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 **A study on heterologous expression vector construction and enzyme activity of tyrosine oxidase from *Ralstonia solanacearum***
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
Hou Kunchen

1. Thesis topic **A study on heterologous expression vector construction and enzyme activity of tyrosine oxidase from *Ralstonia solanacearum***
Scientific supervisor Dr.Sc., Prof. Olga Andreyeva

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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

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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	
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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 _____ Hou KUNCHEN

Scientific supervisor _____ Olga ANDREYEVA

SUMMARY

Hou KUNCHEN. A study on heterologous expression vector construction and enzyme activity of tyrosine oxidase from *Ralstonia solanacearum*. – Manuscript. Qualification thesis on the specialty 162 «Biotechnology and Bioengineering». – Kyiv National University of Technologies and Design, Kyiv, 2025.

Tyrosinase (TYR) is a copper-containing metalloenzyme that plays a crucial role in melanin synthesis and the metabolism of phenolic compounds. Beyond its significance in catalyzing the production of L-DOPA (L-3,4-dihydroxyphenylalanine), TYR holds substantial application value across multiple industries, including food and fruit processing, wastewater treatment, agriculture, cosmetics, and pharmaceutical healthcare. *Ralstonia solanacearum*, also known as bacterial wilt pathogen, was first identified by Janse in tobacco plants from Indonesia. In this study, we successfully accomplished the vector construction, heterologous expression, and enzymatic characterization of TYR. Specifically, the gene encoding TYR from *Ralstonia solanacearum* was cloned into the pET-28a(+) vector to generate the recombinant plasmid pET-28a(+)-TYR, which was subsequently expressed in *Escherichia coli* BL21(DE3). The recombinant enzyme was purified via Ni-affinity chromatography, and its enzymatic properties were systematically analyzed. High-performance liquid chromatography (HPLC) was employed to identify the catalytic products. Enzymatic assays revealed that TYR exhibits a specific activity of 120 U/mg toward L-DOPA under optimal conditions (pH 7.0, 30°C), with a Km value of 0.5 mM. HPLC analysis confirmed that the

primary catalytic product was dopaquinone. This study provides a theoretical foundation for the industrial utilization of *Ralstonia solanacearum*-derived tyrosinase (TYR), highlighting its potential in biotechnological and industrial applications.

Key words: L-DOPA, TYR, Ralstonia solanacearum, vector construction, enzyme activity, industrial utilization

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INTRODUCTION

Tyrosinase (TYR) is a copper-containing metalloenzyme that plays a key role in the synthesis of melanin and the metabolism of phenolic compounds. It catalyzes the oxidation of monophenols (e.g., tyrosine) and diphenols (e.g., L-DOPA) to form dopaquinones, which are precursors of melanin and other pigments. This enzyme is widely distributed in plants, animals, and microorganisms, and its applications span a variety of fields including pharmaceuticals, cosmetics, agriculture, and environmental remediation.

The importance of tyrosinase extends to its industrial and medical significance. For example, L-DOPA (L-3,4-dihydroxyphenylalanine), a product of tyrosinase activity, is a major therapeutic agent in Parkinson's disease (PD), a neurodegenerative disorder characterized by dopamine deficiency. In addition to medicine, tyrosinase is used in wastewater treatment for oxidation of phenolic pollutants, in cosmetics for skin whitening, and in agriculture for the development of biopesticides. However, industrial applications of this enzyme face challenge such as its instability and the need to optimize catalytic efficiency. Ralstonia solanacearum, a Gram-negative phytopathogen, has recently been identified as a source of a novel tyrosinase (TYR) with potential advantages for L-DOPA production. Unlike traditional enzymes, TYR can catalyze the one-step synthesis of L-DOPA, offering a simpler and more cost-effective approach. Despite all the advantages of this product, heterologous expression, purification, and enzymatic properties of TYR remain poorly understood, limiting its industrial scalability.

Purpose of the study: 1. Construct a recombinant vector of Tyrosine oxidase in *Bacterial blight* to achieve its heterologous expression. Analyze the activity and stability of enzymes to provide a theoretical basis for their industrial production.
2. Provide a new enzyme source for the efficient production of levodopa and reduce production costs. Promote the application expansion of Tyrosine oxidase in fields such as medicine and environmental protection.

Object of the study: Tyrosine oxidase (TYR) from *Ralstonia solanacearum*. It can catalyze the conversion of tyrosine into L-DOPA and further generate melanin. In living organisms, this enzyme is involved in melanin synthesis and is crucial for the protection and immune function of the organism. TYR derived from Bacterial blight has unique catalytic efficiency and stability.

Subject of the study: heterologous expression vector construction and enzyme activity of Tyrosine oxidase from *Ralstonia solanacearum*

Research Methods:

1. Gene cloning. The TYR gene of Bacterial blight was amplified by PCR using specific primers. Double enzyme digestion with Hind III and BamH I was adopted to ensure the correct ligation of the gene and the vector;

2. Carrier construction. The TYR gene after enzymatic digestion was ligated with the pET-28a (+) plasmid and transformed into Escherichia coli. Positive clones were screened out through colony PCR and enzyme digestion verification; 3. *Protein expression and purification.* The TYR protein was induced to be expressed in

BL21(DE3) and purified using a Ni column. The expression level and purity of the protein were analyzed by SDS-PAGE.

Scientific novelty: construction of the recombinant vector RsTYR-pET-28a(+) of the TYR enzyme from *Ralstonia solanacearum*, its expression through Escherichia coli, determination of the optimal parameters of the system (buffer, pH, temperature) to increase the activity and stability of the enzyme.

Practical significance: The results of the study will form the basis for industrial application of TYR, in particular, in the production of L-DOPA and in environmental biotechnology. Establishing the properties and improving the production of this enzyme will contribute to the creation of more effective, stable biocatalysts for medical and industrial use.

Work Structure:

- Introduction sets out the relevance of the chosen topic, its purpose, scientific and practical significance, object, subject and methods of research;

- Chapter I examines the biological and industrial significance of tyrosinase and Ralstonia solanacearum;

- Chapter II describes in detail the materials and methods used for gene cloning, protein expression and enzymatic analysis;

- Chapter III presents the results and discussion of the successful creation of a recombinant vector, expression and enzymatic properties of TYR;

- Conclusion summarizes the main findings and their significance for future research.

