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Molecular Cloning, Heterologous Expression, and Characterization of a Neutral Uricase from *Arthrobacter* sp. YHBJ11 in Chaka Salt Lake

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Abstract

Uricase (or urate oxidase) is a key enzyme in purine catabolism, which is usually used to treat gout, hyperuricemia, and tumor lysis syndrome. In this study, an uricase-producing strain (YHBJ11) was isolated from soil in Chaka Salt Lake, Qinghai Province, China. The strain YHBJ11 was identified as *Arthrobacter* sp. by 16S rRNA gene sequence alignment. The uricase gene (*uox-YHBJ11*) of *Arthrobacter* sp. YHBJ11 was amplified by PCR. UOX-YHBJ11 gene was transferred to *E. coli* BL21 for cloning and heterologous expression. Recombinant UOX-YHBJ11 was purified and characterized. The optimum pH and temperature for UOX-YHBJ11 were 7.0 and 25 °C, respectively. It exhibited more than 60% relative activity at pH 5.0-8.0, and more than 50% relative activity at 10-40 °C. The relative enzyme activity was above 60% after incubation at 25 to 37 °C for 80 min. The enzyme was inhibited by Fe^{3+}, Ca^{2+}, Ni^{2+}, Co^{2+}, Ba^{2+}, Mn^{2+}, and Pb^{2+}. Kinetic characterization revealed that its Vmax and Km were 0.30 μmol/min/mg and 0.018 mM. The enzyme was

significantly inactivated by EDTA, SDS, and PSMF. These enzymatic properties indicate that UOX-YHBJ11 has potential applications in uric acid detection kits and pharmaceutical enzymes.

Keywords: Chaka Salt Lake; Uricase-producing strain; *Arthrobacter* sp. YHBJ11; Heterologous expression; Neutral uricase

Background

Uricase (or urate oxidase, EC 1.7.3.3) is the key enzyme of purine metabolism in organisms. Uricase can catalyze the oxidation of uric acid to allantoin, the solubility of which is 147 mg/100 mL and is more than 5 to 10 times that of uric acid, so it can be excluded from the body. Uricase has been identified in many species, including animals, plants, and microorganisms. However, some higher primates such as apes and humans lack functional uricase due to gene mutations, leading to uric acid as the end product of purine metabolism and leading to the development of gouty arthritis when the concentration of uric acid reaches its solubility limit in the blood **[1,2].** In recent years, the prevalence of gout has been increasing. New research has shown that the prevalence rate of gout in the world is 1 to 4%, with an incidence rate of 0.1 to 0.3% **[3].** Recurrent episodes of gout may have adverse effects, such as hypertension, hyperlipidemia, atherosclerosis, chronic kidney disease, osteoporosis, atrial fibrillation, and venous thromboembolism **[4-8].** Currently, there are several types of anti-hyperuricemic drugs, including allopurinol, benzbromarone, febuxostat, oral probenecid, rasburicase, and pegloticase **[9].** The latter two drugs are uricases, which can effectively improve treatment efficiency with fewer adverse reactions and higher safety compared to other anti-hyperuricemic drugs **[10,11].** Uricase is widely distributed among various organisms. Although many sources of uricase have been gradually discovered, microorganisms are one of the important sources of uricase **[12].** However, it is difficult for uricase to be massively produced due to complex purification procedures, high cost, and low yield **[13].** Therefore, it is important to explore new uricase resources for their development and utilization. In this study, a uricase-producing strain named *Arthrobacter* sp. YHBJ11 was isolated from soil samples in Chaka Salt Lake, Qinghai Province, China. The uricase gene (*uox-YHBJ11*) was amplified by PCR. The gene was then cloned and heterologously expressed in *E. coli*. The recombinant uricase (UOX-YHBJ11) was purified and characterized. Enzymatic properties indicated that UOX-YHBJ11 has potential applications in uric acid detection kits and pharmaceutical enzymes.

Materials and Methods

Soil samples

The soil samples were collected from Chaka Salt Lake, Qinghai Province, China (Coordinates: latitude 36.45234°N, longitude 99.51784°E, altitude 3059 m). The sampling depth was 5 to 10 cm. The soil samples were stored in 4 °C refrigerator.

