

# Antibacterial Mechanisms of *Orostachys Cartilaginosa* Cell Cultures: Effect on Cell Permeability and Respiratory Metabolism of *Bacillus Subtilis*

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## Research Article

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# Abstract

*Orostachys cartilaginosa* of Crassulaceae family is a plant native to the Changbai Mountain area, China. Although *O. cartilaginosa* has various medicinal values, its product development and production are restricted by the insufficient resource available. *O. cartilaginosa* cell cultures possess an efficient antibacterial effect against *Bacillus subtilis*, but the underlying mechanism is not clear yet. Therefore, this study investigated the effects of extract from bioreactor cultured *O. cartilaginosa* cells (OE) on *B. subtilis* cell permeability and respiratory metabolism to provide a reference for the further utilization of *O. cartilaginosa* cell cultures. Results showed alkaline phosphatase activity, electrical conductivity, nucleic acid and protein contents in *B. subtilis* suspensions were significantly increased ( $p < 0.01$ ) by OE treatment, indicating the occurrence of cell damage or increase in cell permeability. OE inhibited *B. subtilis* respiration, and the combination groups of OE+iodoacetic acid (IA) and OE+sodium phosphate (SP) showed low superposition rates (approximately 35%), revealing that OE likely affected IA- and SP-represented metabolic pathways. The activities of *B. subtilis* enzymes, specifically, hexokinase and pyruvate kinase in the Embden-Meyerhof (EMP) pathway and glucose-6-phosphate dehydrogenase in the pentose phosphate (HMP) pathway, decreased after OE treatment. This result proved that OE inhibited *B. subtilis* respiration by regulating the EMP and HMP pathways.

## Introduction

*Orostachys cartilaginosa* A. Bor is a species of the *Orostachys* genus, which belongs to the Crassulaceae family (Tian et al. 2019). It is mainly distributed in Changbai Mountain area, China, and the whole plants of this species have various pharmaceutical effects, such as anti-inflammation, antifebrile, hemostatic, antidote, and anticancer effects because they contain polysaccharides, phenolics, flavonoids, steroids, and triterpenoids (Park et al. 1991). However, the raw materials of *O. cartilaginosa* plants are insufficient due to the excessive collection of their wild resources and the incomplete system of artificial cultivation; this situation critically affects production and development of their products (Piao et al. 2017). Cell culture technology is an alternative method for producing plant materials for species that cannot provide materials via the traditional route (Efferth 2019). Therefore, researchers have attempted to establish cell culture systems, and cells of various species, including *O. cartilaginosa*, have been successfully produced in large-scale bioreactors (Zhang et al. 2017; Tian et al. 2019). Nevertheless, to date, cultured cells of *O. cartilaginosa* have been rarely applied because of various reasons, including the lack of information on the various bioactive effects.

Plant bacteriostatic substances have strong antibacterial effects with a wide spectrum, high safety, and low toxicity and side effects (Naz et al. 2015). Studies have indicated that bacteriostatic substances affect bacterial cell walls and membranes (Cui et al. 2018; Dong et al. 2020; Hu et al. 2019). The cell wall, as the outermost layer of microbial cells, can block macromolecular substances from entering the cell and resist osmotic pressure. The cell membrane is the basis of the maintenance of cell integrity and the normal metabolism of substances and energy (Gao et al. 2020). The action of a variety of plant bacteriostatic agents on the cell wall and membrane system results in the destruction of the integrity of

the membrane structure, thereby affecting cell metabolism and changing cell permeability (Song et al. 2020). The main pathways for respiratory metabolism include the tricarboxylic acid cycle (TCA); Embden-Meyerhof (EMP); and the pentose phosphate (HMP) pathway, which is essentially the oxidative metabolic pathway of intracellular sugars (Bajpai et al. 2013; Li et al. 2016; Oliveira et al. 2015). Once respiratory metabolism is inhibited, cells cannot obtain the energy required for basic life needs; this effect leads to cell death indirectly (Liu et al. 2020). The effects of plant bacteriostatic substances from various species on bacterial permeability and respiratory metabolism have been investigated to clarify their antibacterial mechanism (Hu et al. 2019).

*Bacillus subtilis* is a kind of conditional pathogenic bacteria (Masengu et al. 2014). When the resistance of an organism is low, the pathogenic *B. subtilis* can cause several diseases, such as endophthalmitis, adult sepsis and pharyngeal abscess (Luo et al. 2016; Richard et al. 1988; Oggioni et al. 1998). At present, the abuse of chemical antibiotics, the increase of bacterial resistance, and the emergence of multi-drug resistant bacteria have become the common problems in the practical treatment (Li et al. 2020). Therefore, finding a safe and efficient natural substances has become an urgent need. Our previous study found that *O. cartilaginous* cell cultures can efficiently inhibit *B. subtilis* growth, but its underlying mechanism has yet unclear. Therefore, this study investigated effects of extract from bioreactor cultured *O. cartilaginous* cells (OE) on cell permeability and respiratory metabolism of *B. subtilis* to clarify the antibacterial mechanism, and provided a reference for the disease treatment through regulating the key factors or enzymes.