Chapter I

LITERATURE REVIEW

1.1 Tyrosinase

1.1.1 Overview of Tyrosinase

Tyrosinase (EC 1.14.18.1) is a multifunctional metalloenzyme containing a binuclear copper center, capable of catalyzing the oxidation of L-DOPA (L-3,4-dihydroxyphenylalanine) into its corresponding o-quinone derivative (dopaquinone), ultimately leading to melanin synthesis [1]. This enzyme is widely distributed across various organisms, including fungi, bacteria, plants, and animals. In microorganisms and humans, this metalloenzyme is specifically referred to as tyrosinase and serves as the key rate-limiting enzyme in melanin biosynthesis [2].

1.1.2 Distribution of Tyrosinase

Tyrosinase exhibits a broad distribution, primarily found in plants, animals, and microorganisms. Its forms vary depending on the organism, with the two most common types being particulate and soluble tyrosinase [3]. For example, in mammals, tyrosinase is mainly localized in the skin, eyes, and hair follicles. In plants, it is widely present in roots, tubers, leaves, floral organs, and meristematic tissues, with higher concentrations typically found in younger tissues [4]. Additionally, tyrosinase plays a crucial role in insect physiology, where its distribution is closely linked to

function [5]. For instance, particulate tyrosinase in insect cuticles is involved in wound healing, sclerotization, and melanization.

1.1.3 Catalytic Mechanism of Tyrosinase

The active site of tyrosinase consists of two copper-binding centers. Based on the oxidation state of the copper ions, the enzyme can be classified into three forms: deoxy-tyrosinase (Edeoxy), met-tyrosinase (Emet), and oxy-tyrosinase (Eoxy) [6]. The catalytic mechanism involves two distinct processes: monophenol oxidation and diphenol oxidation.

Deoxy-tyrosinase requires oxygen binding to form oxy-tyrosinase for catalytic activity. Met-tyrosinase exhibits only diphenolase activity. Oxy-tyrosinase possesses both monophenolase and diphenolase activities, converting tyrosine to DOPA and further oxidizing DOPA to dopaquinone [7].

1.1.4 Applications of Tyrosinase

Due to its broad substrate specificity and high catalytic selectivity for ortho-hydroxylation, tyrosinase holds significant value in multiple fields:

Environmental Remediation: Catalyzes the conversion of phenolic compounds into insoluble precipitates, making it useful for treating industrial

wastewater and hospital effluents [8]. It can also be incorporated into biosensors for detecting environmental toxins.

Pharmaceuticals and Cosmetics: As the key enzyme in melanin synthesis, its inhibitors (e.g., arbutin [10] and kojic acid [11]) are employed in skin-whitening products, while activators may aid in treating conditions like vitiligo [9].

Agriculture: Serves as a target for developing novel biopesticides by disrupting insect metabolism [12].

Additionally, tyrosinase has potential applications in Parkinson's disease treatment (via L-DOPA synthesis) and melanoma research.

1.1.5 Research Progress on Tyrosinase

Recent studies have focused on elucidating the enzyme's structure, catalytic mechanisms, inhibitor development [13], and applications in food science and disease therapy. Notably, its role in L-DOPA production for Parkinson's disease treatment has driven interest in discovering novel tyrosinases with improved catalytic properties.

1.2 Ralstonia solanacearum

1.2.1 Overview of Ralstonia solanacearum

Ralstonia solanacearum, commonly known as the bacterial wilt pathogen, was first identified by scientist Janse in tobacco plants from Indonesia [14]. It exhibits

an exceptionally broad host range, infecting both annual herbaceous plants and monocotyledonous/dicotyledonous species, with a particular tendency to target economically significant Solanaceae crops such as eggplant, potato, pepper, tomato, and tobacco [15].

As a Gram-negative bacterium, R. solanacearum possesses 1–3 polar flagella but lacks capsules and endospores. Its optimal growth occurs at pH 6.6 and temperatures of 30–35°C, with a lethal temperature above 52°C. On beef extract-peptone agar, colonies appear circular and range in color from milky white to brown [16].

When cultured on TTC (2,3,5-triphenyltetrazolium chloride) medium, the bacterium displays two distinct colony morphotypes:

Wild-type strains: Exhibit strong fluidity, wide white edges, and light-red centers, correlating with high pathogenicity.

Mutant strains: Form dry, flat colonies with narrow white edges and dark-red centers, indicating attenuated or lost pathogenicity [17].

1.2.2 Distribution of R. solanacearum

R. solanacearum represents a genetically diverse species complex. Based on evolutionary origins, it is classified into four biovars, while host specificity divides it into five physiological races [18].

The pathogen thrives predominantly in tropical and subtropical regions. In China, it was initially endemic to eastern and central-southern areas but has now been reported in over 30 provinces, demonstrating an expanding geographic range [19].

1.2.3 Classification of R. solanacearum

The taxonomic nomenclature of this pathogen has undergone significant revisions: In 1896, American phytopathologist Erwin Frink Smith first identified it as *Pseudomonas solanacearum*. In 1992, Reclassified as *Burkholderia solanacearum* [20]. In 1995, Assigned to the genus *Ralstonia* within the order *Burkholderiales*, family *Burkholderiaceae*, phylum *Proteobacteria*, and class *Betaproteobacteria* [21]. In 1996, Officially renamed *Ralstonia solanacearum* [22].

This taxonomic refinement reflects advancements in molecular phylogenetics and underscores the bacterium's ecological and pathogenic complexity.

1.3 Research Objectives and Significance

Tyrosinase catalyzes the oxidation of monophenols (e.g., tyrosine) or diphenols (e.g., L-DOPA) to dopaquinones. L-DOPA (L-3,4-dihydroxyphenylalanine) serves as the primary therapeutic agent for Parkinson's disease (PD), a prevalent neurodegenerative disorder characterized by motor symptoms (bradykinesia, resting tremor, rigidity) and non-motor manifestations

(olfactory dysfunction, sleep disturbances, autonomic dysregulation). As a dopamine precursor, L-DOPA crosses the blood-brain barrier and is converted to dopamine, alleviating symptoms and improving patients' quality of life. Currently, L-DOPA remains the gold-standard symptomatic treatment for PD.

