微生物学报
Acta Microbiologica Sinica
2024, 64(5): 1494–1505
http://journals.im.ac.cn/actamicrocn
DOI: 10.13343/j.cnki.wsxb.20230697

Research Article 研究报告

来源于超嗜热古菌雅氏火球菌(*Pyrococcus yayanosii*) CH1 耐热耐压脯肽酶的酶学性质研究

张欢欢[1,2]，陈柔珂[1]，徐俊[1*]

1 上海交通大学生命科学技术学院 微生物代谢国家重点实验室，上海 200240
2 上海交通大学海洋学院，上海 200240

张欢欢, 陈柔珂, 徐俊. 来源于超嗜热古菌雅氏火球菌(*Pyrococcus yayanosii*) CH1 耐热耐压脯肽酶的酶学性质研究[J]. 微生物学报, 2024, 64(5): 1494-1505.
ZHANG Huanhuan, CHEN Rouke, XU Jun. Characterization of a thermostable and piezotolerant prolidase from the hyperthermophilic archaeon *Pyrococcus yayanosii* CH1[J]. Acta Microbiologica Sinica, 2024, 64(5): 1494-1505.

摘　要：【目的】脯肽酶是一种能从二肽(Xaa-Pro)的 C 末端水解脯氨酸或羟脯氨酸残基的肽酶。对深海来源的雅氏火球菌(*Pyrococcus yayanosii*) CH1 基因组中 *PYCH_07700* 基因编码的蛋白 *Py*prol 的体外酶学性质进行研究，以期发现新型脯肽酶。【方法】在小宝岛热球菌(*Thermococcus kodakarensis*) TS559 中异源表达 *Py*prol。使用二肽 Met-Pro 作为底物，检测重组蛋白的脯肽酶活性。【结果】*Py*prol 的最适温度为 100 °C，最适 pH 为 6.0。*Py*prol 在与 Co^{2+}结合时活性最高，最适的金属离子浓度为 1.2 mmol/L。与 *P. furiosus* 来源的脯肽酶 *Pf*prol 相比，*Py*prol 在更宽的 pH 范围具有活性，并且能够耐受更高浓度的金属离子。*Py*prol 是耐压蛋白，最适静水压为 40 MPa。与常压条件下相比，40 MPa 下，*Py*prol 在 40、70 和 100 °C 均有更高的活性。【结论】来源于深海热液喷口的严格嗜压的超嗜热古菌 *P. yayanosii* CH1 的新型脯肽酶 *Py*prol 具有热稳定和耐压特性。

关键词：超嗜热古菌；雅氏火球菌；脯肽酶；热稳定；耐压

资助项目：国家重点研发计划(2020YFA0906800)；国家自然科学基金面上项目(41976085，42276091)
This work was supported by the National Key Research and Development Program of China (2020YFA0906800) and the National Natural Science Foundation of China (General Program) (41976085, 42276091).
*Corresponding author. E-mail: xujunn@sjtu.edu.cn
Received: 2023-11-14; Accepted: 2024-02-19; Published online: 2024-02-22

Characterization of a thermostable and piezotolerant prolidase from the hyperthermophilic archaeon *Pyrococcus yayanosii* CH1

ZHANG Huanhuan[1,2], CHEN Rouke[1], XU Jun[1*]

1 State Key Laboratory of Microbial Metabolism, School of Life Sciences and Biotechnology, Shanghai Jiao Tong University, Shanghai 200240, China
2 School of Oceanography, Shanghai Jiao Tong University, Shanghai 200240, China

Abstract: [Objective] Prolidase is an enzyme that can hydrolyze proline or hydroxyproline residues from the C-terminal dipeptides (Xaa-Pro). A putative prolidase-encoding gene was identified in the genome of *Pyrococcus yayanosii* CH1 isolated from the deep sea. In this study, we characterized the enzymatic properties of *Py*prol encoded by *PYCH_07700 in vitro*, aiming to find a new prolidase. **[Methods]** *Py*prol was heterologously expressed in the hyperthermophilic archaeon *Thermococcus kodakarensis* TS559. The dipeptide Met-Pro was used as a substrate to test the prolidase activity of the purified recombinant protein. **[Results]** *Py*prol showed the best performance at 100 °C and pH 6.0. *Py*prol binding to Co^{2+} exhibited the maximum activity, and the optimal metal ion concentration was 1.2 mmol/L. *Py*prol had catalytic activity in a wider pH range and can tolerate higher concentrations of metal ions than the prolidase *Pf*prol from *P. furiosus*. *Py*prol was a piezotolerant protein with an optimal hydrostatic pressure of 40 MPa. It exhibited enhanced activities at 40, 70, and 100 °C under 40 MPa, compared with at the atmospheric pressure. **[Conclusion]** *Py*prol is a novel thermostable and piezotolerant prolidase of *P. yayanosii* CH1, which is an obligate piezophilic hyperthermophilic archaeon strain isolated from a deep-sea hydrothermal vent.

Keywords: hyperthermophilic archaeon; *Pyrococcus yayanosii*; prolidase; thermostable; piezotolerant

Prolidase (EC 3.4.13.9) is a peptidase that specifically cleaves the C-terminal proline or hydroxyproline residues of dipeptides. Prolidase has been identified in various organisms to date, including humans[1-2], bacteria[3-4], and archaea[5-7]. In prokaryotes, it is generally believed that prolidase is involved in the proline cycle[8] and bacterial defense against toxins[9]. In humans, prolidase is involved in the degradation of collagen[10]. The activity of prolidase has been found to be abnormally increased in breast cancer[11] and myeloproliferative neoplasms[12]. In addition, the enzyme can also combine with the tumor suppressor p53[13]. So human prolidase is also an important cancer marker. Prolidase has many applications in biotechnology. During cheese fermentation, prolidase can be added to increase the content of proline in the product[14]. In addition, prolidase can be used as an antidote for organophosphorus compounds[15].

Prolidase is a metalloenzyme, and metal ions contribute to stabilizing its structure and anchoring the substrate at the active site[8]. Prolidase always contains two metal-binding cores, and it requires both metal cores to be occupied for full enzymatic activity[16]. The metal-binding site amino acids (Asp-Asp-His-Glu-Glu) are highly conserved[8].

