MINISTRY OF EDUCATION AND SCIENCE OF UKRAINE
KYIV NATIONAL UNIVERSITY OF TECHNOLOGIES AND DESIGN
Faculty of Chemical and Biopharmaceutical Technologies
Department of Biotechnology, Leather and Fur

QUALIFICATION THESIS

on the topic **Analysis of the antioxidant activity of the exopolysaccharide produced by bacterium TB10**
First (Bachelor's) level of higher education
Specialty 162 "Biotechnology and Bioengineering"
Educational and professional program "Biotechnology"

Completed: student of group BEBT-21
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APPROVE
Head of Biotechnology, Leather and Fur Department, Professor,
Dr. Sc., Prof.
_____Olena MOKROUSOVA
«___»_____2025

ASSIGNMENTS
FOR THE QUALIFICATION THESIS
Zhao Lingyu

1. Thesis topic **Analysis of the antioxidant activity of the exopolysaccharide produced by bacterium TB10**

Scientific supervisor Dr. Sc., Prof. Tetiana SHCHERBATIUK

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2. Initial data for work: assignments for qualification thesis, scientific literature on the topic of qualification thesis, materials of Pre-graduation practice

3. Content of the thesis (list of questions to be developed): literature review; object, purpose, and methods of the study; experimental part; conclusions

4. Date of issuance of the assignments 05.03.2025

WORK CALENDAR

№	The name of the stages of the qualification thesis	Terms of performance of stage	Note on performance
1	Introduction	until 11 April 2025	
2	Chapter 1. Literature review	until 20 April 2025	
3	Chapter 2. Object, purpose, and methods of the study	until 30 April 2025	
4	Chapter 3. Experimental part	until 11 May 2025	
5	Conclusions	until 15 May 2025	
6	Draw up a bachelor's thesis (final version)	until 25 May 2025	
7	Submission of qualification work to the supervisor for feedback	until 27 May 2025	
8	Submission of bachelor's thesis to the department for review (14 days before the defense)	28 May 2025	
9	Checking the bachelor's thesis for signs of plagiarism (10 days before the defense)	01 June 2025	Similarity coefficient ____% Citation rate ____%
10	Submission of bachelor's thesis for approval by the head of the department (from 7 days before the defense)	04 June 2025	

I am familiar with the task:

Student _____ Zhao Lingyu

Scientific supervisor _____ Tetiana SHCHERBATIUK

Abstract

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Qualification thesis, specialty 162 "Biotechnology and Bioengineering". Kyiv national university of technologies and design, Kyiv, 2025.

Exopolysaccharide (EPS) are a class of functional polysaccharide compounds synthesized by microorganisms, which have a variety of physiological activities such as regulating immunity, antioxidants, lowering blood lipids and glucose and inhibiting tumors, and are widely used in functional food, medical care and agricultural production. Bacterial exopolysaccharide (EPS), as a natural antioxidant, has attracted much attention for its nontoxicity and good biocompatibility.

This study focuses on bacteria TB10 as the research object, extracting and purifying its exopolysaccharide through fermentation culture, and systematically evaluating its antioxidant activity in vitro and in vivo. The results of in vitro experiments show that the exopolysaccharide of bacteria TB10 has a significant ability to scavenge ABTS, DPPH, OH^-, and O^{2-} free radicals. Among them, the polysaccharide showed good antioxidant effect in the ABTS and superoxide anion systems, and its removal rate could be increased to about 19% when the concentration increased from 0.4 mg/mL to 2 mg/mL. In the DPPH and hydroxyl radical experiments, the antioxidant effect of the polysaccharide was limited, and the removal rate was only higher than 15% when the concentration reached 2 mg/mL, and the removal rate increased slowly after the concentration exceeded 1.6 mg/mL. In vivo experiments using a nematode model further verified the antioxidant effect of the exopolysaccharides of bacteria TB10 in vivo, and found that it can significantly increase the activity of superoxide dismutase (SOD) and catalase (CAT). The experiment shows that the SOD activity and CAT activity of the nematode are dose-dependent with the extracellular polysaccharide concentration of bacteria TB10, and the enzyme activity is higher when the polysaccharide concentration is 200 ug/mL.

The comprehensive analysis shows that the exopolysaccharide of bacteria TB10 effectively alleviates oxidative stress by scavenging free radicals and enhancing the activity of antioxidant enzymes, indicating its potential application value in preventing or alleviating degenerative diseases. This study provides experimental evidence for the development of new natural antioxidants based on bacterial exopolysaccharides and theoretical references for their application in functional foods and medicine.

Key words: extracellular polysaccharide; antioxidant activity; sevage method; nematode model; free radical scavenging

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Introduction

Exopolysaccharide (EPS) are natural macromolecules secreted by microorganisms, renowned for their antioxidant, anti-inflammatory, and immune-regulatory properties. Among them, bacterial EPS stand out due to their structural diversity, biocompatibility, and low toxicity, making them a focal point in biomedical and functional food research [1]. However, the antioxidant mechanisms and molecular targets of many bacterial EPS remain underexplored, particularly for strains like *Klebsiella pneumoniae* (TB10). The antioxidant activity of EPS is closely tied to their chemical composition (e.g., monosaccharide types, glycosidic bonds, molecular weight) and structural modifications (e.g., sulfation, acetylation) [2]. For instance, DZ-1 and DZ-2 from *Lactobacillus plantarum* exhibit free radical scavenging abilities [3], while *Lactobacillus fermentum* EPS protects intestinal cells from oxidative stress [4]. Despite these findings, the antioxidant mechanisms of TB10 EPS remain poorly understood, despite its broad-spectrum antibiofilm activity and potential for functional applications [5].

To address this gap, this study investigates the antioxidant properties of TB10 EPS through a comprehensive approach: In vitro assays (DPPH, ABTS, hydroxyl radical scavenging) to evaluate antioxidant capacity, *C. elegans* models to assess in vivo effects on lifespan, stress resistance, and antioxidant enzyme activity (SOD, CAT). Caenorhabditis elegans belongs to the genus Corynebacterium, a small family of Nematoda. In the field of biological research, Caenorhabditis elegans is a very famous model organism, the only multicellular organism whose somatic phylogeny has been clearly studied, and the first multicellular eukaryotic organism with a clear genome series[6]. Under this background, Caenorhabditis elegans has become an ideal model organism to study the interaction between microorganisms and hosts by virtue of its unique advantages. Under laboratory conditions, nematodes mainly feed on Escherichia coli, but also form symbiotic relationship with some other bacteria, which together constitute the microbial group of nematodes [1].

The scientific novelty lies in linking structure-activity relationships with molecular mechanisms and practical applications, integrating chemical, biological, and genetic analyses to advance understanding of bacterial EPS. This research not only provides a theoretical foundation for developing natural antioxidants in functional foods and pharmaceuticals but also highlights the potential of TB10 EPS as a safe and effective bioactive agent against oxidative stress, addressing critical needs in health and biotechnology.

