

Screening, Identification, and Characterization of a Novel Alkali- Tolerant Chitinase from *Vibrio parahaemolyticus* CH-1

Zhe Wang

Dalian University

Yihan Wan

Dalian University

Jiahong Wu

Dalian University

Lin Pan

Dalian University

Yuqi Wang

Dalian University

Jiao Wu

Dalian University

Youngyan Guo

Dalian University

Xiaohui Wang

wangxiaohui@dlu.edu.cn

Dalian University

Yue Ma

Liaoning Academy of Agricultural Sciences

Research Article

Keywords: Chitin, Chitinase, Alkali-tolerant enzyme, Enzyme activity, Degradation mode

Posted Date: October 16th, 2026

DOI: <https://doi.org/10.21203/rs.3.rs-11160259/v1>

License: © ⓘ This work is licensed under a Creative Commons Attribution 4.0 International License.

[Read Full License](#)

Additional Declarations: No competing interests reported.

Screening, Identification, and Characterization of a Novel Alkali-Tolerant Chitinase from *Vibrio parahaemolyticus* CH-1

Zhe Wang^{1#}, Yihan Wan^{1#}, Jiahong Wu¹, Lin Pan¹, Yuqi Wang¹, Jiao

Wu¹, Youngyan Guo¹, Xiaohui Wang^{1*}, Yue Ma^{2*}

¹School of Life Science and Technology, Dalian University, Dalian 116622,
China

²Institute of Vegetable Research, Liaoning Academy of Agricultural Sciences,
Shenyang 110161, China

[#] These authors contributed equally to this work.

*Corresponding author: Dr. Xiaohui Wang, Dalian 116622

wangxiaohui@dlu.edu.cn; Yue Ma, M.S., Shengyang

110161,386809887@qq.com

Abstract

Chitin is the second most abundant carbon source on Earth and has great potential for producing chitin oligosaccharides. This study aimed to characterize the enzymatic properties and degradation mechanism of the novel low-temperature and alkali-tolerant chitinase VpCHI-1 from *Vibrio parahaemolyticus* CH-1. The VpCHI-1 gene was cloned and heterologously expressed in *Escherichia coli*. Enzyme activity under different temperatures, pH values, metal ions, and chemical reagents was analyzed. Kinetic parameters and degradation products were also determined, and sequence analysis was performed to identify structural domains. VpCHI-1 exhibited maximum activity at 35 °C and pH 8.0, while retaining over 60% activity at pH 11. Fe³⁺ and EDTA activated the enzyme, whereas Zn²⁺ and Cu²⁺ strongly inhibited it. The enzyme showed a k_m of 2.8 mg/mL and a k_{cat} of 178.3 s⁻¹. VpCHI-1 exhibits remarkable alkaline tolerance (>60% activity at pH 11.0) and low-temperature activity, with Fe³⁺ and EDTA serving as potent activators. Notably, the enzyme employs a triple catalytic mechanism combining endochitinase, exochitinase, and β -N-acetylglucosaminidase activities, producing (GlcNAc)₂ and GlcNAc as primary end products. These distinctive biochemical features position VpCHI-1 as a promising biocatalyst for industrial chitin conversion.

Clinical trial number: Not applicable.

Keywords: Chitin; Chitinase; Alkali-tolerant enzyme; Enzyme activity;
Degradation mode

1 Introduction

Chitin, also referred to as chitin, is ubiquitous in nature, occurring in the exoskeletons of crustaceans such as shrimp and crabs, as well as in the cell walls of insects and fungi [1,2]. Two primary configurations of chitin are found in nature: the α -type and β -type. The α -configuration of chitin is the most prevalent form in the natural world [3]. In α -chitin, N-acetylglucosamine units are arranged in two antiparallel chains, whereas in β -chitin, a single parallel chain is present [4]. The structure of β -chitin is comparatively looser than that of α -chitin, making it more susceptible to degradation. Currently, the most commonly used sources for chitin extraction are the shells of shrimp and crabs [5,6].

The breakdown of chitin into chitin oligosaccharides primarily involves the cleavage of its β -1,4 glycosidic bonds, resulting in the formation of small, water-soluble oligosaccharides or monosaccharides that are more readily absorbed and utilized by organisms. Techniques for obtaining chitin oligosaccharides include physical degradation, chemical degradation, and enzymatic hydrolysis [7-10]. Among them, physical degradation method and acidolysis method consume a lot of energy, are extremely unfriendly to the environment, and the products obtained cannot be controlled. In contrast, enzymatic degradation offers a more sustainable approach, with gentle reaction conditions and greater control over the product outcome [11]. Consequently, the exploration and investigation of chitinase enzymes are of paramount importance.

Chitinases are categorized into endochitinases and exochitinases based on their mode of chitin degradation and the types of products they yield [12,13]. Endochitinases typically produce chitobiose (GlcNAc)₂, chitotriose (GlcNAc)₃, and chito-oligosaccharides with higher degrees of polymerization [14,15]. Exochitinases, primarily N-acetylglucosaminidases, cleave chitin oligosaccharides

along the β -1,4 glycosidic bonds, releasing N-acetylglucosamine (GlcNAc) monosaccharides [16,17]. These two types of enzymes often coexist in wild strains, collaborating to degrade chitin into more readily absorbable and utilizable monosaccharides or oligosaccharides, enhancing the strain's ability to exploit chitin as a nutrient source [18,19].

Marine environments, characterized by low temperatures, high pressure, high salinity, and often fluctuating pH, harbor a vast diversity of microorganisms that produce enzymes with unique properties [20]. Among marine bacteria, the genus *Vibrio* is well-recognized for its prominent chitinolytic capability, playing a crucial role in marine chitin cycling [21,22]. However, the industrial application of chitinases is often hampered by several critical limitations: low catalytic efficiency, poor stability under extreme conditions (e.g., high temperature, extreme pH, or high salinity), and high production costs [23]. The majority of currently characterized chitinases originate from mesophilic terrestrial microorganisms and fail to meet the demands of harsh industrial processes. Therefore, the discovery of novel chitinases from extreme environments, such as the ocean, which possess unique traits like cold-adaptation and alkali-tolerance, is of paramount importance for developing robust biocatalysts for chitin bioconversion [22].

Although several marine chitinases from *Vibrio* and *Photobacterium* species have been reported, including the recently characterized PbCHI2 from *Photobacterium* sp. LG-29[24], the enzymatic properties and degradation mechanisms of chitinases from *Vibrio parahaemolyticus* strains remain largely unexplored. In particular, the combination of low-temperature activity, extreme alkaline tolerance, and a triple-mode catalytic mechanism has rarely been documented in a single enzyme from this species.

In this study, a chitinase-producing strain was isolated from the deep-sea sediment of the Bohai Sea using a chitin-containing screening plate. The strain was

identified as *Vibrio parahaemolyticus* CH-1 through morphological observation and 16S rDNA sequence analysis. The chitinase gene VpCHI-1 was subsequently cloned, expressed in *E. coli*, and the recombinant enzyme was purified and biochemically characterized. Here, we report the cloning, heterologous expression, and comprehensive biochemical characterization of VpCHI-1, providing novel insights into its catalytic versatility and potential for industrial applications.

2 Materials and Methods

2.1 Sample source and strain isolation

The chitinolytic strain CH-1 was isolated from deep-sea sediment collected in the Bohai Sea (Dalian, China) using a selective agar medium containing 1 g/L colloidal chitin as the sole carbon source. After incubation at 15°C for 72 h, colonies with clear hydrolysis zones were picked and purified by three rounds of streaking. The strain exhibiting the largest hydrolysis-to-colony diameter ratio (2.14 U/mL at 25°C, measured by the DNS method) was designated CH-1 and used for subsequent analyses [25]. Strain preservation was performed in LB medium with 25% glycerol at –80°C.