The enzymatic properties of prolidase from hyperthermophilic archaea are different from those of other species. While prolidase from humans and *Escherichia coli* preferentially bind to Mn^{2+}[4,17], prolidases from hyperthermophilic archaea have

the highest activity when binding Co^{2+}[5,7]. It is worth noting that a prolidase *Pf*prol of *Pyrococcus furious* can also bind Fe^{2+} under anaerobic conditions[5]. The optimum temperature of prolidases from *P. furious* DSM3638 and *P. horikoshii* OT3 reached 100 °C[5,7], which is the highest among the prolidases studied. Moreover, prolidases from *Pyrococcus* species exhibit excellent thermal stability, for example, the prolidase from *P. furiosus* DSM 3638 and *P. horikoshii* OT3 maintained their activity without significant loss when incubated at 100 °C for 12 h and 8 h, respectively[5,7].

P. yayanosii CH1 was isolated from the sediment sample collected at a depth of 4 100 m in the Mid-Atlantic Ridge[18]. The optimum growth temperature of *P. yayanosii* CH1 is 98 °C and the optimum growth pressure is 52 MPa[19]. So in this regard, *P. yayanosii* CH1 is an obligate piezophilic hyperthermophile and serves as an important model organism for studying the mechanisms of high-pressure adaptation in microorganisms[20]. A putative prolidase encoding gene *PYCH_07700* was found in the genome of *P. yayanosii* CH1. This study will present the results of the characterization of the enzymatic properties of the above-mentioned prolidase, namely *Py*prol. *Py*prol was obtained by heterologous overexpression in *Thermococcus kodakarensis* TS559, which is an agmatine auxotroph strain[21-22]. The effect of hydrostatic pressure on the enzymatic activity of *Py*prol was investigated, which provides clues for expanding the application of this type of prolidase.

1 Materials and Methods

1.1 Strains, plasmids, and culture conditions

The strains and plasmids utilized in this study are outlined in Table 1. *E. coli* DH5α was cultured in Luria-Bertani (LB) medium at 37 °C. *P. yayanosii* A1 was cultivated under anaerobic at 95 °C in TRM medium[23]. *T. kodakarensis* TS559 strains were cultivated in the artificial seawater (ASW-YT) liquid medium supplemented with 1 mmol/L agmatine under anaerobic at 85 °C.

1.2 Bioinformatics analysis

The amino acid sequence of the experimentally characterized prolidase *Pf*prol (WP_011012489.1) of *P. furiosus* was used as a query to BLAST against the genome sequence of *P. yayanosii* CH1 (GenBank accession number: NC_015680.1). Putative prolidases including *Py*prol (WP_013905514.1), *Pf*prol, and other homologous sequences were retrieved from GenBank. The amino acid sequences of obtained prolidases were aligned using ClustalX2 and visualized using ESPript 3.0 (http://espript.ibcp.fr/ESPript/ESPript/). The phylogenetic tree was constructed by the neighbor-joining (NJ) method using MEGA (version 7)[24]. Bootstrap analysis was computed with 1 000 replicates.

Table 1 Strains and plasmids used and constructed in this study

Strains and plasmids	Description	Reference
Strains		
Escherichia coli DH5α	The strain used for gene cloning	
Pyrococcus yayanosii A1	The facultatively piezophilic derivative strain	Li et al.[23]
Thermococcus kodakarensis TS559	Agmatine auxotrophic strain	Santangelo et al.[21]
Δ*PYCH_07700*	*PYCH_07700* deletion strain	This study
Plasmids		
pTE1	A *T. kodakarensis-E. coli* (Tk-Ec) shuttle vector	Song et al.[25]
pTE-*Py*prol	pTE1::P_{gdh}-*PYCH_07700*	This study
pTE-*Pf*prol	pTE1::P_{gdh}-*PF_1343*	This study

1.3 Cloning of prolidase encoding genes

Using the genomic DNA of *P. yayanosii* A1 as a template, the full-length sequence of gene *PYCH_07700* was amplified using primer 0770-kod-F/R (Table 2). Plasmid pTE1 was used as the template DNA and the primer pTE1-F/R (Table 2) was used in PCR to amplify the backbone of this *E. coli-T. kodakarensis* shuttle vector[25]. Using ClonExpress II One Step Cloning Kit (Vazyme, China), gene *PYCH_07700* was ligated to the pTE1 plasmid and transformed into DH5α. The prolidase encoding gene *PF_1343* of *P. furiosus* DSM3638 was cloned into pTE1 using same strategy with changes in PCR primers (1343-kod-F/R) and corresponding template DNA (Table 2). In order to purify the protein, a 12×His tag was added to the C-terminus of *PYCH_07700* and *PF_1343*, respectively. Plasmid constructed with correct insertion of either gene *PYCH_07700* or *PF_1343* was confirmed by colony PCR and DNA-sequencing analysis.

Genetic manipulations of *T. kodakarensis* were carried out under anaerobic conditions. The transformation of *T. kodakaraensis* was performed as previously described[25]. The host strain *T. kodakarensis* TS559 was cultivated in ASW-YT liquid medium supplemented with 1 mmol/L agmatine at 85 °C for 10 h, and the cells were harvested by centrifugation (6 500×*g*, 5 min). The harvested cells were resuspended in 200 μL of 0.1 mol/L $CaCl_2$ and kept on ice for 30 min. Then, 3 μg of plasmid was added to the cell suspension and incubated on ice for 1 h, followed by a heat shock at 85 °C for 45 s and incubation on ice for 10 min. The cell suspension was added to 5 mL ASW-YT liquid medium and incubated at 85 °C for 4 h. The culture was spread onto ASW-YT solid medium without agmatine and cultured at 85 °C until colonies were observed. The positive colonies were confirmed by colony PCR and DNA sequencing analysis.

1.4 Prolidase expression and purification in *Thermococcus kodakarensis*

The recombinant strains were inoculated into ASW-YT medium and cultured at 85 °C for 15 h under anaerobic conditions. Cells were collected by centrifugation at 10 000×*g* for 5 min at room temperature. The cells were resuspended in 50 mmol/L Tris-HCl (pH 8.0) containing 0.5 mol/L NaCl and then crushed by sonication on ice. The supernatant was collected by centrifugation at 10 000×*g* for 30 min at 4 °C. Proteins were purified using Ni-NTA 6FF Sefinose Resin Kit (Sangon, China). Imidazole in the buffer that was used to elute the overexpressed protein was removed using Millipore 10 kDa ultrafiltration tubes. The purified protein was finally stored in 50 mmol/L Tris-HCl (pH 8.0) containing 0.5 mol/L NaCl.