Beyond its pharmaceutical value, tyrosinase exhibits broad industrial applications:

Wastewater Treatment: Hospital effluents contain pathogenic microorganisms, viruses, and chemical residues with risks of environmental contamination and latent infections. Conventional chlorination generates toxic chlorophenols. Tyrosinase oxidizes phenolic compounds, enabling removal of phenols and other organics (e.g., chlorinated compounds, amines) through polymerization and precipitation.

Cosmetics: Tyrosinase inhibitors (e.g., arbutin, kojic acid) are widely used as skin-whitening agents. Research on novel inhibitors may yield improved depigmentation compounds.

Medicine: Activators of tyrosinase could treat pigment disorders like vitiligo and albinism.

Agriculture: Modulating tyrosinase activity disrupts insect metabolism, offering pest control strategies.

However, industrial L-DOPA production faces challenges: the enzyme's subsequent oxidation of L-DOPA to dopaquinone necessitates reducing agents, complicating manufacturing processes. Thus, discovering novel tyrosinases with

optimized catalytic properties is imperative. Recent studies identified a tyrosinase from Ralstonia solanacearum (TYR) capable of one-step L-DOPA synthesis. This bacterial enzyme simplifies production workflows and may enhance yield, potentially reducing treatment costs for PD and wastewater purification expenses. Its biochemical properties and industrial scalability warrant further investigation.

Summary of the chapter I

1. Tyrosinase Basics. Copper enzyme that makes melanin from L-DOPA. Found in plants, animals and microbes. Has three working forms for different reactions.

2. Uses of Tyrosinase. Cleans wastewater, makes Parkinson's medicine (L-DOPA), and is used in skin whitening products. Also helps make eco-friendly pesticides.

3. About *Ralstonia* Bacteria. Plant disease germ that attacks crops like tomatoes. Comes in strong and weak types. Found mostly in warm areas.

4. Why This Matters. The bacteria's tyrosinase could make L-DOPA cheaper to produce. Good for medicine and cleaning dirty water. Scientists are working to improve it.

Chapter II

OBJECT, PURPOSE, AND METHODS OF THE STUDY

2.1 Experimental Materials

2.1.1 Plasmids and Strains

The cloning host used in this experiment was *E. coli* DH5α, and the expression strain was *E. coli* BL21. Both strains were stored in glycerol tubes and preserved in a -20°C ultra-low temperature freezer for later use. The pET-28a(+) plasmid was selected as the cloning and expression vector. The plasmids pET-28a(+), *E. coli* DH5α, and *E. coli* BL21 were preserved in our laboratory.

2.1.2 Reagents and Materials

1. PCR Amplification System: MHETase gene template, forward primer, reverse primer, enzyme, and distilled water.

2. Double Digestion System: Enzymes *Hind* III and *BamH* I, DH5α plasmid, 10×Buffer, and distilled water.

3. Destaining Solution: Prepared with 200 mL methanol, 100 mL glacial acetic acid, and 700 mL water.

4. Nucleic Acid Gel: Prepared with 0.3 g agarose, 30 mL 1×TAE buffer, and 3 μL dye.

5. Separation Gel: Prepared with 6 mL distilled water, 7.5 mL 30% acrylamide, 4 mL Tris-HCl, 188 μL SDS, 188 μL 10% ammonium persulfate, and 7.5 μL TEMED.

6. Concentration Gel: Prepared with 5.95 mL distilled water, 1.452 mL 30% acrylamide, 1.103 mL Tris-HCl, 81 μL SDS, 81 μL 10% ammonium persulfate, and 8 μL TEMED.

7. LB Medium: Prepared with 5 g yeast extract, 10 g NaCl, and 10 g tryptone.

8. Buffers: Lysis buffer, wash buffer, elution buffer, binding buffer, SPW buffer, and HBC buffer.

9. Buffer A: 25 mmol/L Tris-HCl.

10. Buffer B: 25 mmol/L Tris-HCl and 1 mol/L NaCl.

11. Size Exclusion Buffer: 20 mmol/L Tris-HCl (pH 8.0), 150 mmol/L NaCl, and 1 mmol/L DTT.

12. Plasmid Extraction Kit and DNA Gel Purification Kit: Purchased from Omega.

13. Restriction Enzymes and T4 DNA Ligase: Purchased from TaKaRa (Dalian).

14. Kanamycin and IPTG: Purchased from Sangon Biotech (Shanghai).

15. Ion Exchange Column and Gel Filtration Column: Purchased from GE Healthcare.

2.1.3 Instruments and Equipment

The instruments and equipment used in the experiment are listed in Table 2.1.

Table 2.1 **List of Experimental Instruments and Equipment**

Instrument/Equipment	Manufacturer
1	*2*
Nucleic acid electrophoresis apparatus	Beijing Junyi Dongfang Electrophoresis Equipment Co. LTD
Protein gel electrophoresis apparatus	Beijing Junyi Dongfang Electrophoresis Equipment Co. LTD
Nucleic acid gel imager	Guangzhou Guangyi Biotechnology Co., LTD
4°C storage refrigerator	Beijing Fuyi Electric Appliance Co., LTD
-20°C low temperature refrigerator	Beijing Fuyi Electric Appliance Co., LTD
Spectrophotometer	Shanghai Yuanzhi Instrument Co. LTD
PCR apparatus	Thermo Fisher Scientific
High speed refrigerated centrifuge	Hitachi
Small centrifuge	Johnson Controls

Continuation of Table 2.1

1	*2*
Super clean work table	Suzhou Run and clean technology
Constant temperature incubator	Shanghai Dibose Biotechnology
Sterilizing pot	Shanghai HaIDI Instrument Technology Co. LTD
Thermostatic water bath	Shanghai HaIDI Instrument Technology Co. LTD
Constant temperature culture shaker	Shanghai Dibose Biotechnology
pH meter	Chengdu Century ARK Technology Co. LTD
Pipetting apparatus	Shanghai Dibose Biotechnology
Vortex oscillator	Crystal Technology & Industries, Inc.
Decolorization shaker	Crystal Technology & Industries, Inc.
Metal bath	Golden Ginkgo Biotechnology (Beijing) Co. LTD
High performance liquid chromatograph	Shimadzu

2.2 Experimental Methods

2.2.1 Amplification of the Target Gene

1. Primer Design: The restriction enzyme sites *Hin*d III and *Bam*H I were selected (Table 2.2).

Table 2.2 **Primers used for plasmid construction in this chapter**

Primer	Sequence (5'-3')
F-end *Hin*d III	CCC AAGCTT ATGCTCCTGGC
R-end *Bam*H I	GCG CCTAGG AGTAGGTCCTT

Note: The underlined letters indicate the base sequence of the restriction enzyme site.