## Materials And Methods

### Plant materials and extract preparation

*O. cartilaginous* cells were produced by using the method of Piao et al. (2017). Approximately 15 g/L fresh cells were inoculated into a 5 L airlift balloon-type bioreactor containing 4 L of Murashige and Skoog (1962) medium with 3.5 mg/L benzylaminopurine, 0.1 mg/L  $\alpha$ -naphthalene acetic acid and 30 g/L sucrose. The pH of the medium was adjusted to 5.8 prior to autoclaving. The bioreactor was aerated at the flow rate of 400 mL/min air and maintained at 25°C under 30  $\mu\text{mol}/\text{m}^2/\text{s}$  light intensity with a 16 h photoperiod. Cells were harvested after 25 days of bioreactor culture. The cells were washed three times with tap water and placed in a drying oven (50°C) for 48 h.

The dried cells were soaked in 70% (v/v) ethanol (solid:liquid ratio = 1:15) with condensation reflux for 1 h at 60°C. Then, the filtrate was collected after vacuum filtration. The residue was repeatedly extracted twice in accordance with the above conditions. All filtrates were combined together and concentrated by using a rotary evaporator (R201D, Shanghai Shenshun Biological Technology Co., Ltd., Shanghai, China). Afterward, the concentrated extract was freeze-dried (Beijing Boyi Kang Experimental Instrument Co., Ltd., Beijing, China). The dried extract was dissolved and diluted in deionized water, and used in the experiments.

# Bacterial Culture

The bacterial strain of pathogenic *B. subtilis* (CGMCC 1.3358) (China General Microbiological Culture Collection Center, Beijing, China) was uniformly streaked on beef peptone (BP) solid medium (3 g/L beef extract + 10 g/L peptone + 5 g/L sodium chloride + 20 g/L agar, pH 7.0–7.2). After 12 h of incubation at 37°C, the bacterium was transferred to liquid BP medium, and incubated for 12 h on a shaker with continuous shaking (100 rpm) at 37°C, and used in the experiments.

## Determination of electrical conductivity value, alkaline phosphatase activity, and nucleic acid and protein contents

Electronic conductivity (EC) value, and alkaline phosphatase (AKP) activity, nucleic acid and protein contents were determined by using the method of Jin et al. (2020). *B. subtilis* suspension (approximately  $1 \cdot 10^8$  CFU/mL) was centrifuged for 4 min at 5,000 rpm, and the precipitated bacterial cells were collected. After adequately rinsing with phosphate-buffered saline (PBS, pH 7.4), *B. subtilis* cells in the OE group were added with 10 mM PBS and MIC of OE (8 mg/mL) that was previously determined by Tian et al. (2019), and those in the control group were added with same volume of BP liquid medium. After 2 h of incubation (130 rpm) at 37°C, the EC value was measured by using an EC measurement instrument (DDSJ-308A, INESA Scientific Instrument Co., Ltd., Shanghai, China).

A total of 2 mL of *B. subtilis* suspension (approximately  $1 \cdot 10^8$  CFU/mL) was inoculated into 100 mL of liquid BP medium, and OE was added with its MIC. The the same volume of the liquid BP medium was added to the control group. After 2 h of incubation (130 rpm) at 37°C, the mixture was centrifuged for 5 min at 5,000 rpm, and the supernatant was collected to determine AKP activity and nucleic acid and protein contents. Nucleic acid content was determined at an optical density (OD) of 260 nm (UV-2600, Shimadzu Corporation, Kyoto, Japan) according to Jin et al. (2020). The AKP activity and protein content were determined by using an AKP assay kits (Nanjing JianCheng Bioengineering Institute, Nanjing, China) and Coomassie Brilliant Blue (CBB) (Nanjing JianCheng Bioengineering Institute), respectively, in accordance with the manufacturer's instructions. The values of AKP and the protein were measured at 520 (iMark, Bio-Rad Laboratories, Inc., Hercules, CA, USA) and 595 nm (UV-2600, Shimadzu Corporation), respectively.

The intracellular protein content of *B. subtilis* was measured by applying the methods for bicinchoninic acid (BCA) assay and sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis described by Jin et al. (2020). In brief, 1 mL of *B. subtilis* suspension (approximately  $1 \cdot 10^8$  CFU/mL) was inoculated into 100 mL of liquid BP medium and treatment with OE at 1/2MIC in the OE group. The control group was added with the same volume of liquid BP medium. After 2 h of incubation (130 rpm) at 37°C, the mixture was centrifuged at 5,000 rpm for 4 min, and precipitated bacterial cells were collected. The cells were rinsed with PBS (pH 7.4), resuspended in sterile water, and sonicated for 10 min at 200 w (THC, Jining Tianhua Ultrasonic Electronic Instruments Co., Ltd., Jining, China). The mixture was centrifuged (12,000 rpm) for 15 min at 4°C and the supernatant was collected. For the determination of

protein content, 2.5  $\mu\text{L}$  of supernatant, 2.5  $\mu\text{L}$  of double distilled water, 100  $\mu\text{L}$  of BCA were added to a 96-well plate. After reaction at 37°C for 30 min, the OD value of the mixture was measured at 550 nm, and protein content was calculated on the basis of the standard curve of BCA (purity  $\geq 95\%$ ) (Sigma-Aldrich, St. Louis, MO, USA). For SDS-PAGE analysis, the supernatant (100  $\mu\text{L}$ ) was mixed with 25  $\mu\text{L}$  of loading buffer (3 g/L Tris-HCl + 1 g/L sodium dodecyl sulfate + 14.4 g/L glycerine), and the mixture was subjected to gel electrophoresis for 30 min at 80 V and for an additional 1 h at 110 V. The gel was dyed by using CBB R-250 (Nanjing JianCheng Bioengineering Institute), and separated protein bands were observed after 24 h of decoloration with glacial acetic acid, ethanol, and distilled water.