1.5 Prolidase activity assay

Prolidase activity was determined as previously described[5]. The 50 μL reaction mixture contained 50 mmol/L MOPS buffer (pH 7.0), 200 mmol/L NaCl, 5% glycerol, 0.1 mg/mL BSA protein, and 1.2 mmol/L $CoCl_2$. After adding an appropriate amount of protein, react at 100 °C for 5 min to allow the protein to bind to the metal ion. Add Met-Pro at a final concentration of 10 mmol/L and react at 100 °C for 10 min. Add 50 μL of acetic acid to stop the reaction, then add 50 μL of 3% (*W*/*V*) ninhydrin solution to react at 100 °C for 10 min. After cooling to room temperature, use a microplate reader to measure

Table 2 Primers used in this study

Primer	Sequence (5′→3′)
0770-kod-F	CCTAATTTGGAGGGATGAACGTGAAAGATAGAATTAAAAGGCTC
0770-kod-R	TCAGTGATGATGATGATGATGATGATGATGATGATGTATCAGCTCCCGC
1343-kod-F	CCTAATTTGGAGGGATGAACATGAAAGAAAGACTTGAAAAATTAG
1343-kod-R	TCAGTGATGATGATGATGATGATGATGATGATGATGGAGTAGCTCTCTTTCGG
pTE1-F	CATCATCATCATCATCATCACTGAATCCATCACACTGGCGGCCG
pTE1-R	GTTCATCCCTCCAAATTAG

the absorbance at 515 nm. The activity unit of prolidase is defined as the amount of enzyme that releases one micromole of proline per minute.

1.6 Influence of temperature, pH, and metal preference on enzyme activity

The optimum temperature of *Py*prol was determined in the range of 40–100 °C. The optimum pH of *Py*prol was determined in the range of pH 4.0–8.0. The buffer used were 50 mmol/L of CH_3COOH-CH_3COONa (pH 4.0–5.0), NaH_2PO_4-Na_2HPO_4 (pH 6.0–7.0), Tris-HCl (pH 8.0). To determine the optimal metal ion of *Py*prol, *Py*prol was combined with Co^{2+}, Mn^{2+}, Zn^{2+}, Ca^{2+}, Cu^{2+}, Ni^{2+}, and Mg^{2+}, respectively, and then reacted with the substrate. To evaluate the effect of metal ion concentration on the enzyme activity, *Py*prol was combined with Co^{2+} at a final concentration of 0–6 mmol/L and then reacted with the substrate.

1.7 Influence of hydrostatic pressure on enzyme activity

To assess the effect of hydrostatic pressure on *Py*prol activity, prolidase activity assays were performed at 0.1, 10, 20, 30, 40, and 52 MPa at 100 °C, respectively. To evaluate the effect of high hydrostatic pressure on prolidase activity at different temperatures, the prolidase activity under its optimum hydrostatic pressure was measured at 40, 70, and 100 °C, respectively. High pressure was achieved and controlled by adding water through the hand-operated pump that was equipped with a pressure gauge[26-27]. The pin-closure pressure vessels were used in this study (constructed by Nantong Feiyu Petroleum Technology Development Co., Ltd., China).

1.8 Influence of temperature and hydrostatic pressure on enzyme stability

The thermal stability of *Py*prol was determined by incubating the assays at specific temperatures (80, 90, and 100 °C) for different periods (1, 2, 3, 4, and 5 h), and measuring the residual activity. The high hydrostatic pressure stability of *Py*prol was determined by incubating at 80 °C under 20 MPa and 40 MPa for 1 h, and the residual enzyme activity was determined.

1.9 Determination of enzyme kinetics constants

The enzyme reaction rate of *Py*prol was determined in the Met-Pro concentration range of 1–10 mmol/L at 0.1 MPa and 40 MPa, respectively. The reaction was conducted under the condition of 50 mmol/L NaH_2PO_4-Na_2HPO_4 buffer (pH 6.0), 1.2 mmol/L $CoCl_2$, and 100 °C. The results were fitted using the Michaelis-Menten equation in the data analysis and graphing software Origin.

2 Results

2.1 Overexpression and purification of a prolidase *Py*prol from *Pyrococcus yayanosii* in *Thermococcus kodakarensis*

In *P. yayanosii* CH1, the protein *Py*prol encoded by *PYCH_07700* is predicted to function as a prolidase. In *Py*prol, there are two conserved structural domains, namely creatinase_N located at the N-terminus, and peptidase_M24 situated at the C-terminus (Figure S1, data was deposited in the China National Microbiology Data Center, accession No.: NMDCX0000258). BLASTp analysis showed that *Py*prol exhibited high similarity to prolidases from *P. furiosus* DSM3638 and *P. horikoshii* OT3, with amino acid sequence similarities of 76% and 75%, respectively (Figure 1). Pyprol also exhibits a high structural similarity to the prolidase from *P. furiosus* (Figure S2, data was deposited in the China National Microbiology Data Center, accession No.: NMDCX0000259). Multiple alignments of *Py*prol with its homologous proteins revealed that *Py*prol contained the conserved metal-binding sites (Asp-Asp-His-Glu-Glu). These results suggested that *Py*prol was a putative prolidase.