The relevance of the topic stems from the growing demand for natural antioxidants to combat oxidative stress-related diseases and the limitations of synthetic antioxidants (e.g., toxicity, instability). Bacterial EPS, with their diverse structures, biocompatibility, and low toxicity, offer a promising alternative. Additionally, Caenorhabditis elegans (*C. elegans*), as a model organism, provides a cost-effective and efficient system to study antioxidant mechanisms, making this research impactful for both basic and applied sciences [8]. Studies have shown that EPS prolongs the lifespan and enhances the anti-stress ability of nematodes by activating the antioxidant pathway. For example, L et al. found that lentinolysaccharide extends the lifespan of nematodes by 35.3% through the DF-16 pathway [9]. The structure-activity relationship of EPS shows that the composition of monosaccharides (such as mannose and glucose), the type of glycosidic bond (such as α-1,6 bonds), and the molecular weight (lower molecular weight is better) significantly affect the activity. For example, Z et al. studied that the enzymatic hydrolysate of yeast cell wall extends the lifespan by regulating mitochondrial ROS and apoptosis [10]. In terms of application, EPS has been used in the development of anti-aging products. For instance, Si et al. discovered that Lentinula edodes polysaccharides improve the physiological characteristics of nematodes by activating the DF-16 /SKN-1 pathway [11], while the extracellular polysaccharides of heat-inactivated probiotics extend the lifespan by regulating the protein homeostasis and immune response of Caenorhabditis elegans [12].

The purpose of the study is to evaluate the antioxidant properties of TB10 EPS and elucidate its mechanisms (e.g., free radical scavenging, antioxidant enzyme activation),

while the object of the study is TB10 EPS, a bacterial extracellular polysaccharide derived from *Klebsiella pneumoniae.*

The subject of the study includes antioxidant activity, mechanisms of its antioxidant effects, applications in anti-aging, functional foods, and pharmaceuticals, as well as molecular targets (e.g., oxidative stress response genes) [13].

The research methods employ a multi-level approach, combining in vitro experiments (radical scavenging assays), in vivo experiments (*C. elegans* models), and data analysis (statistical evaluation, gene expression, and pathway analysis).

The scientific novelty of the study lies in its first-time investigation of TB10 EPS's antioxidant mechanisms and molecular targets, as well as its integration of in vitro and in vivo models to validate antioxidant activity.

Finally, the practical significance of the results includes functional food development (natural antioxidant additives), pharmaceutical applications (anti-aging drugs, therapies for oxidative stress-related diseases), biotechnology insights (EPS modification strategies), and model organism utility (cost-effective platform for bioactive compound screening).

Chapter I
LITERATURE REVIEW

1.1 Experimental materials

1.1.1 Experimental strain

A strain of *Klebsiella pneumoniae* that produces exopolysaccharide was isolated from discarded tea soup. It was identified and named TB10, and is preserved in the laboratory.

1.1.2 Experimental equipment

Table 1.1 Name of each equipment and its manufacturer

Device name	**Manufacturer**
Refrigerator (BCD-210VBP-J)	Hisense (Beijing) Electrics
High-speed freezing centrifuge (TGL-16M)	Ebend China Ltd.
Constant temperature water bath pot (HH)	Zhongda instrument factory
Constant temperature oscillation incubator	Zhicheng analysis instrument manufacturing co., ltd
JT202N electronic balance	Jingtian instrument co., ltd
Model 752 ultraviolet-visible spectrophotometer	Shanghai yuanxi instrument co., ltd
Chromatographic column (φ26x400 mm and φ16x100 mm)	Jingke industry co., ltd
Computer automatic partial collector (BSZ-100)	Jingke industry co., ltd

1.1.3 Reagent consumables

Table 1.2 Name of each reagent and its manufacturer

Reagent name	**Reagent specification**	**manufacturer**
peptone	AR	Beijing aoboxing biotechnology co., ltd
sea salt	AR	Qingdao yanrui new material co., ltd
yeast extract	AR	Beijing aoboxing biotechnology co., ltd
ammonia sulfate	AR	Tianjin dingshengxin chemical co., ltd

glucose	AR	National medicine group chemical reagent co., ltd
sulphuric acid	AR	Laiyang Economic and Technological Development Zone Fine Chemical Plant
phenol	AR	Guangcheng chemical reagent co., ltd
95% ethanol	AR	Yantai yuandong fine chemical co., ltd
normal butanol	AR	Tianjin Fuyu Fine Chemical Co., Ltd.
Chloroform	AR	Yantai yuandong fine chemical co., ltd
DEAE-52 cellulose	BC	Beijing suolaibao technology co., ltd
sodium chloride	AR	Tianjin dingshengxin chemical co., ltd
sodium hydroxide	AR	National medicine group chemical reagent co., ltd
argentum nitricum	AR	Shanghai, China Reagent No.1 Factory

1.2 Extraction method of bacterial TB10

1.2.1 Crude polysaccharide was extracted from bacteria TB10.

The fermentation broth of bacteria TB10 was preliminarily treated by centrifugation. First, it was centrifuged at 5000 r/min for 20 minutes to remove the bacteria and collect the supernatant. Subsequently, 4 times of 95% ethanol was added to the supernatant, and the mixture was fully shaken and mixed to ensure the full reaction. The mixed solution was refrigerated at 4℃ overnight to promote the precipitation of polysaccharide.

The next day, centrifuge at the speed of 5000 r/min for 20 minutes again, discard the supernatant this time, collect the gray precipitate, and finally get the preliminary extract of EPS-TB10 crude polysaccharide [14].

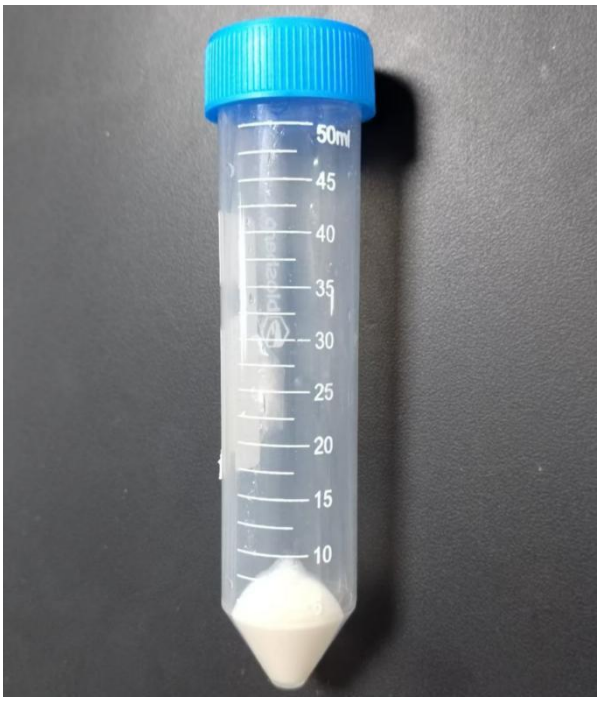

Figure 1.1 Extraction diagram of crude polysaccharide

1.2.2 Elution of EPS-TB10 crude polysaccharide protein by sevage method.

Referring to the experimental method of Yang Jing et al. [16], the dried crude polysaccharides were redissolved in distilled water to prepare a polysaccharide solution of appropriate concentration. Subsequently, add three times the volume of Sevage reagent (with a volume ratio of chloroform to n-butanol of 4:1) to the polysaccharide solution, shake thoroughly for 15 minutes to ensure thorough mixing of the reagent and the solution. The Sevage reagent was separated and removed from the gel-like precipitated impurity proteins using a separatory funnel. This protein removal step is repeated 4 to 5 times until the ultraviolet absorption peaks of the polysaccharide solution at 260 nm and 280 nm disappear, indicating that the impurity proteins have been completely removed. Finally, the polysaccharide solution after protein removal was subjected to vacuum concentration and dried in an oven at 50℃, and finally the crude extracellular polysaccharide of TB10 was obtained.

