

# Study on Identification of the Pressure-Resistant Bacteria Isolated from Coconut Puree and its Lethal Effect with Combined Mild Temperature and Ultrahigh Pressure

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**Abstract**—This research based on fresh coconut and the coconut puree was conducted with the ultra-high pressure (500MPa/ 10min). Two strains of pressure-resistant bacteria in coconut puree were screened out and identified as *Leuconstoc mesenteroides* subsp *mesenteroides* and *Leuconstoc mesenteroides* subsp *dextranicum*. When comparing with constant and intermittent pressure treatments. Treatment with the intermittent pressure of 300MPa-600MPa can greatly improve the lethal effect of pressure-resistant bacteria. The fatality rate on *L. mesenteroides* subsp *mesenteroides* and *L. mesenteroides* subsp *dextranicum* were 92.6% and 89.5%. Lethality goes up significantly along with the temperature's rising. At 65°C, lethality of *L. mesenteroides* subsp *mesenteroides* and *L. mesenteroides* subsp *dextranicum* were 98.9% and 98.2%.

**Keyword** *coconut puree; pressure-resistant bacteria; identification; ultrahigh pressure; lethality*

## I. INTRODUCTION

*Cocos nucifera* L. belong to *Palmaceae* in *Cocos*. It is mainly one of the perennial woody oil crops and energy crops in tropical area. Coconut is a tropical hi-light plant, which are widely distributed in Asia, Africa, Oceania and Americas, especially in the equator distribution in coastal area. Coconut producing area are mainly distributed in the southeast coast of Hainan island in China, which the area and production about 80 percent of the total amount of the country. The coconut is mainly composed of exocarp, mesocarp, endocarp, seed coat, coconut, coconut water and other parts. Coconut, called the solid endosperm, is the main component of coconut.

Fresh coconut contains 35.2% of fat, 3.8% of protein and 40% of moisture content, and a small amount of VB1, VA, VC, VE,  $\beta$ -carotene and polyphenols, which have the benefit of spleen and stomach, prevent hyperlipidemia, protect the cardiovascular system [1-2]. The coconut had wasted in a certain extent because of the large, heavy and high proportion of coconut, added to feeding and carrying is more inconvenient. Turning coconut into puree will promote the fresh-keeping of coconut and is more advantageous to the consumer demand based on original nutritional quality. Coconut is perishable for

the inevitably infection by microorganisms in product processing. The study found that only rely on the separate ultrahigh pressure processing is difficult to achieve the sterilization requirements of coconut puree [3]. In the process of storage, With the residual barotolerant microbes multiply, the nutrient had decreased and the shelf life had shorten. Therefore, studying for barotolerant microbes of coconut puree is of great significance on coconut meat storage and fresh keeping. This paper adopted the ultra-high pressure 500MPa/10min to deal with the coconut puree and the barotolerant microbes in coconut puree were screened out and identified by morphology, physiology, and molecular biology (16SrDNA) method. At the same time, the lethal effect of the combined mild temperature and ultrahigh pressure treatment on the barotolerant microbes was studied. To improve the theoretical basis for the sterilization technology of coconut puree.

## II. MATERIALS AND METHODS

### A. Materials and reagents

Coconut were bought in the wholesale market of Newport Bridge in Haikou, selected the watering sound small, the shell color deeper and the mature moderate of coconut.

Peptone bacteriological and Beef extract were purchased from Guangdong central Kai Microbial Technology Company Limited; Anhydrous glucose, Agar bacteriological, D (+) galactose, glucose, mannose, xylose and maltose, sucrose and arabinose, were bought from the Pharmaceutical Group Chemcial Reagent Company Limited.

Biological reagents, including bacterial genome DNA extraction kit, PCR recycling kit and purification, dNTP Mix, Taq buffer, goldview, DL2000, DNA Ladder Mix, were provided by Tiangen Reagent Company.

### B. The configuration of the main culture medium

Nutrient AGAR medium (NA): peptone 10.0g, beef extract 3.0g, sodium chloride 5.0g, Agar 17.0g, add distilled

water to 1000mL, dissolved and adjusted the pH to 7.2, packaging sterilization (121 °C, 20 min).

