

Enhancement of Enduracidin Accumulation from Sweet Sorghum Juice via Alleviation of Oxidative Damage in *Streptomyces Fungicidicus* M30

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Research

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Abstract

Background: Sweet sorghum juice is the preferred feedstock for enduracidin production, but the fermentation efficiency still need to be further enhanced. Oxygen-mediated microbial cell damage is an effective way to boost cell growth and metabolite accumulation in aerobic fermentation. However, this strategy on enduracidin production from sweet sorghum juice has not been reported yet.

Results: In this study, enduracidin production titer was significantly enhanced when 90 mM Vitamin C was added at 4 d of fermentation. The enhanced T-AOC and antioxidant enzyme activities may help to further understand the mechanism of Vitamin C effect on regulation of enduracidin accumulation in *S. fungicidicus* M30. The effects of chemical preservative methyl paraben on cell growth and enduracidin production were also evaluated, and 0.04 g/L of methyl paraben had no significant inhibitory effect on cell growth and enduracidin production, indicating that the concentrated sweet sorghum juice assisted by methyl paraben for a long-term storage can be used as the substrate for enduracidin production effectively. Finally, based on the group with 0.04 g/L of methyl paraben and 90 mM Vitamin C, a final enduracidin concentration of 1.14 g/L was achieved from clarified sweet sorghum juice by M30 strain.

Conclusions: This work provided a promising strategy to enhance enduracidin production using sweet sorghum juice via adding the antioxidant to boost antioxidant capacity in *S. fungicidicus*.

Background

Enduracidin is a lipopeptide antibiotic being widely used as an excellent animal growth promoter due to its high safety, low toxicity, low residue, and powerful antibacterial effect[1–4]. With growth of the enduracidin industry, the global demand for enduracidin continues to increase. Taking China as an example, the production capacity is up to 4.8 kilo tons in 2016. *Streptomyces* sp. is an attractive robust microbial cell factory for industrial enduracidin production[5, 6]. However, low enduracidin productivity of *Streptomyces* sp. and the expensive glucose used as the carbon substrate both make the fermentation process uneconomical. The future market expansion of enduracidin highlights more efficient and economical enduracidin production approaches.

As a C4 crop, sweet sorghum contains rich fermentable sugars in its stems (such as sucrose, glucose and fructose) and a variety of cultivars and growing characteristics such as high biomass yield and rapid growth, which make it an attractive feedstock for value-added biochemical production. A number of fuels and bio-chemicals have been successfully produced using sweet sorghum juice (SSJ) as feedstocks[7–12]. In a previous study, we have shown that SSJ was the preferred feedstock for enduracidin production with approximately equivalent efficiency to that of glucose, and *S. fungicidicus* M30 can also produce 1.01 ± 0.05 g/L of enduracidin via clarified SSJ and fed-batch strategy[13]. However, the fermentation efficiency of *S. fungicidicus* M30 still needed to be further enhanced for industrial fermentation application.

Currently, several considerable strategies have been conducted to enhancing enduracidin production of *Streptomyces* sp., such as mutation screening [6], pH shift feeding[14] and genetic engineering[15]. Besides, some environmental factors also significantly influence strain growth and antibiotic fermentation efficiency, such as reactive oxygen species (ROS)[16]. As metabolic byproducts, ROS are usually generated in organism cells and maintain ROS level can be achieved via the cleaning of synthesized antioxidant enzymes by cells. However, severe environmental conditions significantly increased ROS accumulation level[17, 18], which broke the stable balance. Excess ROS can cause severe damage to DNA, proteins, carbohydrates and lipids in organism cells[18, 19], which can further influence physiological function of microbial cells. Interestingly, success experiment cases indicated that the external addition of antioxidants can enhance cell growth and microbial fermentation efficiency because of the enhancement of total antioxidant capacity (T-AOC) and the reduction of ROS accumulation in microbial strain[19]. But so far, few studies have focused on the effect of the external addition of antioxidants on the growth and enduracidin production of *Streptomyces* sp.