2.2 Identification of strain CH-1

Identification of strain CH-1 was performed using a combination of morphological and molecular approaches. Colony morphology was observed on screening plates, and cellular morphology was examined by Gram staining and SEM (JSM-6390/LV, JEOL). For SEM, cells were prefixed with 2.5% glutaraldehyde, dehydrated in a graded ethanol series, critical-point dried, and gold-coated prior to imaging at 10.0 kV. The 16S rDNA was PCR-amplified from genomic DNA using the universal primer pair 27F/1492R, and the resulting amplicon was subjected to Sanger sequencing. The obtained sequence (1489 bp) was compared against the NCBI GenBank database using BLAST. A phylogenetic tree was constructed using the neighbor-joining method in MEGA X with 1000

bootstrap replicates.

2.3 Strains and materials

Bacterial DNA extraction kit, DNA restriction enzyme, LA Taq polymerase, DNA purification kit, DNA Ligation Kit were all purchased from TaKaRa Company; IPTG and BCA kits were purchased from Solarbio Company; α -chitin and β -chitin were purchased from Sigma-Aldrich; GlcNAc₁₋₆ standards were purchased from Aladdin; Ni-NTA resin was purchased from Novagen.

The experimental strain *Vibrio parahaemolyticus* CH-1 (GenBank accession number: PV849134.1) was from Liaoning Marine Microbial Engineering Technology Research Center, College of Life Science and Technology, Dalian University.

2.4 Bioinformatics Analysis

The nucleotide and deduced amino acid sequences of VpCHI-1 were analyzed using ExPASy ProtParam for molecular weight and pI prediction, SignalP 6.0 for signal peptide identification, and SMART and NCBI CDD for domain architecture prediction. Multiple sequence alignments with representative GH18 chitinases were performed using Clustal Omega and visualized with ESPript 3.0, and a phylogenetic tree was constructed using MEGA 11 with the neighbor-joining method (1,000 bootstrap replicates). The three-dimensional structure of VpCHI-1 was predicted using AlphaFold3, and molecular docking with (GlcNAc)₆ was performed using AutoDock Vina. The binding pose and intermolecular interactions were analyzed with PyMOL and Discovery Studio Visualizer.

2.5 Cloning, expression and purification

The VpCHI-1 gene was PCR-amplified from the genomic DNA of *V.*

parahaemolyticus CH-1 using primer pairs designed based on the obtained ORF sequence (forward: 5'-AAGCTGACAGCTTGTATGTTTCTGC-3'; reverse: 5'-GCTTTCGCTGTGTTTGCGC-3'), with NdeI and XhoI restriction sites incorporated at the 5' and 3' ends, respectively. The purified PCR product was ligated into the pET-28a(+) vector and transformed into *E. coli* BL21(DE3). Recombinant clones were selected on kanamycin-supplemented plates and verified by colony PCR and DNA sequencing.

For recombinant protein production, a single positive colony was inoculated into LB broth containing 50 µg/mL kanamycin and incubated overnight at 37°C with shaking (180 rpm). This starter culture was diluted 1:100 into fresh LB medium and grown under the same conditions until the optical density at 600 nm reached 0.6–0.8. Protein expression was then induced by the addition of IPTG to a final concentration of 0.15 mmol/L, and the culture was further incubated at 15°C for 16 h with gentle shaking (100 rpm). Bacterial cells were harvested by centrifugation (8,000 ×g, 15 min, 4°C), resuspended in ice-cold lysis buffer (20 mmol/L Tris-HCl, pH 8.0), and disrupted by ultrasonication (500 W output, 20 min on ice). After removing cell debris by centrifugation (8,000 ×g, 15 min, 4°C), the clarified supernatant was applied to a Ni-NTA affinity chromatography column. Following extensive washing with binding buffer, the His-tagged VpCHI-1 protein was eluted with 150 mmol/L imidazole in the same buffer. The purity of the eluted fractions was assessed by 10% SDS-PAGE with Coomassie Brilliant Blue R-250 staining, and the purified protein was dialyzed overnight against 20 mmol/L Tris-HCl (pH 8.0) to eliminate imidazole.

2.6 Colloidal chitin preparation

Weigh 5 g chitin powder and slowly add to 60 mL concentrated HCl, stir at

4 °C overnight. Add 2 L 95% ethanol, stir at room temperature overnight, filter, wash with distilled water until pH neutral, then add 250 mL distilled water to obtain 2% colloidal chitin [26].

2.7 Assay of enzyme activity

When using 2% (w/v) colloidal chitin as substrate, mix 0.2 mL of substrate with 0.8 mL of 50 µg/mL enzyme solution and react for 30 min. Centrifuge the supernatant and add 0.1 mL of DNS reagent, boil for 5 min, add 0.2 mL of distilled water, and measure the absorbance at 520 nm wavelength to calculate the content of released reducing sugars [25]. Let one unit of enzyme activity (U) be the amount of enzyme that releases 1 µmol of reducing sugar every 1 min.

2.8 Optimal temperature and temperature stability

Using 2% colloidal chitin as substrate, it was placed at different temperatures for post-reaction testing. Assuming the maximum enzyme activity was 100%, calculate the relative enzyme activity and determine the optimal reaction temperature. The enzyme solution was stored at different temperatures for 3 hours in advance and then taken out. After rewarming it at 35 °C for 10 minutes, the enzyme solution was measured at 35 °C using 2% colloidal chitin as substrate for its residual enzyme activity. Assuming the maximum enzyme activity is 100%, calculate its relative enzyme activity and determine its temperature stability.

2.9 Optimal pH and pH stability

Using 2% colloidal chitin as the substrate, react in buffer solutions of different pH at 35 °C to detect the enzyme activity and determine the optimal reaction pH. Set the maximum enzyme activity to 100% to calculate the relative enzyme activity. After storing the enzyme at different pH and 4 °C for 3 hours, adjust the pH to 8.0,

react at 35 °C, and measure the residual enzyme activity. Set the maximum enzyme activity to 100% to determine its pH stability.

2.10 Effects of different metal ions and chemical reagents on enzyme activity

Configure metal ions and chemical reagents with different concentrations so that the final concentrations of metal ions in the reaction system are 5 mmol/L and 10 mmol/L respectively. Use 2% colloidal chitin as the substrate, add no metal ions as the blank group, and set the enzyme activity of the blank group to 100%. Observe the effects of different metal ions on the enzyme activity, and use SPSS v26.0 software to analyze the significance ($P \leq 0.05$).

2.11 Enzymatic reaction kinetic analysis

React at pH 8.0 and 35 °C, use colloidal chitin as the substrate, change the substrate concentration (0–15 mg/mL), calculate the initial enzyme activity, and use nonlinear regression to fit the Michaelis-Menten equation to calculate the kinetic parameters.

2.12 Analysis of hydrolysis product

In a 35 °C water bath, use colloidal chitin (2% w/v) as the substrate and react for 2, 4, 6, 8, and 24 hours before sampling. HPLC detection was performed using TSKgel Amide-80 column. The loading volume was 20 µL, the eluent was 70% acetonitrile, the flow rate was 1 mL/min, and the detection wavelength was 195 nm.

3 Results

3.1 Isolation and identification of strain CH-1

A chitinolytic bacterial strain, CH-1, was successfully isolated from marine sediment using a chitin-containing screening plate. The strain formed circular,

smooth, and creamy colonies with clear hydrolysis zones, indicating chitinase secretion (Fig. 1a). Microscopic observation after Gram staining revealed that CH-1 was a Gram-negative, short-rod-shaped bacterium (Fig. 1b). SEM analysis (Fig. 1c) further confirmed its typical *Vibrio*-like morphology: slightly curved rod-shaped cells with a smooth surface, measuring approximately 1.5 μm in length and 0.6 μm in diameter. These morphological characteristics are consistent with the typical description of the genus *Vibrio*.

