyruvate, meanwhile NAD^+ is reduced to NADH by Embden-Meyerhof-Parnas pathway (EMP pathway), and then pyruvate is converted to acetyl CoA by many kind of enzymes. At finally, citric acid which is formed from the reaction of oxaloacetic acid and acetyl CoA enter into citrate cycle (TCA cycle) and generate NADH. When microorganism use citric acid as sole carbon source for growth under aerobic condition, citric acid can directly enters into TCA cycle and emerge NADH by TCA cycle. On the other hand, NADH oxidation gaining a great deal of energy may be achieved in part though the electron transport chain, but this activity can lead to the production of potentially hazardous oxygen reduction products, reactive oxygen intermediates (ROI) including O^{2-}, H_2O_2 and HO [10].

Antioxidant systems which include many enzymes like CAT, superoxide dismutase (SOD), glutathione peroxidase [11, 12] and nonenzymatic antioxidants such as ascorbic acid (VC) [13] are able to resist the damage caused by ROI. There were only several literatures about the effects of carbon sources on antioxidant systems in fungus [14 – 16], but few related reports involved in bacteria.

In the present studies, we systematically analysis the change on CAT yield, the values of antioxidant indexes and intermediate metabolites from *Serratia marcescens* SY08, and to clarify the effect of carbon sources on antioxidant systems and intermediate metabolites synthesis in bacteria.

MATERIALS AND METHODS

Strains and culture conditions

The strain used in the study was identified and designated as *S. marcescens* SYBC08 by 16SrDNA sequence (Genbank Accession no GU188473) and subsequently conserved in China General Microbiological Culture Collection Center (Preserved number CGMCC 3449) [9, 17]. Prior to use, the strain was stored frozen at –70 °C in 20 % glycerol stocks. For seed preparation, the microorganism was inoculated into 50 mL seed

medium (containing 20 g·L^{-1} glucose, 10 g·L^{-1} peptone, 5 g·L^{-1} beef extract, 5 g·L^{-1} NaCl and *p*H 7.2) in 250 mL flasks and cultivated at 30 °C on a rotary shaker at 200 rpm for 12 h. Seed with 4 % size of inoculation (V/V) was inoculated into 7 liters fermentor loading 7 liters fermentation medium (containing corn steep liquor powder 33.8 g·L^{-1}, glucose or citric acid 30 g·L^{-1} and initial *p*H 5.91). The culture condition was carried out at 32.8 °C with a speed at 400 rpm. The aeration rate was 1.5 V·V^{-1}·min^{-1}, and the *p*H was adjusted to 7.0 by addition of 5M HCl.

Preparing samples for the following measurement

A certain amount of fermentation broth was collected at intervals of time during 1-day cultivation, and then the fermentation broth was centrifuged at 10800 x *g* for 10 min to obtain the supernatant and bacterial sludge. The extracellular vitamin C content in the supernatant was measured according to following methods. The bacterial mud was washed by 50 mM Na_2HPO_4-NaH_2PO_4 buffer (*p*H 7.0) for two times, and then the bacterial mud was resuspended in cuvette by using a certain amount of buffer solution. The suspension was disrupted by ultrasonic treatment for 10 min, and then the debris was removed for collecting the supernatant by centrifugation at 10800 x *g* for 10 min. The supernatant was used in measuring the intracellular indexes.

Determination of antioxidant indexes

CAT activity was measured UV-2100 spectrophotometrically (UNICO (Shanghai) Instruments Co., Ltd.) by monitoring the decrease in absorbance at 240 nm caused by the decomposition of H_2O_2 [18]. The molar extinction coefficient of H_2O_2 at 240 nm was 43.6 mM·cm^{-1}. The reaction mixture contained suitable amount of enzyme solution, 30 mM H_2O_2, and 50 mM Na_2HPO_4-NaH_2PO_4 buffer (*p*H 7.0) in a total volume of 4 mL. The linear range of the reaction (30 s) was used to calculate the rate of the reaction, and one unit of CAT activity was defined as the amount of enzyme that required to transform 1 μmol of H_2O_2 to water and oxygen per minute [19]. SOD activity was determinate by the method of the reference [20]. The reaction system as blank control includes 4.5 mL of 0.05 mM Tris-HCl buffer (*p*H 8.2) containing 2 mM EDTA, 4.2 mL of deionized water and 0.3 mL of 10 mM HCl solution. The reaction system as contrasts include 4.5 mL of 0.05 mM Tris-HCl buffer (*p*H 8.2) containing 2 mM EDTA, 4.1 mL of deionized water, 0.3 mL of 10 mM HCl solution with 6 mM pyrogallol and 0.1 mL of enzyme liquid. The assay was carried out at wavelength of 325 nm for 30 s. One Unit was defined as 50 % inhibits of the oxidation rate, and SOD activity was present as U·mg^{-1} protein.