## Determination Of The Inhibitory Rate Of Respiration

The inhibitory rate of respiration ( $I_R$ ) against *B. subtilis* and superposition rate ( $S_R$ ) was determined by measuring dissolved oxygen (DO) (Jin et al. 2020). In brief, 2 mL of *B. subtilis* suspension ( $1 \cdot 10^8$  CFU/mL) was mixed with 10 mL of PBS (pH 7.4) and then, OE with 1/2MIC or 0.5 mg/mL typical respiratory inhibitors of iodoacetate acid (IA), malonate acid (MA), and sodium phosphate (SP), or a combination of both (i.e., OE + IA, OE + MA, and OE + SP). Each mixture was exposed to air for 5 min, and the DO value was measured at 1 min intervals within 10 min by using a DO meter (Shanghai ZhenMai Instruments Co., Ltd., Shanghai, China). The difference in DO values per min was calculated as the respiratory rate.

$I_R$  and  $S_R$  were calculated by using the following equations (Cui et al. 2018).

$$I_R(\%) = \frac{R_0 - R_1}{R_0} \times 100$$

$$S_R(\%) = \frac{R_1 - R_2}{R_1} \times 100$$

$R_0$  is the respiratory rate of the control,  $R_1$  is the respiratory rate of OE or each respiration inhibitor, and  $R_2$  is the respiratory rate of the combination of OE with the respiration inhibitors, and the  $S_R$  of OE with different typical respiratory inhibitors (IA, MA, and SP).

## Determination Of Enzyme Activities

The activities of key enzymes in the EMP pathway (i.e., hexokinase [HK], phosphofructokinase [PFK], pyruvate kinase [PK]), and that in HMP pathway (i.e., glucose-6-phosphate dehydrogenase [G6PD]) were determined by using the relevant assay kits in accordance with the manufacturer's instructions. HK, PFK, and PK assay kits were purchased from Nanning Jiancheng Bioengineering Institute (Nanjing, China), and G6PD assay kit was purchased from Shanghai Yuanye Bio-Technology Co., Ltd. (Shanghai, China). A total of 1 mL of *B. subtilis* suspension ( $1 \cdot 10^8$  CFU/mL) was mixed with 1 mL of OE with 1/2MIC in the OE groups. The control group was added with the same volume of liquid BP medium. The mixture was

incubated (130 rpm) at 37°C for 2 h and centrifuged for 4 min at 5,000 rpm. The supernatant was used to determine enzyme activities at OD of 340 nm (UV-2600, Shimadzu Corporation).

## Statistical analysis

The results were expressed as the mean  $\pm$  standard deviation of six replicates. Statistical analysis was performed via ANOVA followed by Student *t*-test by using GraphPad Prism 8 (Graphpad Software, Inc., San Diego, CA, USA).  $p < 0.05$  was considered significant.

## Results

### Effect of OE on cell permeability of *B. subtilis*

AKP generally locates between bacterial cell membranes and cell walls, and it cannot be identified in a bacterial suspension. Figure 1 shows that AKP activity in *B. subtilis* suspension significantly increased ( $p < 0.01$ ) after 2 h of OE treatment, but did not change in the OE-untreated control group, indicating that the cell walls of *B. subtilis* were probably destroyed by OE treatment, leading the release of the AKP.

The leakage of cell contents was also found in the present study. Figure 2A shows that the EC value of *B. subtilis* suspension was remarkably ( $p < 0.01$ ) elevated in the OE treatment group, but that in the control group did not increase from 0 h to 2 h of incubation. The values of OD<sub>260 nm</sub> in *B. subtilis* suspension significant ( $p < 0.01$ ) increased after OE treatment (Fig. 2B), indicating a leakage of the nucleic acid from *B. subtilis* cells. Furthermore, extracellular and intracellular protein contents were detected to investigate the protein leakage. The high protein contents of *B. subtilis* suspension increased after 2 h of OE treatment, and was obviously higher ( $p < 0.01$ ) than that of the control (Fig. 3A), indicating protein leakage. Intracellular protein content was also identified through BCA assay and SDS-PAGE analysis. The BCA assay showed that OE treatment significantly decreased ( $p < 0.01$ ) the extracellular protein content (Fig. 3B). SDS-PAGE analysis revealed similar result (Fig. 3C) and showed that the protein bands of the OE group were lighter than those of the control group. These results indicated that residual cellular protein contents decreased in correspondence with the increase in extracellular protein content.

### Effect of OE on respiratory metabolism of *B. subtilis*

The inhibitory respiratory degree of OE against *B. subtilis* was not entirely consistent with the three typical inhibitors in the respiration pathways but its  $I_R$  of OE reached over 40% (Fig. 4A). Therefore, the  $S_R$  was determined to identify the respiratory metabolic pathways that was regulated by OE. Figure 4B shows that the  $S_R$  of OE + IA and OE + SP were lower than those of OE + MA, revealing that OE likely regulated the IA- or SP-represented respiratory metabolic pathways. Consequently, the key enzymes in the EMP (i.e., HK, PFK, and PK) and HMP (i.e., G6PD) were identified to prove the above result. After 2 h of OE treatment, HK and PFK activities significantly decreased ( $p < 0.01$ ) but PEK activity did not show an obvious change (Fig. 5). In addition, a significant reduction in G6PD activity was also observed under OE treatment for 2 h (Fig. 6).