In the current study, different concentrations of vitamin C (Vc) were added to the fermentation medium in order to assess its effect on cell growth and enduracidin production of *S. fungicidicus*, and the optimized concentration and addition time of Vc were also conducted. The intracellular antioxidant enzymes activities (SOD: superoxide dismutase; CAT: catalase), T-AOC, and the oxidative damage of cell membrane with and without Vc addition were also determined in order to analyze the possible mechanism. In addition, the inhibitory effect of different concentration of methyl paraben on cell growth and enduracidin accumulation was also investigated. Finally, the enduracidin fermentation efficiency was significantly enhanced via the external addition of Vc when using SSJ as a feedstock. This work provided a promising strategy to enhance enduracidin production using SSJ via adding the antioxidant to boost antioxidant capacity in *S. fungicidicus*.

Results And Discussion

The effect of Vc concentration and addition time on cell growth and enduracidin production

In present study, antioxidant Vc was used to assess its effect on enduracidin production of *S. fungicidicus* M30, and different concentration of Vc (0, 10, 50, 90 mM) were added to the medium at 0 d, 2 d, 4 d and 6 d during enduracidin fermentation. Due to the volumetric change via the addition of Vc, the same volume of distilled water was added. As shown in Fig. 1a, it was indicated that enduracidin accumulation was significantly enhanced when 90 mM Vc was added into fermentation medium at 2 d or 4 d ($p < 0.05$ or 0.01), and the highest enduracidin production was achieved by Vc with the addition time at 4 d. However, addition of 130 mM Vc had a negative effect on enduracidin production at four different phases, indicating that high Vc concentration inhibited enduracidin accumulation in *S. fungicidicus* M30. Similar results have also been reported in previous studies[19, 20]. The result indicates that the addition of 90 mM Vc can promote enduracidin accumulation in *S. fungicidicus* M30.

In addition, complete time profiles of cell growth and enduracidin production by *S. fungicidicus* M30 with the optimized addition amount of 90 mM Vc, were also conducted during all cultivations (Fig. 1b). Considering the existence of insoluble components in medium, cell growth was represented by total intracellular protein as previously reported[21]. The results showed no significant difference in cell growth at any fermentation phases when Vc was added. However, the addition of Vc led to approximately 9.6% increase of obtained enduracidin accumulation compared to that with non-supplemented group after 10 d fermentation. Our results were in accordance with previous studies in which Vc promoted ϵ -poly-L-lysine accumulation in *Streptomyces* sp. AF3-44, but had no significant effect on cell growth[22].

Effects of Vc on the intracellular antioxidant enzymes activities (SOD: superoxide dismutase; CAT: catalase) T-AOC, and the oxidative damage of cell membrane

Previous studies have proved that the external addition of antioxidants can enhance microbial fermentation efficiency due to its positive effects of oxidative protection[19, 23, 24], such as the direct scavenging action by antioxidant substances and indirect scavenging action by the synthesis of antioxidant enzymes[25, 26]. Generally, cellular oxidative protection is mainly achieved through the enhancement of intracellular antioxidant enzymes activities and T-AOC, as well as reduction of membrane damage[27]. Thus, in order to further explore the mechanism of the observed effects of Vc on enduracidin accumulation, we determined intracellular antioxidant enzymes activities (SOD: superoxide dismutase; CAT: catalase), T-AOC, and the oxidative damage of cell membrane level of *S. fungicidicus* M30 intervals of 2 d during the entire fermentation process.

T-AOC is a specific embodiment of the antioxidant system in microbial strains, and improving T-AOC is conducive to metabolite accumulation[19]. As shown in Fig. 2a, with 90 mM Vc supplementation at 4th day of fermentation, the activity of T-AOC of the cultures grown (6 d, 8 d, and 10 d) increased as the culture time went and reached its maximum at 10 d, and the T-AOC values at 6 d fermentation were significantly higher compared to the non-supplemented group ($P < 0.05$), indicating that Vc can greatly improve the antioxidant capacity as intended. In addition, as the first line of defense, intracellular antioxidant enzymes, such as SOD and CAT, can protect cells from ROS damage[28]. It is generally believed that SOD and CAT have a synergistic effect on the oxidative protection in cells[23]. Specifically, SOD can disproportionate $O_2^{\cdot-}$ to H_2O_2 and O_2 , then the H_2O_2 generated by SOD was further scavenged by CAT. As shown in Fig. 2b and 2c, in the non-supplemented group, both SOD and CAT activities reached its peak of 14.2 U/mg protein and 52.8 nmoL/min/mg protein at 4 d fermentation, respectively, and then the activities quickly decreased. However, when Vc was added, the highest SOD activity can reach 22.9 U/mg protein at 6 d, reaching peak activities that was 61% higher than that of non-supplemented group. In case of CAT, the highest CAT activity can reach 112.29 nmoL/min/mg protein at 8 d by the addition of Vc, reaching peak activities that was 1.13 times of that of non-supplemented group. It was suggested that the external addition of Vc can significantly enhance the activities of SOD and CAT, which agreed with previous reports by Sun et al[23]. Cell membrane is an important damage target attacked easily by highly oxidized $\cdot OH$. MDA value can indirectly reflect the damage level of membrane system, and low MDA value indicates high membrane fluidity[27], which could be beneficial to substance transport and