Alkyl hydroperoxide reductase (AhpC) was measured by the method which was described in the article of Mishra et al [21] and slightly modified in the study. The reaction system of 3 mL was composted of reaction liquid of 1.9 mL (50 mM Na_2HPO_4-NaH_2PO_4 buffer (*p*H 7.0) containing 15 mM DTT, 50 mM NaCl and 1.5 mM EDTA), reaction liquid of 1 mL (50 mM Na_2HPO_4-NaH_2PO_4 buffer (*p*H 7.0) containing 6 mM tert butyl hydroperoxide) and 100 μL enzyme solution. The rate of DTT oxidation was measured spectrophotometrically at 310 nm at room temperature, and the change rate of OD (0.001·min^{-1}) was defined as one unit. The enzyme activity was expressed as U·mg^{-1} protein.

NADH oxidase (NOX) activity was measured by the method of Werer et al [22] with slight modification. The reaction system consisted of 0.1 mM Na_2HPO_4-NaH_2PO_4 buffer (*p*H 7) and 0.1 mM NADH. The amount of the enzyme was added into the reaction system for starting the reaction at 30 °C, and the change of absorbance was measured at a wavelength of 340 nm. Unit of enzyme was calculated according to the method of Chen et al [23]. Enzyme activity was expressed as $U \cdot mg^{-1}$ protein.
The levels of total antioxidant capacity **(**T-AOC), malondialdehyde (MDA), H_2O_2 and vitamin C (VC) were determined using commercially available colorimetric assay kits according to the manufacturer's recommended protocol (Beyotime Biotechnology, China).

Determination of intracellular metabolic intermediates

The supernatant which freshly obtained by ultrasonic breaking was placed in boiling water for 2 min immediately, and intracellular concentration of citric acid, succinic acid and pyruvic acid was measured by high performance liquid chromatography (HP1100) that was equipped with chromatography column (ZORBAX SB C18 4.6X150m). Mobile phase (0.1 mM KH_2PO_4 buffer and 5 % methanol (*p*H 2.5)) had a flow rate of 1 $mL \cdot min^{-1}$. The column temperature was maintained at 25 °C and the injection volume was 50 μL of sample liquid. The detection wavelength was set at 210 nm.
The protein concentration was determined by the method according to Bradford [24] with bovine serum albumin as the standard.
All the reagents used were of analytical grade or HPLC purity.

The data analysis

The Tukey test, one of methods for multiple comparisons, was used for statistical significance analyses. The values are the mean of three separate experiments. The *P* values were 2-tailed and $P < 0.05$ was considered to be significant. The data were analyzed using SPSS 12.0 for Windows (SPSS Inc., Chicago, IL).

RESULTS AND DISCUSSION

The variety of CAT yield in *S. marcesces* SYBC08

CAT yield variation was measured during the 0-24 h of incubation and those results showed in Figure 1. The yield of CAT in presence of glucose as sole carbon source was increased with the prolonging of culture time and the highest yield (13680 $U \cdot mL^{-1}$) was obtained at 24 h. The CAT yield in presence of citric acid as sole carbon source increased very rapidly from 4 to 12h, and the enzyme yield was remained at about 20000 $U \cdot mL^{-1}$ from 12 h to 24 h. Those CAT production which the stain grew in presence of citric acids as sole carbon source was approximately 1.47-2.52 folds higher than those in presence of glucose as sole carbon source from 12 h to 24 h ($P < 0.05$).