## Discussion

The rapid development of plant cell culture technology offers the possibility of acquiring sufficient materials from rare and precious plant species. For nearly 20 years, cell culture protocols have been extensively studied to improve culture systems. However, little is known regarding the bioactivity of cultured cells, thus delaying cell application. Previous studies have indicated that *O. cartilaginosa* cell cultures contain phenolics, flavonoids, and polysaccharides (Piao et al. 2017; Zhang et al. 2017) those are known as antibacterial substances. The antibacterial effects of the field-grown plants of *Orostachys* species have been investigated by Ding and Wang (2013), and Ren et al. (2011). Furthermore, the inhibitory effect of *O. cartilaginosa* cultured cells against *B. subtilis* has also been reported in our previous study (Tian et al. 2019). However, the antibacterial mechanisms of *Orostachys* species including field-grown plants and cultured cells have not been investigated to date.

The present study investigated antibacterial mechanisms of *Orostachys* in the first time, and found that OE increased AKP activity in *B. subtilis* suspension (Fig. 1), and the levels of EC, nucleic acid, and protein in *B. subtilis* suspensions increased after OE treatment (Figs. 2 and 3). Most studies regarding antibacterial mechanisms have performed in terms of cell permeability change. For example, Liu et al. (2017) found that baicalin exerts its antibacterial effect by enhancing the cell permeability of *Escherichia coli*, resulting in the extensive leakage of cell contents. Li et al. (2019) indicated that a chitosan-gallic acid derivative disrupts the cell membranes of *E. coli* and *Staphylococcus aureus*, resulting in the cytoplasm leakage and EC increase. The result of the present study implied that *B. subtilis* cell walls were destroyed and cell membrane permeability was enhanced by OE treatment, leading the cell contents (including electrolyte, nucleic acid, and protein) leaked out, which subsequently resulted in inhibition of *B. subtilis* growth or death.

In addition to the cell permeability change, antibacterial mechanisms based on respiration inhibition have also been widely studied. Respiratory metabolic pathways mainly include the TCA, EMP, and HMP pathways; IA, MA, and SP are the typical inhibitors of the TCA, EMP, and HMP pathways, respectively (Jin et al. 2020). The  $S_R$  of the antibacterial substance with a typical respiration inhibitor is often used to identify the respiratory metabolic pathway that involves a specific antibacterial substance (Zheng et al. 2015). A low  $S_R$  indicates that substance has a high probability of affecting a typical inhibitor-represented pathway (Tong et al. 2005). Studies on antibacterial mechanisms in respiratory metabolism have showed that different antibacterial substances are involved in various pathways (Cui et al. 2018; Cui et al. 2019; Hu et al. 2019). The present study found that OE inhibited *B. subtilis* respiration (Fig. 4A), and the groups of OE + MA and OE + SP showed low  $S_R$  (Fig. 4B). These results implied that OE was involved in the EMP and HMP pathways. HK, PFK, and PK are the key enzymes in the EMP pathway (Fig. 5). OE treatment decreased the activities of HK and PK in the present study but did not significantly affect PFK activity, proving that OE participated in the EMP pathway by regulating HK and PK activities. Furthermore, G6PD is the key enzyme of the HMP pathway (Fig. 6), and its activity was decreased by OE treatment. This result therefore indicated that OE also participated in the HMP pathway by regulating G6PD activity. This

involvement indicated that *B. subtilis* respiration was inhibited through an inhibition of key enzymes related to respiratory metabolic pathways.

## Conclusions

OE treatment increased AKP activity, EC value, and nucleic acid and protein contents in *B. subtilis* suspension, indicating the cell damage or increment in cell permeability. *B. subtilis* respiration was inhibited by OE treatment, and low  $S_R$  was found in the combination groups of OE + IA and OE + SP. The HK, PK, and G6PD activities of *B. subtilis* decreased after OE treatment, revealing that OE inhibited *B. subtilis* respiration by regulating the EMP and HMP pathways.

## Declarations

### Funding

This research was supported by the National Natural Science Foundation of China (31660080).

### Conflicts of interest

The authors declare that they have no conflict of interest.

### Ethics approval

Not applicable.

### Consent to participate

The authors declare that they consent to participate.

### Consent for publication

The authors declare that they consent for publication.

### Availability of data and material

Data are available by e-mail on reasonable request.

### Code availability

Not applicable.

**Author' contributions** YXL conducted experiment of cell permeability. XLJ conducted experiment of respiratory metabolite. YNX cultured bacterial strain. XCP designed the experiments. MLL wrote the paper.

