

Full Length Research Paper

The ultrasonic effect on the mechanism of cholesterol oxidase production by *Brevibacterium* sp.

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The effects of ultrasonic radiation on cholesterol oxidase production by *Brevibacterium* sp. are studied in this paper. An ultrasonic wave with low intensity at 20 kHz, 200 W/ cm² was employed to study the effects of irradiation at different lengths of time on the growth of *Brevibacterium* sp. cells. The result showed that the production of cholesterol oxidase from *Brevibacterium* sp. obviously increased, and was about 18.3%, when the culture was radiated for 1 min every 4 h. In order to investigate the action of ultrasonic on cell membrane and to facilitate enzyme excretion, transmission electron microscopy (TEM) was employed. Scanning of the bacterial cells by TEM also first revealed that holes appeared in the cytoplasmic region, then excluded from the cell barrage, and finally self-destructed with higher irradiating dosage.

Key words: Ultrasonic, cholesterol oxidase, permeability, transmission electron microscope.

INTRODUCTION

With the continuous development of biotechnology, biotechnology products have gradually occupied increasing proportions of the national economy, and have become pillars of the high-tech industry. Currently, traditional fermentation cannot meet the demands of modern biological reaction engineering. The continued use of novel scientific research findings within related disciplines has improved the literature on biological reaction technology. At present, research on reaction characteristics (such as enzyme activity, etc) has been studied and has gained a good degree of research results. Effect of ultrasonic treatment on reaction processes has also aroused great interest and attention with the popularity and development of ultrasonic equipment in recent years. This study shows that a suitable intensity of ultrasonic irradiation can create a biological reaction medium which can enhance the efficiency of gene transfer,

increase the permeability and selectivity of cell membranes, and promote the secretion of enzymes and enhance cell metabolism, thus, shortening reaction time, and improving product quality and yields. There are many reports regarding this aspect of research in China and abroad. For example, Pablo Resa studied the monitoring of lactic acid fermentation in culture broth using ultrasonic velocity (Pablo Resa et al., 2007). Under the conditions used, the microorganism was basically homofermentative and sugars were predominantly transformed into lactic acid. A remarkable correlation was found between ultrasonic velocity and bacterial catabolism. These results could be used to model changes occurring during lactic acid fermentation and show the great potential of this non-invasive technique for monitoring biotechnological processes. Liu et al. (2003) studied the influence of ultrasonic stimulation on the growth and proliferation of *Oryza sativa* Nipponbare callus cells. This study aimed to investigate the effect of ultrasonic stimulation on the growth and proliferation of *Oryza sativa* Nipponbare cells (rice callus) in suspension culture. It was found that ultrasonic stimulation could promote the growth and proliferation of *O. sativa* Nipponbare cells in suspension culture with the optimal stimulation of 5 s, while with longer agitation, its

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Abbreviations: TEM, Transmission electron microscopy; COD, cholesterol oxidase; FAD, flavin adenine dinucleotide.

growth and proliferation was inhibited. The mechanism may be that the ultrasound activated or destroyed the cellular structure, such as cell membrane, cytoskeleton and mitochondria in which many enzymes and ion channels are affected. Lo'rincz studied on ultrasonic cellular disruption of yeast in water-based suspensions (Lo'rincz, 2004). It was found that a concentration of 3.2 g/l lyophilised *Saccharomyces cerevisiae* baker's yeast stopped cavitation in the ultrasound field. Then, by using multiples of the aforementioned concentration, the acoustic phenomena occurring in the ultrasound field were monitored and, simultaneously, the survival dynamics of the yeast cells were examined. Physical parameters of the ultrasound field had an essential effect on the acoustic phenomena formed in the sound field and on the threshold levels of their formation.

The stimulation of *Brevibacterium* sp. with a high cholesterol oxidase yield for reducing cholesterol by means of biological technique remains an important task for researchers.

Cholesterol oxidase form *Brevibacterium* sp., a FAD-dependent enzyme that catalyzes the oxidation and isomerization of cholesterol into cholest-4-en-3-one and yields hydrogen peroxide, has been widely applied in clinical assays. Moreover, COD exhibits a potent insecticide activity and can inhibit the growth and generation of Lepidoptera (Purcell et al., 1993; Cho et al., 1995). It has also been reported that COD can degrade food cholesterol efficiently and economically, especially egg yolk cholesterol (Aihara and Watanabe, 1988; Speroulla et al., 1994).