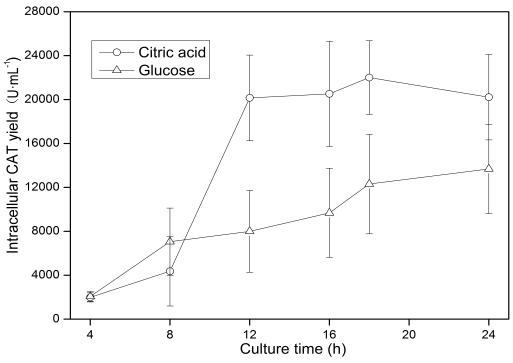

Figure 1. *The time curves of intracellular CAT yield of S. marcescens SYBC08*

The changes of SOD activity in *S. marcesces* SYBC08

The time change curve of SOD activity was assayed and shown in Figure 2. At the initial stage of fermentation, SOD activity in presence of citric acid or glucose as carbon source was not high level. The SOD activity in presence of glucose as sole carbon source was increased during two time periods (from 4 h to 8 h and from 8 h to 24 h) and the maximum SOD activity (15.20 U·mg^{-1} protein) reached at 24 h. With the prolonging of fermentation time, SOD activity in presence of citric acid as sole carbon source was increase first and then decrease, and the maximum SOD activity (21.53 U·mg^{-1} protein), which was significantly higher than 15.20 U·mg^{-1} protein ($P < 0.05$), reached at 8 h of culture time.

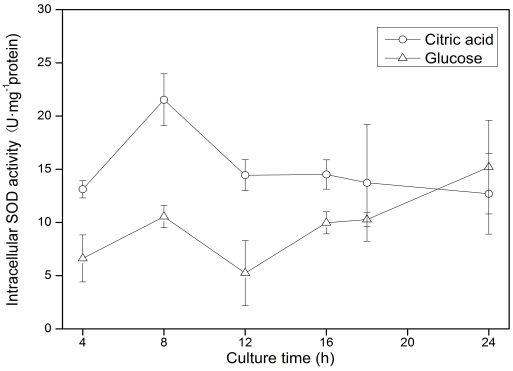

Figure 2*. The time curves of intracellular SOD activity of S. marcescens SYBC08*

The changes of intracellular AhpC activity from *S. marcesces* SYBC08

The AhpC activity was determined at different time, and the result was showed in Figure 3. The AhpC activity in presence of glucose or citric acid sharply increased during the whole cultural time and the highest AhpC activity in presence of citric acid as carbon source (9.19 U·mg^{-1} protein) reached at 24 h of culture time was significantly higher than that in presence of glucose as carbon source (the highest activity was 6.81 U·mg^{-1} protein) ($P < 0.05$).

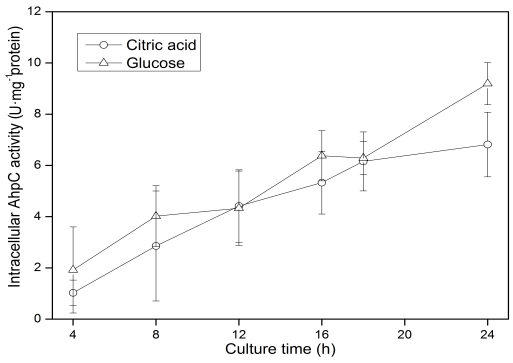

Figure 3. *The time curves of intracellular AhpC activity of S. marcescens SYBC08*

The changes of NOX activity of *S. marcesces* SYBC08

The intracellular NOX activity was measured, as shown in Figure 4. The NOX activities in presence of glucose as carbon source were higher than those in presence of citric acid at all assayed time points (except 8 h) (P<0.05). The NOX activity in presence of citric acid as carbon source was gradually increased from 4 h to 8 h, and the highest enzyme activity (202 U·mg^{-1} protein) was obtained at 8 h of culture time, and then NOX activity slightly decreased from 8 h to 24 h. NOX activity in presence of glucose as carbon source was gradually increased from 8 h to 16 h, and the highest NOX activity (322 U·mg^{-1} protein) was reached at the culture time of 16 h, and then the activity decreased from 16 h to 24 h.

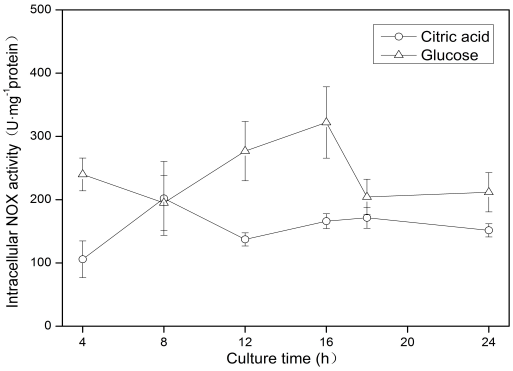

Figure 4. *The time curves of intracellular NOX activity of S. marcescens SYBC08*

The changes of intracellular T-AOC of *S. marcesces* SYBC08

The Figure 5 exhibited the T-AOC time-change curve of *S. marcesces* SYBC08. It was found that T-AOCs in presence of glucose as carbon source were significantly higher than those in presence of citric acid at 12 h and 24 h of fermentation time (P < 0.05). T-AOC in presence of glucose or citric acid was a little change during whole culture period.