energy metabolism in cells. In present study, compared with the non-supplemented group, the addition of Vc in the medium showed lower MDA value (Fig. 2d), indicating Vc can alleviate damage of membrane in *S. fungicidicus*. Meanwhile, Sun et al. also found that the addition of Vc could significantly alleviate damage of membrane in *Streptomyces* sp. AF3-44, which was mainly reason for high-yield ϵ -poly-L-lysine accumulation[23].

Enduracidin production from clarified SSJ by fed batch fermentation in a 250-mL flask

As a potential bioethanol plant, SSJ has been developed to produce different bio-chemicals in recent years. Nevertheless, the biotechnology utilization process is still not commercialized, mainly because of short-term preservation period of sugars in sweet sorghum juice[29]. Interestingly, a combination of evaporation (400 g/L of sugar concentration) and chemical preservation (0.4 g/L of methyl paraben) for long-term storage of fresh SSJ has been developed, and only 5.0% of fermentable sugars in SSJ were consumed after a 90-day preservation at room temperature[11], making SSJ more attractive as low-cost alternative substrate for bio-chemicals production. In this case, chemical preservative, such as methyl paraben, was used to extend the storage period of SSJ, but methyl paraben had a broad spectrum antibacterial agent[30], which could influence the microbial metabolism and metabolite accumulation. Hence, before using the concentrated SSJ assisted by methyl paraben for a long-term storage for bio-chemicals production, the inhibitory effect of different concentration of methyl paraben on microbial cell growth and metabolite accumulation need to be evaluated.

Correlating with the previous experiment results that a combination of evaporation (400 g/L of sugar concentration) and 0.4 g/L of methyl paraben had a better storage performance of fresh SSJ. In a previous study, we also have showed that the optimized sugar concentration of clarified SSJ was 40 g/L for enduracidin production by *S. fungicidicus* M30. Thus, 0.04 g/L, 0.08 g/L and 0.12 g/L of methyl paraben were added into clarified SSJ (40 g /L reducing sugar), respectively. As shown in Fig. 3, the results showed that no significant difference on cell growth and enduracidin accumulation was observed at any fermentation phases when 0.04 g/L of methyl paraben was added compared to that with non-supplemented group. Based on the group with 0.08 or 0.12 g/L of methyl paraben, the reduction on cell growth and enduracidin production was more obvious along with the increase in methyl paraben concentration ($p < 0.01$). Therefore, adding 0.04 g/L of methyl paraben into clarified SSJ showed the best enduracidin production performance by *S. fungicidicus* M30, indicating that the concentrated SSJ assisted by methyl paraben for a long-term storage can be used as the substrate for enduracidin production effectively.

Based on the group with 0.04 g/L of methyl paraben and 90 mM of Vc, enduracidin accumulation using clarified SSJ was further evaluated. As shown in Fig. 4, 1.14 g/L of enduracidin was obtained after 11 d fermentation, leading to approximately 15% increase of obtained enduracidin accumulation compared to that with no Vc supplemented group ($P < 0.05$). Thus, this study provided a complete industrial chain for enduracidin production from enhancing the production capacity of microbial strain, combined with

evaluating the inhibitory effects of chemical preservative for long-term storage of SSJ, to efficient microbial metabolite accumulation.