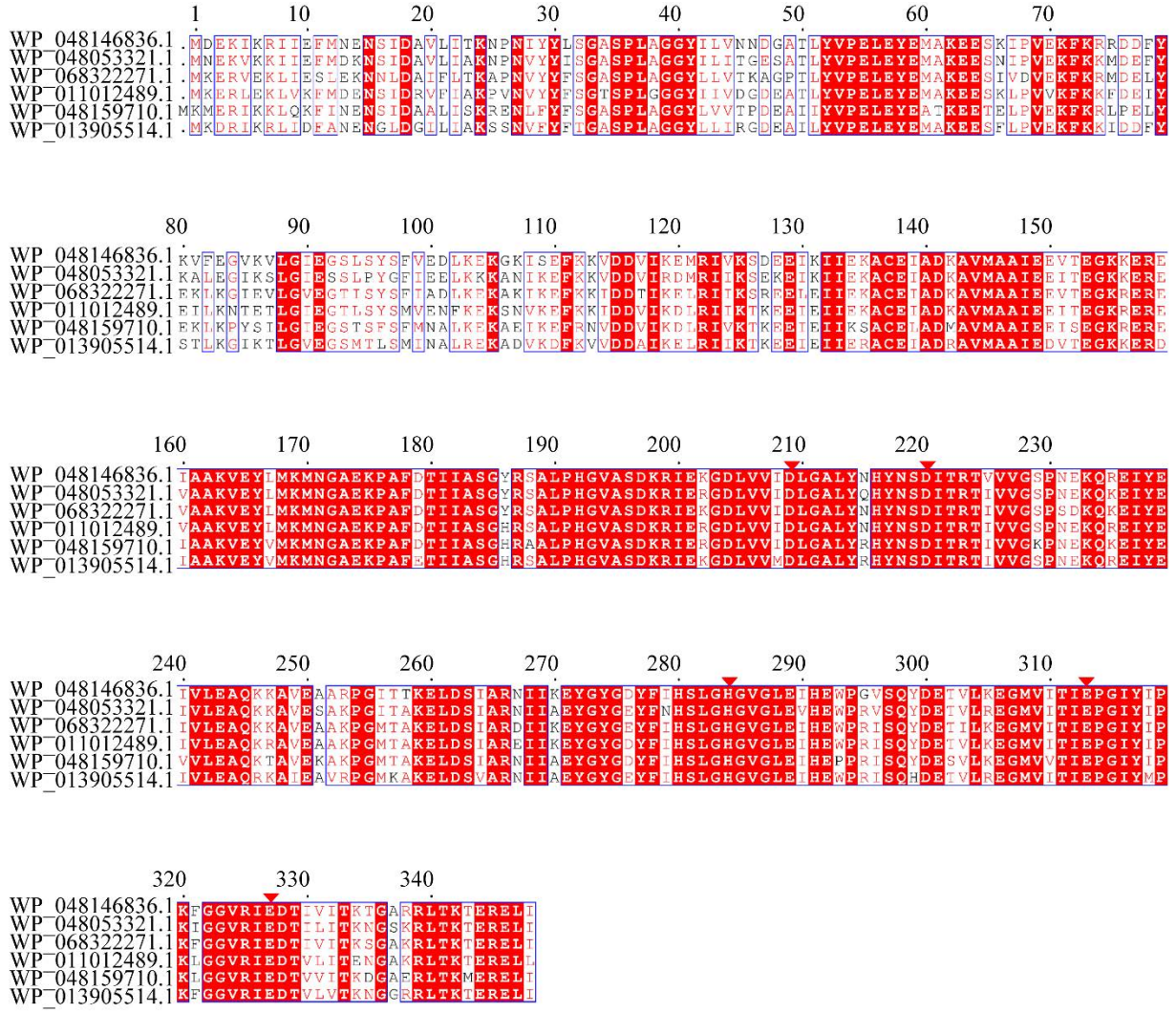

Figure 1 Multiple amino acid sequence alignment of prolidase from hyperthermophilic archaea. Including prolidase from *Pyrococcus abyssi* GE5 (WP_048146836.1), *P. horikoshii* OT3 (WP_048053321.1), *P. kukulkanii* (WP_068322271.1), *P. furiosus* DSM3638 (WP_011012489.1), *Thermococcus barophilus* MP (WP_048159710.1), *P. yayanosii* CH1 (WP_013905514.1). The regions with red shading and red lettering indicate conserved residues. Red triangles indicate the conserved metal binding sites (Asp-Asp-His-Glu-Glu).

To overexpress the prolidase from *P. yayanosii* CH1 in *T. kodakarensis* TS559, *PYCH_07700* gene fragment was cloned into a shuttle vector plasmid pTE1 in the downstream region of the glutamate dehydrogenase (P_{gdh}) promoter from *P. furiosus* DSM3638 (Figure 2A). The recombinant strains containing plasmids pTE-*Py*prol and pTE-*Pf*prol were cultured in ASW-YT medium at 85 °C for 15 h, and cells were harvested by centrifugation. The cells were crushed by sonication, and the supernatants were collected by centrifugation. The prolidases were purified by using nickel-charged resin. The purified proteins were analyzed using SDS-PAGE (Figure 2B), and the results showed that the molecular weight of the recombinant protein was approximately 43 kDa which was consistent with the theoretical relative molecular weight.

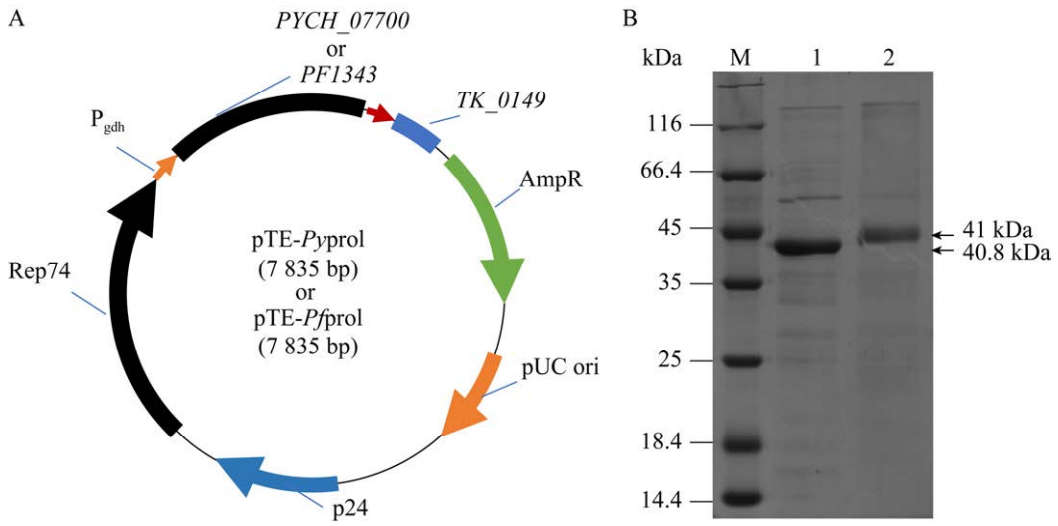

Figure 2 Overexpression of *Py*prol and *Pf*prol in *Thermococcus kodakarensis* TS559. A: The map of the shuttle plasmid pTE1 used to overexpress *Py*prol and *Pf*prol in *T. kodakarensis*, Rep74 and p24 from the *T. nautilus* 30-1 plasmid pTN1, replication origin (pUC ori) and ampicillin antibiotic marker (AmpR) from *Escherichia coli* plasmid pUC19, *TK_0149* from *T. kodakarensis* and encodes an arginine decarboxylase. B: SDS-PAGE of *Py*prol and *Pf*prol purified from *T. kodakarensis* TS559, Lane M: Protein Marker, Lane 1: Purified recombinant His-tagged *Py*prol, Lane 2: Purified recombinant His-tagged *Pf*prol.