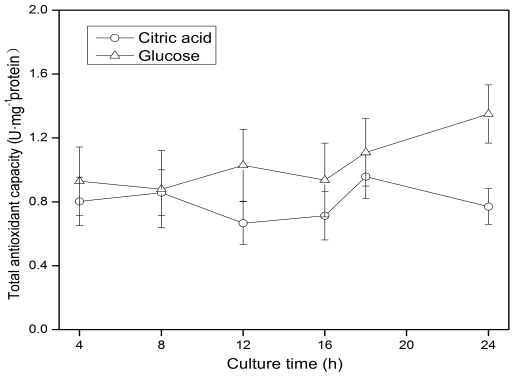

Figure 5. *The time curves of intracellular T-AOC of S. marcescens SYBC08*

The changes of intracellular and extracellular VC content in *S. marcesces* SYBC08

As shown in Figure 6a, the intracellular VC content in presence of glucose as sole carbon was higher than that in presence of citric acid as carbon source during the fermentation process (from 8 h to 24 h) ($P < 0.05$). The intracellular VC content in presence of glucose as sole carbon source was increased from 4 h to 8 h. The intracellular VC content in presence of glucose or citric acid as carbon sources display the lowest contents (114.18 μg·mg^{-1} protein and 32.05 μg·mg^{-1} protein) at 4 h of culture time.

The extracellular VC content time-change curve of *S. marcesces* SYBC08 was shown in Figure 6b, and there was no significant difference in the VC content between in presence of glucose and of citric acid from 4 h to 8 h. The extracellular VC content gradually increased during cultural time from 8 h to 24 h, while the extracellular VC content in presence of glucose as carbon source gradually decreased during cultural time from 8 h to 24 h, finally, the extracellular VC content in presence of citric acid was significantly lower that in presence of glucose from 8 to 24 h ($P < 0.05$).

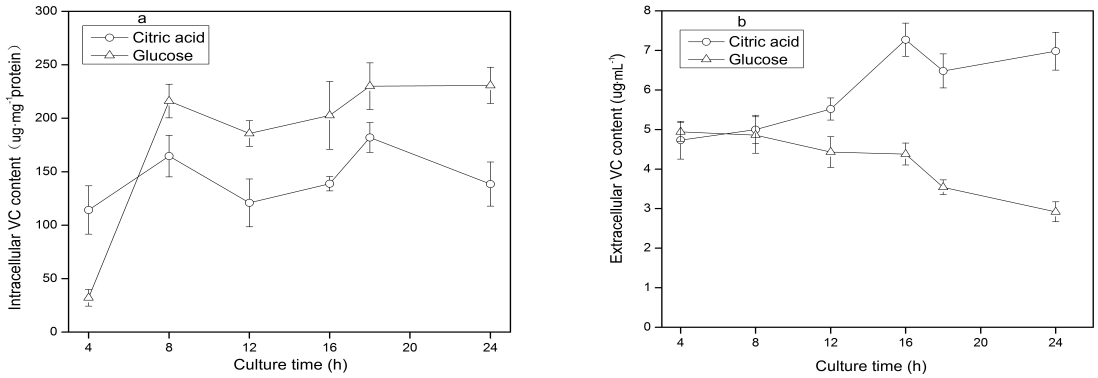

Figure 6. *The time curves of intracellular (a) and extracellular (b) VC contents of S. marcescens SYBC08*

The changes of intracellular H_2O_2 content from *S. marcesces* SYBC08

As shown in Figure 7, the H_2O_2 content in presence of citric acid or glucose as the carbon source was increased from 4 h to 8 h, the H_2O_2 content in presence of citric acid or glucose as the carbon source was little change or decreased during other time periods. The H_2O_2 content in presence of citric acid (0.91 mol·mg^{-1} protein) at 8 h of culture time was higher than that in presence of glucose (0.66 mol·mg^{-1} protein) ($P < 0.05$), but the result was opposite at 24 h of culture time ($P < 0.05$).