The 16S rDNA gene (1489 bp) was successfully amplified (Fig. 2a). Phylogenetic analysis based on the 16S rDNA sequence showed that strain CH-1 shared 99% similarity with *Vibrio parahaemolyticus* NBRC 12711 (Fig. 2b). The sequence was deposited in GenBank under accession number PV849134.1. Therefore, the strain was definitively identified as *Vibrio parahaemolyticus* CH-1.

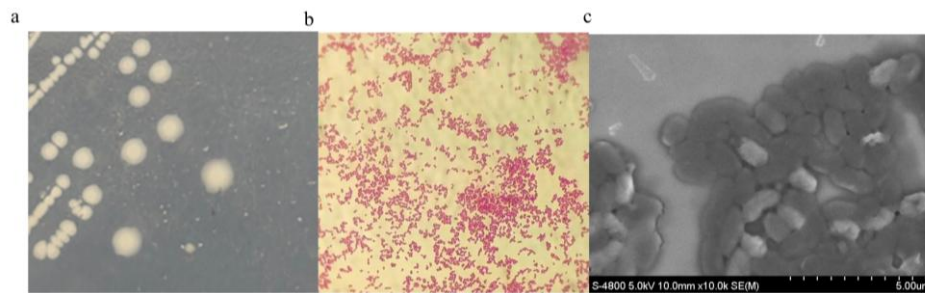


Fig.1. Morphological characteristics of strain CH-1. (a) Colony morphology on screening plate; (b) Gram staining (1000 $\times$); (c) Scanning electron microscopy image.

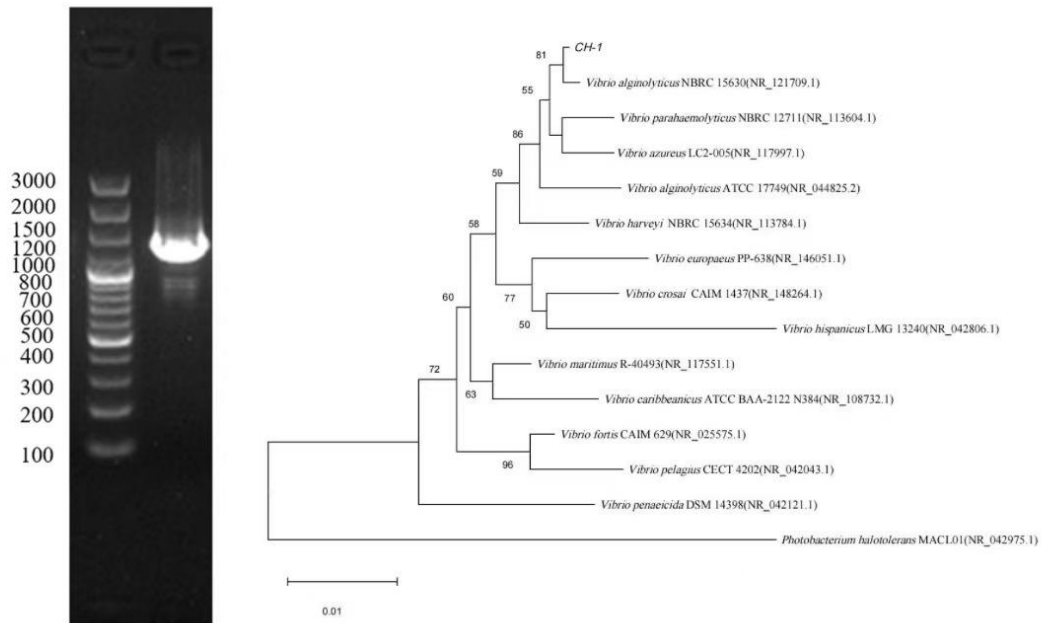


Fig. 2. (a) 16S rDNA PCR amplification; (b) Phylogenetic tree based on 16S rDNA sequence.

3.2 Gene cloning and bioinformatics analysis

The chitinase gene VpCHI-1 was successfully amplified from the genomic DNA of *Vibrio parahaemolyticus* CH-1. Sequencing revealed that the ORF consists of 2,465 bp (Fig. 3), encoding a polypeptide of 821 amino acids with a predicted molecular mass of 90.3 kDa and an isoelectric point of 4.3.

Domain analysis using SMART and NCBI CDD revealed a multi-modular architecture comprising an N-terminal signal peptide (residues 1–24), a Glyco_18 catalytic domain (residues 29–378) harboring the conserved GH18 catalytic motif (DxDxE), three tandem PKD domains (residues 392–472, 482–560, and 568–649), and a C-terminal ChtBD3 domain (residues 724–821) (Fig. 4). In contrast to PbCHI2 from *Photobacterium* sp. LG-29, which contains an additional PKD domain and a longer C-terminal extension, VpCHI-1 possesses a distinct domain organization with three linearly arranged PKD domains, suggesting different

substrate-binding dynamics and environmental adaptation strategies within the same GH18 family[24]. The presence of the signal peptide indicates that VpCHI-1 is a secreted enzyme. The three tandem PKD domains, which are characteristic of several marine bacterial chitinases, have been reported to contribute to cell protection against environmental stress [27,28].

Phylogenetic analysis placed VpCHI-1 within the marine *Vibrio* clade of GH18 chitinases, where it clustered closely with homologs from *Vibrio harveyi* and *Vibrio cholerae* [21] (Fig.5). Multiple sequence alignment with representative GH18 chitinases (Figs.6) demonstrated that VpCHI-1 fully conserves the characteristic functional motifs within its catalytic domain. Specifically, key catalytic residues, including Asp392, Asp394, and Glu498 within the conserved DxExE motif, along with their flanking regions, strictly conform to the canonical proton-transfer mechanism of GH18 hydrolases. Within the substrate-binding cleft, several evolutionarily conserved aromatic (e.g., Tyr391) and polar residues establish a hydrophobic microenvironment and a hydrogen-bonding network critical for stabilizing the substrate. Furthermore, overall sequence conservation patterns indicate that VpCHI-1 exhibits the highest sequence identity to bacterial GH18 subfamily A members, reinforcing its classification as a canonical GH18 chitinase. The tertiary structure predicted by AlphaFold3 adopts the classic (β/α)₈ TIM-barrel fold characteristic of GH18 family proteins (Fig. 7). Molecular docking simulations with (GlcNAc)₆ showed that the hexasaccharide ligand is stabilized through hydrophobic interactions with Tyr391, hydrogen bonds with Ser204, Asp392, Arg463, and Glu498, as well as a salt bridge with Arg463, yielding a calculated binding free energy of -7.2 kcal/mol. Collectively, these structural and molecular interactions elucidate the catalytic mechanism and substrate recognition features of VpCHI-1.