Nutrient broth (NB) medium: peptone10.0g, beef extract3.0g, distilled water 1000mL. Adjusted pH to 7.2, packaging sterilization (121 °C, 20 min).

Plate count AGAR (PCA): tryptone5.0g, yeast extract2.5 g, glucose1.0g, Agar15.0g, distilled water 1000 mL, dissolved and adjusted the pH to 7.0±0.2, packaging sterilization (121 °C, 20 min).

### C. Instruments and equipment

HPP. L3-600/0.6 ultrahigh pressure equipment, Tianjin Huatai Senmiao Company Limited; SW-CJ-1 type fd bench, Suzhou Antai air technology company., LTD;DZ-500/2 s type vacuum packaging machine, Shandong Zhucheng leading machinery co.,LTD; IKA A11Basic analysis using grinding machine, Germany IKA company; AL-204 electronic analytical balance, mettler Toledo instrument (Shanghai) co., LTD.; ZEALWAYGR60DA autoclave, Xiamen micro instrument co.,LTD.;LRH-150-B biochemical incubator, medical apparatus and instruments factory in Guangdong province; Nikon ECKIPSE Ci-s/Ci-L microscope, Nanjing HengQiao instrument co.,LTD.; RDY-SP1Z nucleic acid electrophoresis apparatus, Beijing RongYang classic science and technology co., LTD.A200 gene amplification, Hangzhou lang base scientific instrument co., LTD.;JY04S-3C gel imaging system, Beijing Oriental electrophoresis equipment co., LTD.

### D. Test method

#### 1) The coconut puree of ultrahigh pressure processing

The coconut puree with the vacuum packaged was placed in the pressure medium of the ultra-high pressure vessel,at 500MPa under 10 min. Each sample was carried out for three parallel tests[3].

#### 2) The separation and purification of barotolerant microbes

The coconut puree with processing of ultra-high pressure were homogenized according to the GB4789-2010. Drawing the 200uL homogenate in the PCA medium plate, coating by triangle glass rod, and each dilution gradient of homogeneous liquid coated three plates, cultivated 24-48h at37°C.The bacterial colony was picked on NA medium plate by the sterile toothpicks, crossed and purified repeatedly, the single colony was obtained. The number of the obtained strains was kept at 4°C for reserve [4].

#### 3) Morphology observation of barotolerant microbes

Colony morphology: observed and recorded each colony morphology of nutrient AGAR plate by means of macroscopic. Including colony size, shape, convex surface, edge conditions, colony morphology, surface gloss, color of the colony and colony transparent degree, etc.

Cell form: the strains were separated by the gram staining, bacteria morphology observed by optical microscope and record observations.

#### 4) Physiological and biochemical characteristics of the experiment

Determination of physiological and biochemical characteristics in accordance with the "berger bacteria identification manual" eighth edition (Buchanan et al,1984) and "common bacteria system identification manual".

#### 5) Molecular identification of barotolerant microbes

16SrDNA amplified primers were common primers for bacteria, the forward primer was P1: 5'-AGAG TTT GAT CCTGGTCAGAACGCT-3,40pmol; the reverse primer was P6: 5'-T ACG GCT ACC TTG TTA CGA CTTACCCCC-3',40pmol, the primer was synthesized by Sangon biological engineering. Using 25mL amplification system for PCR amplification [5-7].

#### 6) Sequencing and analysis

After the purification, transformed, the PCR product was obtained and sent to Sangon biological engineering for sequencing. The Blast analysis of the sequence and the GenBank database showed that the results of identification of 16S rDNA [8].

### E. The lethal effect of different pressure mode on barotolerant microbes

#### 1) The preparation of bacterium suspension

The barotolerant microbes was inoculated in a sterile nutrient broth medium, cultivated 24-48h at30°C,200r/min. A small amount of activated bacteria was absorbed and the blood cell counting plate was used to carry the microscope, the initial concentration of the bacteria suspension was about 10<sup>8</sup>cfu/mL. Drawing the 10mL bacteria suspension in the sterile polyethylene bags, vacuum packaging [4].