1.3 Purification method of bacterial TB10

The analysis was conducted by referring to the experimental method of Wang Chunlei, and ion exchange chromatography was used for purification. In this experiment, the DEAE-52 cellulose filler was treated by staged activation: Firstly, it

was subjected to alkaline activation treatment with 0.5 mol/L NaOH solution for 1 hour. Then, it was subjected to acid activation treatment with 0.5 mol/L HCl for 45 minutes. Finally, it was balanced with 0.01 mol/L Tris-HCl buffer solution (pH 8.0) until the conductivity was ≤50 μS/cm. When loading the column by wet method, the compression rate of the column bed should be strictly controlled to be ≤8%. The uniformity of column loading was verified by funnel test (the contact time difference of the exudate should be ≤2 seconds) [17]. The gradient elution program was set to a linear gradient of 0-2 mol/L NaCl (120 minutes), and the polysaccharide content was monitored in real time simultaneously using an ultraviolet detector (λ=490 nm). The main peak components (absorbance >0.3 and the change rate of three consecutive tubes ≤5%) were collected through an automatic partial collector (accuracy ±0.1 mL), and finally polysaccharide components with a purity of ≥95% (HPLC single-peak area ratio) were obtained, with a total recovery rate of more than 85%.

1.4 Quantitative analysis

1.4.1 Preparation of standard curve

Weigh 10 mg of anhydrous glucose at 105℃ and dissolve it to 100 mL to obtain 0.1 mg/mL stock solution, and dilute the stock solution to 0.2 mg/ml, 0.4 mg/ml, 0.6 mg/ml, 0.8 mg/ml, 1.0 mg/ml and 1.2 mg/mL as a series of standard solutions.

1.4.2 Phenol-sulfuric acid chromogenic method

Take 2 mL of standard solution with different concentrations, add 1 mL of 5% phenol and 5 mL of concentrated sulfuric acid in turn, mix well, let stand at room temperature for 30 minutes, and measure the absorbance at 490 nm wavelength.

1.4.3 Sample Pretreatment

Firstly, acid hydrolysis was carried out. After the crude polysaccharide was dissolved, 6 mol/L HCl was added and hydrolyzed at 100℃ for 6 hours to generate monosaccharide. After cooling, the pH was adjusted to neutral with 10% NaOH to avoid residual acid interfering with color development. Then dilute to constant volume, and dilute the hydrolysate by 1000 times (take 10 mL filtrate to constant volume to 100

mL, and then take 10 mL to dilute to 100 mL) to eliminate the color error caused by too high concentration.

1.5 Statistical analysis data

Excel table is used for data statistics. In order to improve the analysis efficiency of experimental data, Excel is used to sort out experimental data.

1.6 Results and discussion

1.6.1 Glucose standard curve

According to the experimental method in 1.4, the linear regression equation between glucose concentration and absorbance is established. The standard curve is: y=0.0049x+0.0044, R^2 =0.9966, and the linear fitting is good.

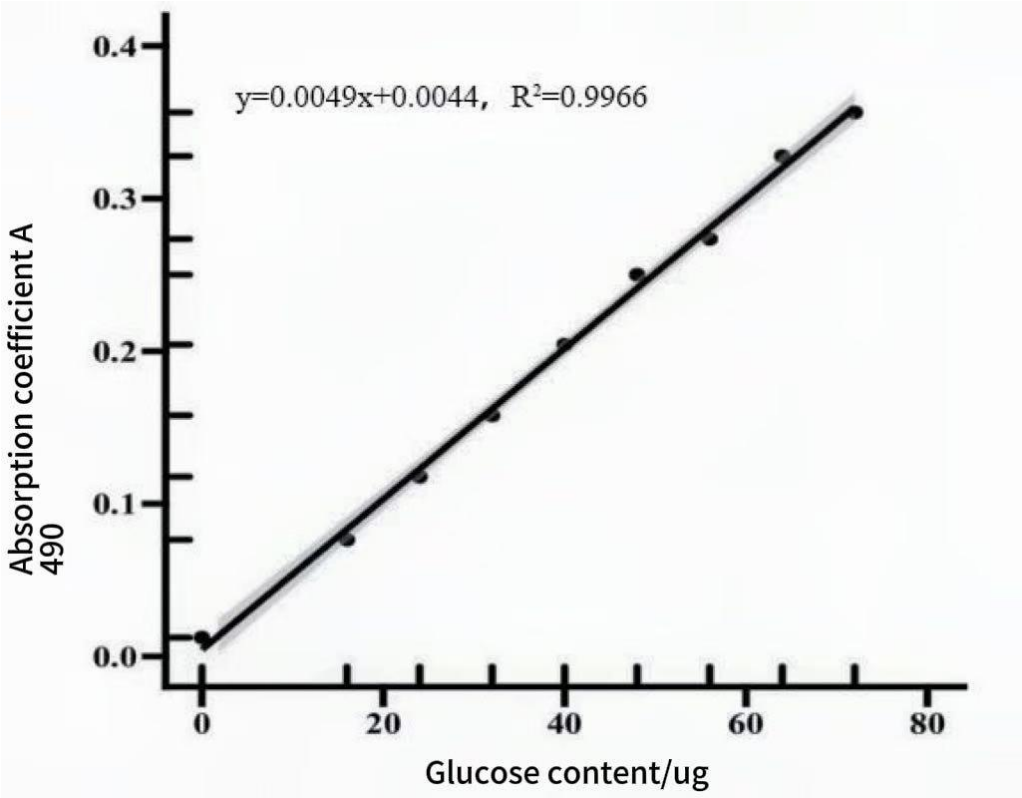

Figure 1.2 Glucose standard curve

1.6.2 EPS-TB10 output analysis

The purified polysaccharide was measured by phenol-sulfuric acid chromogenic method, and the absorbance of three groups of EPS-TB10 in different time periods was measured and recorded.

Substitute the data into the regression equation (y=0.0049x+0.0044, R^2 =0.9966) to calculate the polysaccharide concentration:

X=(Y-0.0044)/0.0049, that is, yield = concentration × dilution multiple × volume of fermentation broth [18]. Three groups of EPS-TB10 concentrations were obtained. The average value of the three groups of data was calculated to obtain the following extracellular polysaccharide content curve graph.

