



OPEN Characterization of a novel halophilic and thermostable multifunctional cellulase from Ebinur Salt Lake

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Cellulase is essential for cellulose hydrolysis and is used across industries such as food, feed, textiles, biofuel, and biomass pretreatment. However, its utility is restricted by high temperatures and salt concentrations. This study identified a novel cellulase gene (named *c5-cel4*) from Ebinur Salt Lake in Xinjiang, China using metagenomic technology. Its amino acid sequence has a 90.97% similarity to the GH5 family cellulase of *Microbulbifer litoralis* (WP_250463697.1). The gene was expressed in *Escherichia coli*, and the recombinant protein, C5-CEL4, was purified via Ni-NTA affinity chromatography. C5-CEL4, secreted extracellularly (0.886 U/mL), revealed a protein size 14 KDa smaller than predicted, with mass spectrometry indicating a truncated C-terminal of 135 amino acid residues. Optimal activity was observed at 50 °C and pH 7.0, maintaining over 80% activity at 40–65 °C and 45–50 °C for 2 h. The enzyme's half-life was 60 min at 55–60 °C, retaining over 90% activity after 24 h in pH 5.0–12.0 buffers. C5-CEL4 showed activity against CMC-Na, bagasse xylan, and beech xylan, with k_{cat} values of 98.20 s⁻¹ and 12.32 s⁻¹ for CMC-Na and bagasse xylan, respectively. It also hydrolyzed wheat bran and maize stalks into reducing sugars. Remarkably, C5-CEL4 exhibited high salt tolerance, maintaining 100% activity in 0.5 M–5.0 M NaCl and after 9 months in 5.0 M NaCl. It retained over 90% activity in ionic liquids (BMIM-Ac, EMIM-Cl, BMIM-BF₄) at 50% concentration and showed resistance to heavy metal ions (Co²⁺, Cu²⁺, Ag⁺, Mn²⁺, Pb²⁺, and Ni²⁺) and inhibitors (PMSF, DTT, Tween80, and SDS). In conclusion, C5-CEL4 is a robust cellulase with heat, alkali, salt, ionic liquid, and inhibitor resistance, alongside cellulase and xylanase activity, presenting significant potential for feed, food, and bioenergy applications.

Keywords Ebinur Salt Lake, Metagenome, Halophilic, Multifunctional cellulase, Extracellular recombinant cellulase

Cellulose, a renewable organic macromolecular polysaccharide, is the most prevalent component in plant biomass¹. The efficient degradation of cellulose by cellulolytic enzymes is a crucial step in the transformation of lignocellulosic materials. The complete hydrolysis of cellulose into glucose necessitates the synergistic action of endo-1,4- β -glucanase (EC 3.2.1.4), exo-1,4- β -glucanase (EC 3.2.1.91), and β -glucosidase (EC 3.2.1.21)². Among these enzymes, endoglucanases target the amorphous regions within the cellulose molecule, initiating random cleavages that render the polymer more amenable to further hydrolysis by other cellulolytic enzymes, and are primarily responsible for cleaving internal glycosidic bonds³. Currently, cellulases find extensive applications across various industries, including pulp and paper, biofuels, food and beverage processing, textiles, animal feed, bio-nanomaterials, and phenolic compounds⁴. However, these enzymes are particularly vulnerable to

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denaturation under elevated temperatures and in saline or alkaline industrial environments, making thermal stability and salinity tolerance of paramount importance. Although numerous thermostable cellulases exhibit tolerance to salinity and polyextremophilic, only a few possess true halotolerance and the capability to maintain catalytic efficiency in high-salt conditions^{5,6}. Consequently, the identification of salinity-tolerant cellulases is advantageous for cost reduction and efficiency enhancement in industrial processes.

Saline soil, characterized by its hypersaline nature, represents a significant reservoir for various salt-tolerant cellulases. Ionic liquids (ILs), defined as nonvolatile salts that are liquid below 100 °C, have demonstrated efficacy in solubilizing cellulose, hemicellulose, and lignin from plant biomass at moderate temperatures, thereby enhancing enzymatic hydrolysis⁷. These ILs exhibit minimal contamination, high thermal and chemical stability, high polarity, and very low toxicity. However, the activity of most commercial cellulases is compromised or inhibited by even small concentrations of ILs⁸. Consequently, the identification and engineering of new enzymes with improved IL tolerance are essential for advancing lignocellulosic bioprocesses that incorporate ILs.

As one of the planet's most abundant renewable resources, cellulose's effective utilization is pivotal for mitigating the energy crisis and reducing environmental pollution⁹. The direct utilization of cellulose is hindered by its crystalline structure and the protective role of lignin¹⁰. Cellulase, a potent biocatalyst, hydrolyzes cellulose into monosaccharides such as glucose, facilitating innovative resource utilization in sectors including energy, food, and chemicals. The enzymatic hydrolysis of lignocellulose by cellulases not only enables the efficient valorization of substantial amounts of agricultural waste but also mitigates the environmental impact associated with incineration and other conventional waste treatment methods. Despite the promising potential of cellulases, challenges remain in hydrolyzing complex substrates, including high production costs and suboptimal hydrolysis efficiency¹¹. Furthermore, only a small fraction (0.1%–1%) of microorganisms can be cultured in laboratory conditions^{12,13}. Metagenomic technologies, however, offer significant advantages in screening novel cellulases¹⁴. The integration of metagenomic and gene cloning techniques facilitates the discovery of extremophilic enzymes from harsh environments, which is anticipated to address the challenges of high production costs and enhance the hydrolysis efficiency of complex lignocellulosic substrates, thereby increasing sugar yields.