#### 2) The treatment of different pressure mode on barotolerant microbes

Constant pressure treatments: the bacteria suspension with the vacuum packaged was placed in the pressure medium of the ultra-high pressure vessel, respectively in 300MPa and 600MPa pressure under 10 min at 30°C.

Intermittent pressure treatments: the bacteria suspension with the vacuum packaged was placed in the pressure medium of the ultra-high pressure vessel, started with a lower pressure (300MPa at 30°C) treatment for 5min, followed by a higher pressure (600MPa at 30°C) for another 5min.

### F. The lethal effect of different processing temperature on barotolerant microbes

The bacteria suspension with the vacuum packaged was placed in the pressure medium of the ultra-high pressure vessel, At the pressure of 300MPa-600MPa, respectively in 35°C,45°C,55°C,65°C under 10min, which pressure time of 300MPa and 600MPa was 5 min.

### G. The determination of the fatality rate

According to GB4789.2-2010, microbial counts were determined by the method of plate coating, and the fatality rate was calculated [9].

## III. RESULTS AND ANALYSIS

### A. Isolation and purification of barotolerant microbes

The coconut puree with Vacuum packed was treatment for 500MPa under 10min at 30°C, the homogeneous liquid was coated in PCA medium, and cultivated 24-48h at 37°C. The morphology of colonies was separated from

the surface of the medium, and the colonies were numbered WS1 and WS2 respectively. Colony morphology, cell shape as shown in figure 1, figure 2

Figure1 and figure 2 showed that the strain WS1 colonies were spherical, and the diameter was 0.5-1mm, the surface of the colony was sticky and rose, the color was white. The strain WS2 colonies were spherical, and the diameter was 1-2mm, the surface of the colony was smooth and rose, the color was hoary. The strain WS1 and WS2 were gram-positive bacteria and the cell morphology were in pairs and streptococcal by microscopic examination of smearing and gram staining.

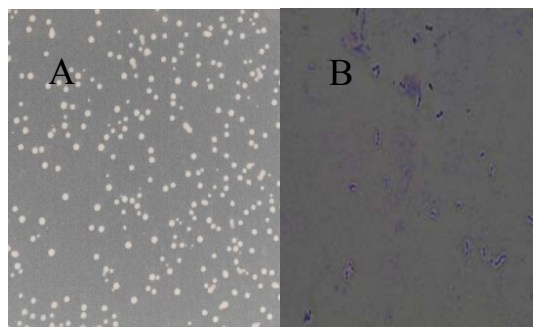


Figure 1. Photographs of colonies(A) and cells(B) of the strain WS1

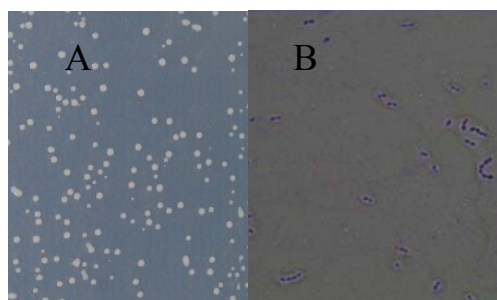


Figure 2. Photographs of colonies(A) and cells(B) of the strain WS2

#### B. Physiological and biochemical characteristics of barotolerant microbes

The physiological and biochemical identification of the strain WS1 and WS2 was carried out by the physiological and biochemical test of the Berger's bacterial identification manual. The results were shown in Table 1.

From the table 1, the catalase of the strain WS1 and WS2 were all negative and the strains were non acidification, but freezing milk and fermenting galactose, fructose, maltose, mannose, xylose, sucrose and glucose. The glucan was produced in the identification of the culture medium. The arabinose was fermented by the strain WS1 that it can be produced the acid, and the strain WS2 was not. Through the comparison of Berger's bacterial identification manual, the WS1 and WS2 were identified as *Leuconostoc spp.*

TABLE1 RESULT OF BIOCHEMICAL AND PHYSIOLOGICAL IDENTIFICATION OF WS1 AND WS2

project	WS1	WS2
catalase	—	—
litmus milk	—	—
galactose	+	+
fructose	+	+
maltose	+	+
mannose	+	+
xylose	+	+
sucrose.	+	+
glucose	+	+
arabinose	+	—
glucan	+	+

Comment:“+”positive reaction; “—”negative reaction

#### C. The sequences analysis of 16Sr DNA

##### 1) The extraction of genomic DNA

Genomic DNA was extracted from bacterial genomic DNA rapid extraction kit and detected by 1%gaerose gel electrophoresis. The results were shown in Figure3. There is no impurity in the rubber hole, the band has no interference, the length of the DNA fragment was about 1500bp,and the DNA was better that can be amplified by PCR.