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## Figures

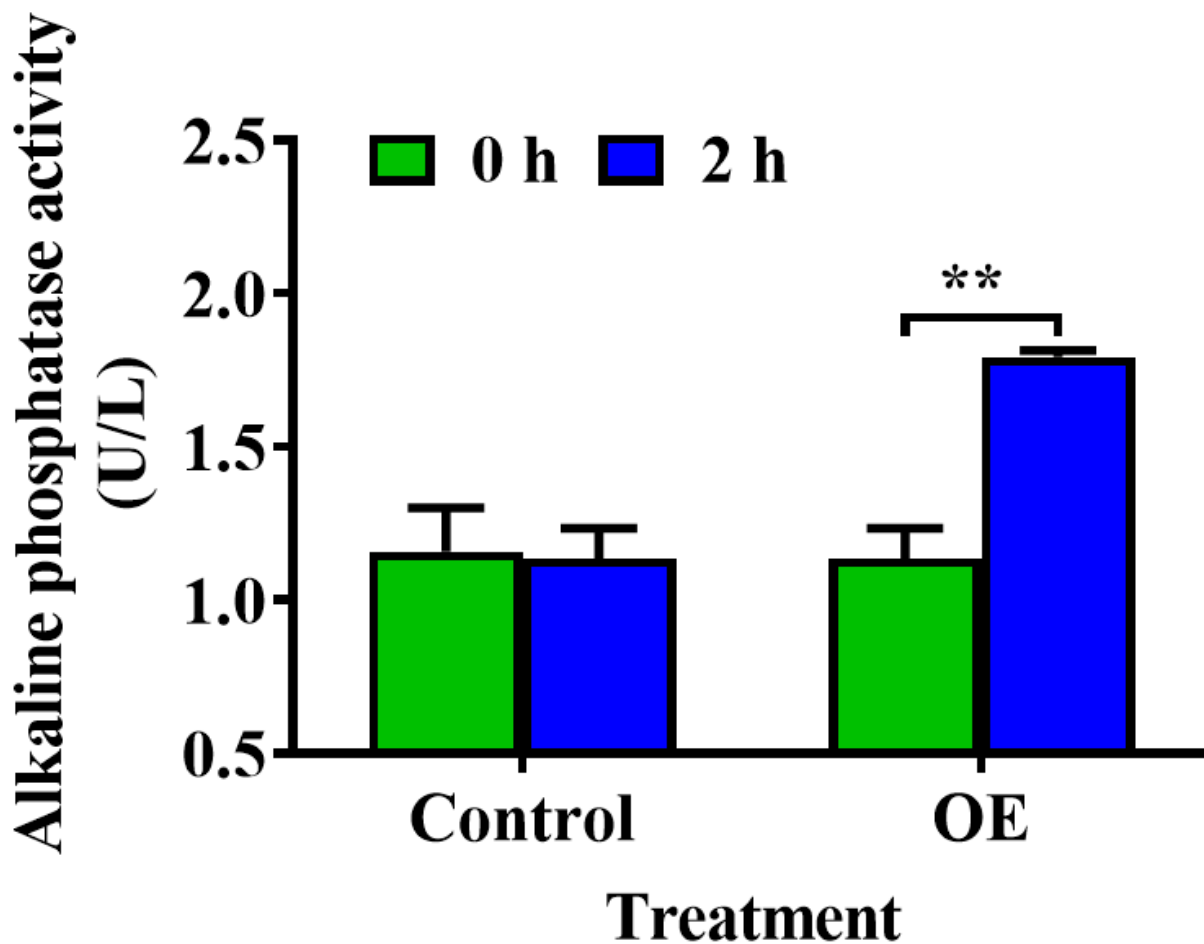
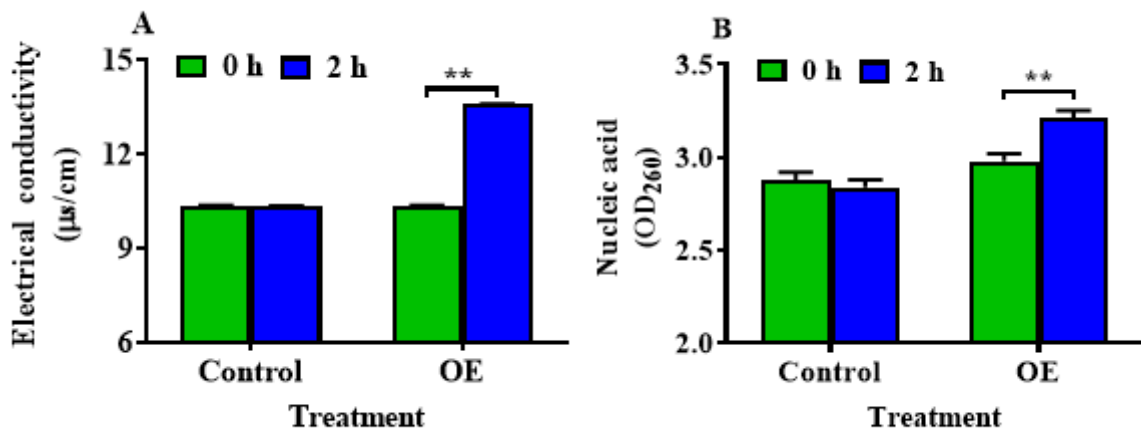