Strain, plasmid, and media

Urate Basic (UB) medium ($MgSO_4$ 0.5g/L, $NaNO_3$ 2g/L, $K_2HPO_4{\cdot}3H_2O$ 0.5g/L, KH_2PO_4 0.5g/L, pH 7.5, urate 2g/L, 2% agar) was used for isolating uric acid degrading bacteria. *Escherichia coli* DH5α and *E. coli* BL21(DE3) were used for gene cloning and protein expression. Blunt zero plasmid (TransGen Biotech, China) and pET28a were used for the cloning vector and expression vector, respectively. LB medium (Yeast 5g/L,

peptone 10g/L, NaCl 5g/L) was used to cultivate *E. coli*. LB-urate medium (LB medium with 2g/L urate) was used to screen and detect the activity of uricase-producing strains.

Reagent

Primers (Beijing Qingke Biotechnology Co., Ltd.); DNA Taq enzyme (Shenggong Bioengineering Co., Ltd.); Plasmid Extraction kit and DNA Extraction Kit (Sangong Bioengineering Co., Ltd.); SDS polyacrylamide gel (Arnold Biotechnology Co., Ltd.); working fluid and stock solution (0.01% uric acid, 1 mmol / L EDTA, 0.001% Triton X-100, pH 8.5, 50 mmol/L boric acid buffer, frozen in a brown bottle); Sodium citrate buffer (pH 3.0-8.0); Glycine sodium hydroxide buffer (pH 8.0-10.0); PBS (pH 7.6).

Screening and detection of uricase-producing strain

10g of the soil sample was added to the conical flask containing 250 mL sterile water. After shaking at 25 °C and 200 rpm for 2 h, the sample was diluted into 3 gradients (10^{-3}, 10^{-4}, and 10^{-5}) for coating on UB medium plates. The plates were incubated at 25 ºC for 3 d. The single colony was selected from UB medium plates to pure culture. The purified colony was inoculated on the center of the UB medium plate to observe its transparent ring. According to the diameter ratio of the transparent ring (diameter ratio of transparent ring = diameter of transparent ring/colony diameter), the strain's ability to produce uricase was preliminarily determined.

Identification of uricase-producing strain

The single clone of the uricase-producing strain was added to a PCR tube with 50 μL TE buffer and lysed the cells at 98 °C for 30 min. After cooling at 12 °C and centrifuge 12000×g for 10 min, the supernatant was the DNA template for PCR. The strain's 16S rDNA was amplified using the following primers: 27F：5′-AGAGTTTGATCCTGGCTCAG-3′ and 1492R：5′-GGTTACCTTGTTACGACTT-3′. PCR was performed under the following conditions: 94 °C for 4 min, 32 cycles of 94 °C for 30 s, 55 °C for 35 s, 72 °C for 1 min 30 s, and final extension at 72 °C for 10 min. PCR product size was determined by agarose gel electrophoresis. The PCR product was sequenced by Tsingke Biotechnology Limited Company. The sequencing results were compared against the NCBI database and submitted to GenBank (http://www.ncbi.nlm.nih.gov/genbank), and MEGA7 software was used to construct the phylogenetic tree **[14].**

Gene amplification and vector construction

DNA of the screened strain was extracted by a genomic DNA extraction kit (Sangong Bioengineering Co., China). The primers were designed according to the uricase genes of *Arthrobacter*. The following pair of primers were used, **CAAATGGGTCGCGGATCCGAA**ATGAAATGAGCAGCAAGATCATCCT and **GTGCTCGAGTGCGGCCGCAAG**TTAGCAGAAGCCGGCGATGC. The highlighted sequences represent the homologous recombinant fragment with the pET28a vector, which had been previously digested using EcoRI and Hind III. The open reading frame of the uricase gene was amplified by TransStarFastPfu Fly DNA Polymerase (TransGen Biotech, China). The PCR program consisted of denaturation at 95 °C for 3 min, followed by 30 cycles at 98 °C for 20 s, 65 °C for 30 s, and 72 °C for 45 s, and then a final incubation at 72 °C for 5 min for the final extension. The PCR product was linked with pET28a using the *p*EASY-Uni Seamless Cloning and Assembly Kit (TransGen Biotech, China) to yield the expression plasmid *pET28a-uox-YHBJ11*.