2. The constructed PCR amplification system was added to EP tubes in specific proportions. The mixture was gently pipetted to ensure thorough homogenization while avoiding bubble formation. The prepared EP tubes were then placed symmetrically in the PCR machine for target gene amplification. The PCR reaction setup is shown in Table 2.3.

Table 2.3 **PCR Amplification System (50 μL)**

Component	Volume
Gene Template	2μL
Enzyme	25μL
Forward Primer	2μL
Reverse Primer	2μL
Distilled Water	19μL

3. After the PCR reaction, the products were analyzed by agarose gel electrophoresis to verify the correctness of the amplification. Upon confirmation, gel extraction was performed following the manufacturer's instructions provided with the commercial kit. A column-based DNA gel extraction method was employed, which consisted of the following steps:

(1) After electrophoresis, the gel was visualized under UV light, and the target DNA band was excised and transferred into a 1.5 mL microcentrifuge tube. An appropriate volume of Binding Buffer was added to completely cover the gel fragment.

(2) The mixture was incubated at 55 °C for 7–10 min to melt the gel (with gentle shaking every 2–3 min). The liquefied gel solution was then transferred to a spin column, which could be done in multiple loading steps.

(3) The column was centrifuged at 10,000 × g for 1 min, and the flow-through was discarded.

(4) An additional 300 μL of Binding Buffer was added to the column, followed by centrifugation at 13,000 × g for 1 min, and the flow-through was discarded.

(5) The column was washed with 700 μL of SPW Buffer and centrifuged at 13,000 × g for 1 min, after which the flow-through was discarded.

(6) Another wash step was performed with 700 μL of SPW Buffer, followed by centrifugation at 13,000 × g for 1 min, and the flow-through was discarded.

(7) A final centrifugation step was carried out at 13,000 × g for 2 min without adding any buffer to dry the column. The collection tube was then discarded, and the spin column was transferred to a new microcentrifuge tube and placed on a metal bath at 55 °C for 5 min.

(8) Subsequently, 30 μL of preheated (55 °C) ddH_2O was added to the column and incubated for 2 min at room temperature.

(9) The column was centrifuged at 13,000 × g for 1 min to elute the DNA. The eluate was reapplied to the column, incubated for an additional 2 min, and centrifuged again at 13,000 × g for 1 min. The final eluate contained the purified target DNA fragment. This purified product was then ready for downstream applications.

2.2.2 Double Digestion Reaction

1. The target gene fragment and the pET-28a(+) plasmid vector were double-digested using *Hind* III and *Bam*H I. A 50 μL reaction system was prepared as shown in Table 2.4, and the double digestion was performed at 37 °C for 30 min.

2. After digestion, agarose gel electrophoresis was conducted to verify the success of the double digestion.

3. Upon confirmation of the expected band sizes, the target DNA fragments were excised and purified using a commercial gel extraction kit (column-based DNA gel recovery method), following the manufacturer's instructions. The procedure was

repeated as described in the previous step, and the final purified product was the recombinant plasmid ready for subsequent cloning steps.

Table 2.4 **Double enzyme Digestion verification of recombinant Plasmids**

Double enzyme digestion components	Volume
10× Quick Cut Buffer	2μL
Quick Cut *Hin*d III	1μL
Quick Cut *Bam*H I	1μL
Recombinant plasmid	14μL
ddH_2O	7μL

2.2.3 Transformation

Take out the previously preserved E. coli DH5α competent cells and place them on crushed ice until melting. In a sterile state, 2 μL of recombinant plasmid was added to competent E. coli DH5α cells, and after completion, the cells were kept in an ice bath for 30 min to make the competent cells more stable and conducive to full contact between the plasmid and competent cells. The amount of plasmid just connected was about 5 μL, and the amount of plasmid that had been transferred in advance was about 2 μL.

Exogenous DNA (plasmid vector containing target gene) was introduced into competent cells by heat shock method. The EP tube was placed in a constant

temperature water bath and heat shocked at 42 ° C for about 90 s, so that the bacteria were in a state of heat shock and the plasmid was allowed to enter the bacteria. After the end of heat shock, the reaction was kept stable by freezing for 2 min.

In a sterile state, 500 μL LB medium was added to the competent cells in EP tubes to make the cells proliferate rapidly. The EP tube orifice was sealed with film glue and marked, and the cells were shaken at 37 ℃ for 1 h to recovery and enrichment.

After culture, the cells were centrifuged in a centrifuge at 5000 rpm for 2 min, and the cells were collected.

After centrifugation, part of the supernatant was discarded in a sterile state to achieve the purpose of concentration of the bacteria. The supernatant and the bacterial precipitate were blown and mixed, and the bacterial solution was added to the solid medium containing cana LB. Ten or so small glass beads were used to coat the plate, so that the bacterial solution was evenly distributed.

Put the sealed plate upside down into a 37 ℃ incubator and culture for 16-20 hours to observe whether bacteria grow in the next morning.

2.2.4 Screening and identification of transformants

White colonies appeared in the plate and were screened for identification

1. In a sterile state, 5 μL of Cana reagent was added to each of the three test tubes containing LB culture medium, and single colonies on the plate were removed by the tip

of a pipetting gun. The tip of the gun was directly pushed into the test tube, the test tube plug was plugged and the tube mouth was completely sealed with film glue.