Conclusions

The addition of 90 mM Vc resulted in significant improvement in enduracidin production. The enhanced T-AOC and antioxidant enzyme activities may help to further understand the mechanism of Vc effect on regulation of enduracidin accumulation in *S. fungicidicus* M30. The effects of chemical preservative methyl paraben on cell growth and enduracidin production were also evaluated, and 0.04 g/L of methyl paraben had no significant inhibitory effect on cell growth and enduracidin production, indicating that the concentrated SSJ assisted by methyl paraben for a long-term storage can be used as the substrate for enduracidin production. Finally, based on the group with 0.04 g/L of methyl paraben and 90 mM Vc, a final enduracidin concentration of 1.14 g/L was achieved from clarified SSJ by M30 strain.

Materials And Methods

Strains and media

S. fungicidicus M30 was used for enduracidin production, and was cultivated on agar plates at 28 °C for 7 d, as described in a previous report[31]. The seed culture medium and conditions of propagation were prepared according to our previous study[31]. The seed medium included 30 g/L glucose, 30 g/L corn steep liquor, 20 g/L CaCO₃, 8 g/L NaCl, 5 g/L yeast extract, with the pH of the medium adjusted to 7.5. The fermentation medium comprised 40 g/L glucose, 40 g/L soluble starch, 30 g/L corn gluten meal, 20 g/L corn steep liquor, 15 g/L NaCl, 15 g/L CaCO₃, 5 g/L NH₄Cl, 1 g/L arginine, and 0.2 g/L KH₂PO₄, with the pH of the medium adjusted to 6.9–7.1.

Investigation of the effects of Vc addition on cell growth and enduracidin production by *S. fungicidicus* M30

1 mL spore suspension of *S. fungicidicus* M30 (3×10^7 cells/mL) was inoculated into 50 mL of seed medium and cultured at 28 °C on a SPH-311D rotary incubator (Shanghai Shiping Laboratory Equipment Co., Ltd, China) at an agitation speed of 220 rpm for 2 d[31]. Subsequently, The 4% (v/v) seed medium was transferred into 50 mL of fermentation medium at 50 °C and 220 rpm. Before using, 2M Vc was sterilized by 0.22- μ m sterile filtration, and supplemented to fermentation medium at a final concentration of 0, 10, 50, 90 and 130 mM, and the addition time were 0, 2, 4 and 6 d, respectively, and the pH was adjusted to the value in fermentation stage with 2 M NaOH. The control group was added with the same volume sterile water.

Determination of key enzymes activities involving in antioxidant capacity of *S. fungicidicus* M30

Before determining key enzymes activities involving in antioxidant capacity, the mycelia with different fermentation period were first harvested by centrifuging 200 μ L fermentation broth at 8000 r for 10 min,

and washed twice with 0.2% KCl. Then, the mycelia was suspended in 1 mL extracting solution and broken by JY92-IIN ultrasonic disrupter (Xinzhi Biotechnology Co., Ltd, Ningbo, China), and the intracellular T-AOC, catalase (CAT), malondialdehyde (MDA), superoxide dismutase (SOD), and total soluble protein of the cells (BCA) were determined according to the manufacturer's instructions using the corresponding assay kits (Suzhou Keming Bio-Technology Co. Ltd., Suzhou, China).

Enduracidin Production From Clarified Ssj By Fed Batch Fermentation

Certain amounts of the preservative methyl paraben (0.04 g/L, 0.08 g/L and 0.12 g/L) were added into the clarificated SSJ and were mixed well. Clarificated SSJ was prepared according to our previous report. Then 40 g/L clarificated SSJ was used carbon source instead of 40 g/L glucose in fermentation medium, and other ingredients remained the same.

A fed-batch fermentation strategy was conducted with adding 0.04 g/L methyl paraben in fermentation medium, coupling with clarificated SSJ as a carbon source. After 4 days of fermentation, a final concentration of 90 mM Vc was added into fermentation broth, and the control group was added with the same volume sterile water. The pH was adjusted to the value in fermentation stage with 2 M NaOH. After 5 days of fermentation, addition of clarificated SSJ increased the reducing sugar concentration to 15–20 g/L.

Analytical Methods

According to a previous study, total reducing sugar concentration was determined by 3, 5-dinitrosalicylic acid method (DNS method)[32]. The treatment of fermentation broth and the determination of enduracidin concentration were followed by the method proposed by Mou et al.[33]

Abbreviations

Vc

Vitamin C; Sweet sorghum juice:SSJ; T-AOC:total antioxidant capacity; CAT:catalase; MDA:malondialdehyde; SOD:superoxide dismutase; BCA:total soluble protein of the cells

Declarations

Acknowledgements

Not applicable.