Expression and purification of UOX-YHBJ11

The recombinant plasmid was transformed into *E. coli* BL21 (DE3) for *uox-YHBJ11* expression. The positive clone was inoculated on the center of the LB-UB medium plate (LB medium with 1% urate, 50 μg/mL kanamycin, 0.1 mM IPTG, 2% agar) to test its transparent ring. Transformants were cultured in 200 mL LB

broth containing 50 µg/mL kanamycin at 37 °C and 200 rpm. To induce the expression of recombinant uricase, 0.2 mL of 100 mM IPTG (Isopropyl β-D-1-thiogalactopyranoside) was added to the cell suspension when absorption (600 nm) reached 0.6. Afterwards, the suspension was incubated at 25 °C with shaking at 200 rpm for 6 h. Induced cells were harvested by centrifugation at 10,000 × g, 4 °C for 15 min and cell lysates were treated with ultrasonication. After centrifugation, cell-free extracts were purified using the Ni-chelating affinity column (Histrap, TransGen Biotech, China) according to the method previously reported by Yin et al. (2017) **[15].** Purified uricase was loaded at 10% SDS-PAGE (Sodium dodecyl sulfate-polyacrylamide gel electrophoresis). Protein bands were stained by Coomassie brilliant blue dye R-250. Protein concentrations were determined with Bradford Protein Assay Kit (Order NO. C503031, Sangon Biotech, China) using bovine serum albumin as the standard.

Uricase assay

The purified recombinant UOX-YHBJ11 activity was assayed by using urate as a substrate. The amount of urate degradation was determined by UV spectrophotometry at 290 nm. The standard uric acid curve was shown in the attached Figure S1, the concentration of uric acid (X) = absorbance of $\lambda_{290\ nm}$ (Y)/12.241. One unit (U) of uricase activity was identified as the amount of enzyme releasing 1 µmol reducing urate per min. Three parallel experiments were carried out.

Biochemical characterization

The optimum pH was determined by incubating purified UOX-YHBJ11 in various buffers ranging from pH 3.0 to 10.0 (Sodium citrate buffer, pH 3.0-8.0; Glycine sodium buffer, pH 8.6-10.0). The optimum temperature was determined by measuring uricase activity at various temperatures (0-50 °C) at optimum pH. To measure thermostability and pH stability, residual uricase activity was measured after incubation of purified UOX-YHBJ11 at pH 3.0 to 10.0 for different times (12 and 24 h) and at different temperatures (20, 25, 30, and 37 °C) for different times (0-160 min), respectively. To evaluate the effects of metal ions and chemical reagents on the activity of UOX-YHBJ11, 1 mM of various metal ions such as KCl, $MgSO_4$, $FeCl_3$, $CaCl_2$, $NiSO_4$, $CoCl_2$, $BaCl_2$, $MnCl_2$, $Pb(NO_3)_2$, $ZnSO_4$ and $AlCl_3$, 1% EDTA (Ethylene Diamine Tetraacetic Acid), SDS and PMFS (Phenylmethylsulfonyl Fluoride) were added individually to the reaction system. Control conditions were tested using the same process described above without any additives to the reaction mixture. The kinetic constants of UOX-YHBJ11 were determined using different urate concentrations (0.01 to 0.1 µmol/mL) at optimum pH and temperature for 5 min. The *Km* (Michaelis-Menten constant) and *Vmax* (maximum reaction velocity) were calculated by the Lineweaver-Burk plot.

Results

Screening and molecular identification of uricase-producing strains

A total of 15 isolates for uricase production were screened. The strain YHBJ11 had the largest transparent ring on uricase assay media compared to other strains, and a diameter ratio of 2.40. The 16S rDNA sequence of strain YHBJ11 (Genbank Accession NO.: ON351058.1) showed 99.85% similarity to *Arthrobacter sp.* Axa17 (Genbank Accession NO.: JQ977288.1) and *Arthrobacter nitroguajacolicus* strain T-8 (Genbank Accession NO.: HQ202843.1). As observed in Figure 1, phylogenetic analysis of 16S rDNA sequences revealed YHBJ11 clustered with *Arthrobacter* sp. strains. This indicated that strain YHBJ11 was the strain of *Arthrobacter*, which designated *Arthrobacter* sp. YHBJ11.