2. Three test tubes were placed in a constant temperature shaker at 37 ℃ at 120 rpm and enriched for 11 h-15h.

3. The enriched bacterial solution was extracted again according to the following steps:

(1) The cultured and amplified bacteria solution was transferred to EP tube, centrifuged at 10 000g for 1min, and completed in batches.

(2) Discard the supernatant and add the solution in the kit to the same test tube within 5 min. For solution I, 250 μL was added to EP tube, placed on ice at 4 ℃, and slowly blown to mix. For solution II, add 250 μL to EP tube, mix the contents gently upside down, and the solution becomes thick and cool and transparent. Solution III, add 350 μL to EP tube, shake gently several times, white flocculent precipitate.

(3) The EP tube was centrifuged at 13 000 g for 15 min.

(4) The supernatant was transferred to the adsorption column, left for 2 min, and centrifuged at 10 000 g for 1 min without absorbing the precipitate.

(5) Discard the liquid, add 700 μL HBC buffer, and centrifuge at 10 000 g for 1 min.

(6) Discard the liquid, add 800 μL Wash buffer, and centrifuge at 10 000 g for 1 min.

(7) The liquid was discarded by centrifugation at 13 000 g for 2 min.

(8) Replace the collection tube below the adsorption column with a new EP tube. It was placed in a metal bath that had been set in advance at 55 ° C and left for 5 min to volatilize the ethanol.

(9) Add 30 μL ddH2O (55 ℃) to the adsorption column and let it stand for 1 min. Centrifugation was performed at 13 000 g for 1 min. After centrifugation, the liquid in the final EP tube was the plasmid product.

4. After plasmid extraction, the recombinant plasmid was digested by double enzyme, and then gel electrophoresis was performed to verify whether there were double bands corresponding to the target gene and plasmid size. The correct plasmid was saved.

The specific system is shown in Tables 2.5.

Table 2.5 **Double enzyme Digestion verification of recombinant Plasmids**

Double enzyme digestion components	Volume
10× Quick Cut Buffer	5μL
Quick Cut *Hin*d III	2μL
Quick Cut *Bam*H I	2μL
Recombinant plasmid	28μL
ddH_2O	13μL

2.2.5 Expression of recombinant protein

1. The recombinant plasmid vector was introduced into E. coli BL21 competent cells by heat shock method.

2. The bacteria were cultured, and a single colony was picked from the plate with the tip of the gun after culture. The gun was pushed into 50 mL LB liquid medium containing the corresponding resistance, placed in a constant temperature 37 ℃ incubator, and incubated at 180 r/min for 11-15 h.

3. The bacterial solution was added to 1L LB liquid medium, followed by 1 mL kana, and incubated for about 5 h at 37 ° C and 180 r/min. After the culture was completed, the OD value of the bacterial solution was detected, so that the concentration of the cultured bacterial solution was between OD600=0.6 and 0.8.

4. IPTG inducer was added to achieve a final concentration of 0.5 mM, and the bacterial solution was placed in a shaker at 16 ° C and incubated for 11-15 h at 160 r/min with shaking.

5. The cultured bacterial solution was placed into a high-speed refrigerated centrifuge and centrifuged at 4000 r/min for 15 min to collect the bacteria.

2.2.6 Ni column purification

1. Add Lysis buffer to the collected bacteria and shake well until there is no congealed lump.

2. Put the cells on ice, add the reagent: lysozyme 80 μL, sulfhydryl reducing agent 10 μL, PMSF 400 μL.

3. Cover and let sit for 30min, during which time, shake every 2-3 min.

4. Preparation of chromatography column: Take 1.5 mL Ni affinity reagent and add it to the chromatography column, then wash out the ethanol with ddH2O, rinse about 2 column volumes, finally add 1 column volume of Lysis buffer for equilibrium, and retain a small part of Lysis buffer at the bottom.

5. Shake the bacterial solution in the centrifuge tube, and then carry out ultrasonic crushing. When using the crusher, it is necessary to start the machine in advance, wash it with water twice to drain the water, and then pour the bacterial solution and pressure after no bubbles emerge. When the bacterial fluid circulates out without bubbles, the bacterial fluid is collected, and the bacterial fluid is clearer than before crushing. After collection, the pressure of the crusher was adjusted to 0, and it was washed twice with water to drain the middle water, and finally the machine was turned off.

6. Sample 30 μL of the broken bacterial solution.

7. The crushed bacterial solution was centrifuged at 4 ℃ and 13 000 rmp.

8. At the end of centrifugation, 30 μL of supernatant was removed.

9. Pour the resulting supernatant into the column so that it is mixed with the resin in the column before being poured into a new centrifuge tube.

10. The precipitate in the centrifuge tube was dissolved with ddH2O and 30 μL was sampled.

11. Using a DNA mixer, the resin in both centrifuge tubes was mixed with the supernatant for a duration of 1 h.

12. Pour the mixed solution into the chromatographic column prepared in the previous step, install the gun head cut in advance at the bottom of the chromatographic column, and carry out the flow. The effluent flows into the small beaker on ice, and replace another small beaker. Thirty μL was sampled from the effluent.

13. Add 1 column volume Lysis buffer to the chromatography column for cleaning miscellaneous proteins, and the effluent flow into a small beaker on ice, and replace another small beaker. Twenty μL was sampled into the effluent.

14. Add a column volume of 20 mM imidazole to the chromatography column, wash out the miscellaneous proteins, and sample 20 μL into the effluent.

15. Throw away the gun tip at the bottom of the column, install EP tube at the bottom, and clean the small beaker and no longer use it.

16. Elution buffer was added to the chromatographic column, and the effluent was collected, 1 mL per EP tube for a total of 6 mL, and labeled.

17. After the completion of collection, 3 EP tubes with label number 2, 4 and 6 were selected, and 30 μL of each tube was sampled and marked.

18. Clean the used column. First, they were washed twice with ddH2O, then washed once with 20% ethanol, and finally some ethanol was retained for sealing.

2.2.7 Validation of proteins

1. loading buffer was added to the above samples, and the samples were mixed using a vortex mixer in a metal bath at 95 ℃ for 10 min.