2.2 Optimal temperature, optimal pH, and metal ion preference of *Py*prol

The activity of *Py*prol was determined by measuring its ability to cleave the Met-Pro dipeptide substrate over a temperature range of 40–100 °C (Figure 3A). The results indicated that the optimal temperature for *Py*prol activity was 100 °C, and there was a notable decrease in activity when the temperature dropped below 70 °C. The activity of *Py*prol was determined over a pH range of 4.0–8.0 (Figure 3B). The optimal pH for *Py*prol activity was found to be 6.0, and a significant decline in activity was observed at pH 4.0. When *Py*prol was bound to different metal ions (Figure 3C), significant variations in activity were observed. *Py*prol exhibited the highest activity when bound to Co^{2+}. It also retained 93% of the activity observed when bound to Co^{2+} when it was bound to Mn^{2+}. However, the binding of Zn^{2+}, Ca^{2+}, Cu^{2+}, Ni^{2+}, and Mg^{2+} resulted in almost undetectable prolidase activity. The results demonstrated that *Py*prol exhibited maximum activity when bound to 1.2 mmol/L Co^{2+}, but even when bound to 0.6 mmol/L Co^{2+}, it retained 81% of the maximum activity (Figure 3C). Additionally, we observed that *Py*prol retained 29% activity even in the absence of additional metal ions, compared to that of when 1.2 mmol/L Co^{2+} was added. This may be attributed to the binding of trace amounts of Co^{2+} from the culture medium to *Py*prol.

2.3 Effect of hydrostatic pressure on *Py*prol activity

The activity of *Py*prol was found to be higher under high hydrostatic pressure compared to atmospheric pressure. At 40 MPa, *Py*prol exhibited the highest activity (Figure 3D). Furthermore, the extent of enhancement in *Py*prol activity varied at different temperatures under high hydrostatic pressure. At 40, 70, and 100 °C, compared to 0.1 MPa, *Py*prol activity increased by 67%, 31%, and 24%, respectively (Table 3). This indicated that high hydrostatic pressure has a significant impact on *Py*prol activity at lower temperatures, especially at 40 °C. Notably, at 40 °C and 40 MPa, the specific activity of *Py*prol

reached 967 U/mg, which is close to the specific activity observed at 70 °C and 0.1 MPa (1 120 U/mg). Similarly, we observed that the activity of *Pf*prol was higher at 20 MPa compared to atmospheric pressure. Additionally, compared to 0.1 MPa, at 20 MPa, the enzyme activity of *Pf*prol at 40, 70, and 100 °C increased by 47%, 27%, and 24%, respectively.

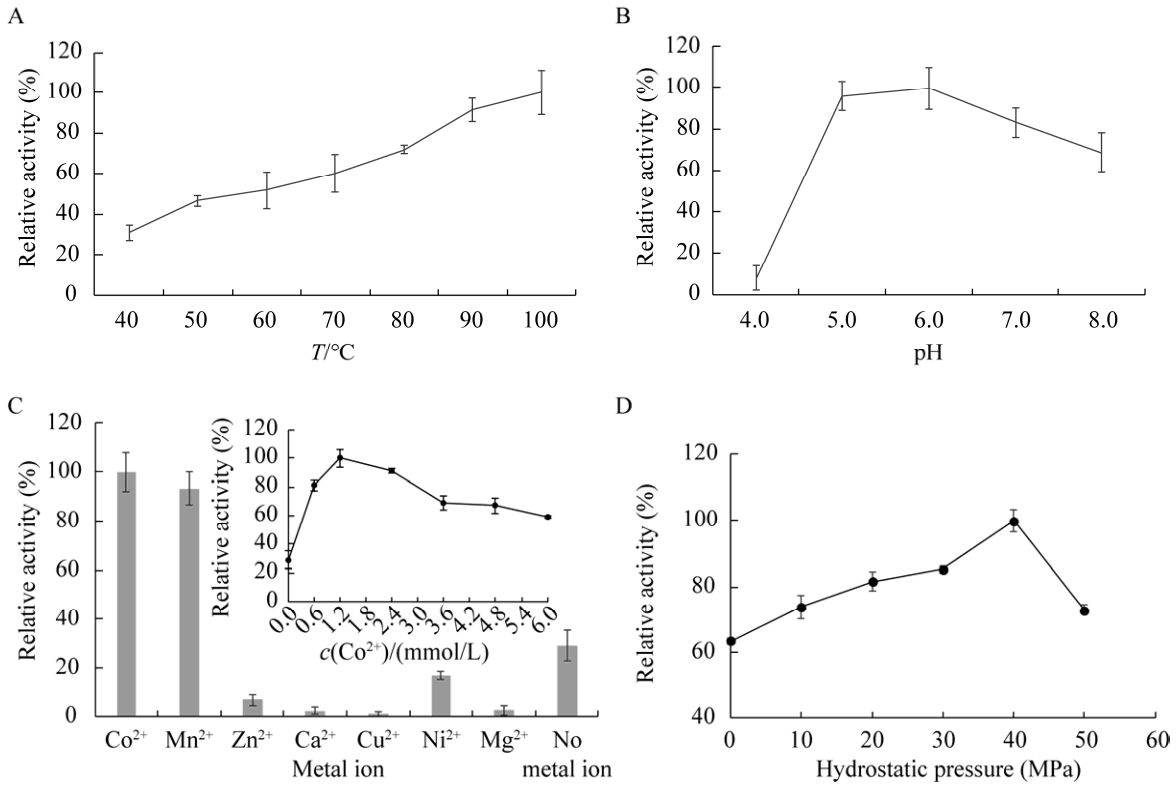

Figure 3 Characterization of *Py*prol. A: The effects of temperature on the activity of *Py*prol. B: Effects of pH on the activity of *Py*prol, CH_3COOH-CH_3COONa (pH 4.0–5.0), NaH_2PO_4-Na_2HPO_4 (pH 6.0–7.0), Tris-HCl (pH 8.0). C: Effects of metal ions on the activity of *Py*prol. D: Effects of hydrostatic pressure on the activity of *Py*prol.

Table 3 The effect of high hydrostatic pressure on *Py*prol and *Pf*prol activity at different temperatures

Protein	*T*/°C	Hydrostatic pressure (MPa)	Specific enzyme activity (U/mg)
*Py*prol	40	0.1	578
*Py*prol	40	40.0	967
*Py*prol	70	0.1	1 120
*Py*prol	70	40.0	1 471
*Py*prol	100	0.1	1 857
*Py*prol	100	40.0	2 309
*Pf*prol	40	0.1	38
*Pf*prol	40	20.0	56
*Pf*prol	70	0.1	899
*Pf*prol	70	20.0	1 142
*Pf*prol	100	0.1	1 977
*Pf*prol	100	20.0	2 469

2.4 Kinetic constants

We used Met-Pro as the substrate and determined the kinetic constants of *Py*prol at 0.1 MPa and 40 MPa. The results revealed that the K_m values of *Py*prol at 0.1 MPa and 40 MPa were similar, but at 40 MPa, *Py*prol exhibited a higher V_{max} value compared to that at 0.1 MPa (Table 4). The k_{cat}/K_m value of *Py*prol at 40 MPa was 1.16 times higher than that at 0.1 MPa.