Authors' contributions

WH and LBZ designed the experiments. LL performed the whole experiments. JHC and WJL analyzed the data. WH and LBZ wrote the paper.

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Availability of data and materials

All data generated or analyzed during this study are included in this article.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

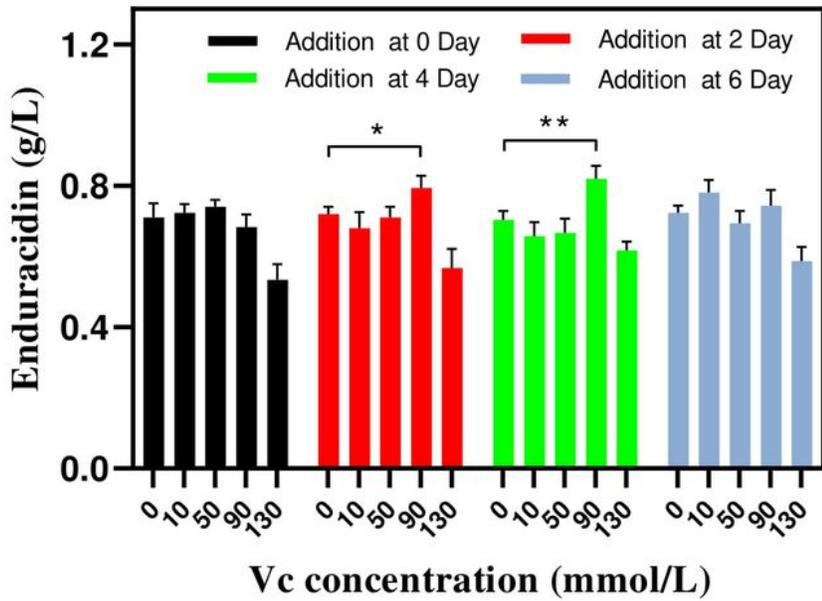
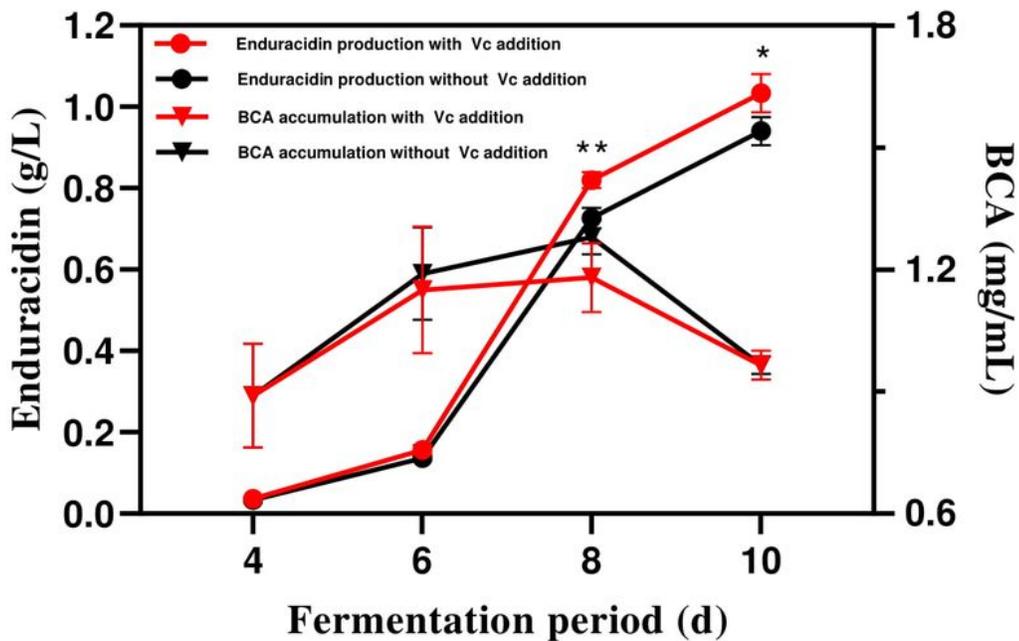
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Figures

a**b****Figure 1**

(a) The effect of Vc concentration and addition time on cell growth and enduracidin production. (b). Enduracidin production and cell growth when 90 mM Vc was added at 4 d of fermentation. The error bars in the figure indicate the standard deviations of three parallel replicates, and * $p < 0.05$ indicated a significant difference and ** $p < 0.01$ indicated a highly significant difference.