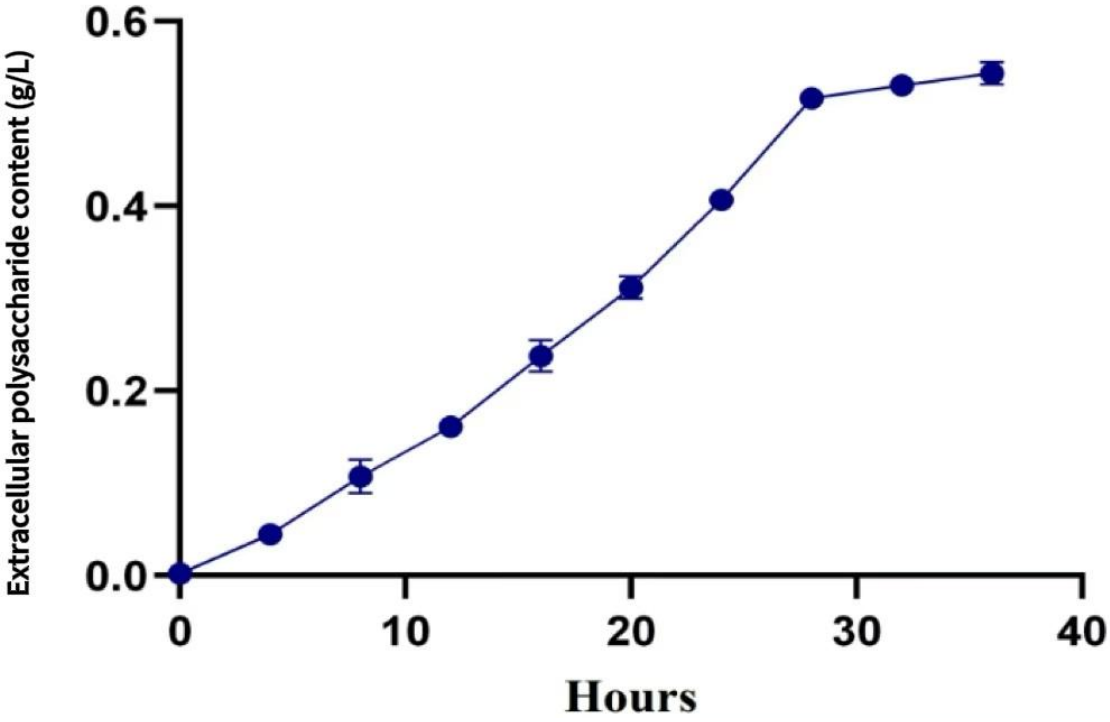

Figure 1.2 Curve graph of extracellular polysaccharide content of TB10

The variation trend of extracellular polysaccharide content with time can be analyzed through the graph, and the highest yield is 36 hours, and the concentration of extracellular polysaccharide is 0.54 g/L.

Summary of the chapter I

1. This chapter of the study took the TB10 strain as the research object and systematically constructed the standardized extraction process of its extracellular polysaccharide (EPS-TB10) for the first time.

2. It has a certain degree of innovation in the extraction method, combining the traditional ethanol precipitation method with the improved Sevage deproteinization process, effectively improving the purity and yield of polysaccharides.

3. During the research process, DEAE-52 ion exchange chromatography technology was adopted to achieve the separation and purification from crude extracts to high-purity polysaccharides, and a complete polysaccharide purification control system was established.

4. The concentration of the purified polysaccharide samples was determined by the phenol-sulfuric acid colorimetric method. The results showed that the polysaccharide content reached 0.54 g/L, indicating that this extraction and purification method had good effect and repeatability.

5. In conclusion, this chapter has successfully established an efficient extraction and purification process for Exopolysaccharide of the TB10 strain, laying a solid foundation for subsequent structural analysis, functional research, and application development.

Chapter II

OBJECT, PURPOSE, AND METHODS OF THE STUDY

2.1. Experimental materials

2.1.1 Experimental strains

In this experiment, the extracellular polysaccharide (EPS-TB10) extracted from the second chapter was stored in 0.02% NaN_3 solution at 4℃.

2.1.2 Experimental equipment

Table 2.1 Name of each equipment and its manufacturer

Device name	**manufacturer**
UV-2600 ultraviolet spectrophotometer	Beijing Puxi General Instrument Co., Ltd.
High-speed freezing centrifuge (TGL-16M)	Ebend China Ltd.
Constant temperature water bath pot (HH)	Zhongda instrument factory

2.1.3 Reagent consumables

Table 2.2 Name of each reagent and its manufacturer

Reagent name	**Reagent specification**	**manufacturer**
monosodium phosphate	AR	Tianjin kemiou chemical reagent co., ltd
disodium hydrogen phosphate	AR	Tianjin kemiou chemical reagent co., ltd
potassium ferricyanide	AR	National medicine group chemical reagent co., ltd
trichloroacetic acid	AR	Sinopharm group chemical reagents are
Ferric chloride hexahydrate	AR	National medicine group chemical reagent co., ltd
ferrous sulfate	AR	National medicine group chemical reagent co., ltd
Reduced iron powder	AR	Tianjin dingshengxin chemical co., ltd
sulphuric acid	AR	Laiyang Economic and Technological Development Zone Fine Chemical Plant
salicylic acid	AR	National medicine group chemical reagent co., ltd

absolute ethyl alcohol	AR	Tianjin Fuyu Fine Chemical Co., Ltd. National Medicine Group
30% hydrogen peroxide	AR	Chemical reagent co., ltd
Tris	AR	Shenggong Bioengineering (Shanghai) Co., Ltd.
muriatic acid	AR	Laiyang Economic and Technological Development Zone Fine Chemical Plant
Phloroglucinol	AR	Tianjin hengxing chemical reagent manufacturing co., ltd
ABTS(2,2'- diazo-bis (3- ethyl-benzothiazoline -6- sulfonic acid))	AR	Beijing suolaibao technology co., ltd
DPPH(1,1- diphenyl -2- trinitrophenylhydrazine)	AR	Beijing suolaibao technology co., ltd
NBT (nitroblue tetrazole)	AR	Beijing suolaibao technology co., ltd
Pure vitamin c	AR	Beijing suolaibao technology co., ltd

2.2. Experimental methods

2.2.1 ABTS free radical scavenging experiment

Referring to the improved method of Re et al. [19] Prepare 7 mmol/L ABTS stock solution and mix it with 2.45 mmol/L $K_2S_2O_8$. React in the dark for 16 hours to prepare the ABTS+ cationic radical solution. Take 0.1 mL of the polysaccharide solution to be tested (0.1-2.0 mg/mL) and mix it with 2.4 mL of $ABTS^+$ working solution (absorbance 0.70±0.02 at 734 nm). After the reaction at room temperature in the dark for 6 minutes, measure the absorbance at 734 nm. Anhydrous ethanol was used as the sample substrate of the ABTS cation radical working solution, dimethyl sulfoxide was selected as the blank control, and 0.10 g/L VC solution was used as the positive control experimental group at the same time.

Calculation formula of clearance rate: clearance rate (%) = [1-(a sample-a blank)/(a control-a blank)] × 100.

2.2.2 DPPH free radical scavenging experiment

The method of Tan Yun et al. [20] was adopted: 0.2 mL of the sample solution (0.05-1.0 mg/mL) was mixed evenly with 2.8 mL of 0.1 mmol/L DPPH methanol solution. After the reaction in the dark for 30 minutes, the absorbance at 517 nm was measured. Anhydrous ethanol was used as the sample substrate of the DPPH radical working solution, dimethyl sulfoxide was selected as the blank control, and 0.10 g/L VC solution was used as the positive control experimental group simultaneously.

Calculation formula of clearance rate: clearance rate (%) = [1-(a sample-a blank)/a control] × 100.

2.2.3 Hydroxyl radical scavenging experiment

Referring to the method of Liu Dan et al. [21]: In a 3 mL reaction system containing 0.1 mmol/L $FeSO_4$, 0.1 mmol/L H_2O_2, 0.1 mmol/L salicylic acid and polysaccharide solutions of different concentrations, the absorbance at 510 nm was measured after a water bath reaction at 37 ° C for 30 minutes. The scavenging ability of hydroxyl radicals is expressed by the clearance rate of salicylic acid. Anhydrous ethanol was used as the sample substrate of the hydrogen peroxide working solution, dimethyl sulfoxide was selected as the blank control, and 0.10 g/L VC solution was used as the positive control experimental group simultaneously.