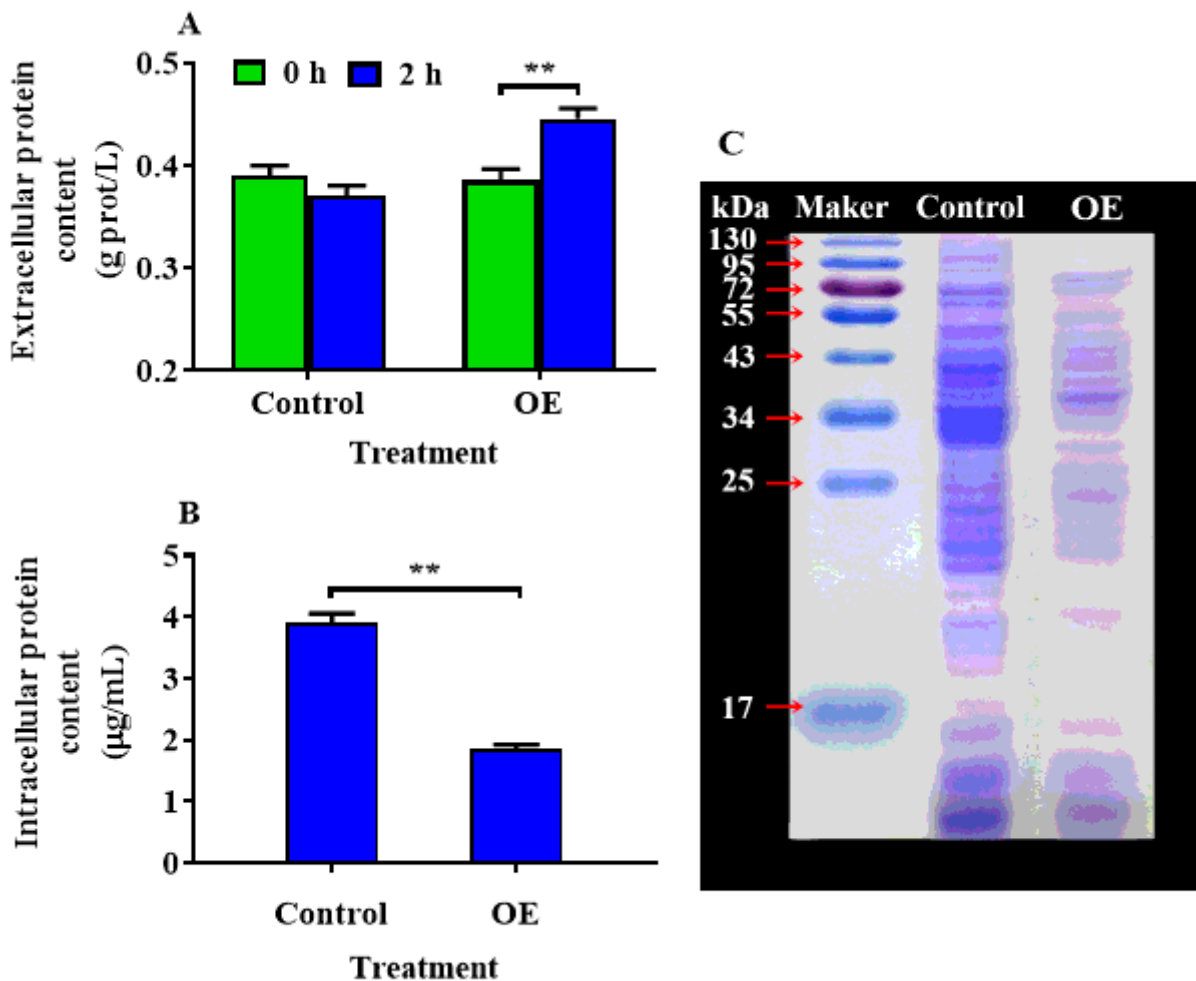
Figure 1

Effect of extract from *Orostachys cartilaginea* cell cultures (OE) on alkaline phosphatase activity of *B. subtilis* suspension. Data are the mean  $\pm$  standard deviation (n = 6). \*\*indicates the values were considered to be significant at  $p < 0.01$  versus the control group.