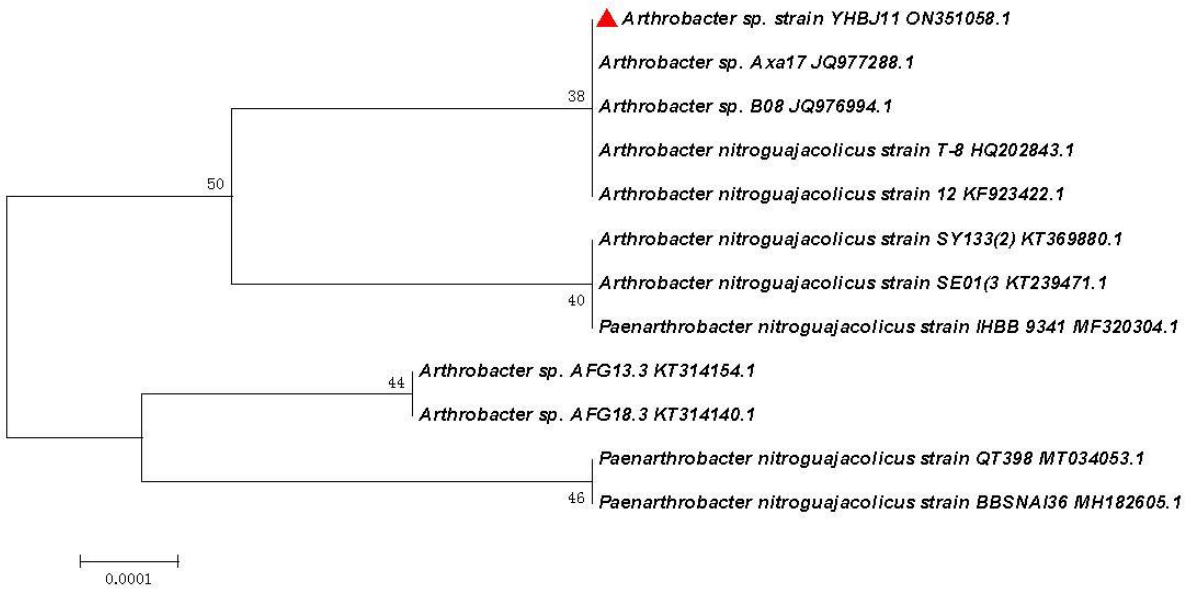

Figure 1: Phylogenetic tree of *Arthrobacter* sp. YHBJ11 comparison with 16S rDNA sequence homology.

Cloning and expression of uricase gene

The PCR product (≈900bp) was connected with pET28a to construct the recombinant plasmid *pET28a-uox-YHBJ11*. The recombinant plasmid was transformed into *E. coli* BL21 (DE3) for UOX-YHBJ11 expression. The uricase activity of wild strain YHBJ11 and positive clone *E. coli* BL21/*pET28a-uox-YHBJ11* were identified by UB and LB-UB media plates, respectively **(Figure 2).** The diameter ratio of the transparent ring of UOX-YHBJ11 was 1.3 times that of the wild strain, which proved that uricase gene expression was higher in *E. coli* than in the wild strain.