Calculation formula of clearance rate: clearance rate (%) = [1-(a sample-a blank)/(a control-a blank)] × 100.

2.2.4 Superoxide anion scavenging experiment

Referring to the method of Liu Bing et al. [22], make slight adjustments: In a 200 μL reaction system, 20 μL of polysaccharide sample solutions with different concentrations (1, 2, 4, 6, and 8 mg/mL) were mixed with 180 μL of 50 mmol/L Tris-HCl buffer solution with pH 8.2 in a 96-well plate, and preheated at 37 ° C for 20 min. Subsequently, 20 μL of preheated 3 mmol/L catechol solution was added, mixed well, and the absorbance value at 325 nm was measured and recorded as D_0. Replace the

catechol solution with distilled water. The operation is the same as D0 and is denoted as D_1. Replace the polysaccharide sample solution with distilled water. The operation is the same as D_0 and is denoted as D_2. The 0.10 g/L VC solution was used as the positive control experimental group.

Calculation formula of clearance rate: clearance rate (%)=$[1-(D_1-D_2)/D_0]\times100$.

2.3. Results and Discussion

2.3.1 ABTS free radical scavenging ability

Based on the measurement results, using vitamin C as the control and setting the activity of 100 μg/mL vitamin C to 100%, a comparison curve graph of the clearance activity of ABTS between EPS-10 and Vc was drawn, as shown in Figure 2.1.

It can be analyzed through the curve graph that the polysaccharide shows a significant dose dependence (R^2=0.962) within the concentration range of 0.4-2 mg/mL, and the clearance rate gradually increases from 5.97% to 19.05%. The clearance rate of vitamin C (Vc) reached 97.16% at 0.4 mg/mL, indicating that the clearance efficiency of Vc was higher.

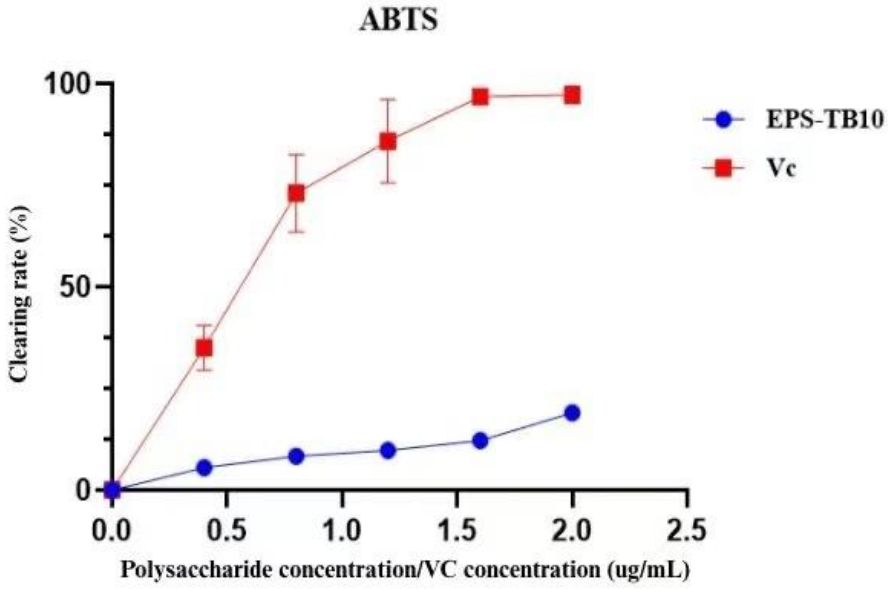

Figure 2.1 Comparison curves of the clearance capabilities of ABTS between EPS-TB10 and Vc

2.3.2 Free radical scavenging capacity of DPPH

Based on its measurement results, a comparison curve graph of the clearance activity of EPS-10 and Vc on DPPH was drawn, as shown in Figure 2.2.

In the DPPH system, the clearance rate of polysaccharides reached 12.923% at 1.6 mg/mL. However, as the concentration continued to increase to 2 mg/mL, the clearance rate only increased by 1.5%, showing an obvious plateau period. In contrast, Vc achieved a clearance rate of 97.01% at 2 mg/mL, and its clearance efficiency was higher than that of polysaccharides throughout the entire concentration range (the clearance rate ratio was 0.78-0.95).

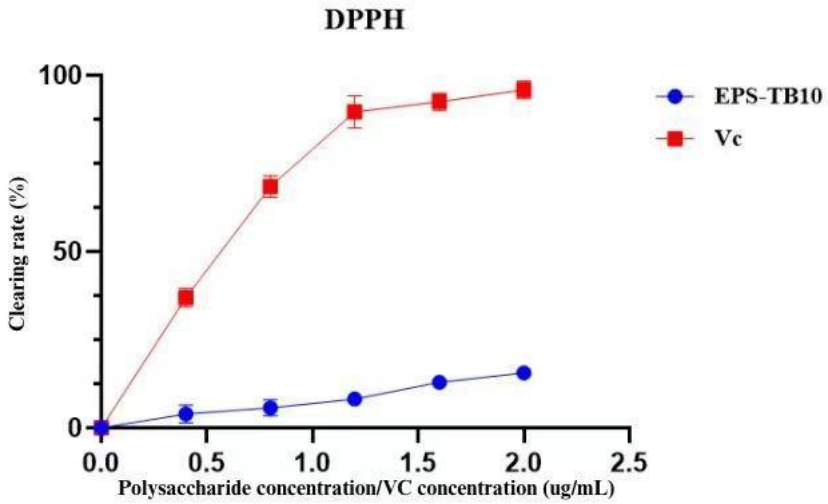

Figure 2.2 Comparison curves of the clearance capabilities of EPS-TB10 and Vc for DPPH

2.3.3 Hydroxyl radical scavenging ability

Based on the measurement results, a comparison curve graph of the scavenging activity of hydroxyl radicals by EPS-10 and Vc was drawn, as shown in Figure 2.3.

Polysaccharides exhibit nonlinear scavenging characteristics in the hydroxyl radical system. When the clearance rate was between 0.8 mg/mL and 1.2 mg/mL, it showed a downward trend, which might be related to the conformational changes of polysaccharide molecules. The clearance rate can be as high as 15.6% at most. The clearance rate of Vc steadily increased throughout the entire concentration range, reaching 98.47% at 2 mg/mL, and its clearance efficiency was 12%-18% higher than that of polysaccharides.

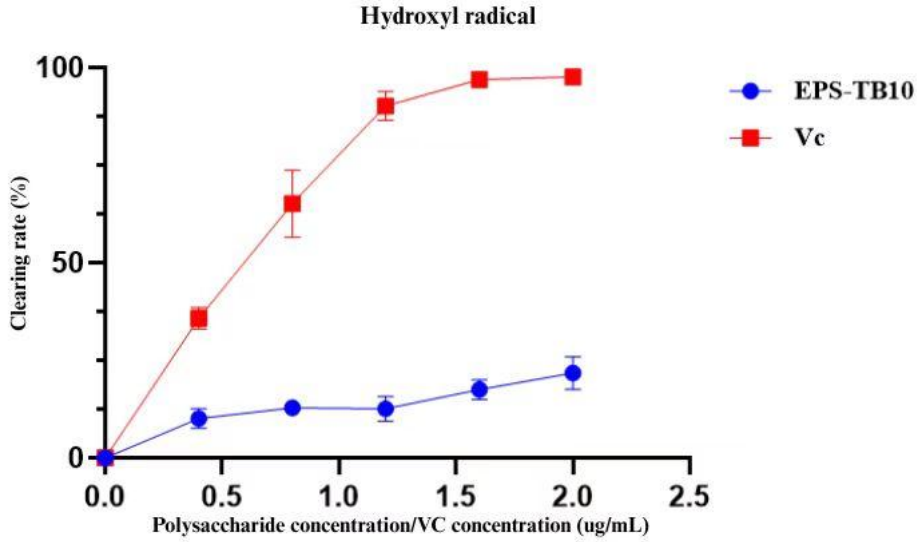

Figure 2.3 Comparison curve of the scavenging ability of hydroxyl radicals between EPS-TB10 and Vc

2.3.4 Superoxide anion removal capacity

Based on the measurement results, a comparison curve graph of the scavenging activity of EPS-10 and Vc for superoxide anions was drawn, as shown in Figure 2.4.