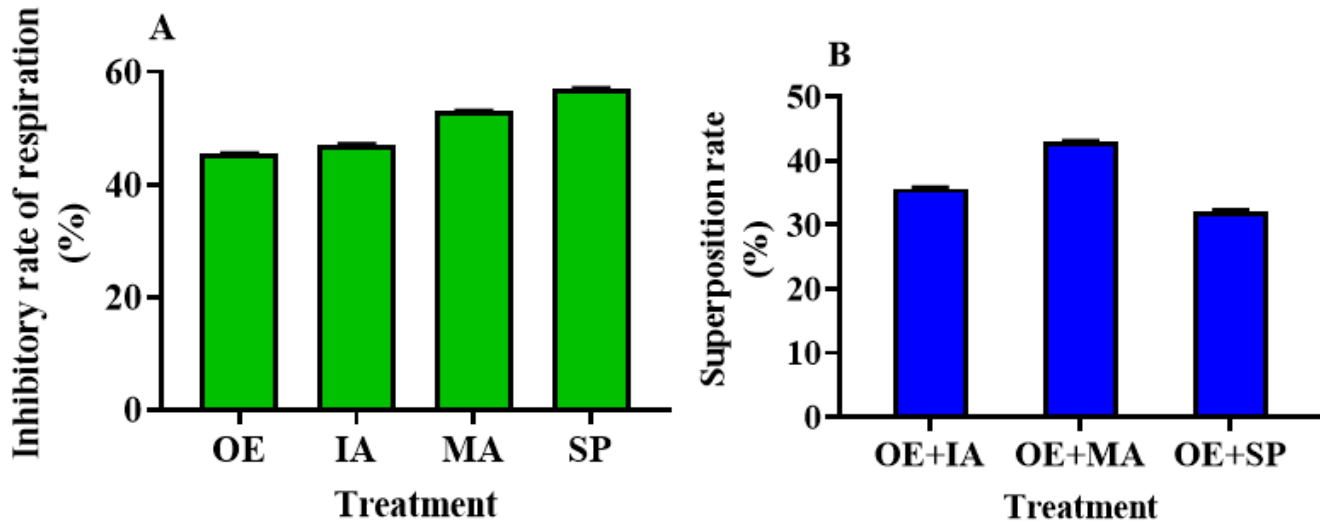
**Figure 2**

Effect of extract from *Orostachys cartilaginosa* cell cultures (OE) on electronic conductivity (A) and nucleic acid content (B) in *B. subtilis* suspension. Data are the mean  $\pm$  standard deviation (n = 6). \*\*indicates the values were considered to be significant at  $p < 0.01$  versus the control group.



**Figure 3**

Effect of extract from *Orostachys cartilaginosa* cell cultures (OE) on protein leakage. A, protein content in *B. subtilis* suspension. B, protein content in *B. subtilis* cells. C, gel electrophoresis image of proteins in *B. subtilis* cells. Data are the mean  $\pm$  standard deviation (n = 6). \*\*indicates the values were considered to be significant at  $p < 0.01$  versus the control group.



**Figure 4**

Effect of extract from *Orostachys cartilaginosa* cell cultures (OE) on respiratory metabolism of *B. subtilis*. A, respiration inhibitory rate. B, superposition rate. Data are the mean  $\pm$  standard deviation (n = 6). Iodoacetic acid (IA), malonic acid (MA), and sodium phosphate (SP) are typical inhibitors of the respiratory metabolic pathways.