COD from different microorganisms contain different physicochemical properties and substrate specificity. A mutant DGCDG2 from *Brevibacterium* sp., which was isolated previously from soil and subjected to genetic improvement, showed extracellular COD production with cholesterol as the inducer and supplementary carbon source (Wenming, 2000).

In this article, *Brevibacterium* sp. was treated by imposing ultrasonic waves during the submerged fermentation course. The industrial microorganism treated ultrasonically and its relevant biological behaviors have been reported in the past (Wood et al., 1997; Jeong et al., 2000; Cowan et al., 1999; Dazhong et al., 1996). The products from *Brevibacterium* sp. subjected to ultrasonic treatment do not have any medical side effects, and the treatment can also increase product yield.

MATERIALS AND METHODS

Microorganism

Brevibacterium sp. DGCDC-2, preserved by this laboratory, was used throughout this study.

Culture conditions

The agar medium consisted of 0.3% beef extract, 0.5% NaCl, 1%

peptone, 2% agar. pH was adjusted to 7.5 with 0.1 M NaOH. The seed culture medium consisted of 0.3% beef extract, 0.5% NaCl, 1% peptone, 2% agar. pH was adjusted to 7.5 with 0.1 M NaOH. The fermentation medium consisted of 2% glucose, 0.15% cholesterol, 0.75% yeast extract, 0.1% NaCl, 0.01% CaCl₂, 0.2% CH₃COONH₄, 0.02% K₂HPO₄, 0.005% MgSO₄·7H₂O, 0.001% FeSO₄·7H₂O, 0.05% Tween-80, pH was adjusted to 7.5 with 0.1 M NaOH. After autoclaving for 20 min, 50 ml aliquots of culture medium were inoculated with 5 ml activated seed culture and subjected to incubation in a 500 ml Hinton's flask at 30°C for 36 h in an orbital shaker.

Ultrasonic treatment

The culture medium of *Brevibacterium* sp. was treated by ultrasonic. Irradiation was started after incubating for 12 h. The culture medium was irradiated once every 4 h. The time periods of irradiation were 20 min, 10 min, 5 min, 2 min, 1 min, 30 s, 10 s and 5 s based on the requirements of our study. After sonication, the culture medium was continuously incubated at 30°C for a period of time without sonication.

Measurement of cholesterol oxidase

50 µL crude enzyme were incubated with 3 mL solution A(4-Amino-antipyrine 1 mmol/L; phenol 6 mmol/L; Sodium azide 0.2 g/L; peroxidase 5000 U/L; potassium phosphate buffer 25 mmol/L, pH = 7.5) and 150 µL solution B(cholesterol 8.26 mg/mL; Triton X-100, 4.26%; isopropanol for solvent) for 5 min at 37°C, then were boiled, and were measured by spectrophotometry at 500 nm.

$$\text{Enzyme activity (U/mL)} = 0.1315A_{500} \times 3.2 \times 20 \div 5 = 1.6832 \times A_{500}$$

Dry weight of the organism

After incubation, 10 mL even culture medium samples were centrifuged for 10 min at 8000 rpm, and then weighed after drying at 80°C for 24 h. Dry mycelia weight (w1) was obtained and converted into: w1 × 100 (g/L).

Electron microscope investigation

The wet cells of *Brevibacterium* sp. and the ultrasonic-treated cells were coated with gold sputter coater for 3 min, to increase the electron conductivity. The sample was then studied using the transmission electron microscope.

RESULTS AND DISCUSSION

Effect of ultrasonic on COD production

The culture medium of *Brevibacterium* sp. was treated by ultrasonic during the course of incubation. After incubation, enzyme activity was measured by spectrophotometry at 500 nm (Figure 1).