Polysaccharides show a good linear relationship in the superoxide anion system, and the clearance rate reaches 31.07% at 2 mg/mL.

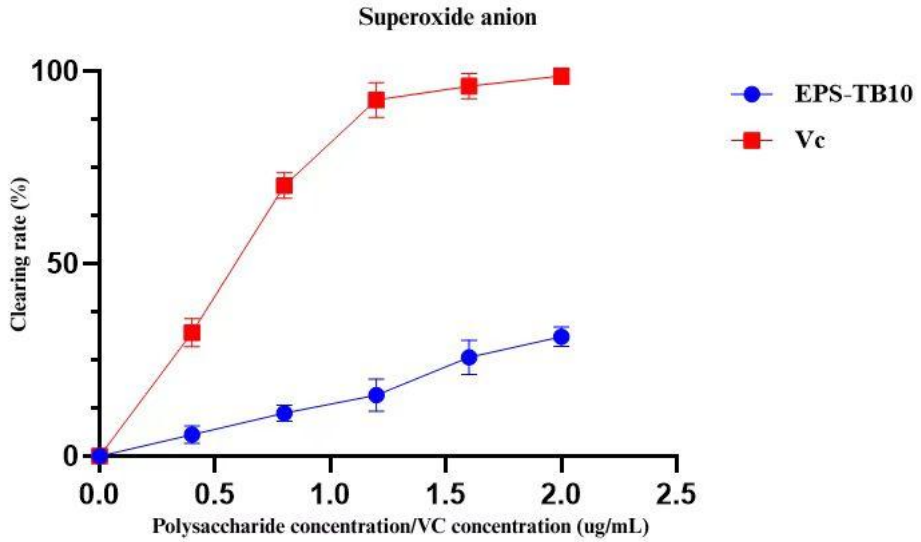

Figure 2.4 Comparison curve of the removal capacity of superoxide anions by EPS-TB10 and Vc

Summary of chapter II

1. This chapter mainly focuses on the in vitro antioxidant capacity detection of Exopolysaccharide of TB10 (EPS-TB10). Four common antioxidant activity evaluation models, namely ABTS, superoxide anion, DPPH and hydroxyl radical scavenging assay, are adopted to comprehensively assess its ability to scavene free radicals.

2. The experimental results show that EPS-TB10 exhibits certain antioxidant activity in the ABTS radical and superoxide anion system. Its clearance rate gradually increases as the concentration increases from 0.4 mg/mL to 2 mg/mL, up to approximately 19% at most.

3. Compared with vitamin C (VC), there is still a significant gap in the antioxidant capacity of EPS-TB10. At the same concentration, the clearance rate of vitamin C exceeded 97%, approximately five times that of polysaccharides, demonstrating the high efficiency of synthetic antioxidants in eliminating free radicals.

4. In the DPPH and hydroxyl radical scavenging experiments, the antioxidant effect of EPS-TB10 was more limited. When the concentration reached 2 mg/mL, the scavenging rate was only slightly higher than 15%, and after the concentration exceeded 1.6 mg/mL, the increase in the scavenging rate tended to level off, indicating that there was a concentration dependent upper limit for its antioxidant effect.

5. It is worth noting that in the hydroxyl radical experiment, the clearance rate of EPS-TB10 decreased within the concentration range of 0.8-1.2 mg/mL. This might be due to the aggregation of polysaccharide molecules under high concentration conditions, resulting in the occlusion of active sites and thus affecting its effective binding with free radicals.

Although the overall antioxidant capacity is not as good as that of synthetic antioxidants, the number of experiments is 6. It is indicated that EPS-TB10 has a linear upward trend in the clearance of superoxide anions with the increase of concentration, indicating that it has certain natural antioxidant potential.

7. To sum up, although EPS-TB10 has relatively weak antioxidant activity at present, as a polysaccharide from natural sources, it has good safety. In the future, its activity can be further enhanced through structural modification (such as sulfation, acetylation) or

by being used in combination with other antioxidant components, providing a theoretical basis for the development of natural functional foods or health products.

Chapter III
EXPERIMENTAL PART

3.1. Experimental materials

3.1.1 Experimental Subjects

The wild-type elegans (*C. elegans* N_2) was selected.

3.1.2 Experimental equipment

Table 3.1 Name of each equipment and its manufacturer

Device name	manufacturer
Constant temperature incubator	Zhongda instrument factory
High-speed freezing centrifuge (TGL-16M)	Ebend China Ltd.

3.1.3 Reagent consumables

Table 3.2 Name of each reagent and its manufacturer

Reagent name	Reagent specification	manufacturer
NGM basal medium	AR	Beijing suolaibao technology co., ltd
t-BHP（2 mmol/L，Sigma）	AR	Beijing suolaibao technology co., ltd
M9 buffer	AR	Beijing suolaibao technology co., ltd
SOD kit	AR	Beijing suolaibao technology co., ltd
CAT kit	AR	Beijing suolaibao technology co., ltd

3.2. Experimental methods

3.2.1 Nematode Culture and Synchronization

Refer to Tang Min's [24] experimental method and make modifications. In this experiment, *Caenorhabditis elegans* N_2 was used as the model organism, and the L4-stage larvae were simultaneously cultured by the egg-laying isolation method (20°C, 48 h). The synchronization operation adopted the modified lysis method: The nematodes were transferred to M9 buffer containing 0.5% sodium hypochlorite (containing 1 mM $MgSO_4$) to lyse into worms. After centrifuge at 3000 rpm for 1 minute, they were

washed three times with pre-cooled M9 and inoculated into fresh NGM medium (containing 15 mg/L cholesterol, pH 6.0) for continued culture for 24 hours. In the pretreatment stage, three polysaccharide treatment groups of 100 ug/mL, 200 μg/mL and 300 μg/mL were set up. The control group used 0.1% DMSO (final concentration) as the solvent control.

3.2.2 Thermal stress model

In the heat stress experiment, the pretreated nematodes were transferred to a preheated 35°C incubator (Thermo Scientific, USA), and the survival status of 40 nematodes was randomly detected by capillary picking method every 2 hours (the head swinging frequency was less than once per 10 seconds), and the death and survival number of *C. elegans* were observed and recorded for 14 hours.