2.5 Thermal stability and high hydrostatic pressure stability

*Py*prol was incubated under different temperatures and hydrostatic pressure conditions, followed by the measurement of prolidase activity. The results (Figure 4A) showed that after incubating *Py*prol at 80 °C and 90 °C for 5 h, it retained 64% and 55% of its activity, respectively. However, after incubating *Py*prol at 100 °C for 1 h, its activity decreased to only 41% **(**Figure 4A**)**. On the other hand, after incubating *Py*prol at 20 MPa and 40 MPa for 1 h, it still retained 88% and 78% of its activity, respectively **(**Figure 4B**)**.

3 Discussion

In this study, we reported the characterization of a prolidase *Py*prol from *P. yayanosii* CH1. To the best of our knowledge, this is the first report about a prolidase that exhibits higher activity under high hydrostatic pressure than under atmospheric pressure.

Previous studies have found that the native prolidase (N-prol) purified from *P. furiosus* and the recombinant prolidase (R-prol) expressed in *E. coli* showed no significant differences in enzymatic properties[5]. However, when Met-Pro was used as the substrate, the k_{cat}/K_m value of R-prol was 1.64 times higher than that of N-prol[5]. To avoid the host background effect, we chose hyperthermophilic archaeon *T. kodakarensis*, which is a close relative of *P. yayanosii* CH1, as the surrogate host to overexpress the prolidase *Py*prol[22]. As shown in a previous report, successful expression of the soluble hydrogenase I (SHI) from *P. furiosus* has been achieved in *T. kodakarensis* TS559[25]. We anticipated that the prolidase obtained from *T. kodakarensis* TS559 may exhibit enzymatic properties more similar to the native prolidase from *P. yayanosii*.

For comparison, we also cloned the prolidase encoding gene (*PF_1343*) of *P. furiosus* and overexpressed it in *T. kodakarensis* TS559 (Figure 2B). For both *Py*prol and *Pf*prol, the prolidase activity

Table 4 The kinetic constants of *Py*prol

Hydrostatic pressure (MPa)	K_m (mmol/L)	V_{max} (μmol/(min·mg))	k_{cat} (s^{-1})	k_{cat}/K_m (L/(mmol·s))
0.1	2.5	2 722	2 238	895
40	2.4	3 100	2 481	1 034

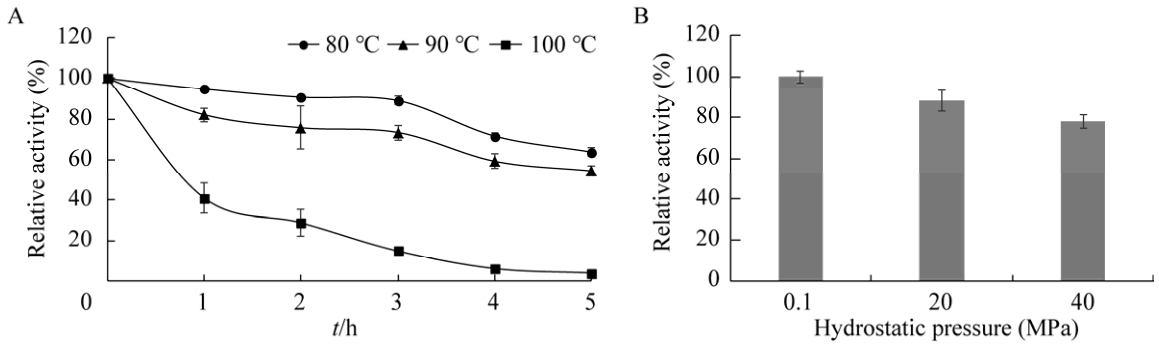

Figure 4 The thermal and high hydrostatic stability of *Py*prol. A: Determination of *Py*prol thermal stability, the enzyme was incubated at 80, 90, and 100 °C for 1, 2, 3, 4 and 5 h. B: Determination of *Py*prol high hydrostatic stability, the enzyme was incubated at 80°C, at 20 and 40 MPa for 1 h.

was higher under high hydrostatic pressure than at of under atmospheric pressure (Table 3). At 40 °C and 0.1 MPa, the specific enzyme activity of *Py*prol (578 U/mg) is 15.21 fold of that of *Pf*prol (38 U/mg). This indicated that at 40 °C, *Py*prol has greater potential in practical applications.

A phylogenetic analysis of *Py*prol indicates that prolidase from diverse hyperthermophiles, including that from three genus of *Thermococcaceae*, *Sulfolobus*, *Archaeoglobus* and *Thermotoga* are located within the same branch (Figure 5). It is interesting that the prolidase from *T. barophilus* MP, which was the first true hyperthermophilic piezophilic archaeon isolated[28], showed close relationship with prolidases from the *Pyrococcus* genus. It is commonly believed that enzyme activity decreases under high hydrostatic pressure[29]. However, there were reports that proteases derived from *Methanocaldococcus jannaschii* exhibited activity 3.4 times higher at 50 MPa 125 °C compared to 10 MPa 125 °C[30]. As well as that, we found that the activity of *Py*prol at high hydrostatic pressure was significantly higher than that at atmospheric pressure. Moreover, we observed that *Pf*prol exhibits a 25% increase in enzyme activity at 20 MPa compared to 0.1 MPa (Table 3). It is indicated that the prolidase from a deep-sea microbe may have a potential similarity in their enzymatic properties.