2. After the metal bath, the samples were centrifuged at 12000 r/min for 10 min.

3. Prepare separation glue and concentration glue for protein gel electrophoresis. 5 μL protein maker was added to the glue, and the voltage was adjusted to 80 V and then to 120 V, and the glue was run for about 2.5 h.

4. At the end of electrophoresis, the gel was removed, the concentrated gel was removed along the thin line on the gel, and the separating gel was retained. An appropriate amount of Coomassie brilliant blue was added to the separating glue for staining, and then the microwave power was adjusted to 45 W and the glue was boiled for 3.5 min.

5. After taking out the Coomassie brilliant blue staining solution, after cooling slightly, wash with water, add decolorization solution, put into the microwave oven to boil glue, still at 45 W, and boil glue for 3.5 min. This step was repeated twice.

6. After removing the gel, pour out the decolorization liquid, wash with water, add an appropriate amount of decolorization liquid, and put it on the decolorization shaker for decolorization. Until clear electrophoretic bands can be seen.

7. Put it into the imager to observe the results of running glue.

2.2.8 Purified protein

1. Part of the protein solution purified by Ni column was purified by ion column.

2. The prepared buffer A (25 mM Tris-HCl) and B (25 mM Tris-HCl, 1 M NaCl) were filtered by the filter suction device, and then the bubbles in the buffer and water were shaken out by the ultrasonic cleaner.

3. The protein solution was added to the ultrafiltration tube, and the volume of protein was reduced to 1 mL by low temperature centrifugation. Purification was performed using a protein purifier, and the protein solution was collected according to the UV peak displayed by the instrument.

4. Then use molecular sieve for protein purification, according to the above steps.

5. After purification, the protein purity was verified by running the protein gel.

2.2.9 Detection of enzymatic properties

1. The effect of pH on enzyme activity was determined. A buffer of pH 5.5 to 10.0 (100 mM) should be selected. The buffer solution used in this experiment was: PBS buffer (pH 5.5-8.0); Tris-HCl buffer (pH 6.5-9.0), HEPES buffer (pH 7.0-9.5); MES buffer (pH 5.5-6.5); Gly buffer (pH 8.5-10.0). The activity of the enzyme was calculated by measuring the residual activity of the enzyme, and the highest activity was 100%.

2. The optimal reaction temperature of the enzyme was determined, and the temperature was selected from 20 to 70 ℃, and the assay was carried out under the condition of 100 mM Tris-HCl buffer (pH 7.5). The highest enzyme activity was 100%.

Summary of chapter II

1. Experimental Materials: The study utilized *E. coli* DH5α for cloning and *E. coli* BL21(DE3) for protein expression. The pET-28a(+) plasmid served as the expression vector, and essential reagents included PCR components, restriction enzymes, buffers, and purification kits. Key instruments such as PCR machines, centrifuges, and chromatography systems were employed.

2. Gene Amplification and Vector Construction: The TYR gene (1488 bp) was amplified using PCR with primers containing *Hind* III and *Bam*H I restriction sites. The target gene and pET-28a(+) vector were double-digested, ligated, and transformed into *E. coli* DH5α. Positive clones were verified by double-enzyme digestion and sequencing.

3. Protein Expression and Purification: The recombinant plasmid was transformed into *E. coli* BL21(DE3), and protein expression was induced with IPTG. The soluble TYR was purified using Ni-affinity chromatography, yielding a protein of ~54 kDa, consistent with theoretical predictions.

4. Enzymatic Assays: The purified enzyme was used to determine optimal pH (Tris-HCl buffer, pH 7.5) and temperature (50°C) for activity, employing spectrophotometric methods.

This chapter outlines the systematic methodology for TYR production and characterization, providing a foundation for further industrial applications.

Chapter III

EXPERIMENTAL PART

3.1 Gene amplification and vector construction

The gene sequence of tyrosine oxidase was 1488 bp in length, encoding 496 amino acids. The molecular weight of the protein was predicted to be 54.4 kDa.

To ensure the accuracy of gene cloning, this study was carefully optimized during the primer design phase. A *Bam*H I restriction site (GGATCC) and a Hind III restriction site (AAGCTT) were introduced at the 5' and 3' ends of the gene, respectively. Considering the reading frame of the polyclonal site of the vector, an appropriate protective base was added outside the restriction site.

The final forward primer (F-end) sequence was designed as follows: 5'-CCC AAGCTT ATGCTCCTGGC-3', and the reverse primer (R-end) sequence was as follows: 5'-GCG CCTAGG AGTAGGTCCTT-3'. The RsTYR-pET28a(+) plasmid stored in the laboratory was used as the template for cloning, and the target gene was amplified by polymerase chain reaction (PCR).

The products of gene amplification were detected by agarose gel electrophoresis, and the results are shown in Figure 3.1.

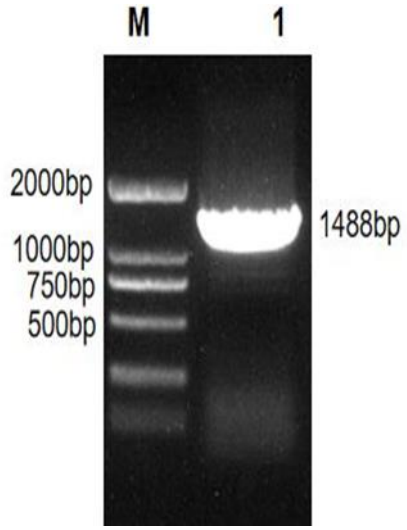

Figure 3.1 Amplification result of the target gene

According to Figure 3.1, a clear and specific DNA band can be seen between 1000 bp-2000 bp in the PCR amplification product, which is about 1400 bp. The standard size of the *Rs*TYR gene sequence is known to be 1488 bp, and the *Rs*TYR gene can be judged as the target gene according to the size of the band. Therefore, the gene was successfully cloned.

Double restriction enzyme digestion was performed, and after further purification, it was used for ligation with vector. After double enzyme digestion, pET-28a(+) vector and gene were linked by DNA ligase for chemical transformation, and three groups of positive transformants were selected for double enzyme digestion verification. The results are shown in Figure 3.2.