In this research, a novel cellulase gene, named *c5-cel4*, was extracted from metagenomic sequences sourced from the substrate of Ebinur Salt Lake in Xinjiang, China. The gene was cloned, a recombinant plasmid was engineered, and then introduced into *Escherichia coli*. The enzymatic activity of the recombinant cellulase was evaluated through heterologous expression and protein purification. Findings indicated that the enzyme exhibits thermostability, alkali resistance, halophilicity, and tolerance to ionic liquids, highlighting its potential utility in cellulase applications within the papermaking, textile, food, feed, and biofuel industries.

Materials and methods

Sample collection and metagenomic sequencing

Samples were collected from Ebinur Salt Lake in Xinjiang, China (45°09'35" N, 83°53'21" E), where the surface temperature was approximately 7.8 °C and the pH was 8.49. Bottom sediment samples were immediately frozen on dry ice for subsequent DNA extraction and metagenomic sequencing. DNA was extracted using the PowerSoil Kit (MOBIO, USA). Metagenomic sequencing was carried out by Suzhou GENWIZ using a HiSeq 2500 platform, and sequence data were analyzed via the IMG server (<https://img.jgi.doe.gov/cgi-bin/mer/main.cgi>).

Cellulase gene prediction and sequence analysis

A cellulase gene sequence, designated *c5-cel4*, was identified from the metagenomic database through functional prediction. The nucleotide sequence of the *c5-cel4* gene was deposited in GenBank (accession NO.: PV014881). The amino acid sequence was translated using the ExPASy-Translate tool, and the protein sequence was analyzed using the BLASTp program (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>). Multiple sequence alignments and homology modeling were performed using RCSB PDB, SWISS-MODEL, AlphaFold Protein Structure Database, MEGA 7, and ESPript 3.0. Signal peptides were predicted using SignalP (<http://www.cbs.dtu.dk/services/SignalP/>), and protein domains were predicted using InterPro (ebi.ac.uk). Phylogenetic analysis was conducted using MEGA 7, with phylogenetic trees constructed via maximum likelihood and Poisson correction models.

Cloning and heterologous expression of C5-CEL4 gene

Primers for amplifying the full-length *c5-cel4* gene were designed using Primer 5.0: *TFH-c5-cel4-F* (CATCATCATCATCATGAAATGATATTCGGCGCAGGTGCCAG) and *TFH-c5-cel4-R* (GTGCTCGAGTGCGGCCGCAAGTCAGTTGGCGCTGCATTCCGGC). PCR amplification was performed with TransStarFastPfu Fly DNA polymerase (TransGen Biotech, China). The underlined sequences represent regions amplified by BamTech. The PCR protocol involved initial denaturation at 95 °C for 3 min, followed by 29 cycles of 98 °C for 20 s, 55 °C for 30 s, and 72 °C for 2 min, with a final extension at 72 °C for 10 min. PCR products were verified by 1.0% agarose gel electrophoresis (120 V, 25 min) and visualized under UV light. Bands matching theoretical sizes were sequenced, purified, and recovered using a gel extraction kit as per the manufacturer's instructions. The PCR product was cloned into the *pSHY211* vector¹⁵ using the pEASY-Uni Seamless Cloning and Assembly Kit (TransGen Biotech, China) to create the expression plasmid *pSHY211-c5-cel4*. *Escherichia coli* DH5α was employed for cloning and heterologous expression.

The strain *E. coli* DH5α-*pSHY211-c5-cel4* was initiated in 15 mL LB broth with 100 µg/mL kanamycin at 37 °C and 180 RPM overnight to prepare the seed culture. A 5% inoculum of this seed culture was then transferred into a mixed medium consisting of 200 mL Glycerol-based salt medium (Glycerol 20 g/L, (NH₄)₂SO₄ 4 g/L, MgSO₄·(7H₂O) 1.2 g/L, CaCl₂ 0.3 g/L, K₂HPO₄ 1 g/L, KH₂PO₄ 1 g/L, NaNO₃ 1 g/L, pH 7.0) and 100 mL LB (yeast extract 5 g/L, peptone 10 g/L, NaCl 10 g/L, pH 7.0) with 100 µg/mL kanamycin, incubated at 37 °C and

180 RPM for 36 h with continuous shaking. The culture was centrifuged at 4,000 RPM for 30 min to harvest the supernate, which served as the extracellular crude enzyme solution.