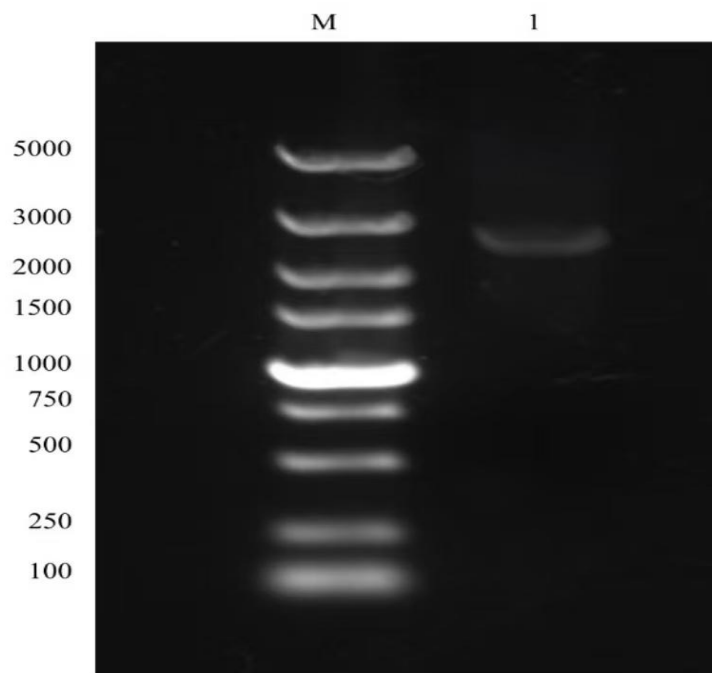


Fig.3. Target gene amplification test of clone-positive strains. Lane M, DNA Marker; Lane 1, PCR amplification fragment of strain E.coli BL21(DE3)-pET28a-VpCHI-1.



Fig. 4. Domain architecture of VpCHI-1 predicted by SMART.

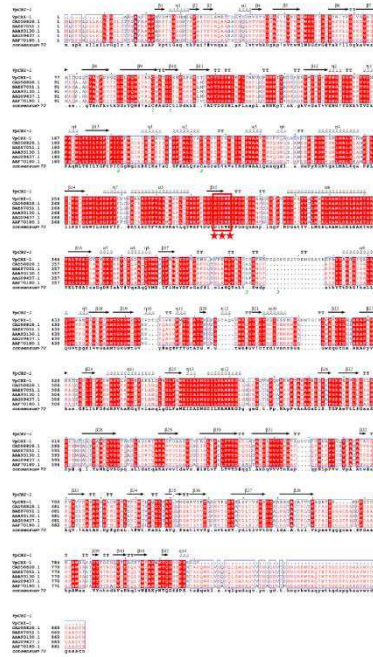


Fig. 6.The sequence comparison among VpCHI-1 and other chitinase from GH18 family.

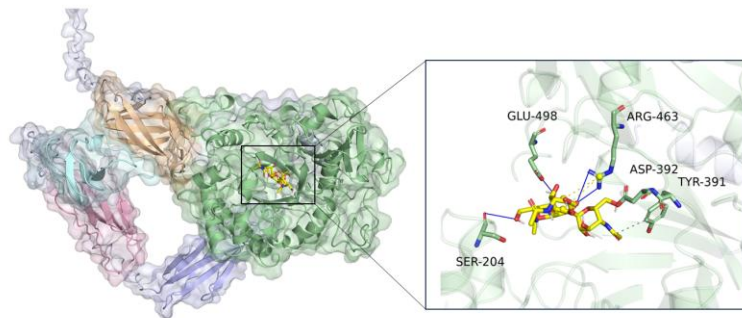


Fig. 7. AlphaFold3-predicted three-dimensional structure of VpCHI-1 and molecular docking with (GlcNAc)₆.

3.3 Purification of recombinant chitinase VpCHI-1

Chitinase was successfully cloned from the *Vibrio parahaemolyticus* strain CH-1 and purified using a nickel column chromatography method. As demonstrated by SDS-PAGE electrophoresis (Fig. 8), the purification process resulted in a higher purity enzyme solution. The specific enzyme activity of chitinase VpCHI-1, after concentration, was 2.47 U/mg. Following purification, the specific enzyme activity increased significantly to 17.62 U/mg, representing a 7.13-fold enhancement. This purified enzyme solution is now suitable for use in subsequent experiments due to its improved purity and activity.

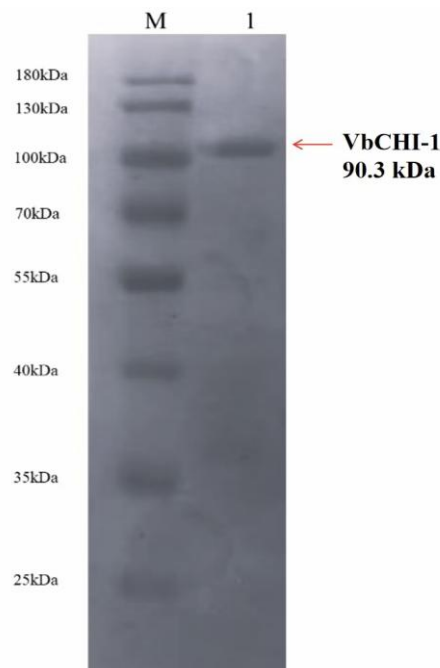


Fig. 8. Electropherogram of recombinant chitinase VpCHI-1 after purification. Marker molecular weight sizes from top to bottom are 180 kDa, 130 kDa, 100 kDa, 70 kDa, 55 kDa, 40 kDa, 35 kDa, 25 kDa, 15 kDa, 10 kDa.

3.4 Optimum operating temperature and temperature stability of recombinant chitinase VpCHI-1

As depicted in Fig. 9, chitinase VpCHI-1 reaches its peak activity at 35 °C

when it acts on colloidal chitin. The enzyme retains 80% of its peak activity from 25 °C to 50 °C, with a steep decline in activity above 50 °C. At 70 °C, the relative activity is only 13%, and it falls to 15% at 0 °C. This indicates that chitinase VpCHI-1 is well-adapted for low-temperature enzymatic processes.

Under the colloidal chitin substrate conditions, chitinase VpCHI-1 exhibits optimal temperature stability at 25 °C to 40 °C. Following storage at temperatures ranging from 0 °C to 40 °C for 3 hours, the enzyme retains over 80% of its relative activity. The enzyme also maintains about 60% of its relative activity when stored at 50 °C, but its activity decreases rapidly above this temperature. At 55 °C, the residual enzyme activity is only 18%. These results indicate that chitinase VpCHI-1 possesses a degree of thermal stability, although its activity is sensitive to higher temperatures.

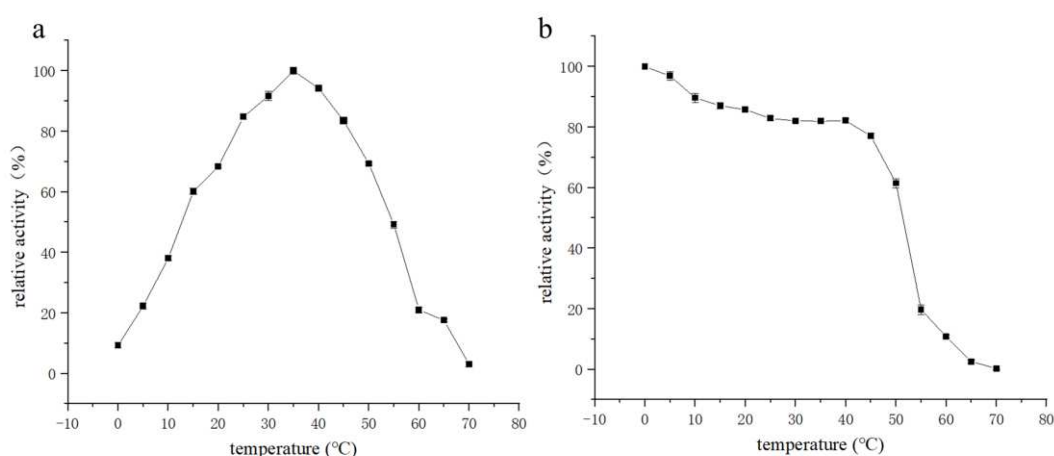


Fig. 9. Optimum temperature and temperature stability of chitinase VpCHI-1. (a) shows the optimal temperature analysis; (b) shows the temperature stability analysis.