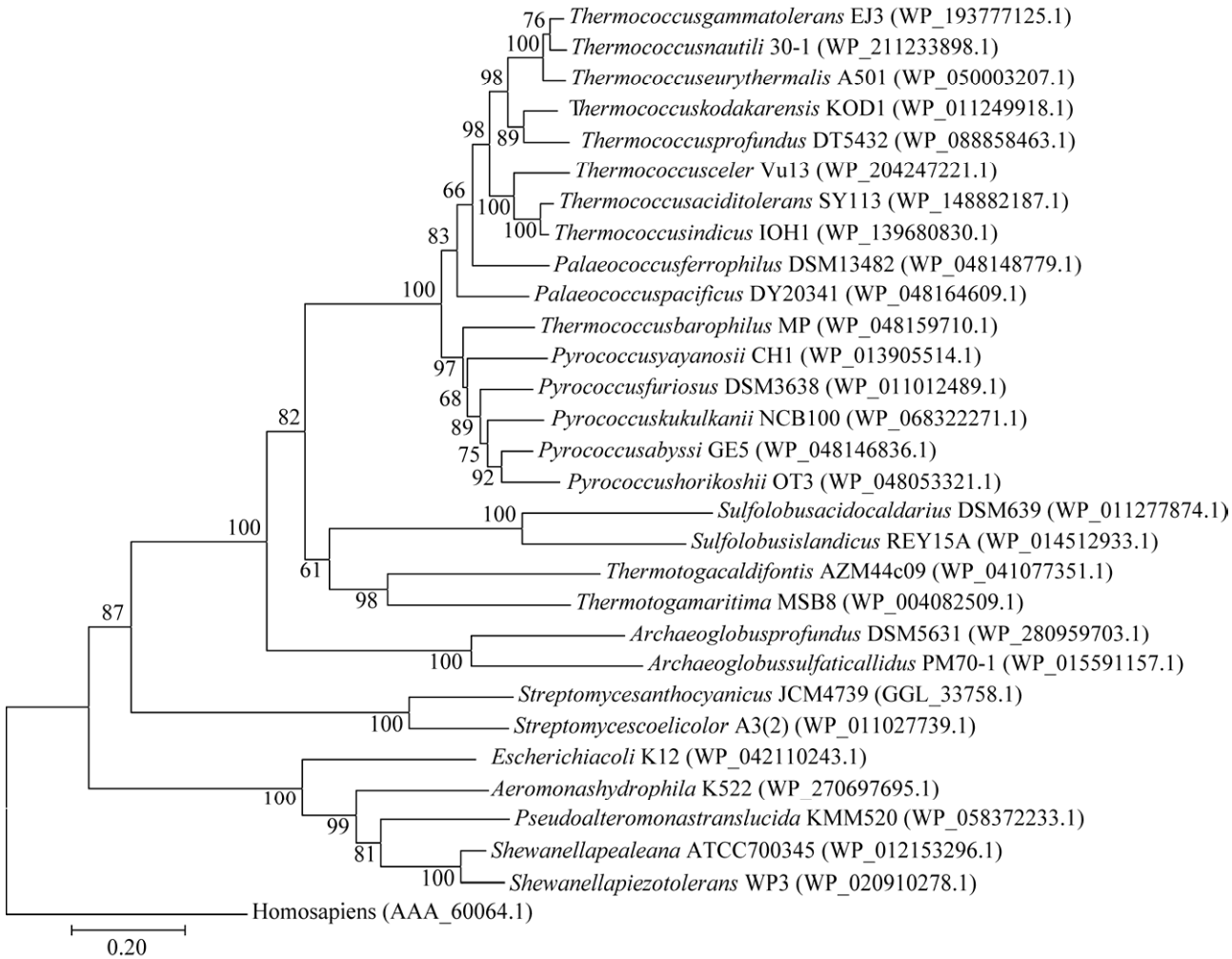

Figure 5 Phylogenetic analysis of prolidases, a neighbor-joining phylogenetic tree of prolidases and closely related proteins. Amino acid sequences of other enzymes were obtained from GenBank (http://www.ncbi.nlm.nih.gov/). Sequence alignment was performed using ClustalW, and the tree was created using MEGA version 7.0.

P. yayanosii CH1 lacks a complete pathway for synthesizing proline[31], indicating the need to acquire proline from the external environment. The unique cyclic structure of proline makes the peptide bonds surrounding the proline residue resistant to degradation, and prolidase is one of the few enzymes in organisms capable of degrading proline residues[32]. The higher activity of *Py*prol under high hydrostatic pressure compared to atmospheric pressure suggests its potential role in proline acquisition for *P. yayanosii* CH1 under high hydrostatic pressure conditions. We deleted *PYCH_07700* in *P. yayanosii*, but no significant decrease in biomass was observed under high hydrostatic pressure (result not shown). Whether functional compensation by other enzymes capable of degrading proline residues in *P. yayanosii* exist or not will be the focus of future experiments.

参考文献

[1] LUPI A, DELLA TORRE S, CAMPARI E, TENNI R, CETTA G, ROSSI A, FORLINO A. Human recombinant prolidase from eukaryotic and prokaryotic sources. Expression, purification, characterization and long-term stability studies[J]. The FEBS Journal, 2006, 273(23): 5466-5478.

[2] BESIO R, ALLEVA S, FORLINO A, LUPI A, MENEGHINI C, MINICOZZI V, PROFUMO A, STELLATO F, TENNI R, MORANTE S. Identifying the structure of the active sites of human recombinant prolidase[J]. European Biophysics Journal, 2010, 39(6): 935-945.

[3] FERNÁNDEZ-ESPLÁ MD, MARTÍN-HERNÁNDEZ MC, FOX PF. Purification and characterization of a prolidase from *Lactobacillus casei* subsp. casei IFPL 731[J]. Applied and Environmental Microbiology, 1997, 63(1): 314-316.

[4] WEAVER J, WATTS T, LI PW, RYE HS. Structural basis of substrate selectivity of *E. coli* prolidase[J]. PLoS One, 2014, 9(10): e111531.

[5] GHOSH M, GRUNDEN AM, DUNN DM, WEISS R, ADAMS MW. Characterization of native and recombinant forms of an unusual cobalt-dependent proline dipeptidase (prolidase) from the hyperthermophilic archaeon *Pyrococcus furiosus*[J]. Journal of Bacteriology, 1998, 180(18): 4781-4789.

[6] WILLINGHAM K, MAHER MJ, GRUNDEN AM, GHOSH M, ADAMS MW, FREEMAN HC, GUSS JM. Crystallization and characterization of the prolidase from *Pyrococcus furiosus*[J]. Acta Crystallographica Section D, 2001, 57(Pt 3): 428-430.

[7] THERIOT CM, TOVE SR, GRUNDEN AM. Characterization of two proline dipeptidases (prolidases) from the hyperthermophilic archaeon *Pyrococcus horikoshii*[J]. Applied Microbiology and Biotechnology, 2010, 86(1): 177-188.

[8] KITCHENER RL, GRUNDEN AM. Prolidase function in proline metabolism and its medical and biotechnological applications[J]. Journal of Applied Microbiology, 2012, 113(2): 233-247.

[9] VYAS NK, NICKITENKO A, RASTOGI VK, SHAH SS, QUIOCHO FA. Structural insights into the dual activities of the nerve agent degrading organophosphate anhydrolase/prolidase[J]. Biochemistry, 2010, 49(3): 547-559.