Purification of intracellular and extracellular Recombinant cellulase C5-CEL4

The biomass was isolated, resuspended in 25 mL Phosphate Buffered Saline (pH 7.6) with 10 mM imidazole, and sonicated in an ice-water bath for cell disruption. The lysate was centrifuged at 4 °C and 12,000 RPM for 20 min, and the supernate was collected as the intracellular crude enzyme solution. Both intracellular and extracellular enzyme solutions were filtered and applied to an equilibrated Ni-NTA affinity chromatography column (HisTrap, TransGen Biotech, China).

The Ni-NTA affinity chromatography column was resuspended in a 500 mL conical flask and agitated at ≤ 15 °C at 150 RPM for 1.5 h to facilitate protein binding. Intracellular and extracellular proteins were purified and characterized for recombinant cellulase as described by Yin et al. (2017)¹⁶. Protein concentrations were quantified with a Bradford assay kit (Order NO. C503031, Sangon Biotech, China), using bovine serum protein as the standard. Purified C5-CEL4 enzyme was resolved on a 12% SDS-PAGE, and protein bands were visualized with Coomassie Brilliant Blue R-250. Cellulase activity in the gel was determined via an enzyme profiling method. Specifically, samples were loaded onto a 12% SDS-acrylamide gel containing 0.5% (w/v) CMC-Na. Post-electrophoresis, the gel was treated with 2.5% (v/v) Triton X-100 (pH 7.0) for 30 min, soaked in 200 mM phosphate buffer for 15 min, and incubated in phosphate buffer (pH 7.0) at 45 °C for 1 h. The gel was then stained with 0.2% (w/v) Congo red for 10 min at 25 °C, and excess dye was removed with 1 M NaCl until clear active bands appeared against the gel background.

Analysis of extracellular Recombinant cellulase C5-CEL4 by mass spectrometry

The protein bands were excised into 1 mm³ pieces and placed into 1.5 mL Eppendorf tubes. They were decolorized with a 50% ACN (acetonitrile)-50% 50 mM NH₄HCO₃ (ammonium bicarbonate) solution for 10–30 min, followed by aspiration and disposal of the solution. This step was repeated until the pellets became colorless. Subsequently, 1000 μ L of 100% ACN was added, incubated for 30 min until the pellets turned white and compact, then the ACN was discarded, and the pellets were air-dried.

For reduction and alkylation, 100 μ L of 10 mM DTT (dithiothreitol) was added to the sample and incubated in a 56 °C water bath for 1 h. The supernate was removed and discarded. Then, 100 μ L of 20 mM IAM (iodoacetamide) was added, and the sample was incubated in the dark at room temperature for 1 h, followed by removal and disposal of the supernate. The sample was then treated with 500 μ L of decolorizing solution, vortexed, and the solution was aspirated and discarded. Another 1000 μ L of 100% ACN was added, and the pellets were incubated until they turned white and compact, then the ACN was discarded, and the pellets were air-dried.

Next, 50 μ L of trypsin (25 ng/ μ L in 50 mM NH₄HCO₃) was added, and the sample was sealed and incubated at 37 °C for 16 h. For peptide extraction, 100 μ L of extraction solution (5% trifluoroacetic acid (TFA)-50% ACN-45% water) was added, and the sample was incubated at 37 °C for 1 h. The sample was sonicated for 5 min, centrifuged for 5 min, and the supernate was transferred to a new Eppendorf tube. This extraction process was repeated once, and the combined extracts were dried via vacuum centrifugation. Post-digestion, peptides were desalted using a desalting column and dried in a vacuum centrifugal concentrator at 45 °C.

Data acquisition was performed using an Easy-nLC 1200 coupled with a Q Exactive™ Hybrid Quadrupole-Orbitrap™ Mass Spectrometer. Chromatographic separation involved mobile phase A (0.1% formic acid in water) and mobile phase B (0.1% formic acid, 80% ACN in water) with a gradient: 0–2 min, 4–8% B. 2–35 min, 8–28% B. 35–55 min, 28–40% B. 55–56 min, 95% B. and 56–66 min, 95% B. The flow rate was set at 0.6 μ L/min through a column of 150 μ m i.d. \times 170 mm, packed with Reprosil-Pur 120 C18-AQ 1.9 μ m particles.

Mass spectrometry parameters for full MS were: resolution 70,000, AGC target 4e8, maximum IT 20 ms, scan range 300–1800 m/z, and data type profile. For dd-MS2: resolution 17,500, AGC target 1e5, maximum IT 50 ms, TopN 20, and stepped NCE 30. Data analysis was conducted using Byonic 4.2.2 (<https://www.proteinmetrics.com/products/byonic>) software.