3.5 Optimum pH and pH stability analysis of recombinant chitinase VpCHI-1

The enzyme chitinase VpCHI-1 exhibits its highest activity at pH 8.0. At pH

7.0, its activity is only 60% of its maximum, and it maintains substantial activity only when the pH is greater than 8.0, with relative activity decreasing to around 20% at pH 3.0. In contrast, the enzyme demonstrates remarkable stability in alkaline conditions, with over 90% of its relative activity retained at pH 9.0 and above 60% at pH 11.0. This indicates that chitinase VpCHI-1 is an alkaliphilic enzyme, as supported by pH stability analysis. After storage under alkaline conditions, the enzyme retains 69% of its activity even at pH 11.0. However, at pH values below 7.0, the enzyme's residual activity drops below 20%, and at pH 3.0 or 4.0, its activity is almost completely lost (as shown in Fig. 10). These findings further confirm that chitinase VpCHI-1 is particularly stable in alkaline environments.

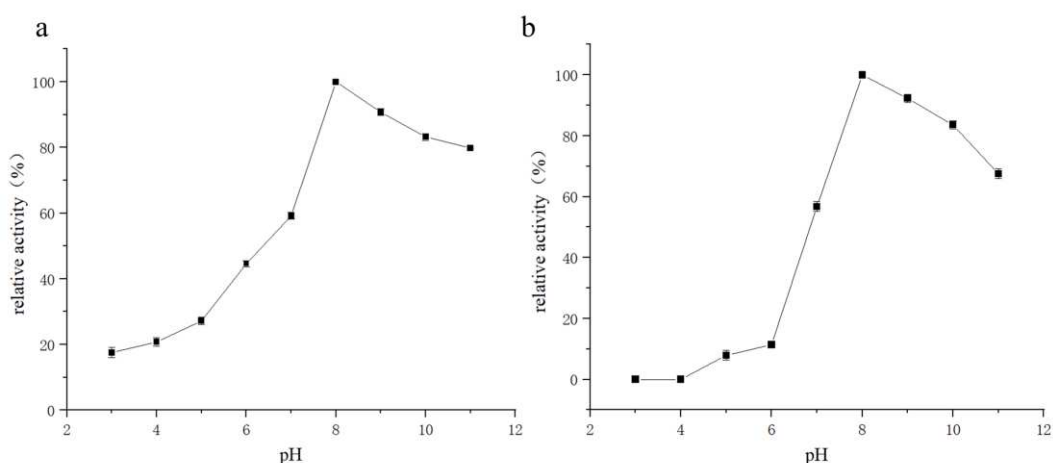


Fig. 10. Optimum pH and pH stability analysis of chitinase VpCHI-1. (a) shows the optimal pH analysis; (b) shows the pH stability analysis.

3.6 Effects of metal ions and chemical reagents on the activity of chitinase VpCHI-1

As depicted in Fig. 11, the presence of Zn^{2+} and Cu^{2+} was found to have a highly detrimental effect on chitinase VpCHI-1 activity, irrespective of the

concentration. These metal ions caused a significant decrease in enzyme activity, with levels dropping below 10% at all tested concentrations.

Conversely, Fe^{3+} and EDTA were observed to significantly enhance chitinase VpCHI-1 activity. At 5 mmol/L, these compounds induced a substantial increase in enzyme activity, with levels rising by over 50%. Tween-20 also showed significant activation at 10 mmol/L.

Li^+ , Na^+ , K^+ , and Triton X-100 displayed a minor inhibitory effect at 5 mmol/L, but the relative enzyme activity remained above 80%, indicating that the enzyme is tolerant to certain salts. Ca^{2+} ions demonstrated an activating effect at low concentrations, with the enzyme activity increasing slightly.

It is important to note that, except for Tween-20 and Fe^{3+} , most chemical reagents used in this study showed an inhibitory effect on the enzyme at high concentrations. SDS and urea strongly inhibited enzyme activity, likely due to protein denaturation, while DTT had minimal effect on enzyme activity.

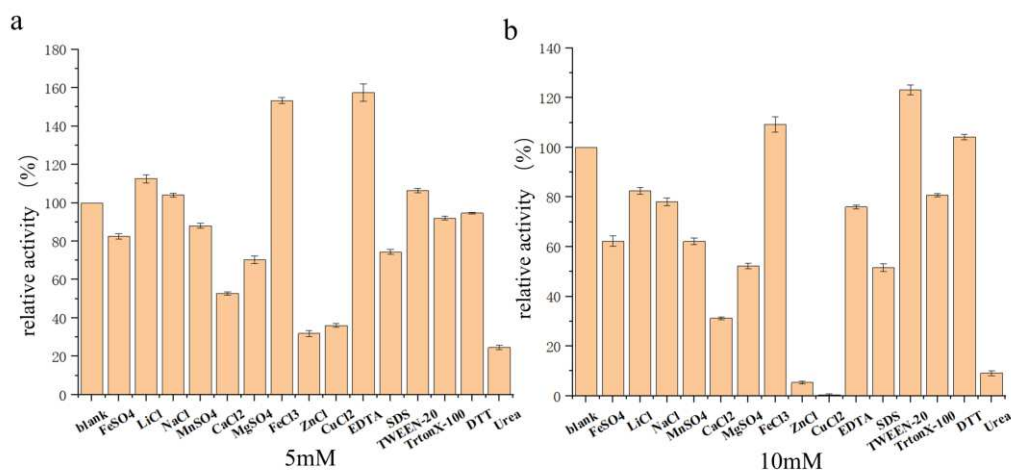


Fig. 11. Effect of different concentrations of metal ions and chemical reagents on the activity of chitinase VpCHI-1.

3.7 Analysis of kinetic parameters of chitinase VpCHI-1

By varying the substrate concentration and employing the Michaelis-Menten equation, kinetic parameters were derived to describe the kinetic behavior of the enzymatic reaction. The concentration of the substrate [S] was measured in milligrams per milliliter.

The kinetic constants K_m and K_{cat} for chitinase VpCHI-1 were calculated to be 2.8 mg/mL and 178.3 s^{-1} , respectively, as depicted in Fig. 12 and Table 1. These values provide insights into the affinity of the enzyme for its substrate and its catalytic efficiency. The catalytic efficiency (K_{cat}/K_m) was calculated to be 63.68 $s^{-1} \cdot mL/mg$.

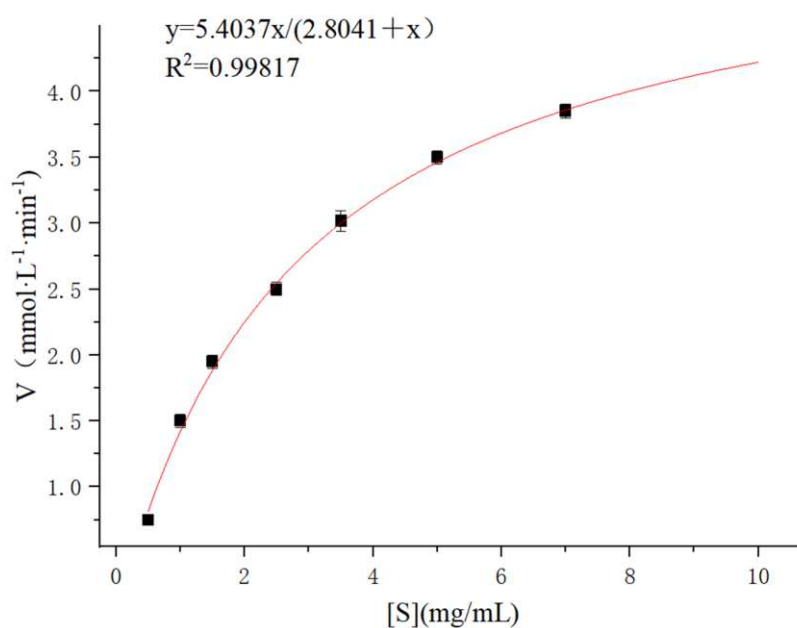


Fig. 12. Graph of the kinetics of the enzymatic reactions of chitinase VpCHI-1.