1 2 M

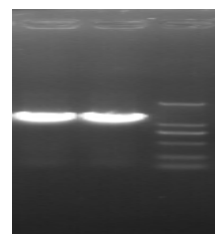


Figure 3. The extraction of genomic DNA of strain WS1 and WS2  
M: DL2000 marker 1-2: The extraction of genomic DNA of strain WS1 and WS2

##### 2) The amplification and detection of 16S rDNA

The results of PCR amplification were showed in Figure 4, the length of the amplified sequence was 1500bp, with the size of expected to, After the purification, transformed, the product was obtained and sent to Sangon biological engineering for sequencing.

1 2 M

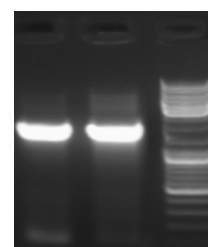


Figure 4. PCR amplification of 16S rDNA of strain WS1 and WS2  
M: DNA Ladder Mix make 1-2: Mean PCR result of strain WS1 and WS2

### 3) Phylogenetic and homology analysis of 16SrDNA sequences

The Blast analysis of the sequence and the GenBank database showed that the sequence of 16SrDNA of the strain WS1 similarity of the *Leuconostoc mesenteroides subsp mesenteroides* reached 99% in GenBank. The strains of WS2 and *Leuconostoc mesenteroides subsp dextranicum* of 16 SrDNA sequence similarity as high as 99%.According to the homology analysis results, the strain WS1 was identified as *Leuconostoc mesenteroides subsp mesenteroides* and the strain WS2 was *Leuconostoc mesenteroides subsp dextranicum*.

Two strains of barotolerant microbes in coconut puree were screened out and identified as *Leuconostoc mesenteroides subsp mesenteroides* and *Leuconostoc mesenteroides subsp dextranicum* by morphological features, physiology and molecular biology (16SrDNA) method. Molecular phylogenetic tree based on 16Sr DNA sequence WS1 and WS2 were showed in Figure 5

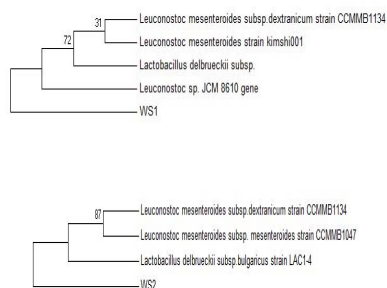


Figure 5. Molecular phylogenetic tree based on 16Sr DNA sequence WS1 and WS2

### D. The lethal effect of different pressure mode on barotolerant microbes

Figure 6 showed that different pressure mode on *Leuconostoc mesenteroides subsp mesenteroides* (A) and *dextranicum*(B) have better lethal effect. Lethal effect of intermittent pressure treatments on strains is superior to constant pressure treatment under the same condition, when comparing with constant and intermittent pressure treatments. Treatment with intermittent pressure of 300-600MPa can greatly improve the lethal effect of compression strain. The fatality rate on *Leuconostoc mesenteroides subsp mesenteroides* and *Leuconostoc mesenteroides subsp dextranicum* were 92.6% and 89.5%.

Analysis of the mechanism that is: The stress of the cell membrane and the cell wall were strengthened from low to high pressure and the process of booster and unloading repeatedly, which made the cell more vulnerable and destroyed [10].Therefore, choosing the combination of intermittent pressure as a sterilization way for ultra-high pressure treatment on coconut puree.