Molecular cloning and functional comparison of GH5 family DNA fragments of C5-CEL4

Primer 5.0 was employed to design concatenated primers: *c5-cel4-gh5-F2* (CATCATCATCATCATCATGAA ATGATATTCGGCGCAGGTGC) and *c5-cel4-gh5-R2* (GTGCTCGAGTGCGCCGCAAGTCAGCTGGAGC TCGAAGAGGAA) to amplify the *c5-cel4-gh5* gene. The *c5-cel4-gh5* gene was cloned, expressed, and purified analogously to *c5-cel4*. Monoclonal strains of C5-CEL4 and C5-CEL4-GH5 were cultured in a mixed liquid medium comprising 6 mL of basal salts and 3 mL of LB broth with 100 μ g/mL kanamycin. Each strain was inoculated into three tubes as replicates and incubated at 37 °C with shaking at 180 RPM for 36 h. Post-incubation, cultures were chilled at 4 °C, centrifuged at 12,000 RPM for 20 min, and the supernate was harvested as the extracellular crude enzyme solution. *Escherichia coli* biomass was collected, resuspended in 5 mL of Phosphate Buffered Saline (pH 7.6), disrupted by ultrasonic fragmentation in an ice-water bath, and centrifuged at 4 °C, 12,000 RPM for 20 min to obtain the intracellular crude enzyme solution. The enzymatic activities of C5-CEL4 and C5-CEL4-GH5 in both intracellular and extracellular fractions were compared.

Enzyme activity assay

Activity against CMC was determined by measuring the release of reducing sugar, with 1% (w/v) CMC as substrate, by the 3,5-dinitrosalicylic acid (DNS) assay. One unit (U) of CMCase activity was defined as the amount of enzyme to release 1 μ mol glucose-equivalent reducing sugars per minute¹⁷. The assay involved adding

10 μL of purified enzyme to 90 μL of an optimal pH buffer containing 1% CMC-Na, incubating at the optimal temperature for 30 min, and terminating the reaction with 150 μL of DNS. The mixture was heated at 90 °C for 10 min for color development, cooled, and 150 μL was transferred to a 96-well plate for absorbance measurement at 540 nm using a microplate reader. Each experiment was conducted in triplicate with one control.

Biochemical characterization

The optimal pH of purified C5-CEL4 was determined across a pH range of 3.0–10.0 using citrate-disodium phosphate buffer for pH 3.0–8.0 and glycine-sodium hydroxide buffer for pH 8.0–10.0. The optimal temperature was identified by measuring enzyme activity at temperatures from 20 to 75 °C under the optimal pH conditions. Thermal and pH stability were assessed by measuring residual enzyme activity after incubation at various temperatures (40, 45, 50, 55, and 60 °C) for time intervals (0, 20, 40, 60, 80, 100, and 120 min), and across a pH gradient (3.0–13.0) for exposure durations of 4 °C, 12 h, and 24 h.

Influence of salts, ionic liquids, metal ions, and chemical reagents on C5-CEL4

The optimal concentration of NaCl (ranging from 0 to 5.0 M) was established at 50 °C and pH 7.0. The purified enzyme solution was incubated at 4 °C in varying salt concentrations (0–5.0 M) for nine months, after which the residual enzyme activity was assessed without a salt removal procedure. The surface electrostatic potential of C5-CEL4 was predicted using AlphaFold Protein Structure Database and PyMOL 2.6 software. The influence of different ionic liquids (1-butyl-3-methylimidazole tetrachloroborate BMIM-BF₄, 1-butyl-3-methylimidazole acetate BMIM-Ac, and ethyl-3-methylimidazolium chloride EMIM-Cl) in varying concentrations (1%, 2.5%, 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, and 50% w/v) on enzyme activity was investigated at 50 °C and pH 7.0. The purified enzyme solution was incubated at 4 °C for 24 h in the aforementioned ionic liquid solutions (1%, 5%, 10%, 20%, 30%, 40%, 45%, and 50% w/v) at pH 7.0, and the residual enzyme activity was measured.

The effects of metal ions, chemical reagents, and ionic liquids on C5-CEL4 activity were studied using 1 mM and 10 mM concentrations of various metal ions (Na⁺, K⁺, Mg²⁺, Fe³⁺, Ca²⁺, Zn²⁺, Co²⁺, Cu²⁺, Ag⁺, Mn²⁺, Pb²⁺, and Ni²⁺), and 0.1% and 1% concentrations of different chemical reagents (EDTA, PMSF, DTT, Tween 80, SDS, CTAB—corresponding to ethylenediaminetetraacetic acid, phenylmethylsulfonyl fluoride, dithiothreitol, polysorbate-80, sodium dodecyl sulfate, and cetyltrimethylammonium bromide, respectively). Additionally, 1% and 10% concentrations of various alcohols (methanol, ethanol, isopropanol, and β -mercaptoethanol) were incorporated into the enzyme solution reaction system. Enzyme activity was compared to the control (untreated enzyme solution, set as 100%).

Substrate specificity and kinetic constants of C5-CEL4

Experiments were conducted under controlled conditions identical to those described above, excluding any additives in the reaction mixture. Substrate specificity of C5-CEL4 intracellular and extracellular enzymes was examined using CMC-Na, bagasse xylan, beechwood xylan, corn cob xylan, Avicel, and cellobiose (each at 1% w/v). Kinetic constants of C5-CEL4 intracellular and extracellular enzymes were determined using varying concentrations of CMC-Na and bagasse xylan (2–20 mg/mL), incubated for 5/10 minutes at 50 °C and pH 7.0. The Michaelis-Menten constant (K_m) and the maximum reaction velocity (V_{max}) were calculated.