The incubation was carried out for both the treated strains and the untamed strain. The results shown in Figure 1 indicate that COD production during ultrasonic action was affected, even though the COD yields were higher than those of the parent strains. When ultrasonic treatment lasted for a longer time, the cell death rate

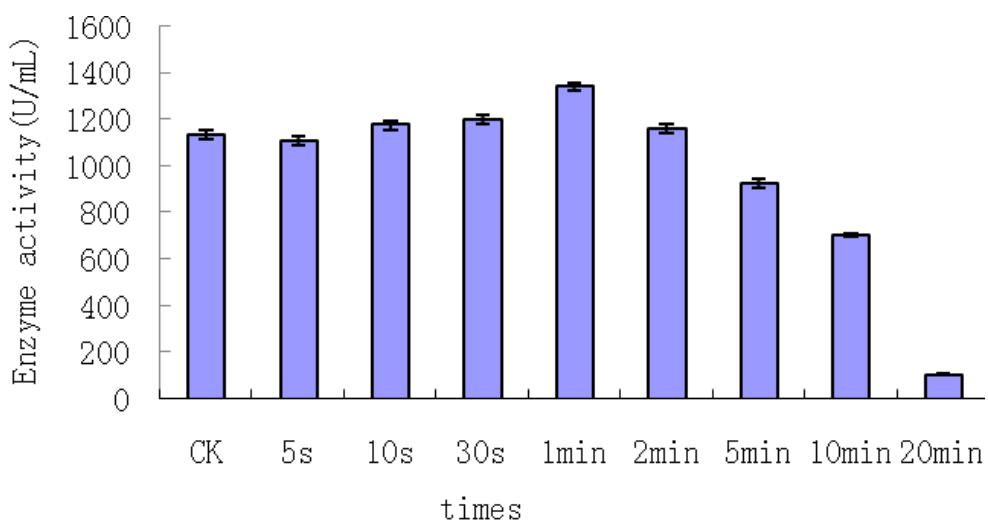


Figure 1. Effect of ultrasonic on COD production.

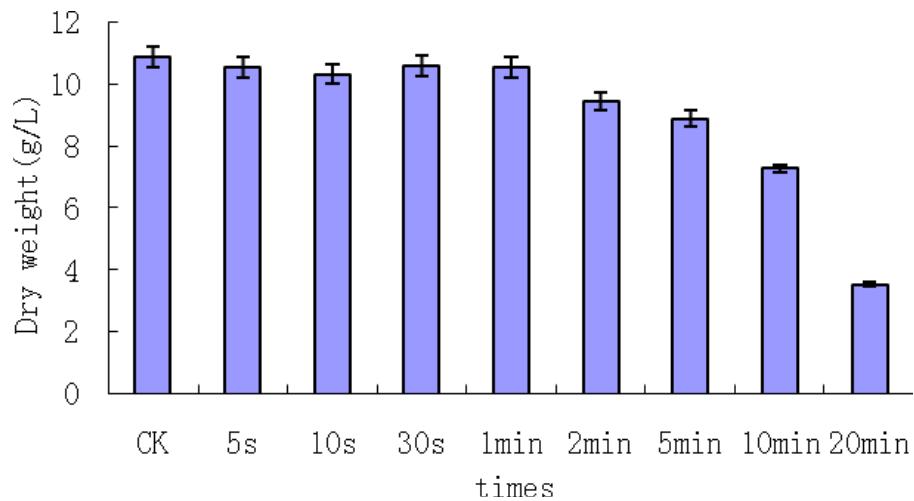


Figure 2. Effect of ultrasonic on the dry weight of the organism.

increased, and caused decreasing yields of COD. Nonetheless, the yield obviously increased, and was about 18.3%, when the culture medium was radiated for only 1 min.

Effect of ultrasonic on the dry weight of the organism

The culture samples were centrifuged for 10 min at 8000 rpm, and were then weighed after drying at 80 °C for 24 h. The dry mycelia weight (w_1) was measured (Figure 2).

As shown in Figure 2, the treatment time could affect the dry cell weight. When ultrasonic treatment lasted for a longer time, cell viability decreased continuously during the fermentation period. When the ultrasonic treatment time exceeded a certain value, cell viability gradually decreased from the starting point of the ultrasonic treat-

ment. If ultrasonic treatment time exceeded 2 min, the dry mycelia weight obviously decreased, indicating that some of the cells died. Irradiation at a shorter length of time could have also led to the decrease of cell viability, but the increase in viable cell counts was observed in static incubation after ultrasonic treatment. High cell viabilities were obtained when combining sonicated fermentation with static incubation.