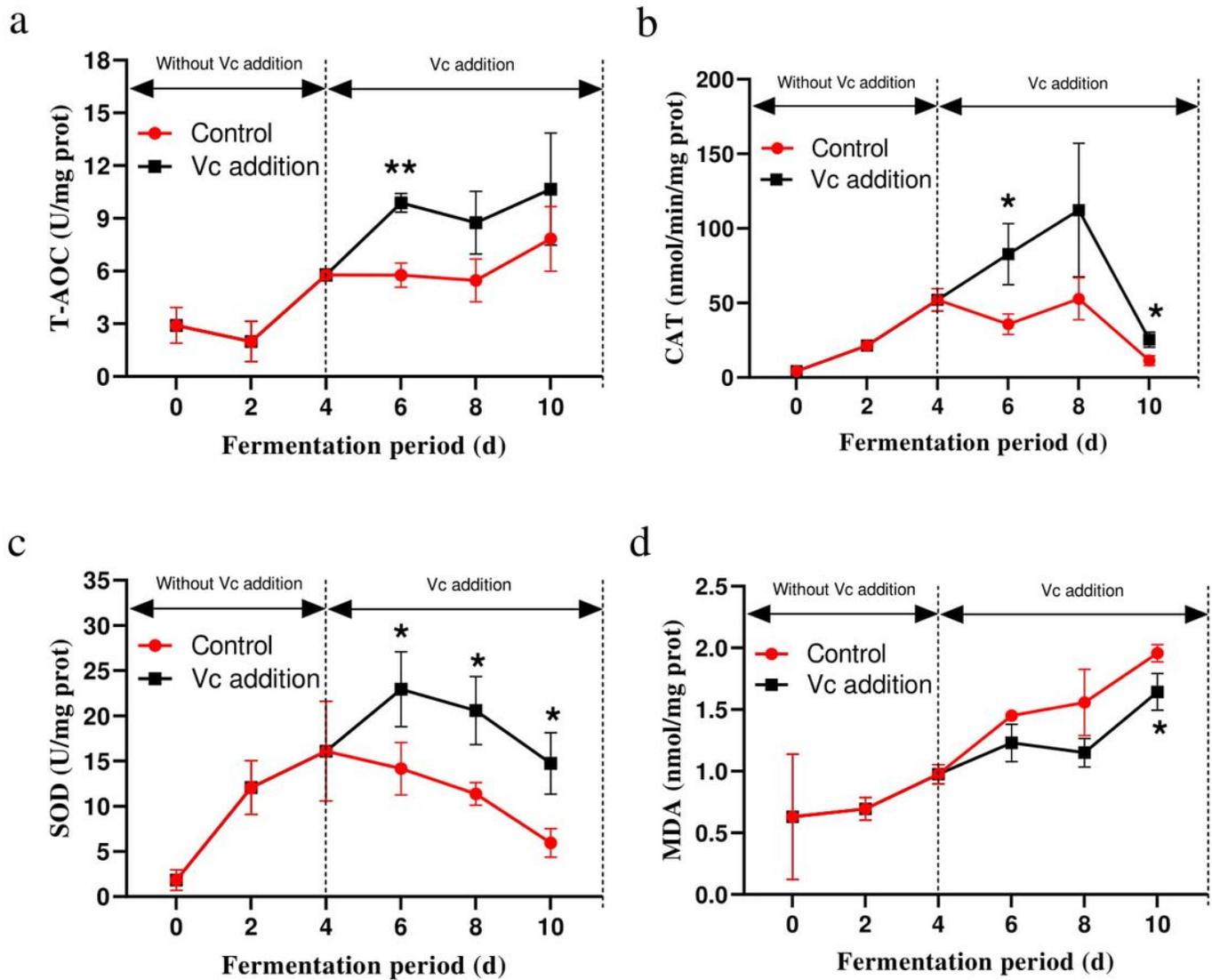


Figure 2

Effects of Vc on total antioxidant capacity and key antioxidant enzyme activities: (a) T-AOC; (b) CAT; (c) SOD; (d) MDA. The error bars in the figure indicate the standard deviations of three parallel replicates, and *p < 0.05 indicated a significant difference and **p < 0.01 indicated a highly significant difference.