TLC analysis

A reaction mixture containing 1% CMC-Na and 10 μg of purified enzyme was incubated under optimal pH and temperature conditions for 2 h. Hydrolysis products were analyzed using thin-layer chromatography (TLC) on silica gel 60 plates (Merck, Darmstadt, Germany). The solvent system used was 1-butanol/acetic acid/water (2:1:1 v/v/v). Sugars were detected post-treatment at 120 °C for 10 min with a freshly prepared 5% (v/v) H₂SO₄ ethanol spray. Sugar standards included glucose (G1), cellulosic disaccharide (G2), cellulosic trisaccharide (G3), and cellulosic tetrasaccharide (G4).

Utilization of C5-CEL4 in the enzymatic degradation of agricultural straw

The raw substrates of wheat bran and corn stalks obtained from the market were washed with tap water to eliminate impurities and then air-dried. The dried substrates were ground and sieved. A 5 g portion of the substrate was mixed with 100 mL of distilled water and subjected to a water bath at 90 °C for 4 h. Post-treatment, the substrate was rinsed with distilled water until the rinse water was clear. The substrate was then dried in an oven at 50 °C for subsequent use. The dried substrate was divided into four 0.2 g portions, placed into 10 mL tubes, and combined with 4 mL of a pH 7.0 citric acid-disodium hydrogen phosphate buffer. The mixture was stirred thoroughly and left to soak overnight. The supernate was centrifuged at 12,000 RPM for 15 min, and the supernate was discarded. To three experimental groups, 0.05 mg/mL of C5-CEL4 was added to the substrate, while the control group received no enzyme solution. All groups were adjusted to a final volume of 5 mL with the same buffer. The mixtures were shaken well and incubated at the optimal temperature, with reducing sugar yield monitored every 2 h.

Statistical analysis

Experiments were performed in triplicate unless otherwise noted, and the results were expressed as mean values. Statistical analyses were conducted using SPSS 20.0, with results presented as mean \pm SEM. One-way ANOVA followed by Tukey's test was used to compare multiple groups (0.01 < p-value < 0.05).

Results

Metagenomic sequencing and sequence analysis of C5-CEL4

Metagenomic sequencing of DNA extracted from the sediment of Ebinur Salt Lake yielded a comprehensive dataset comprising 4.2 Gbp and including 32,394 contigs longer than 500 bp. This dataset enabled the identification of 135,361 unique genes, among which 15 were predicted to be cellulase genes. Based on gene integrity and sequence novelty, a new cellulase gene, named *c5-cel4*, was identified. The gene was sequenced, and the absence of a stop codon within the ORF (Open Reading Frame) was confirmed. The *c5-cel4* gene is 1,347 bp long, encoding 448 amino acids, and lacks a signal peptide, indicating cytoplasmic localization. The deduced protein, without a signal peptide, comprises 448 amino acids with a calculated molecular weight of 47.54 kDa and a theoretical pI of 4.51.

Comparative analysis with NCBI data showed a 90.97% similarity between C5-CEL4 and GH5 family cellulases from *Microbulbifer litoralis*, based on the expressed amino acid sequences. Phylogenetic analysis indicated that C5-CEL4 clusters with a cellulase from *Microbulbifer halophilus* (GenBank: WP 265722981.1) with 85% support, identifying it within the glycosyl hydrolase family (Supplementary Figure S1). This protein shares 70% similarity with GH5 family endoglucanases from *Cellvibrio japonicus* (PDB: 8BQA, 8BQC). Multiple sequence alignments demonstrated high homology of C5-CEL4 with GH5 family cellulases 8BQA, 8BQC, 8C10, 4M1R, and endoglucanase 1EGZ (Supplementary Figure S2). Structural analysis confirmed that C5-CEL4 contains a catalytic domain typical of GH5 cellulases (Fig. 1a). Homology modeling via SWISS-MODEL yielded a GMQE value of 0.94 with GH5 endoglucanases from *Microbulbifer rhizosphaerae*. The protein exhibits a conserved (β/α)₈ TIM barrel fold with eight loops around the active site, contributing to its catalytic versatility, substrate specificity, thermal stability, and pH stability (Fig. 1b).

Heterologous expression, and purification of C5-CEL4

The *c5-cel4* was cloned into the *pSHY211* C-His vector (with 6 His tags) and confirmed by sequencing. Recombinant C5-CEL4 was secreted extracellularly, with optimal fermentation conditions at 37 °C for 36 h, yielding an enzyme specific activity of 0.886 U/mL (Fig. 3a). Both intracellular and extracellular crude enzyme solutions were purified using a Ni-NTA affinity chromatography column, and SDS-PAGE analysis showed a ~33 kDa band for both, which differed from the theoretical size (47.54 kDa) by about 14 kDa. (Fig. 2, The original figure is in Supplementary Figure S3.). Sequence analysis indicated the presence of a His tag at the N-terminus but not at the C-terminus, which lacked a stop codon. Consequently, the C-terminus was selected for protein sequencing analysis.