3.2.3 Oxidative stress model

Referring to the experimental methods of Guan Siyu et al. [25], nematodes were selected respectively and placed in 96-well plates, with 5 nematodes placed in each well and 2 Wells set in each group. Add 1 mL of PBS solution to each well, which contains 1 μL of 30% H_2O_2. Record the number of surviving nematodes every 30 minutes and keep observing until 5.5 hours. All experiments were set up with 3 independent replicates (n=3), and the data were expressed as mean ±SEM. One-way ANOVA (ANOVA, Tukey multiple comparisons, *$p<0.05$ was considered significant difference) was performed using GraphPad Prism 9.0.

3.2.4 Determination of antioxidant enzymes

Referring to the experimental methods of Ning Yue et al. [26] for modification, after culturing the nematodes for two days in the life experiment, the nematodes were transferred to a 10 mL centrifuge tube with phosphate buffer solution and the volume was adjusted to 3 mL.

Subsequently, the nematode tissues were subjected to fragmentation treatment with a power of 800 W and a duration of 8 minutes. After the crushing is completed, the tissue fluid is centrifuged and separated, and the supernatant is collected as the enzyme solution extract. Next, the collected supernatant (enzyme extract) should be

appropriately diluted according to the protein concentration determined by the BCA kit to ensure that the protein content in each well is within the detection range. The enzyme activities of the nematodes were determined respectively by the SOD kit and the CAT kit.

When determining the activity of SOD enzymes, the superoxide anion reactive oxygen species detection kit (DHE) used is a kit that uses DHE as a fluorescent probe to rapidly and sensitively detect superoxide anion reactive oxygen species within cells. Refer to the SOD kit manual to prepare the reaction system. Mix the prepared reaction system evenly and immediately incubate it in a 37°C constant temperature incubator in the dark for 20 minutes. After the incubation is completed, add the stop solution (the stop buffer provided by the kit) to stop the reaction. The absorbance value was measured at the specified wavelength (usually 450 nm) using an enzyme-linked immunosorbent assay (ELISA) reader, and the SOD enzyme activity was calculated based on the standard curve.

When conducting the determination of CAT enzyme activity, the CAT detection kit used is based on the fact that catalase (CAT) can catalyze peroxide specific gas (H_2O_2) to produce water (H_2O) and oxygen (O_2) for the detection of catalase activity. Mix the prepared reaction system evenly and immediately incubate it in a 37°C constant temperature incubator in the dark for 20 minutes. After the incubation is completed, add the stop solution to stop the reaction. The absorbance value was determined at the specified wavelength (usually 240 nm) using an enzyme-linked immunosorbent assay (ELISA) reader, and the CAT enzyme activity was calculated based on the standard curve.

3.3. Results and Discussion

3.3.1 Analysis of Heat Stress Results

The analysis was conducted using GraphPad Prism 9.0, and the experimental figure 3.1 of the survival rate of nematodes under heat stress was obtained.

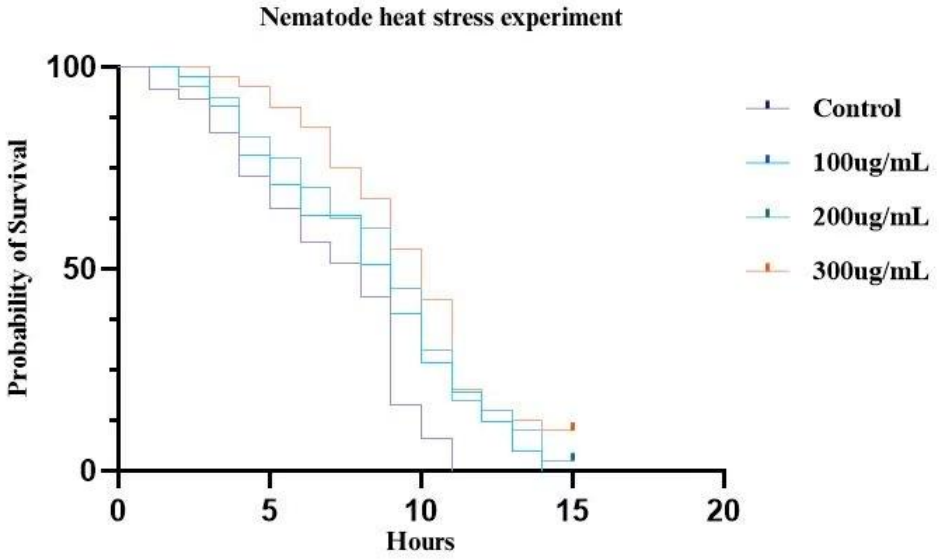

Figure 3.1 Experimental diagram of the effect of EPS-TB10 on the survival rate of nematodes under heat stress

In the heat stress experiment, the 200 ug/mL polysaccharide treatment group significantly increased the survival rate of nematodes ($p<0.05$). With the increase of EPS-TB10 concentration, the death rate of nematodes slowed down. When the concentration of EPS-TB10 reached 300 ug/mL, 4 nematodes were still alive after 15 hours, prolonging the lifespan of the nematodes.

3.3.2 Analysis of Oxidative Stress Results

The analysis was conducted using GraphPad Prism 9.0, and the experimental figure 3.2 showing the effect of EPS-TB10 on the oxidative stress survival rate of nematodes was obtained.

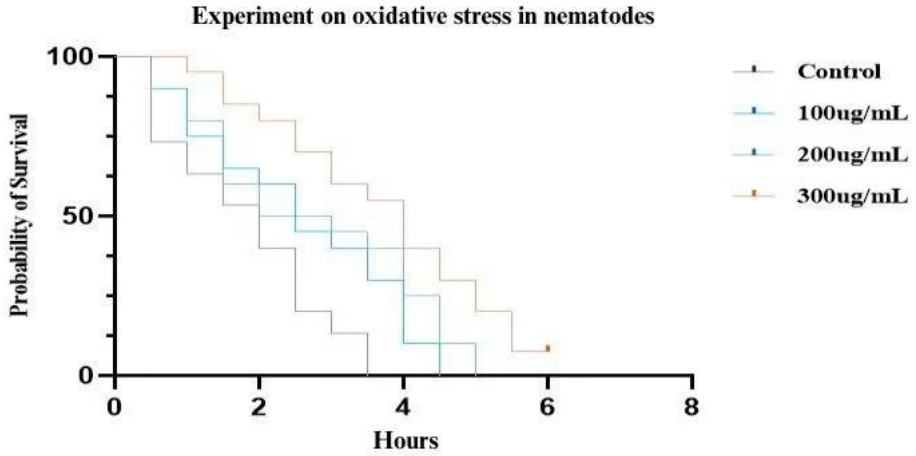

Figure 3.2 Experimental diagram of the effect of EPS-TB10 on the oxidative stress survival rate of nematodes

In the oxidative stress experiment, within 6 hours, as the concentration of EPS-TB10 increased, the survival time of nematodes also gradually increased. When EPS-TB10 reached 300 ug/mL, three nematodes were still alive at 5.5 hours. The experiment successfully captured the dynamic changes of ROS in the nematodes and further verified the antioxidant effect of polysaccharides.

3.3.3 The influence of antioxidant enzyme activity in Nematodes

The levels of CAT and SOD both represent the antioxidant capacity of the body. The higher their levels, the stronger the antioxidant capacity. As shown in Figure 3.3, both the SOD activity and CAT activity in nematodes have a certain dose-dependent effect relationship with the extracellular polysaccharide concentration of bacterial TB10. When the concentration of EPS-TB10 reaches 200 ug/mL, its enzyme activity is relatively high. The results show that EPS-TB10 can enhance the antioxidant capacity of nematodes.