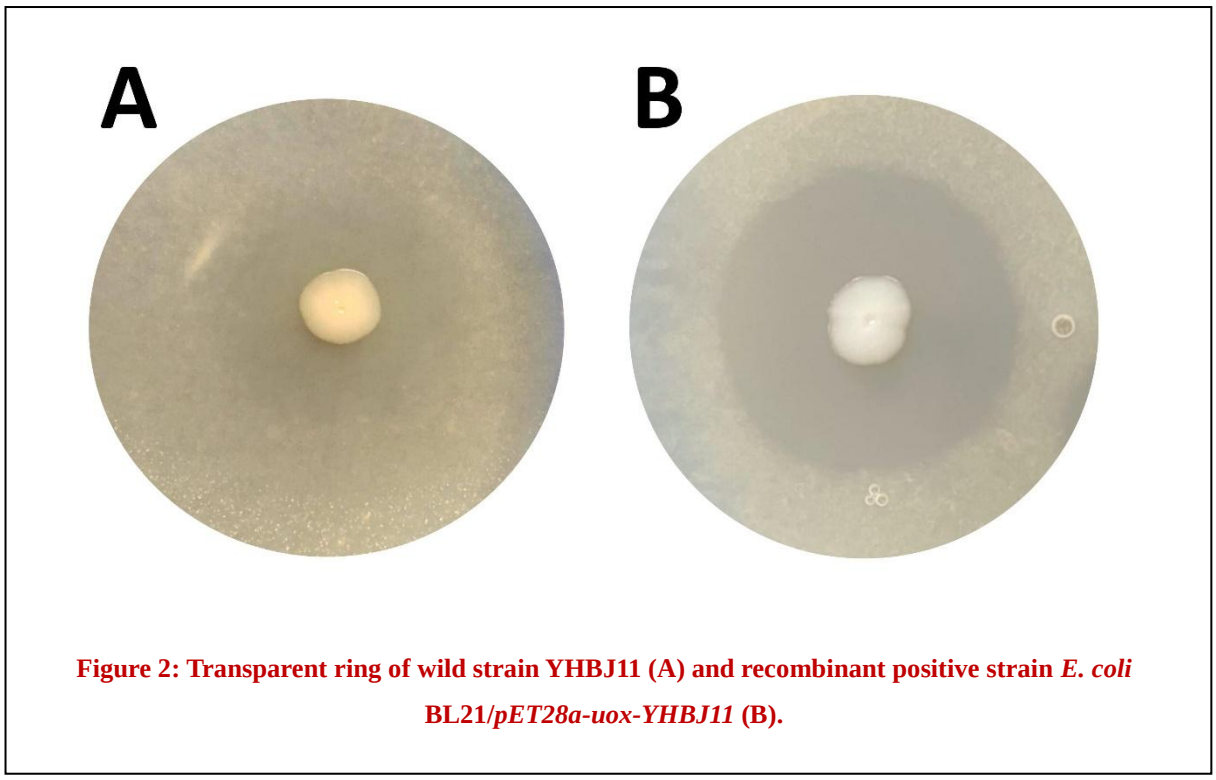

Figure 2: Transparent ring of wild strain YHBJ11 (A) and recombinant positive strain *E. coli* BL21/*pET28a-uox-YHBJ11* (B).

Nucleotide sequence analysis of the complete UOX-YHBJ11 gene revealed 909 bp ORF encoding a uricase protein of 302 amino acid residues. No signal peptide sequence was found. The nucleotide sequence of the UOX-YHBJ11 gene was submitted to GenBank under accession number OP858897. The amino acid sequence of UOX-YHBJ11 showed 98.01% and 97.68% similarity to uricase (GenBank NO.: TQS93009.1) from *Arthrobacter* sp. TS-15 and uricase (GenBank NO.: NWL11305.1) from *Paenarthrobacter nitroguajacolicus*. The theoretical calculated molecular size and theoretical pI of UOX-YHBJ11 were 33.84 kDa and 5.35, respectively. The target protein was purified by Ni-NTA resin affinity chromatography. The purified protein showed a single band of ~34 kDa against the protein marker on 10% SDS-PAGE **(Figure 3)**, which was consistent with the theoretical molecular weight.