The LC-MS/MS-based C-terminal sequencing¹⁸ of the C5-CEL4 protein revealed its terminal sequence as IVRGWDGGGGSSSSSSSSSS, ending at amino acid 314 (Supplementary Figure S4). Consequently, the theoretical molecular weight and isoelectric point of the expressed protein were 34.12 kDa and 4.69, respectively. This premature termination led to a truncated protein, with its actual molecular mass matching the theoretical value (34.12 kDa), confirming the successful expression and purification. Zymography detected cellulase activity, displayed as a clear band on a red background (Fig. 2). Due to the premature termination, the C-terminal region (residues 315 to 448) containing the CBM domain was absent. Primers were designed to amplify the catalytic domain (C5-CEL4-GH5), to obtain the recombinant target protein C5-CEL4-GH5 (Supplementary Figure S5). Activity assays revealed similar intracellular activities for C5-CEL4 and C5-CEL4-GH5, though their extracellular activities differed significantly. The extracellular activity of C5-CEL4-GH5 was notably low, likely due to the absence of the sequence necessary for secretion in *Escherichia coli* (Fig. 3b).

The influence of temperature and pH on C5-CEL4 activity

The optimal temperature for both intracellular and extracellular pure enzyme activities is 50 °C, maintaining above 80% activity between 35 °C and 60 °C (Fig. 4a). The optimal pH for both activities is 7.0, with over 70% of maximal activity preserved within a pH range of 6.0 to 8.0 (Fig. 4b). Thermal stability analysis indicates that C5-CEL4 retains 100% activity after 2 h at 40 °C and 74% after the same period at 50 °C, with a half-life of 70 min at 55 °C and 60 °C (Fig. 4c). pH stability analysis shows the enzyme maintains over 50% of its initial activity across a pH range of 3.0 to 13.0. Following incubation at 4 °C for 12 and 24 h, the enzyme's activity remains above 80% within the pH range of 4.0 to 12.0 (Fig. 4d).

Impact of salts and ionic liquids on C5-CEL4

As depicted in Fig. 5a, the optimal NaCl concentration for C5-CEL4 enzymatic activity ranged from 2.5 to 3.0 M, with activity levels exceeding 100% even at a near-saturated concentration of 5.0 M. The enzyme maintained its activity across a broad NaCl concentration spectrum from 0.5 to 5.0 M. Salinity tolerance analysis revealed that C5-CEL4 preserved approximately 100% or higher activity at NaCl concentrations between 0.5 and 5.0 M after prolonged exposure of 9 months at 4 °C (Fig. 5b). Furthermore, the enzyme exhibited near 100% activity when 1 mM or 10 mM of NaCl, NaNO₃, or Na₂SO₃ were incorporated into the reaction mixture, suggesting that these sodium salts did not significantly impact enzymatic function (Supplementary Table S1). As shown in Fig. 6a, b, the higher prevalence of acidic amino acids on the enzyme's surface contributes to a negative overall electrostatic potential, which enhances hydrophilicity and reduces hydrophobicity. In contrast, the GH5-family cellulase GH5_4¹⁹ (PDB code: 6XSU) from *Ruminococcus flavefaciens* has a lower amount of acidic amino acids on its surface (Fig. 6c, d). The GH5-family cellulase is structurally similar to our enzyme. This modification improves the enzyme's water-binding capacity and prevents aggregation in high-salt environments²⁰. C5-CEL4's activity increased to over 110% in the presence of 1%-10% EMIM-Cl ionic liquids (Fig. 5c) and maintained over 60% relative activity at 20% ionic liquid concentration. Additionally, the enzyme retained about 90% or greater activity and was marginally activated following 24 h of incubation in 40% ionic liquids (Fig. 5d). These results confirm that C5-CEL4 is resilient to both salts and ionic liquids.