3.8 Study on the degradation mechanism of chitinase VpCHI-1

Chitinases exhibit diverse mechanisms for chitin degradation, resulting in chitin oligosaccharides of various polymerization degrees. By employing chitinases to degrade colloidal chitin, the degradation pattern can be elucidated by examining

the types of products generated. As evidenced by Fig. 13, colloidal chitin degraded by chitinase VpCHI-1 predominantly appeared as (GlcNAc)₂ and GlcNAc, with (GlcNAc)₃ appearing as a transient intermediate.

Based on the observed product profile, chitinase VpCHI-1 is classified as possessing a triple catalytic mechanism. The production of (GlcNAc)₃ in the early stage suggests endochitinase activity, primarily cleaving internal glycosidic bonds. The subsequent decrease of (GlcNAc)₃ and accumulation of (GlcNAc)₂ and GlcNAc indicate exochitinase and β -N-acetylglucosaminidase activities. This suggests that chitinase VpCHI-1 may possess both endonuclease and exonuclease properties, with a complete degradation mechanism.

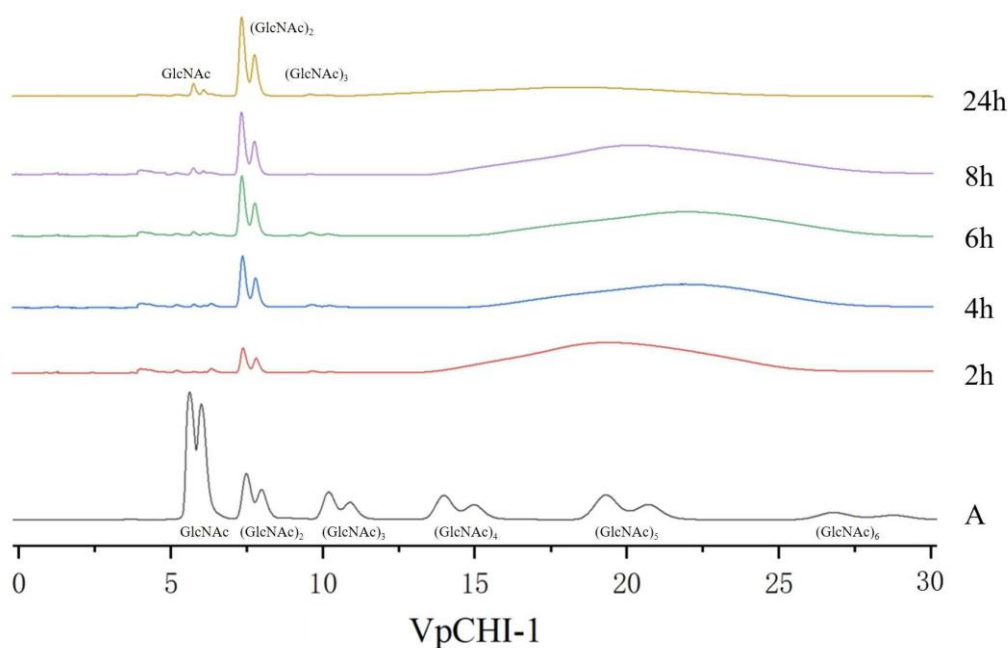


Fig. 13. HPLC analysis of the hydrolysis products of colloidal chitin by chitinase VpCHI-1. Line A is the standard peak of (GlcNAc)₁₋₆, which represents the standard peak of GlcNAc₁-GlcNAc₆ in sequence; Lines B–F represent the product formation after degrading the substrate for 2 h, 4 h, 6 h, 8 h, and 24 h respectively.

4 Discussion

In this study, a novel chitinase-producing strain CH-1 was successfully

isolated from the deep-sea sediment of the Bohai Sea and identified as *Vibrio parahaemolyticus*. The chitinase gene VpCHI-1 from this strain was cloned, expressed, purified, and its enzymatic properties were characterized.

Bioinformatics analysis revealed that VpCHI-1 is a multi-domain GH18 family chitinase, comprising an N-terminal signal peptide, a Glyco_18 catalytic domain with the conserved DxDxE motif, three tandem PKD domains, and a C-terminal ChtBD3 domain (Fig. 4). The presence of the signal peptide confirms that VpCHI-1 is a secreted enzyme, consistent with its functional role in extracellular chitin degradation. The strict conservation of the catalytic residues Asp392, Asp394, and Glu498, as identified by multiple sequence alignment, supports the conclusion that VpCHI-1 employs the substrate-assisted catalytic mechanism characteristic of GH18 family hydrolases.

Chitinase VpCHI-1 retains enzyme activity at low temperatures, indicating it is a low-temperature enzyme with a degree of thermal stability. This characteristic aligns with previously reported chitinases from cold-adapted strains [29]. In comparison, chitinases from *Bacillus subtilis* JN032305, which has an optimal temperature of 50 °C [30], and other characterized chitinases with optimal temperatures above 45 °C [31], both exhibit higher optimal temperatures. Chitinase VpCHI-1's optimal temperature is lower than these previously characterized chitinases. Although it has a higher optimal temperature than chitinases ChiA and ChiC from the strain *Pseudoalteromonas* sp. DL-6 [29], VpCHI-1's temperature stability is superior, with minimal activity loss at 25 °C and 30 °C. Chitinase VpCHI-1's enhanced stability and low-temperature activity make it well-suited for practical industrial applications. The observed temperature characteristics of VpCHI-1 are likely influenced by the environmental conditions in which the strain thrives. This insight suggests that the enzyme's properties may be adapted to the specific growth environment of the *Vibrio parahaemolyticus* strain.

The optimal pH for chitinase VpCHI-1 is 8.0, which is consistent with the pH range of the Bohai Sea, where the strain is isolated. The sea's pH typically ranges from 7.86 to 8.30, which may contribute to the enzyme's ability to exhibit optimal activity within this alkaline range. This pH preference suggests that chitinase VpCHI-1 has adapted to the specific environmental conditions of the Bohai Sea. Furthermore, the stability of VpCHI-1 under alkaline conditions is superior to many previously reported chitinases, indicating a higher resistance to pH changes in an alkaline environment. This is in contrast to other chitinases isolated from different environments, such as *Paenibacillus* sp. from soil [32]. These differences highlight the relationship between the optimal pH and stability of chitinases and the environment from which they are isolated. The pH preference and stability of chitinases appear to be shaped by the ecological niches in which they evolved, reflecting the adaptations necessary for their survival and activity.

Fe^{3+} and EDTA both showed significant activating effects on chitinase VpCHI-1. The activation by EDTA suggests that the enzyme's activity may be inhibited by trace heavy metals present in the reaction mixture, and EDTA relieves this inhibition by chelating these metals. This property is advantageous for applications where the enzyme would be used in crude reaction mixtures containing metal ions. The activation by Tween-20 is also significant, as this non-ionic surfactant enhances enzyme activity by improving the dispersion of the hydrophobic chitin substrate, increasing the enzyme-substrate contact area. Heavy metal ions such as Zn^{2+} and Cu^{2+} have a strong inhibitory effect on chitinase VpCHI-1. It is inferred that heavy metal ions may have certain toxicity to chitinase proteins [33]. The molecular docking results provide a structural explanation for these observations: the active-site residues Tyr391, Asp392, Arg463, and Glu498 form a network of hydrophobic interactions, hydrogen bonds, and a salt bridge with the substrate, and disruption of these interactions by metal ions or denaturants would directly impair catalytic

efficiency. Research has revealed that chitinase VpCHI-1 demonstrates a degree of salt tolerance, which is likely due to its marine origin and the higher salt concentration in oceanic environments compared to other habitats. This adaptation allows the enzyme to function effectively in environments with varying salinity levels.