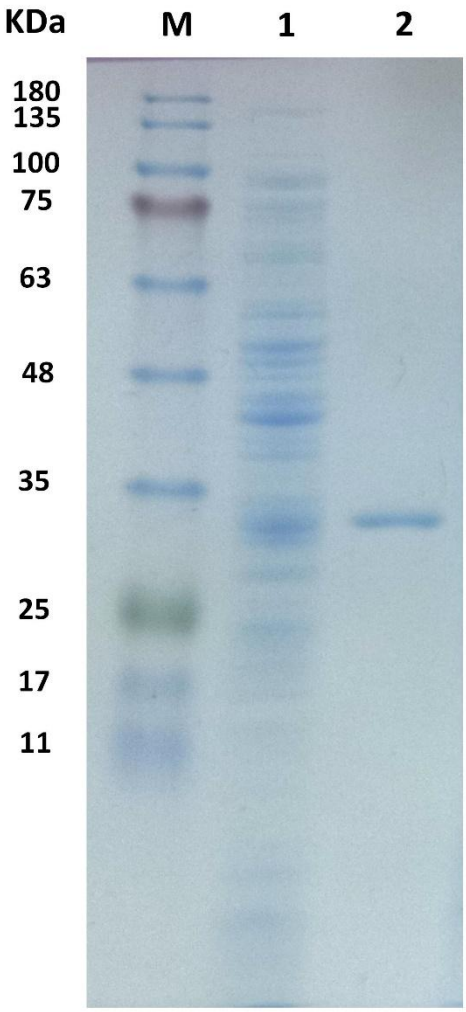

Figure 3: SDS-PAGE analysis of recombinant UOX-YHBJ11 produced by *E. coli* BL21. Lane M, protein molecular weight marker, mass indicated on the left; lane 1, total protein in IPTG-induced *E. coli* BL21/*pET28a-uox-YHBJ11*; lane 2, purified UOX-YHBJ11.

Effect of temperature and pH on UOX-YHBJ11

The optimum reaction pH and temperature for UOX-YHBJ11 activity were 7.0 and 25 °C, respectively. Over 60% of maximal activity was observed in the pH range of 5.0 to 8.0 **(Figure 4A)**, and over 50% of maximal

activity was observed at 10 °C and 40 °C (**Figure 4B**). The pH stability analysis revealed that it retained more than 50% of its initial activity after incubation at pH 3-5 for 24 h and about 60% residual activity after incubation at pH 7.0 for 24 h (**Figure 4C**). Thermostability analysis showed that UOX-YHBJ11 retained 50% activity after incubation at 20, 25, 30, and 37 °C for 100 min, and its half-life at 65 °C and 70 °C were about 38 min and 5 min (**Figure 4D**).