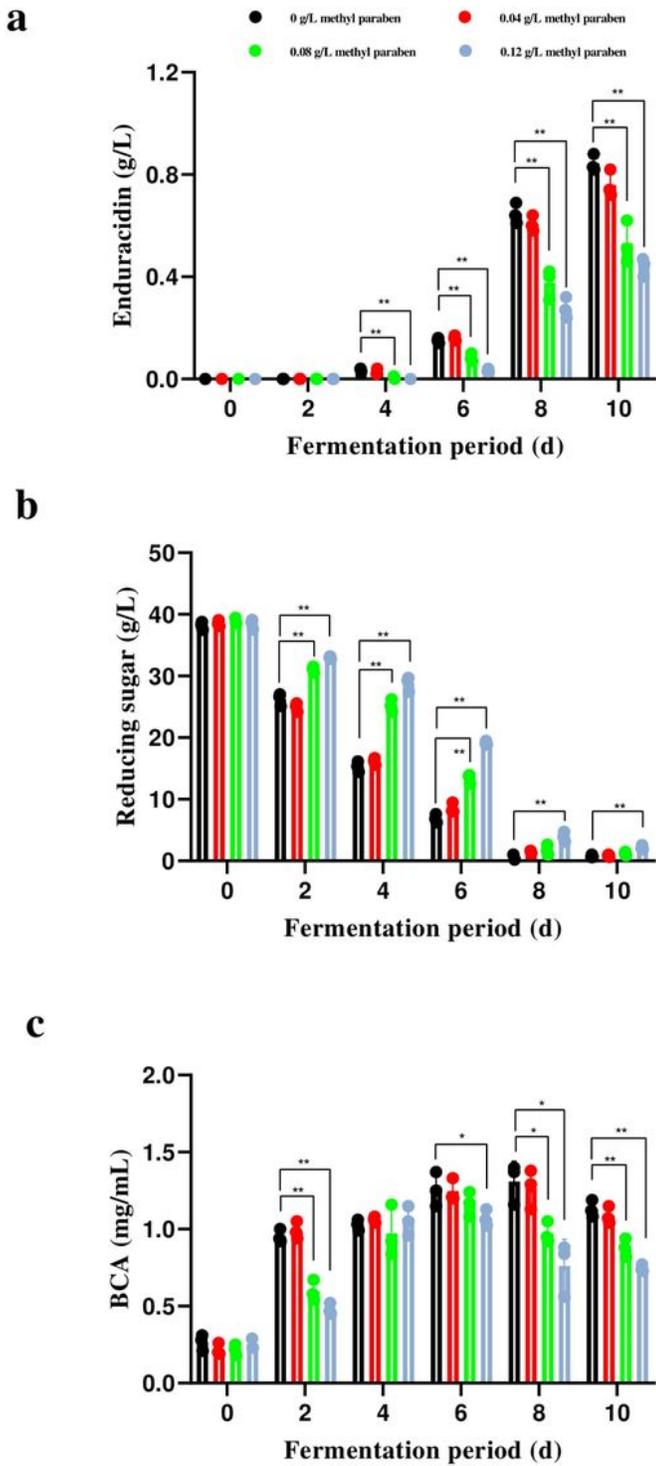
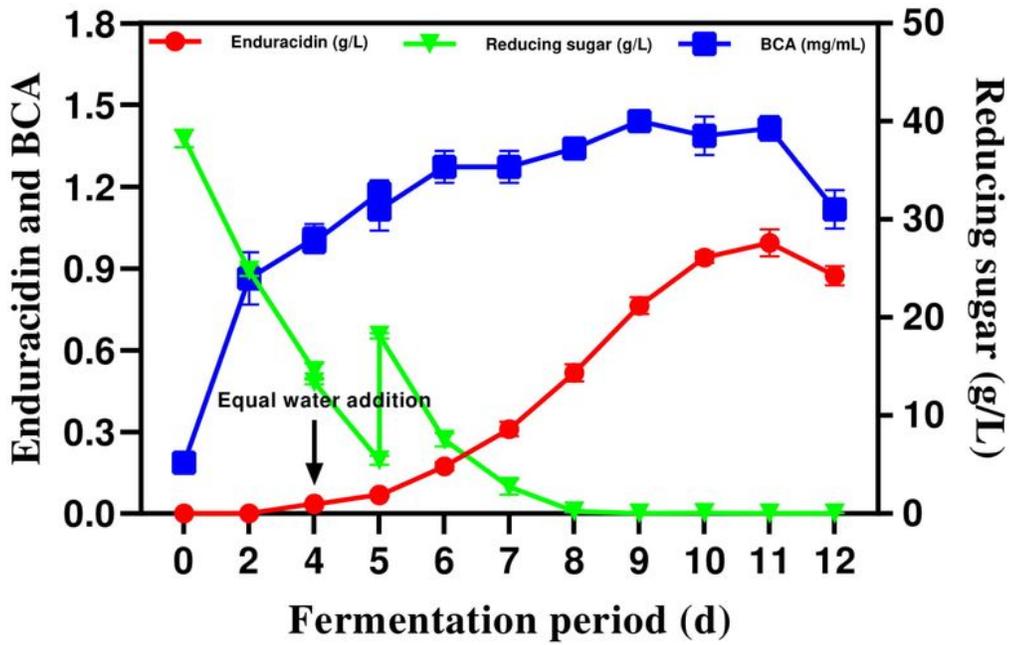