SDS and urea have been found to have an inhibitory effect on chitinase VpCHI-1, which is typical since most studies have shown that SDS and urea can inhibit the activity of many enzymes [34]. The inhibition by SDS and urea suggests that the enzyme's catalytic activity depends on proper protein folding and hydrophobic interactions, which are disrupted by these denaturing agents. The three tandem PKD domains identified in VpCHI-1 warrant particular attention. Although originally discovered in human polycystic kidney disease proteins, PKD domains have been increasingly recognized in bacterial chitinases from marine and soil environments [27,28]. The linear arrangement of three PKD domains between the catalytic GH18 domain and the C-terminal ChtBD3 may provide conformational flexibility, allowing the enzyme to adapt to the irregular surface of crystalline chitin. Moreover, PKD domains have been implicated in cellular protection against environmental stresses, including osmotic and pressure stress [27], which is consistent with the deep-sea origin of *V. parahaemolyticus* CH-1. The C-terminal ChtBD3 domain likely functions as an anchor, tethering the enzyme to the chitin surface while the GH18 domain carries out processive hydrolysis [21].

Comparative analysis with the recently reported PbCHI2 from *Photobacterium* sp. LG-29 [24], reveals both shared and distinguishing features between these two marine GH18 chitinases. Although both enzymes originate from the same Bohai Sea sedimentary environment, exhibit optimal activity at 35°C and pH 8.0, and demonstrate remarkable alkaline stability—reflecting common adaptive strategies to the marine ecosystem—several key biochemical differences underscore their

distinct enzymatic identities. For instance, VpCHI-1 retains over 80% activity at 25–40°C but drops sharply above 50°C, whereas PbCHI2 maintains >80% residual activity even after 3 h of incubation at 50°C, indicating superior thermostability of the latter. In terms of metal ion responsiveness, VpCHI-1 is significantly activated by Fe^{3+} and EDTA (>50% enhancement), while PbCHI2 responds primarily to Mn^{2+} , Ca^{2+} , and Mg^{2+} , suggesting distinct active-site metal coordination environments between the two enzymes. More notably, VpCHI-1 exhibits a triple catalytic mode encompassing endochitinase, exochitinase, and β -N-acetylglucosaminidase activities, as evidenced by the transient accumulation of $(\text{GlcNAc})_3$ during hydrolysis, whereas PbCHI2 functions predominantly as an exochitinase with chitobiosidase and β -N-acetylglucosaminidase activities, without detectable endochitinase activity. Structurally, VpCHI-1 contains three tandem PKD domains between the catalytic domain and ChtBD3, while PbCHI2 possesses an additional fourth PKD domain, a difference that may contribute to variations in substrate-binding affinity and processivity. Collectively, these comparative findings not only distinguish VpCHI-1 as a novel biocatalyst with unique properties but also expand our understanding of the functional diversity of marine GH18 chitinases within closely related taxonomic groups.

In summary, the characterization of VpCHI-1 underscores the remarkable evolutionary adaptation of deep-sea microbial enzymes to their native marine environments. The combination of cold tolerance, alkaline stability, and a multi-domain GH18 architecture endows VpCHI-1 with distinct biocatalytic advantages, particularly in the efficient conversion of chitin-rich waste into value-added products like $(\text{GlcNAc})_2$ and GlcNAc. Furthermore, the structural insights provided by AlphaFold3 prediction and molecular docking establish a solid framework for future protein engineering aimed at enhancing catalytic efficiency and stress tolerance. Overall, VpCHI-1 represents a highly promising and sustainable

biocatalyst for eco-friendly marine waste management and broader industrial applications.

Acknowledgements

This work was supported by the National Natural Science Foundation of China Youth Science Fund Project (31500039); Dalian Youth Science and Technology Star Project (2017RQ155); Liaoning Revitalization Talents Program(XLYC2502013);Liaoning Provincial Key Research and Development Program(Grant No. 2024JH2/102500017);Supported by the Natural Science Foundation of Liaoning Province (20180550728).

Conflict of Interest Statement

The authors declare no conflict of interest.

5 References

[1] Jiang WX, Li PY, Chen XL, Zhang YZ, Qin QL, Su HN, et al. A pathway for chitin oxidation in marine bacteria. *Nat Commun.* 2022;13:5899.

[2] Brown HE, Esher SK, Alspaugh JA. Chitin: A "Hidden Figure" in the fungal cell wall. In: Latgé JP, Editor. *The Fungal Cell Wall: An Armour and a Weapon for Human Fungal Pathogens*. Cham: Springer International Publishing; 2020. p. 83–111.

- [3] Hajji S, Younes I, Ghorbel-Bellaaj O, Hajji R, Rinaudo M, Nasri M, et al. Structural differences between chitin and chitosan extracted from three different marine sources. *Int J Biol Macromol*. 2014;65:298–306.
- [4] Baharlouei P, Rahman A. Chitin and chitosan: Prospective biomedical applications in drug delivery, cancer treatment, and wound healing. *Mar Drugs*. 2022;20:460.
- [5] Moussian B, Schwarz H, Bartoszewski S, Nüsslein-Volhard C. Involvement of chitin in exoskeleton morphogenesis in *Drosophila melanogaster*. *J Morphol*. 2005;264:117–30.
- [6] Courtade G, Aachmann FL. Chitin-active lytic polysaccharide monooxygenases. In: Yang Q, Fukamizo T, Editors. *Targeting Chitin-containing Organisms*. Singapore: Springer; 2019. p. 115–29.
- [7] Borić M, Vicente FA, Jurković DL, Santos E, Božić M, Kokol V, et al. Chitin isolation from crustacean waste using a hybrid demineralization/DBD plasma process. *Carbohydr Polym*. 2020;246:116648.
- [8] Fang L, Yang XG, Liu QF, Qu D, Wang MY, Yuan SP, et al. Homogeneous deacetylation and degradation of chitin in NaOH/urea dissolution system. *Int J Biol Macromol*. 2021;189:391–397.
- [9] Yan B, Yang H, Zhang N, Zhang L, Liu X, Wang Y, et al. Microwave-assisted depolymerization of chitin and chitosan extracted from crayfish shells waste: A

sustainable approach based on graphene oxide catalysis. *Int J Biol Macromol.* 2023;251:126296.

[10] Zhang Q, Duan L, Li Y. Positive effects and mechanism of ultrasound on chitin preparation from shrimp shells by co-fermentation. *Ultrason Sonochem.* 2022;88:106066.

[11] Michel Benedykt, Struszczyk-Swidorska K, Li XK, Szczesna-Antczak M, Daroch M. Enzymatic modifications of chitin, chitosan, and chitooligosaccharides. *Front Bioeng Biotechnol.* 2019;7:243.

[12] Liu Y, Jiang Z, Ma J, Zhang W, Liu J, Single S, et al. Biochemical characterization and structural analysis of a β -N-acetylglucosaminidase from *Paenibacillus barengoltzii* for efficient production of N-acetyl-D-glucosamine. *J Agric Food Chem.* 2020;68:5648–57.

[13] Igarashi K, Uchihashi T, Uchiyama T, Koivula A, Wada M, Samejima M, et al. Two-way traffic of glycoside hydrolase family 18 processive chitinases on crystalline chitin. *Nat Commun.* 2014;5:3975.

[14] Wieczorek AS, Schmidt O, Chatzinotas A, von Bergen M, Gorissen A, Kolb S. Ecological functions of agricultural soil bacteria and microeukaryotes in chitin degradation: A case study. *Front Microbiol.* 2019;10:2650.