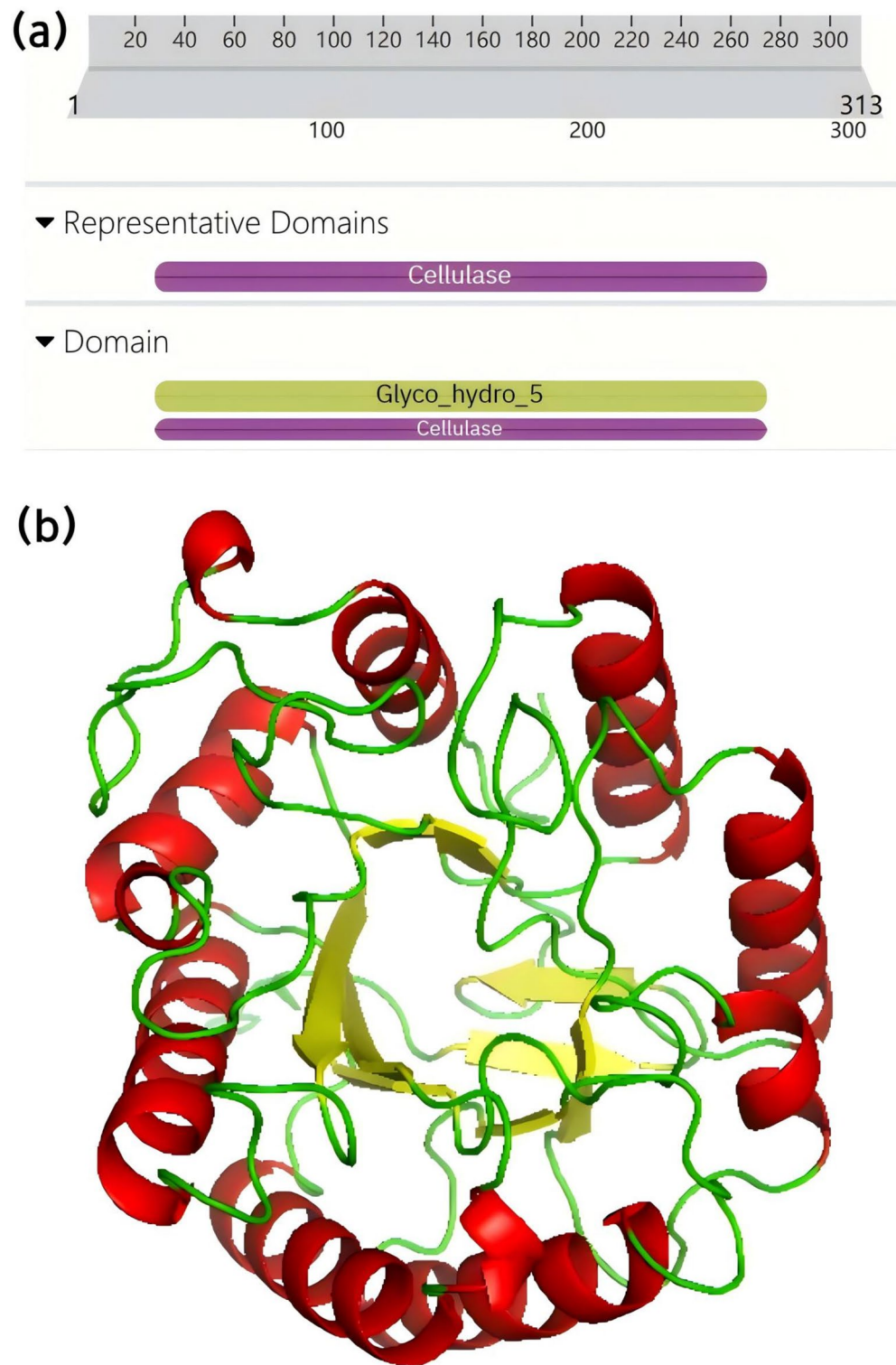


Fig. 1. Protein structure analysis of C5-CEL4. **(a)**, structural domain prediction of the protein sequence of C5-CEL4 using InterPro. The structure describes a cellulase catalytic structural domain (CD). **(b)**, GH5 family cellulase C5-CEL4, homology modeled on the SWISS-MODEL server (<https://swissmodel.expasy.org/interactive>) using a *Microbulbifer rhizosphaerae*-derived GH5 family endoglucanase as a template (UniProt accession no: A0A7W4WCK3).