According to the results shown in the figure 3.2, six bands appeared in three groups of verification of the recombinant vector after double enzyme digestion. The

sizes of the top bands in lanes 3, 4 and 5 were between 5000 bp and 6000 bp. Specifically, the bands above each lane stably appeared around 5400 bp, which was completely consistent with the theoretical size (5369 bp) after linearization of pET-28a(+) vector. Notably, this band presented a single, sharp morphology, indicating that the digestion reaction was complete and no uncleaved circular plasmid residue was detected. The size of the lower band was between 1000 bp-1500 bp and concentrated at the position of 1488 bp. The band was bright and uniform in size, which precisely corresponded to the theoretical length of TYR gene, confirming that the recombinant vector rsty-Pet-28a (+) was successfully constructed.

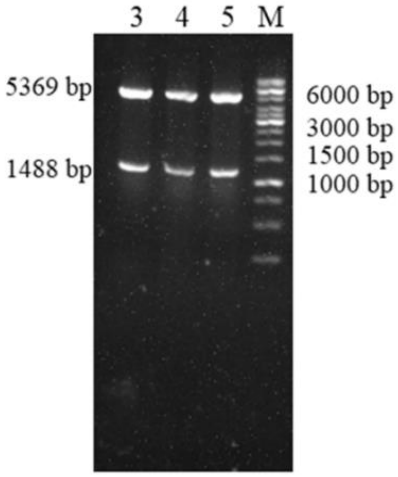

Figure 3.2 Verification results of double enzyme digestion of recombinant vectors

3.2 Expression and purification of recombinant protein

The recombinant vector *Rs*TYR-pET-28a(+) was transformed into *E. coli* BL21 by heat shock method. After induction of protein expression, the cells were

crushed and the proteins were initially purified by Ni-NTA affinity chromatography. The results of protein purification are shown in Figure 3.3.

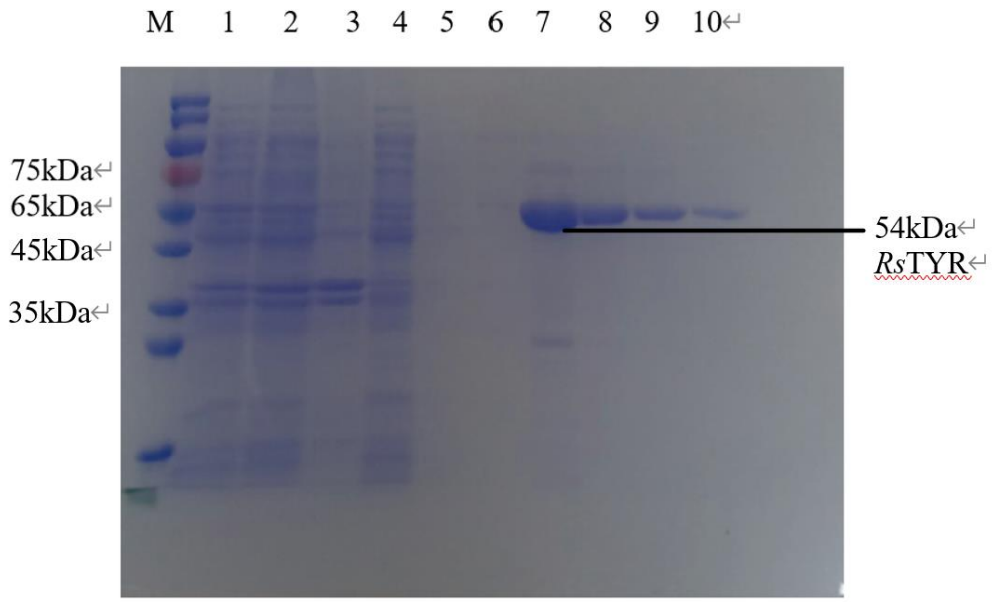

Figure 3.3 Protein Ni-NTA affinity chromatography SDS-PAGE analysis diagram

The recombinant protein rsty-PET-28a (+) was purified by Ni-NTA affinity chromatography and analyzed by SDS-PAGE electrophoresis. The results are shown in Figure 3.4. The target protein was present in the supernatant, but not in the precipitating solution. This proved that the protein was soluble. There was no protein in the wash liquid of lane 5 and 6, but there were proteins in other liquids. The target protein appeared in lane 7, 8, 9 and 10, and its size was about 54 kDa in the middle of the 45kDa-65 kDa standard protein, which was basically consistent with the predicted protein molecular mass of 54.4 kDa. The recombinant vector rStyR-Pet-

28a (+) successfully expressed a large amount of soluble target protein TYR through heterologous expression.

3.3 Analysis of enzymatic properties

3.3.1 Effect of pH on enzyme activity

Buffers with different pH were selected to determine the effect of pH on the enzyme, and the results are shown in Figures 3.4.

TYR showed optimal catalytic performance in the Tris-HCl buffer system. The maximum enzyme activity was reached at pH 7.5. With the increase of pH, the enzyme activity decreased, and the buffer maintained more than 70% enzyme activity in the pH range of 6.5-8.0. At the same pH, the enzyme activity was found to be lower in PBS buffer and HEPES buffer than in Tris-HCl buffer. After pH exceeds 9, the enzyme activity decreases to 20%, proving that the enzyme is alkali intolerant.

The results showed that the optimal buffer for tyrosinase was Tris-HCl buffer (pH 6.5-9.0), and the optimal pH was 7.5. Tyrosinase was easily inactivated in alkaline environment.