Figure 3

The inhibitory effect of different concentration of methyl paraben on cell growth and enduracidin production: (a) enduracidin; (b) Reducing sugar; (c) BCA. The error bars in the figure indicate the standard deviations of three parallel replicates, and * $p < 0.05$ indicated a significant difference and ** $p < 0.01$ indicated a highly significant difference.

a



b

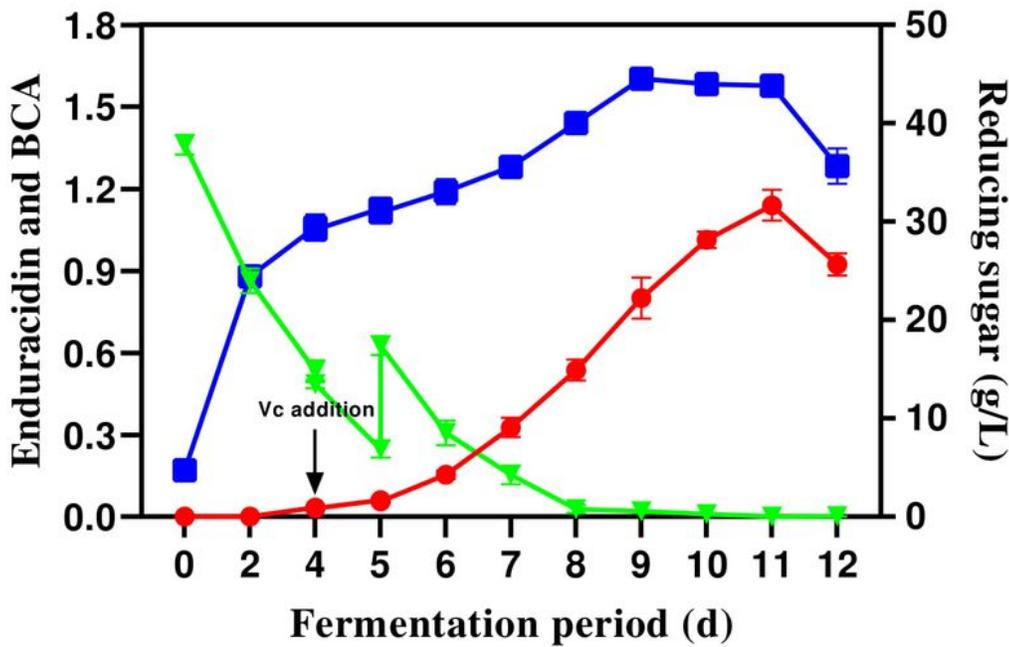


Figure 4

(a) Enduracidin fermentation using SSJ without supplementation of Vc; (b) Enduracidin fermentation using SSJ with supplementation of 90 mM Vc.