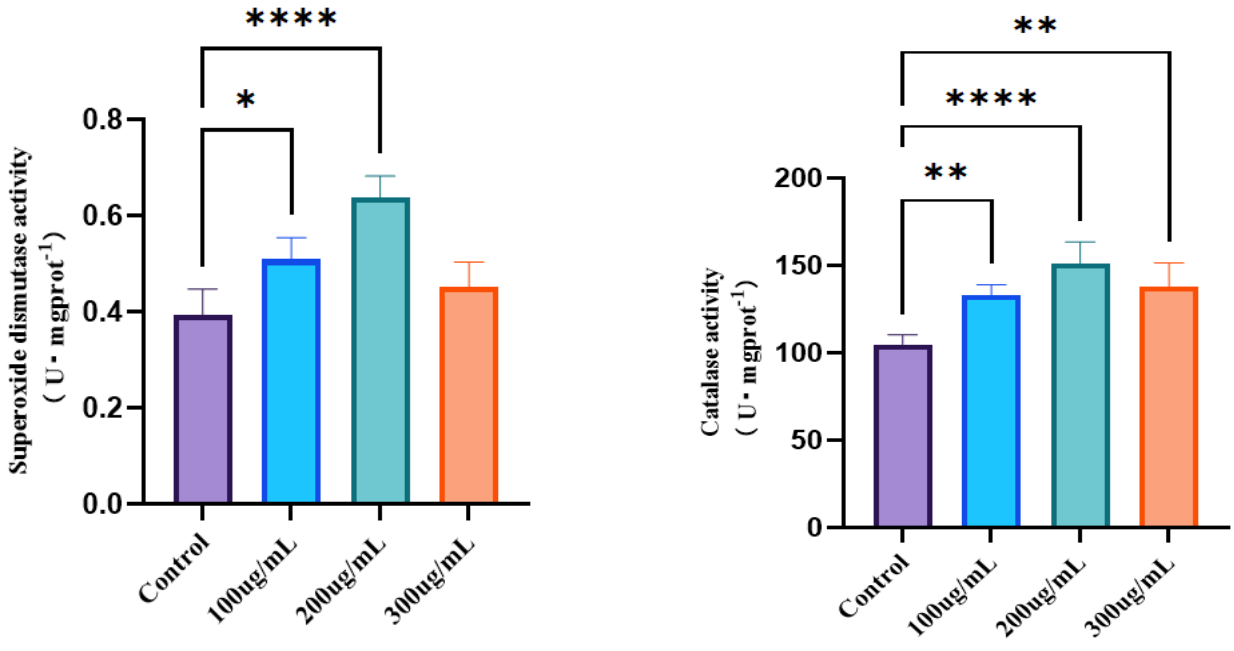

Figure 3.3 Analysis of the effect of EPS-TB10 on the activity of antioxidant enzymes in nematodes and its significance

(a) Superoxide dismutase activity (b) Catalase activity

Summary of chapter III

1. Two stress methods - oxidative stress environment and heat stress environment - were adopted in this chapter to simulate oxidative stress conditions in vivo and to observe the effect of EPS-TB10 on the survival time of nematodes, thereby evaluating their antioxidant capacity [27].

2. The experimental results showed that when the concentration of EPS-TB10 was 300 μg/mL, the death rate of nematodes was the slowest and the survival time was the longest under the stress environment, indicating that EPS-TB10 has good antioxidant activity in vivo.

The effect of EPS-TB10 on the activity of antioxidant enzymes was verified by detecting the activity levels of CAT (catalase) and SOD (superoxide dismutase) in nematodes.

3. The results indicated that EPS-TB10 could significantly increase the activity of antioxidant enzymes in nematodes, and the activity of antioxidant enzymes reached the maximum value when the polysaccharide concentration reached 200 μg/mL, suggesting that EPS-TB10 has the ability to regulate and enhance the antioxidant system.

4. Based on the above results, the experiments in this chapter successfully verified the antioxidant function of EPS-TB10 in vivo through the nematode model, providing an experimental basis for its subsequent application in functional foods or drugs.

Conclusion

1. This study innovatively selected a strain of extracellular polysaccharide-producing bacteria TB10 (*Klebsiella pneumoniae*) isolated from waste tea soup, and took its extracellular polysaccharide EPS-TB10 extracted in the laboratory as the research object, which has good potential for resource reuse and biological activity development.
2. In terms of extraction and purification, a full-process control system from crude to high-purity products was established through research: Firstly, the initial extraction was carried out by the traditional ethanol precipitation method; Subsequently, the modified Sevage method was adopted to remove protein impurities and improve the purity of polysaccharides; Further purification is achieved through DEAE-52 ion exchange chromatography technology. Finally, the content of polysaccharides was determined by the phenol-sulfuric acid colorimetric method, with a result of 0.54 g/L, and the precise determination of the molecular weight of polysaccharides was achieved.
3. In the in vitro antioxidant function assessment, four common antioxidant models, namely ABTS, superoxide anion, DPPH and hydroxyl radical scavenging assay, were systematically adopted in the study. The results show that EPS-TB10 exhibits good antioxidant activity in the ABTS and superoxide anion removal systems, and shows a concentration-dependent linear enhancement trend in the superoxide anion removal experiment, indicating that it has certain natural antioxidant potential. However, the effect was limited in the DPPH and hydroxyl radical scavenging experiments. When the concentration reached 2 mg/mL, the scavenging rate was only slightly higher than 15%, and there was a phenomenon that the scavenging rate tended to be saturated or even decreased with the increase of concentration, which might be related to the occlusion of active sites caused by polysaccharide aggregation.

Compared with synthetic antioxidants such as vitamin C (VC), EPS-TB10 has a relatively weak antioxidant capacity. However, its hydrogen donor effect and metal chelating ability suggest that its antioxidant mechanism has a certain scientific basis.

4. In vivo experiments, the research team used the model organism Caenorhabditis elegans to verify the antioxidant activity of EPS-TB10: Under oxidative stress and heat stress environments, EPS-TB10 significantly prolonged the survival time of nematodes, especially with the most obvious effect at a concentration of 300 μg/mL. Meanwhile, by detecting the activities of CAT (catalase) and SOD (superoxide dismutase), it was found that EPS-TB10 could effectively enhance the antioxidant enzyme activities in nematodes, especially when the enzyme activity reached the peak at a concentration of 200 μg/mL.The experimental results show that EPS-TB10 can enhance the ability of nematodes to resist oxidative stress and has the potential effect of delaying aging.

5. To sum up, this study has made significant progress in aspects such as the optimization of the extraction process of EPS-TB10, structural analysis, and the evaluation of antioxidant functions in vivo and in vitro: A standardized extraction and purification process has been successfully constructed, providing methodological support for subsequent research; The antioxidant potential of EPS-TB10 in both in vitro and in vivo environments was revealed. Although its antioxidant effect is still inferior to that of synthetic antioxidants, its natural source, good biocompatibility and modifiability provide a theoretical basis for its application in functional foods, health products or drugs. Subsequently, it can be considered to further enhance its antioxidant performance and expand its application prospects through structural modification (such as sulfation, acetylation) or combined use with other antioxidant components.

This research not only provides new ideas and experimental basis for the preparation and functional study of Exopolysaccharide from bacteria, but also contributes valuable reference cases to the development of the fields of waste resource utilization and green biomanufacturing.

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