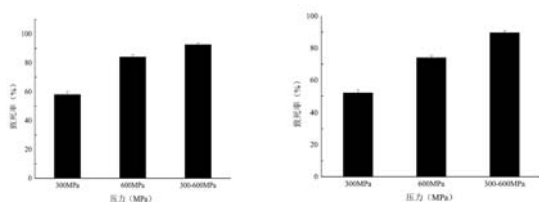


Figure 6. The lethal effect of different pressure mode on *Leuconostoc mesenteroides subsp mesenteroides* (A)and *dextranicum*(B)

### E. The lethal effect of different processing temperature on barotolerant microbes

In coordination with temperature were 35°C, 45°C, 55°C, 65°C and combined with the 300MPa-600MPa pressure on *Leuconostoc mesenteroides subsp mesenteroides* (A) and *dextranicum*(B),the results as showed in Figure7. Lethality goes up significantly along with the temperature increasing and when the temperature is above 55°C, the lethality increases smoothly. At 65, lethality of *L. mesenteroides subsp mesenteroides* and *L. mesenteroides subsp dextranicum* were 98.9% and 98.2%. Kaletunc researched that the cell structure of *Leuconostoc spp* has changed, when the processing of 500MPa,35°C,15min.Such as beaded structure of *Leuconostoc spp*. was destroyed and the part of the cell surface forming processes, besides, the precipitation was formed within cells and the destruction of cell membrane resulted in the decrease of  $Mg^{2+}$  concentration [11].Thus, choosing the 35°C, 45°C, 55°C as processing temperature for ultra-high pressure treatment on coconut puree.

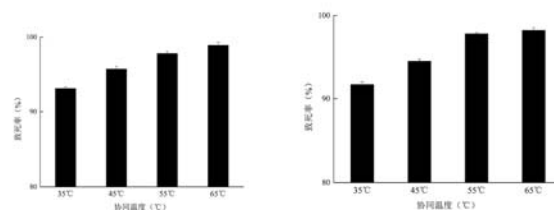


Figure 7. The lethal effect of different processing temperature on *Leuconostoc mesenteroides subsp mesenteroides* (A)and *dextranicum*(B)

## IV. DISCUSSION

This article isolated the two strains of barotolerant microbes from the coconut puree, which was treated by ultra-high pressure. The two strains were *L.mesenteroides subsp mesenteroides* and *L.mesenteroides subsp dextranicum*. Research found that the *L.mesenteroides subsp* and *Weissella viridescens* were typical barotolerant microbes in meat products [12]. In addition, there were a lot of studies on the barotolerant microbes. Such as, Feng research found that the barotolerant microbes of salted dunk including *Staphylococcus warneri*, *Staphylococcus epidermidis*, *Enterococcus faecalis* and *Bacillus cereus*, which the corrosion capability of *Bacillus cereus* was the strongest [13]. Li also found that *Bacillus pumilus* of pickle had a strong compressive ability.

Lethal effect of intermittent pressure treatments on strains is superior to constant pressure treatment under the same condition, when comparing with constant and intermittent pressure treatments. Treatment with intermittent pressure of 300MPa-600MPa can greatly improve the lethal effect of compression strain.The fatality rate on *Leuconostoc mesenteroides subsp mesenteroides* and *Leuconostoc mesenteroides subsp dextranicum* were 92.6% and 89.5%. Lethality goes up significantly along with the temperature increasing and when the temperature is above 55°C, the lethality increases smoothly. At 65, lethality of *L. mesenteroides subsp mesenteroides* and *L. mesenteroides subsp dextranicum* were 98.9% and 98.2%. Therefore, Choosing

the combined mild temperature and ultrahigh pressure intermittent sterilization of coconut puree

## V. CONCLUSION

1. Two strains of barotolerant microbes in coconut puree were screened out and identified as *Leuconostoc mesenteroides subsp mesenteroides* and *Leuconostoc mesenteroides subsp dextranicum* by morphological features, physiology and molecular biology (16SrDNA) method.

2. Combined mild temperature and ultrahigh pressure intermittent of coconut puree has significant sterilization effect. And the stress effect was strengthen from intermittent pressure way which had a process of step-up and step-down. The greater the pressure, the higher the temperature, the stronger the lethal effects

## ACKNOWLEDGMENT

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