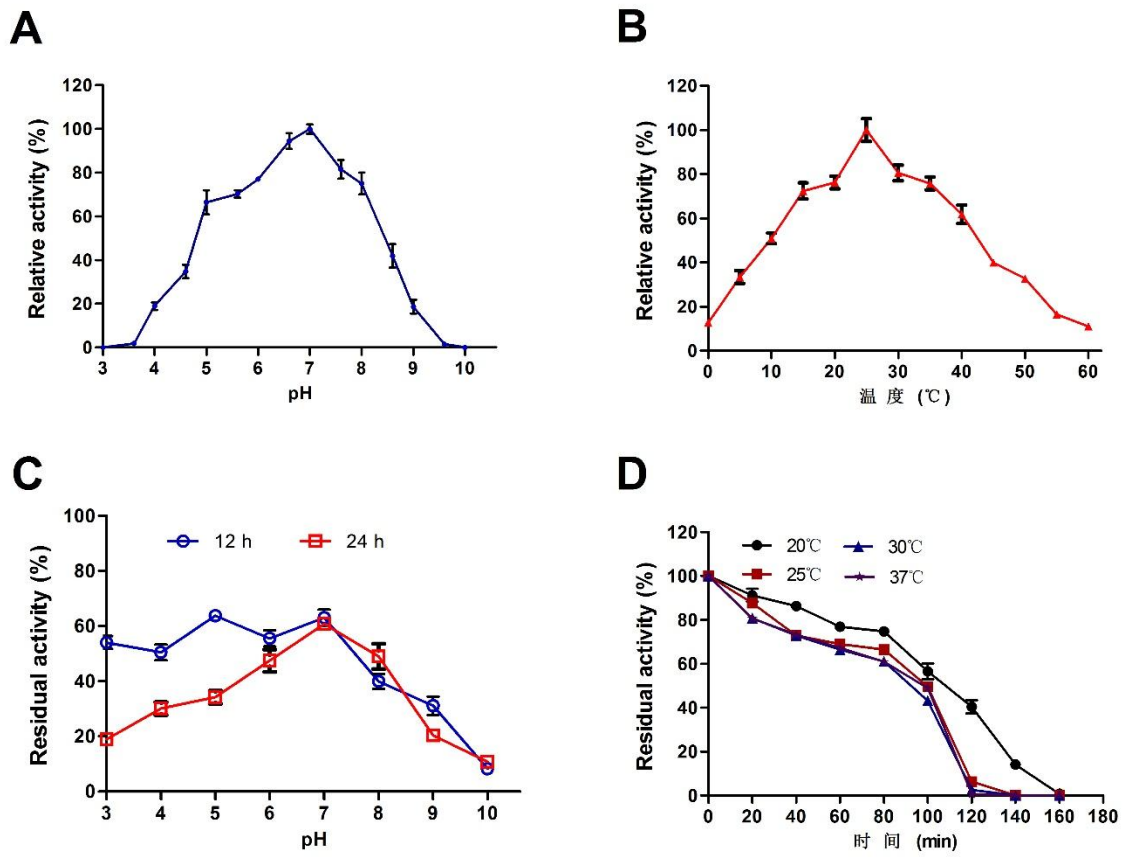

Figure 4: Effects of temperature and pH on the activity and stability of recombinant UOX-YHBJ11. A, pH effect on the activity of UOX-YHBJ111. **B,** Temperature effect on the activity of UOX-YHBJ111. **C,** The effect of pH on stability. **D,** The effect of temperature on stability at different temperatures (20, 25, 30 and 37 °C) for 0, 20, 40, 60, 80, 100, 120, 140 and 160. The primary activity was taken as 100%. Each value in the figure represents mean ± SD (n = 3). 100% = 0.16 ± 0.01 U/mg.

Effect of metal ions and chemical agents on UOX-YHBJ11

The effects of metal ions and inhibitors on enzyme activity are shown in **Figure 5**. K^+ and Mg^{2+} had no influence on uricase activity UOX-YHBJ11, while Fe^{3+}, Ca^{2+}, Ni^{2+}, Co^{2+}, Ba^{2+}, Mn^{2+} and Pb^{2+} significantly inhibited its activity. Among them, Ni^{2+} and Co^{2+} almost inhibited all uricase activity of UOX-YHBJ11. It was slightly inhibited by EDTA, SDS, and PSMF, and the relative enzyme activity was less than 30%.

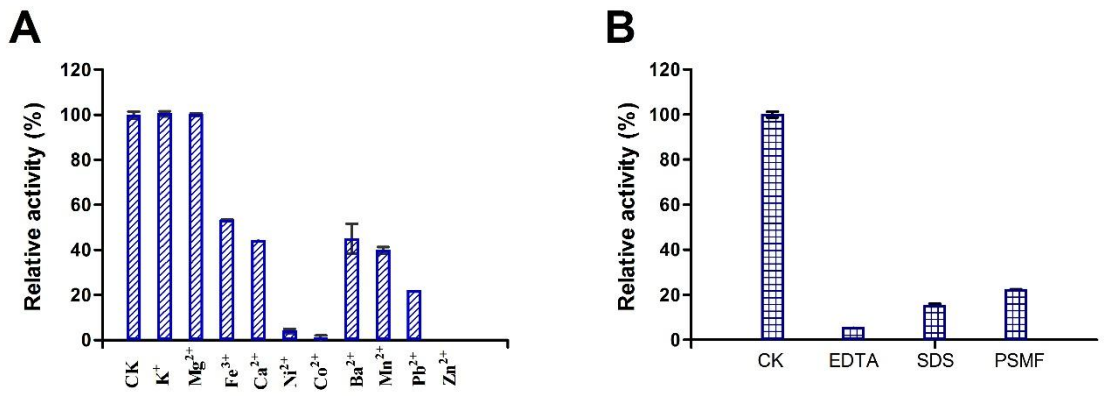

Figure 5: Effects of ion and inhibitor on the activity of UOX-YHBJ11.