Exploration of the mechanism

When the cells were treated by ultrasonic, the permeability of the cell membrane changed. In order to investigate the action of ultrasonic treatment on cell membrane and to facilitate enzyme excretion, transmission electron microscopy (TEM) was used. The scanning of the bacterial

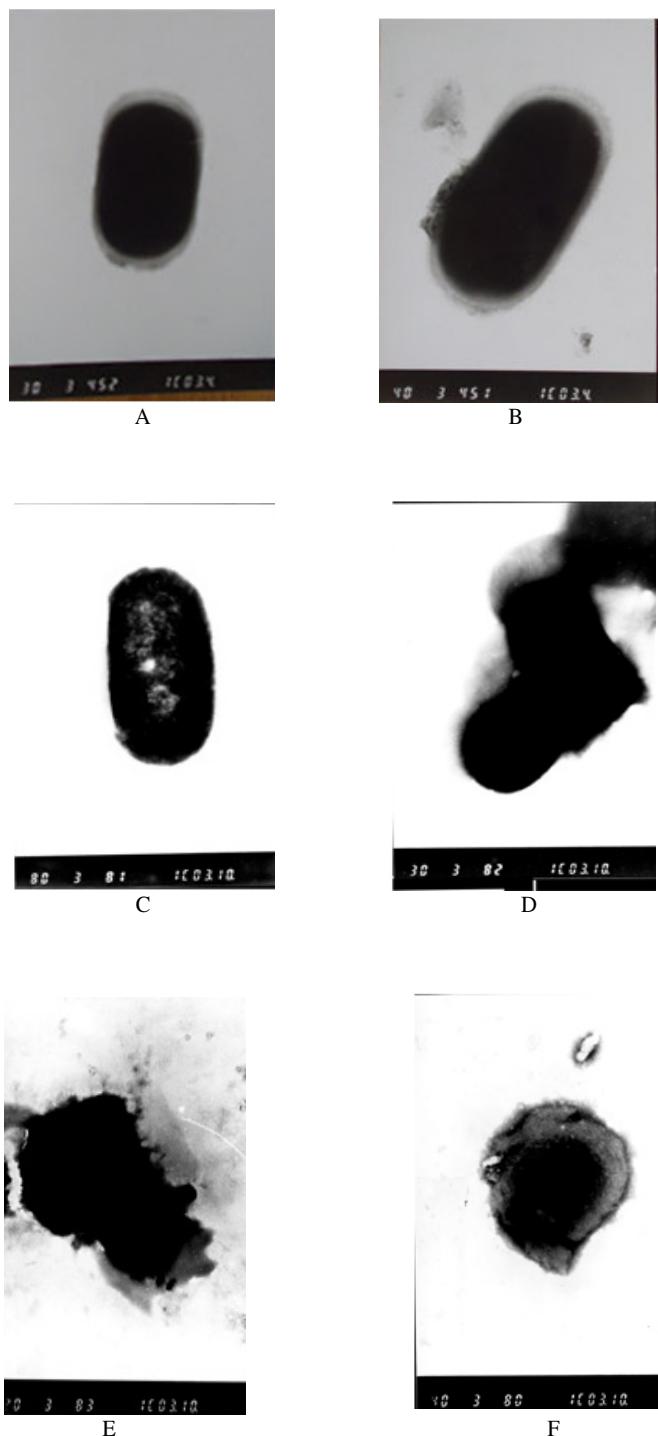


Figure 3. Transmission electron microscopy (TEM) of *Breibacterium* sp., untreated (A) and treated by ultrasonic at 30°C for 20 min (B), 30 min (C), 40 min (D), 50 min (E), 60 min (F).

cells also first revealed that holes appeared in the cytoplasmic region, then excluded from the cell barrage, and finally self-destructed with higher irradiating dosage. A smaller ultrasonic irradiating power injured the microbial

cells, and changed the permeability of the cell membrane to promote the excretion of metabolites of industrial microorganisms.