[10] MISIURA M, MILTYK W. Current understanding of the emerging role of prolidase in cellular metabolism[J]. International Journal of Molecular Sciences, 2020, 21(16): 5906.

[11] PALKA JA, PHANG JM. Prolidase in human breast cancer MCF-7 cells[J]. Cancer Letters, 1998, 127(1/2): 63-70.

[12] NOWICKA J, NAHACZEWSKA W, OWCZAREK H, WOŹNIAK M. The decrease in prolidase activity in myeloproliferative neoplasms[J]. Advances in Clinical and Experimental Medicine, 2012, 21(6): 767-771.

[13] YANG L, LI Y, BHATTACHARYA A, ZHANG YS. PEPD is a pivotal regulator of p53 tumor suppressor[J]. Nature Communications, 2017, 8: 2052.

[14] COURTIN P, NARDI M, WEGMANN U, JOUTSJOKI V, OGIER JC, GRIPON JC, PALVA A, HENRICH B, MONNET V. Accelerating cheese proteolysis by enriching *Lactococcus lactis* proteolytic system with lactobacilli peptidases[J]. International Dairy Journal, 2002, 12(5): 447-454.

[15] THERIOT CM, DU XL, TOVE SR, GRUNDEN AM. Improving the catalytic activity of hyperthermophilic *Pyrococcus* prolidases for detoxification of organophosphorus nerve agents over a broad range of temperatures[J]. Applied Microbiology and Biotechnology, 2010, 87(5): 1715-1726.

[16] DU XL, TOVE S, KAST-HUTCHESON K, GRUNDEN AM. Characterization of the dinuclear metal center of *Pyrococcus furiosus* prolidase by

analysis of targeted mutants[J]. FEBS Letters, 2005, 579(27): 6140-6146.

[17] BESIO R, BARATTO MC, GIOIA R, MONZANI E, NICOLIS S, CUCCA L, PROFUMO A, CASELLA L, BASOSI R, TENNI R, ROSSI A, FORLINO A. A Mn(II)-Mn(II) center in human prolidase[J]. Biochimica et Biophysica Acta, 2013, 1834(1): 197-204.

[18] ZENG X, BIRRIEN JL, FOUQUET Y, CHERKASHOV G, JEBBAR M, QUERELLOU J, OGER P, CAMBON-BONAVITA MA, XIAO X, PRIEUR D. *Pyrococcus* CH1, an obligate piezophilic hyperthermophile: extending the upper pressure-temperature limits for life[J]. The ISME Journal, 2009, 3(7): 873-876.

[19] BIRRIEN JL, ZENG X, JEBBAR M, CAMBON-BONAVITA MA, QUÉRELLOU J, OGER P, BIENVENU N, XIAO X, PRIEUR D. *Pyrococcus yayanosii* sp. nov., an obligate piezophilic hyperthermophilic archaeon isolated from a deep-sea hydrothermal vent[J]. International Journal of Systematic and Evolutionary Microbiology, 2011, 61(12): 2827-2881.

[20] LI Z, SONG QH, WANG YZ, XIAO X, XU J. Identification of a functional toxin-antitoxin system located in the genomic island PYG1 of piezophilic hyperthermophilic archaeon *Pyrococcus yayanosii*[J]. Extremophiles, 2018, 22(3): 347-357.

[21] SANTANGELO TJ, CUBONOVÁ L, REEVE JN. *Thermococcus kodakarensis* genetics: TK1827-encoded beta-glycosidase, new positive-selection protocol, and targeted and repetitive deletion technology[J]. Applied and Environmental Microbiology, 2010, 76(4): 1044-1052.

[22] TAKEMASA R, YOKOOJI Y, YAMATSU A, ATOMI H, IMANAKA T. *Thermococcus kodakarensis* as a host for gene expression and protein secretion[J]. Applied and Environmental Microbiology, 2011, 77(7): 2392-2398.

[23] LI XG, FU L, LI Z, MA XP, XIAO X, XU J. Genetic tools for the piezophilic hyperthermophilic archaeon *Pyrococcus yayanosii*[J]. Extremophiles, 2015, 19(1): 59-67.

[24] KUMAR S, STECHER G, TAMURA K. MEGA7: molecular evolutionary genetics analysis version 7.0 for bigger datasets[J]. Molecular Biology and Evolution, 2016, 33(7): 1870-1874.

[25] SONG YH, LIU MX, XIE LP, YOU C, SUN JS, ZHANG YPJ. A recombinant 12-his tagged *Pyrococcus furiosus* soluble[NiFe]-hydrogenase I overexpressed in *Thermococcus kodakarensis* KOD1 facilitates hydrogen-powered *in vitro* NADH regeneration[J]. Biotechnology Journal, 2019, 14(4): e1800301.

[26] LI SK, XIAO X, LI JY, LUO JX, WANG FP. Identification of genes regulated by changing salinity in the deep-sea bacterium *Shewanella* sp. WP3 using RNA arbitrarily primed PCR[J]. Extremophiles, 2006, 10(2): 97-104.

[27] YAYANOS AA, van BOXTEL R. Coupling device for quick high-pressure connections to 100 MPa[J]. Review of Scientific Instruments, 1982, 53(5): 704-705.

[28] MARTEINSSON VT, BIRRIEN JL, REYSENBACH AL, VERNET M, MARIE D, GAMBACORTA A, MESSNER P, SLEYTR UB, PRIEUR D. *Thermococcus barophilus* sp. nov., a new barophilic and hyperthermophilic archaeon isolated under high hydrostatic pressure from a deep-sea hydrothermal vent[J]. International Journal of Systematic Bacteriology, 1999, 49(2): 351-359.

[29] EISENMENGER MJ, REYES-DE-CORCUERA JI. High pressure enhancement of enzymes: a review[J]. Enzyme and Microbial Technology, 2009, 45(5): 331-347.

[30] MICHELS PC, CLARK DS. Pressure-enhanced activity and stability of a hyperthermophilic protease from a deep-sea methanogen[J]. Applied and Environmental Microbiology, 1997, 63(10): 3985-3991.

[31] XU J, LIU LP, XU MJ, OGER P, WANG FP, JEBBAR M, XIAO X. Complete genome sequence of the obligate piezophilic hyperthermophilic archaeon *Pyrococcus yayanosii* CH1[J]. Journal of Bacteriology, 2011, 193(16): 4297-4298.

[32] CUNNINGHAM DF, O'CONNOR B. Proline specific peptidases[J]. Biochimica et Biophysica Acta, 1997, 1343(2): 160-186.