[15] Martin F, Kohler A, Murat E, Balestrini R, Schueffler A, Zampieri E, et al. Functional genomics gives new insights into the ectomycorrhizal degradation of chitin. *New Phytol.* 2023;238:845–58.

- [16] Kaushal R, Kumar M, Upadhyay A, Dhakar PK, Verma V, Pruthi N. Response surface bioprocess provenance for enhanced chitinase production by *Thermomyces dupontii* for translation of chitinous waste to short-chain chitooligosaccharides. *Biocatal Agric Biotechnol*. 2024;55:102980.
- [17] Vengadaramana P, Surenthery K, Amarchand MC. *Pseudomonas* species-derived chitinase mass multiplication, production cost analysis, and marketing: As a biocontrol agent for crop protection. In: Singh HB, Vaishnav A, Editors. *Industrial Microbiology Based Entrepreneurship*. Singapore: Springer; 2022. p. 179–92.
- [18] Dong H, Fa J, Yan M, Zhang Y, Wang L, Liu X, et al. Development and analysis of chitin/cellulose reinforced galactomannan fluidic hydrogel for drug delivery application. *J Colloid Interface Sci*. 2025;686:701–10.
- [19] Zhang J, Xue S, Zhu X, Zhang Y, Wang X, Tan H, et al. Emerging chitin nanogels/rectorite nanocomposites for safe and effective hemorrhage control. *J Mater Chem B*. 2019;7:5096–103.
- [20] Parrilli E, Tedesco P, Fondi M, Michaud L, Lo Giudice A, de Pascale D, et al. The art of adapting to extreme environments: The model system *Pseudoalteromonas*. *Phys Life Rev*. 2021;36:137–61.
- [21] Suginta W, Sirimontree P, Sritho N, Ohnuma T, Fukamizo T. The chitin-binding domain of a GH-18 chitinase from *Vibrio harveyi* is crucial for chitin-chitinase interactions. *Int J Biol Macromol*. 2016;93:1111–7.

[22] Das S, Dey P, Roy D, Tripathy S, Banerjee IE. N-Acetyl-D-glucosamine production by a chitinase of marine fungal origin: A case study of potential industrial significance for valorization of waste chitins. *Appl Biochem Biotechnol.* 2019;187:407–23.

[23] Zhao XQ, Xu XN, Chen LY. Production of enzymes from marine actinobacteria. *Adv Food Nutr Res.* 2016;78:137–51.

[24] Wang Z, Zhao Z, Pan L, Zhang Y, Guo Z, Yang W, Feng L, Wang X. Cloning, expression, purification, and mutant construction of the chitinase PbCHI2 gene from *Photobacterium* sp. LG-29. *Arch Microbiol.* 2025;207(11):272.

[25] Deng JJ, Liu Y, Li C, Wang X, Zhang Y, Zhao L, et al. Heterologous expression and characterization of an antifungal chitinase (chit46) from *Trichoderma harzianum* GIM 3.442 and its application in colloidal chitin conversion. *Int J Biol Macromol.* 2019;134:113–21.

[26] Sun J, Wang W, Ying Y, Zhang X, Liu Y, Wang L, et al. *Pseudomonas profundus* sp. nov., isolated from deep-sea water. *Int J Syst Evol Microbiol.* 2018;68:1776–80.

[27] Mohan Krishna M, Appa Rao P. Catalytic efficiency of a multi-domain transglycosylating chitinase from *Enterobacter cloacae* subsp. *cloacae* (ecchi2) is influenced by polycystic kidney disease domains. *Enzyme Microb Technol.* 2020;143:109702.

- [28] Yabushita F, Kudo T, Seito T, Kobayashi M, Lin CY, Johnson MD, et al. Role of the polycystic kidney disease domain in matriptase chaperone activity and localization of hepatocyte growth factor activator inhibitor-1. *FEBS J.* 2022;289:3422–39.
- [29] Wang XH, Zhao Y, Zhao XM, et al. Screening, identification and spectrogram analysis of marine low temperature chitinase strains. *Acta Agric Boreali-Occident Sin.* 2014;23:92–7.
- [30] Pan M, Li J, Lv X, Zhang C, Wang Y, Liu X, et al. Molecular engineering of chitinase from *Bacillus* sp. DAU101 for enzymatic production of chitooligosaccharides. *Enzyme Microb Technol.* 2019;124:54–62.
- [31] Yang SQ, Fu X, Yan QJ, Jiang ZQ, Liu Y. Biochemical characterization of a novel acidic exochitinase from *Rhizomucor miehei* with antifungal activity. *J Agric Food Chem.* 2016;64:461–9.
- [32] Du JH, Duan S, Miao JY, Zhang Y, Wang X, Liu Y, et al. Purification and characterization of chitinase from *Paenibacillus* sp. *Biotechnol Appl Biochem.* 2021;68:30–40.
- [33] Sirimontree P, Fukamizo T, Suginta W. Azide anions inhibit GH-18 endochitinase and GH-20 Exo β -N-acetylglucosaminidase from the marine bacterium *Vibrio harveyi*. *J Biochem.* 2015;159:191–200.

[34] Kumar M, Kumar P, Das P, Singh S, Srivastava A, Tomar RS, et al. Draft genome of *Streptomyces* sp. strain 130 and functional analysis of extracellular enzyme producing genes. *Mol Biol Rep.* 2019;46:5063–71.

Figure Legends

Fig.1. Morphological characteristics of strain CH-1. (a) Colony morphology on screening plate; (b) Gram staining ($1000\times$); (c) Scanning electron microscopy image.

Fig. 2. (a) 16S rDNA PCR amplification; (b) Phylogenetic tree based on 16S rDNA sequence.

Fig.3. Target gene amplification test of clone-positive strains. Lane M, DNA Marker; Lane 1, PCR amplification fragment of strain *E.coli* BL21(DE3)-pET28a-VpCHI-1.

Fig. 4. Domain architecture of VpCHI-1 predicted by SMART.

Fig. 5. Phylogenetic tree of VpCHI-1 with GH18 family members.

Fig. 6. The sequence comparison among VpCHI-1 and other chitinase from GH18 family.

Fig. 7. AlphaFold3-predicted three-dimensional structure of VpCHI-1 and molecular docking with (GlcNAc)₆.

Fig. 8. Electropherogram of recombinant chitinase VpCHI-1 after purification. Marker molecular weight sizes from top to bottom are 180 kDa, 130 kDa, 100 kDa, 70 kDa, 55 kDa, 40 kDa, 35 kDa, 25 kDa, 15 kDa, 10 kDa.

Fig. 9. Optimum temperature and temperature stability of chitinase VpCHI-1. (a) shows the optimal temperature analysis; (b) shows the temperature stability analysis.

Fig. 10. Optimum pH and pH stability analysis of chitinase VpCHI-1. (a) shows the optimal pH analysis; (b) shows the pH stability analysis.

Fig. 11. Effect of different concentrations of metal ions and chemical reagents on the activity of chitinase VpCHI-1.

Fig. 12. Graph of the kinetics of the enzymatic reactions of chitinase VpCHI-1.

Fig. 13. HPLC analysis of the hydrolysis products of colloidal chitin by chitinase VpCHI-1. Line A is the standard peak of (GlcNAc)₁₋₆, which represents the standard peak of GlcNAc₁-GlcNAc₆ in sequence; Lines B–F represent the product formation after degrading the substrate for 2 h, 4 h, 6 h, 8 h, and 24 h respectively.

Table 1 Enzyme kinetics parameters of VpCHI-1

Enzyme	K_m (mg/mL)	K_{cat} (s^{-1})	$K_{cat}/K_m(s^{-1}/(mg/mL))$
VpCHI-1	2.8	178.3	63.68