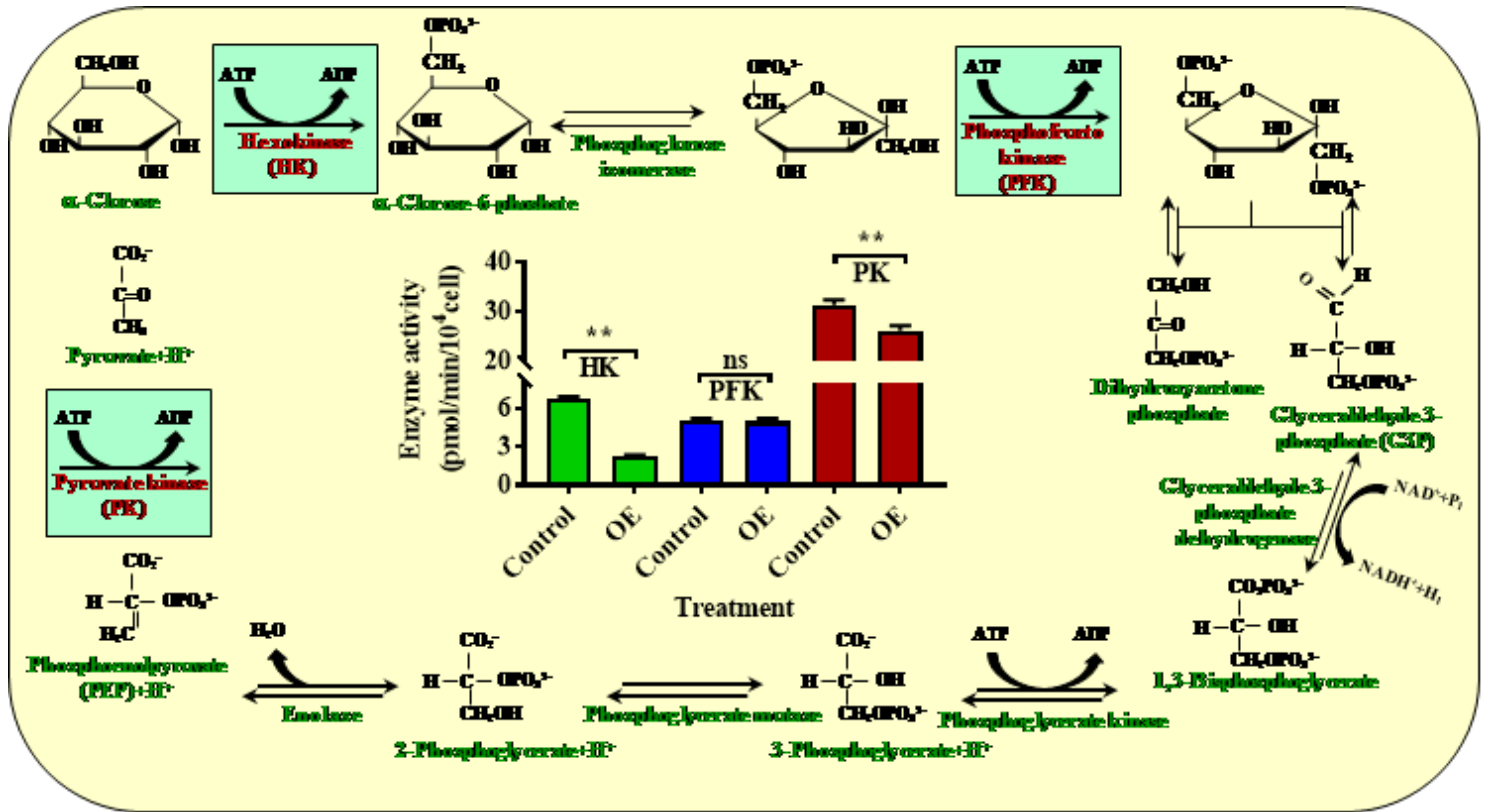
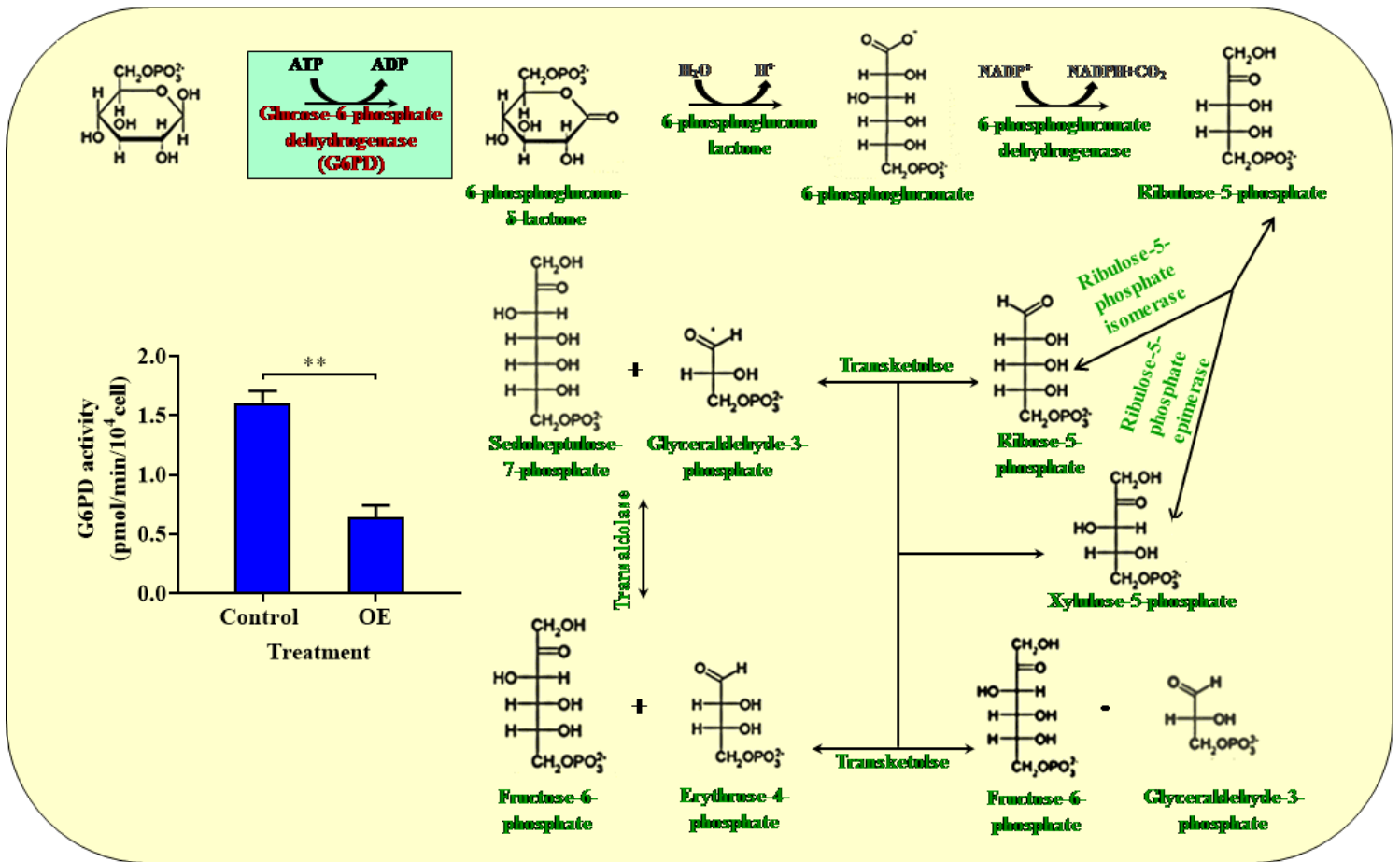


Figure 5

Embden Meyerhof Pathway and effect of extract from *Orostachys cartilaginosa* cell cultures (OE) on activities of hexokinase (HK), phosphofruktokinase (PFK), and pyruvate kinase (PK) of *Bacillus subtilis*. Data are the mean  $\pm$  standard deviation (n = 6). \*\*indicates the values were considered to be significant at p < 0.01 versus the control group.



**Figure 6**

Hexose Monophosphate Pathway and effect of extract from *Orostachys cartilaginous* cell cultures (OE) on activity of 6-phosphate 1-dehydrogenase (G6PD) of *Bacillus subtilis*. Data are the mean  $\pm$  standard deviation (n = 6). \*\*indicates the values were considered to be significant at p < 0.01 versus the control group.