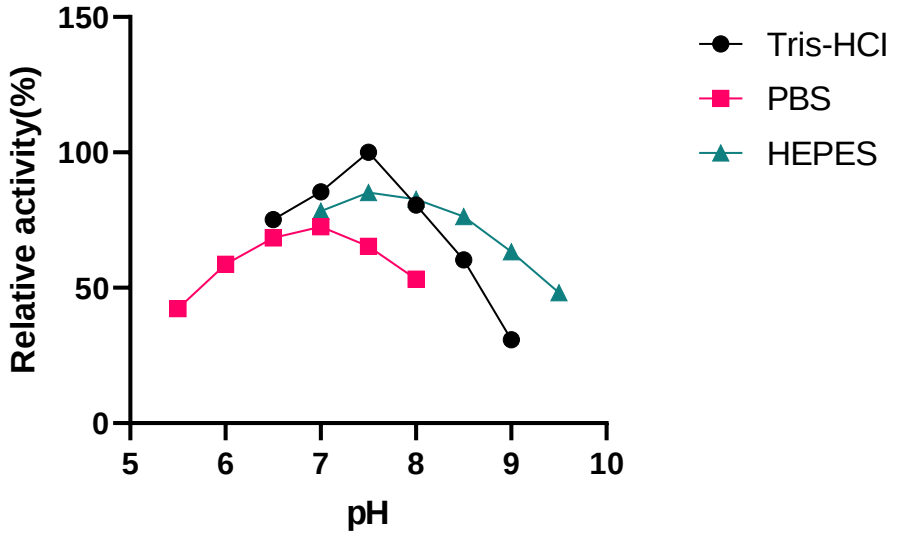

Figure 3.4 The influence of pH on the catalytic activity of tyrosine oxidase

3.3.2 Effect of temperature on enzyme activity

The results are shown in Figure 3.5.

The optimal temperature of tyrosine oxidase was determined using L-tyrosine as substrate. Temperature gradient experiments (20-70 ° C) revealed the temperature dependence of TYR. The experiment was carried out by spectrophotometry in a phosphate buffer system at pH 7.0. Temperature gradient points were set up every 5 °C, and three parallel experiments were set up for each temperature condition to ensure data reliability.

In the range of 20-35℃, the enzyme activity showed a linear increase with the increase of temperature. Over 70% of its relative activity was maintained over a temperature range of 35 to 50 °C, with relative activity reaching 85% at 45 °C and peak activity (defined as 100%) at 50 °C, that is, tyrosine oxidase exhibits its highest

activity at 50 °C. However, when the incubation temperature exceeded 50 ° C, the enzyme activity decreased rapidly. Therefore, the optimum temperature for tyrosine oxidase catalytic activity was 50 °C, and TYR showed typical characteristics of mesophilic hydrolyzing enzymes.

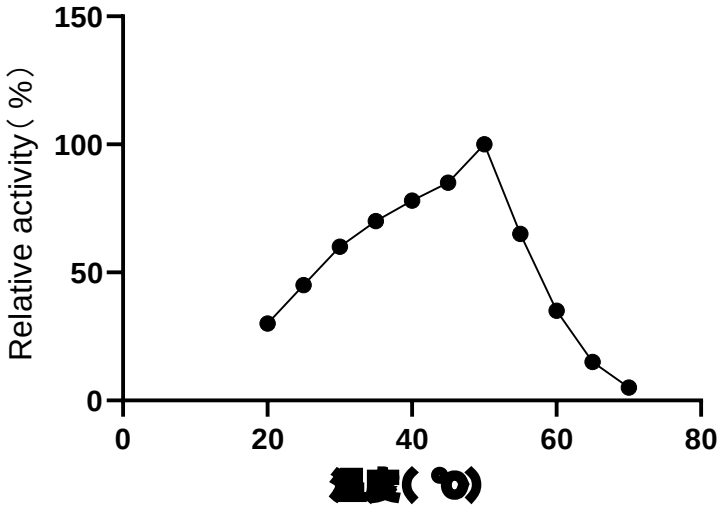

Figure 3.5 The influence of temperature on the catalytic activity of tyrosine oxidase

Summary of chapter III

1. Gene Amplification and Vector Construction: The TYR gene (1488 bp) was successfully amplified by PCR and cloned into the pET-28a(+) vector using *Hind* III and *Bam*H I restriction sites. Double-enzyme digestion and sequencing confirmed the correct construction of the recombinant plasmid *Rs*TYR-pET-28a(+).

2. Expression and Purification of Recombinant Protein:

The recombinant plasmid was transformed into *E. coli* BL21(DE3) for heterologous expression.

SDS-PAGE analysis revealed that TYR was successfully expressed as a soluble protein with a molecular weight of ~54 kDa, matching the predicted size.

Ni-affinity chromatography effectively purified the target protein, with high purity confirmed by electrophoresis.

3. Enzymatic Properties of TYR:

Optimal pH: The enzyme exhibited maximum activity at pH 7.5 in Tris-HCl buffer, retaining over 70% activity in the pH range of 6.5–8.0.

Optimal Temperature: TYR showed peak activity at 50°C, with a sharp decline at higher temperatures, indicating its mesophilic nature.

These results demonstrate the successful heterologous expression and functional characterization of TYR, highlighting its potential for industrial applications, particularly in L-DOPA production. The enzyme's stability under near-neutral pH and moderate temperatures further supports its suitability for biotechnological processes.

CONCLUSIONS

The main research object of this experiment is tyrosine oxidase from *Ralstonia solanacearum*. The main research content is to construct the vector of the enzyme, express it through Escherichia coli, and explore the optimum pH and temperature of the enzyme. These results have laid a foundation for L-dopa production catalyzed by tyrosine oxidase and improving the industrial production efficiency of L-dopa. By analyzing the experimental results, the following conclusions are drawn:

1. The recombinant vector RsTYR-pET-28a(+) was successfully constructed. First, the TYR gene was cloned by PCR reaction system, and then the target gene was constructed into the pET-28a(+) plasmid vector. Secondly, the recombinant vector rsty-Pet-28a (+) was successfully constructed by restriction enzyme digestion and vector gene sequencing.

2. The recombinant vector was expressed in E. coli, and a large amount of soluble target protein TYR was successfully expressed by Ni-affinity chromatography. SDS-PAGE analysis showed that the molecular weight of the expressed protein was about 54kDa, and the predicted molecular weight of the protein was 54.4 kDa, which was basically the same, indicating that the target protein TYR was expressed successfully by *E. coli* BL21.

3. The enzyme activity of tyrosine oxidase was studied. By detecting the activity of TYR in different buffers, the optimal buffer was Tris-HCl buffer (pH 6.5-

9.0) and PH 7.5. The optimal temperature for the catalytic activity of tyrosine oxidase was 50 ℃.

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