Kinetic constants of UOX-YHBJ11

UOX-YHBJ11 exhibited 0.16 ± 0.01 U/mg uricase activity. Using Lineweaver-Burk plots, the Kcat, Km, and Vmax of UOX-YHBJ11 were calculated by using the urate as substrates. These results are shown in Table 1.

Table 1: Kinetic parameters of UOX-YHBJ11.

Substrate	Vmax (μmol/min/mg)	Km (mM)	Kcat (S^{-1})	Kcat/Km
urate	0.30	0.018	0.17	9.4

Discussion

Microorganisms are an important focus of uricase research for their diverse species, abundant resources, rapid breeding, and more active metabolism **[16].** It's been more than 70 years since researchers began studying microorganisms' uricase in lowering uric acid. Currently, uricase has been extracted from *Bacillus fastidiosus* **[17],** *Aspergillus flavas* **[18],** *Microbacterium* sp. **[19]** and *Pseudomonas aeruginosa* **[20].** Although natural uricase extracted from microorganisms has been used in commercial production, there are still some unresolved issues, such as low enzyme production yields, long subculture cycle, high cost, high cost and susceptibility to pathogens etc. **[21].** Recombinant uricase, which has higher safety, can make up for these shortcomings. Krystexxa (or pegloticase), a recombinant uricase conjugated to polyethylene glycol (PEG), was commonly used for the treatment of chronic gout **[22].**

Here, the strain *Arthrobacter* sp. YHBJ11 with high uricase activity was isolated from soil samples from Chaka Salt Lake and the diameter ratio of the transparent ring was 2.40. Heterologous expression of wild strains was used for high-level expression of YHBJ11. The diameter ratio of the transparent ring of UOX-YHBJ11 was 1.3 times that of the wild strain, which proved that uricase was a high level expressed in the recombinant strain, which was similar to other studies **[23].** The uricase from *Arthrobacter globiformis* showed stability in 60 °C and pH 8.5-11, and was not inhibited by Fe^{3+} and EDTA **[24].** However, UOX-YHBJ11 exhibited stability at pH 5-7 and 25 °C, and was significantly inhibited by Fe^{3+} and EDTA. This indicates that there are obvious

differences in enzymatic properties between them. An uricase from the thermophilic microorganism *Thermobispora bispora* exhibited a higher thermostability that retained >90% enzymatic activity in a temperature range of 60-70 °C, whereas its optimum temperature and pH were 65 °C and 8.0, respectively **[25].** A uricase from *Kluyveromyces marxianus* retained nearly 80% of its activity after incubation at 40 °C for 90 h, and its optimal pH was 9.0 **[26].** Nevertheless, a uricase from *Deinococcus radiodurans* showed more than 80% enzyme activity over a pH range of 8.0 to 9.0 in a temperature range of 20-40 °C and maintained almost 100% activity after incubation at 25 °C and 37 °C for 24 h **[25].** However, only 50% of residual activity was remained at physiological pH **[25].** In this study, UOX-YHBJ11 exhibited more than 60% relative activity at pH 5.0-8.0, and more than 50% relative activity at 10-40 °C. Moreover, it retained more than 60% relative enzyme activity after incubation at 25 to 37 °C for 80 min, suggesting that UOX-YHBJ11 was a thermostable enzyme. These enzymatic properties indicate that YHBJ11 has potential applications in uric acid detection kits and pharmaceutical enzymes.

Author Contributions

Y.R.Y. and L.H.L. conceived the study. X.Y. was responsible for the isolation of uricase-producing strain. S.W. amplified the uricase gene. H.Y.L. was responsible for the cloning and heterologous expression of uricase gene. Q.Q.L. purified the recombinant protein. Y.G.Z. measured enzymatic activity. W.H. performed data analysis. W.H., X.Y., Y.R.Y., and L.H.L. wrote the manuscript. All authors discussed the results and commented on the manuscript. All authors read and approved the final manuscript.

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Compliance with Ethical Standards

Conflict of interest: The authors declare that they have no indirect or direct conflict of interest.

Ethical approval: This article does not contain any studies related to human participants or animals.

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