The fresh cells had a smooth and intact surface as shown in Figure 3A. The cells that underwent ultrasound treatment for 20 min (Figure 3B), still had a few cells intact. Among this small number of cells that maintained their integrity, the hyphal cell surfaces contains much cell debris left by the cells that broke down. From the simple observation it is obvious that the morphological view has not changed. Any changes that may not have been seen by TEM might be traced after the restoration of the original static culture and topography. For more detailed observations, we also performed further experiments. As shown in Figure 3C, the permeabilized cells showed the morphological difference in that they had relatively small holes compared to the fresh cells (Figure 3A). When the cells were treated for 40 min (Figure 3D), the small holes became bigger, then the cytoplasm excluded from the cell barrage, and finally resulted in a highly dispersed outer structure (Figure 3E). After further increasing the dosage, the cytoplasm continued to leak, and finally the cells self-destructed (Figure 3F) following higher irradiating dosage. A small energy of ultrasonic irradiation would have injured the microbial cells, and changed the permeability of the cell membrane to promote excretion of metabolites of industrial microorganisms.

Ultrasonic treatment has a strong biological effect on microorganisms. The mechanism is very complex, but the major effect might be cavitation. Cavitation is a series of dynamic courses: vibration, enlargement, shrinking, and even collapse may occur when using ultrasonic treatment. These courses happen when small air bubbles (vapor bubbles, or holes) are formed in the liquid phase. Cavitation bubbles shrink under heat insulation conditions, and even instantly collapse. In the twinkling, the super high temperature of 5000°C and several thousands atmospheres may cause bubbling, and association with the powerful shockwave or shooting flow may lead to the damage of the cell wall and the reversible change in permeability of the cell membrane. Thus, the mass transfer of substrate substances and secretion of products may be increased. This may also impose hydrostatic pressure on the cell membrane, consequently breaking the cell membrane and speeding up the secretion of products.

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REFERENCES

- Pablo R, Toma's B, Luis E (2007). Monitoring of lactic acid fermentation

- in culture broth using ultrasonic velocity. *J. Food Eng.* 78: 1083-1091.
- Lo'rincz A (2004). studied on Ultrasonic Cellular Disruption of Yeast in Water-based Suspensions. *Biosyst. Eng.* 89: 297-308.
- Purcell JP, Greenplate JT, Jennings MG (1993). Cholesterol oxidase: a potent insecticidal protein active against Boll weevil larvae. *Biochem. Biophys. Res Commun.* 196: 1406-1413.
- Cho HJ, Choi KP, Yamashita M (1995). Introduction and expression of the *Streptomyces* cholesterol oxidase gene (Cho A), a potent insecticidal protein active against Boll weevil larvae, into tobacco cells. *Appl. Microbiol. Biotechnol.* 44: 133-138.
- Aihara H, Watanabe K (1988). Degradation of cholesterol in egg yolk by *Rhodococcus equi* no. 23. *J. Food Sci.* 53: 659-660.
- Speroulla C, Tran VH, Maurie AT, Robert GB (1994). Enzymatic degradation of egg yolk cholesterol. *J. Food Prot.* 57: 908-912.
- Wenming J (2000). Production and properties of cholesterol oxidase from *Bre_idbacterium* sp., PhD Dissertation, Wuxi University of Light Industry, Wuxi, People's Republic of China.
- Wood BE, Aldrich HC, Ingram LO (1997). Ultrasound stimulates ethanol production during the simultaneous saccharification and fermentation of mixed waste office paper. *Biotechnol. Prog.* 13(3): 232-237.
- Jeong Yoon KU, Sung OP, Bong SN (2000). Inactivation of peroxidase by hurdle technology. *Food Sci. Biotechnol.* 9(2): 124-129.
- Cowan SE, Black J, Keasling JD White RM (1999). Ultrasonic flexural plate wave sensor for detecting the concentration of settling *E. coli* W3110 cells. *Analyt. Chem.* 71(16): 3622-3625.
- Dazhong W, Sakakibara M, Kondoh N Suzuki K (1996). Ultrasound enhanced lactose hydrolysis in milk fermentation with *Lactobacillus bulgaricus*. *J. Chem. Technol. Biotechnol.* 65(1): 86-92.