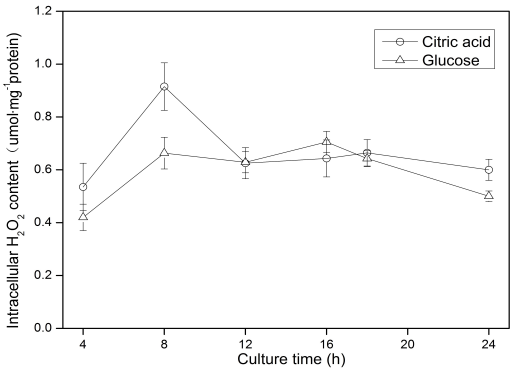

Figure 7. *The time curves of intracellular H_2O_2 content variations of S. marcescens SYBC08*

The changes of intracellular MDA content of *S. marcesces* SYBC08

The MDA content in presence of glucose or citric acid as carbon source was determined at certain hours during culture process of 24 h, as shown in Figure 8. The MDA content in presence of citric acid as carbon source was higher than that in presence of glucose as carbon source at the culture time of 4 h ($P < 0.05$), and MDA content between them was no significant difference at other culture time. The MDA content in presence of glucose as carbon source keep relatively high lever from 4 h to 8 h, and then MDA content was gradually decline from 8 h to 24 h. The MDA content in presence of citric acid as carbon source was increased from 4 to 8 h, and then was decreased from 8 to 24 h.

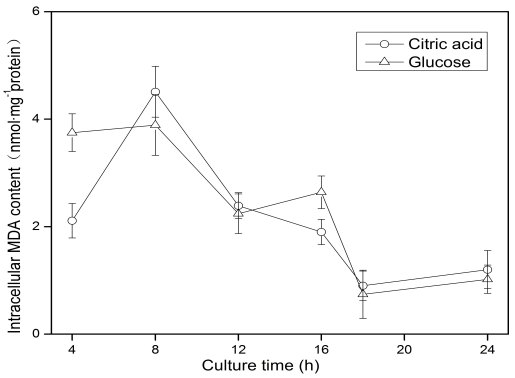

Figure 8. *The time curves of intracellular MDA content of S. marcescens SYBC08*

The changes of intracellular metabolites from *S. marcesces* SYBC08

The contents of pyruvic acid, succinic acid and citric acid were measured during culture time of 24 h by high-performance liquid chromatography, and the result was shown in Figure 9 (a-c). Pyruvate content in presence of citric acid as the carbon source decreased first and then gradually increased. The maximum pyruvate content (4.98 μg·mg^{-1} protein) was found at the culture time of 4 h (Figure 9a). The content of pyruvic acid in presence of glucose as carbon source increased first, and then decreased, and the maximum value was 4.19 μg·mg^{-1} protein (Figure 9a).

The succinic acid contents in presence of citric acid as carbon were higher than those in presence of glucose as carbon source at whole culture time points (expect 4 h and 8 h) (Figure 9b). The succinic acid content in presence of citric acid as carbon source firstly decreased and then increased from 8 h to 24 h (Figure 9b). The highest value (81.83 μg·mg^{-1} protein) was achieved at 24 h of culture time (Figure 9b). The succinic acid content in presence of glucose as carbon source was increased during two culture time phase (from 4 h to 16 h and from 16 h to 24 h), and the maximum succinic acid content was 119.7 μg·mg^{-1} protein at 24 h of culture time (Figure 9b).

As shown in Figure 9c, citric acid content in presence of citric acid or glucose as the carbon source was the lowest value at 4 h of culture time, and the maximum values were 110.93 μg·mg^{-1} protein and 213.22 μg·mg^{-1} protein at 24 h of fermentation time, respectively (Figure 9c).

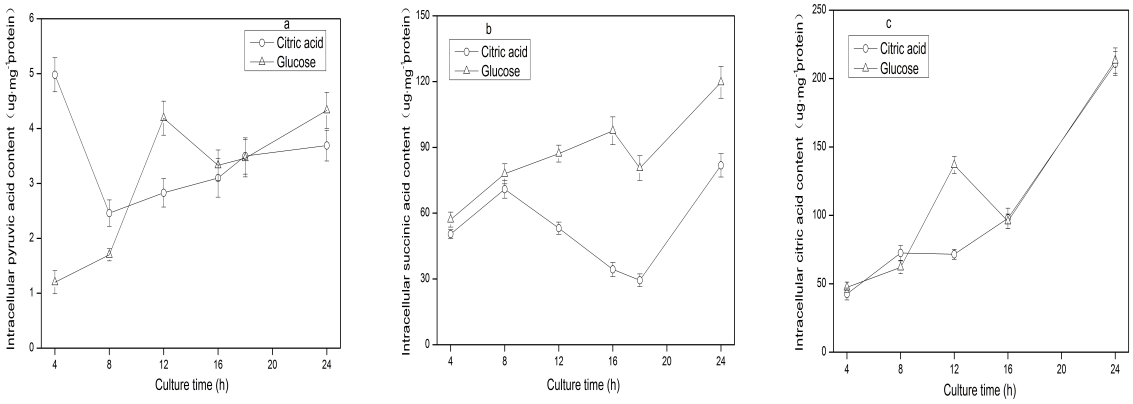

***Figure 9**. The time curves of intracellular contents of pyruvic acid (a), succinic acid (b) and citric acid (c) of S. marcescens SYBC08*

The analysis of those results

Under aerobic conditions，microorganism using glucose or citric acid as sole carbon source by metabolic pathway (including TCA cycle or EMP pathway) generated NADH and synthetized intermediate metabolites, and then the electron transport of NADH is oxidized by the respiratory chain, and the activity of the respiratory chain can generated potential redox products, such as O_2, H_2O_2, and OH [15]. OH is the most active oxygen species [25] and can act directly on lipids to form hydroperoxide. MDA is a marker of lipid peroxidation level in cell membrane, and its

level usually reflects lipid peroxidation, indirectly reflect the degree of injury of the cells [26, 27]. In the study, the MDA content (0-6 nmol·mg^{-1} protein) in presence of two different carbon sources is not high at the whole cultural time (general value is dozens of nmol·mg^{-1} protein under oxidant stress) and gradually decreased from 8 h to 24 h. The result indicated that the degree of injury of the cells was low and was decreased during late culture period. The possible reasons for the decreasing of MDA content were related to the increasing of biosynthesis of CAT and AhpC. AhpC, found in many prokaryotic microorganisms, such as *Escherichia coli*, *Pseudomonas aeruginosa*, *Xanthomonas* [28 – 30], can removal low concentration of H_2O_2, while CAT can removal relatively high concentration of H_2O_2 [28 – 30]. The reason was that the catalytic efficiency of AhpC is much higher than that of CAT [28 – 30]. SOD catalyzes superoxide radicals into H_2O_2, and part H_2O_2 was catalyzed into H_2O and oxygen by CAT and AhpC. OH mainly triggered the formation of MDA was generated from remaining H_2O_2 by the Fenton reaction, so the high CAT yield and AhpC activity lead to a low MDA content.

Those CAT yield which the strain grew in presence of citric acids as sole carbon source was approximately 1.47-2.52 folds higher than those in presence of glucose as sole carbon source from 12 h to 24 h ($P < 0.05$). The possible reason was that NOX activity, VC content and T-AOC in presence of glucose as carbon source was higher than those in presence of citric acid as carbon source at the culture time from 12 h to 24 h. NADH oxidases which are also flavo-enzymes that oxidize NADH to NAD^+ and reduce the electron transport of NADH oxidized by the respiratory chain, thereby maybe decrease H_2O_2 production [31]. Microbial antioxidant systems are mainly included antioxidant enzymes, such as SOD, CAT, glutathione-S-transferase (GST) [11, 12] and non enzymatic antioxidant systems [13], such as vitamins, amino acids, protein. Ascorbic acid is an important metabolite for most living organisms, present in millimolar concentrations and well known for its antioxidant properties [15]. In previous studies indicated VC could decreased significantly H_2O_2-induced DNA damage and deduced H_2O_2 level [32, 33]. T-AOC is associated with free radical scavenging capacity and can be used as an integrative marker of the total antioxidant capacity of the body and the protective ability of the non-enzymatic antioxidant defense system [34]. This probably suggests that non-enzymatic antioxidant defense system also take part in removing part H_2O_2. In total, NOX activity, VC content and T-AOC play a supplementary role in reducing the H_2O_2 production and lead to lower CAT yield in presence of glucose as sole carbon source.

CONCLUSIONS

When *S. marcesces* SYBC08 was grown in presence of citric acid as sole carbon source or glucose as sole carbon source, the different varieties in the value of antioxidant indexes and metabolic intermediates between them were revealed during culture time of 24 h. Furthermore, the possible reason which obtained higher CAT yield in presence of critic acid than that in presence of glucose was preliminarily analyzed.

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