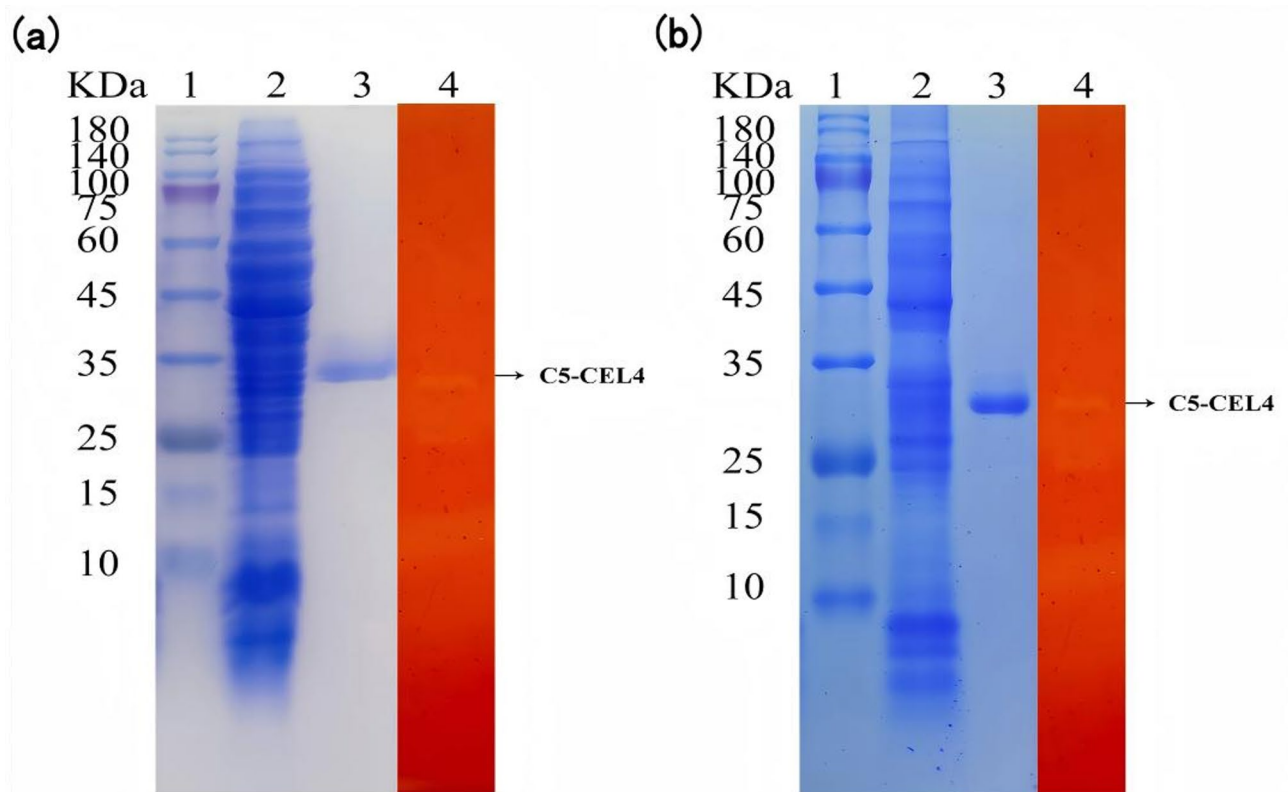


Fig. 2. SDS-PAGE analysis and enzyme activity profiling of recombinant C5-CEL4 intracellular and extracellular purified enzyme. **(a)**, extracellular purified enzyme. **(b)**, intracellular purified enzyme. Lane 1, protein molecular weight marker. Lane 2, *E. coli* DH5 α /pSHY211-*c5-cel4* lysate. Lane 3, purified C5-CEL4. Lane 4, zymogram of purified enzyme.

Influence of metal ions and chemical reagents on C5-CEL4

As presented in Supplementary Table S1, C5-CEL4 activity was significantly enhanced by 1 mM Co²⁺ (114.4 ± 1.1%), 1 mM Mn²⁺ (135.6 ± 8.7%), and 10 mM Mn²⁺ (212.4 ± 0.4%). Conversely, the enzyme was moderately inhibited by 1 mM and 10 mM concentrations of K⁺, Mg²⁺, Fe³⁺, Ca²⁺, Zn²⁺, Cu²⁺, Ag⁺, and Ni²⁺, and exhibited about a 9% reduction in activity with 10 mM Pb²⁺. Specifically, Co²⁺ increased activity by approximately 14%, whereas Mn²⁺ significantly boosted activity by roughly 2.1-fold. The inhibitors EDTA, PMSF, and DTT at 0.1% concentration had negligible effects on C5-CEL4. However, EDTA and PMSF at 1.0% concentration mildly inhibited the enzyme. At 0.1%, Tween 80 enhanced enzymatic activity by about 37% and showed slight activation at concentrations up to 1%. In contrast, SDS and CTAB at both 0.1% and 1.0% concentrations slightly inhibited enzyme activity. Methanol, ethanol, and isopropanol at 1% and 10% concentrations had no significant effect on enzyme activity. The enzyme retained 76% activity in 1% β -ME but was completely inactivated at 10% concentration.

Substrate specificity and kinetic constants of C5-CEL4

The substrate specificity and kinetic constants of C5-CEL4 were detailed in Supplementary Table S2 and Table 1. Both extracellular and intracellular C5-CEL4 demonstrated activities for CMC-Na (24.36 ± 0.23 U/mg and 19.6 ± 3.47 U/mg), bagasse xylan (13.24 ± 0.61 U/mg and 2.5 ± 0.77 U/mg), and beechwood xylan (14.06 ± 0.53 U/mg and 1.48 ± 2.01 U/mg), respectively, while showing no activity for corn cob xylan, Avicel, and cellobiose. The V_{max}, K_m, K_{cat}, and K_{cat}/K_m values for extracellular C5-CEL4 with CMC-Na were 158.73 μ mol/min/mg, 45.03 mg/mL, 98.20 S⁻¹, and 2.18, respectively. For intracellular C5-CEL4 with CMC-Na, these values were 357.14 μ mol/min/mg, 101.71 mg/mL, 220.95 S⁻¹, and 2.17, respectively. The kinetic parameters V_{max}, K_m, K_{cat}, and K_{cat}/K_m for extracellular C5-CEL4 with bagasse xylan were 15.55 μ mol/min/mg, 16.05 mg/mL, 12.32 S⁻¹, and 0.77, respectively. However, these parameters were not determined for intracellular C5-CEL4 with bagasse xylan.

Thin-layer chromatography (TLC) analysis of CMC-Na hydrolyzed by C5-CEL4

The hydrolysis products of CMC-Na by C5-CEL4 were analyzed using TLC, as illustrated in Fig. 7. Results indicated that C5-CEL4 cleaved cellulose oligosaccharide chains randomly, producing cellulotriose (G3) and other fiber oligosaccharides.

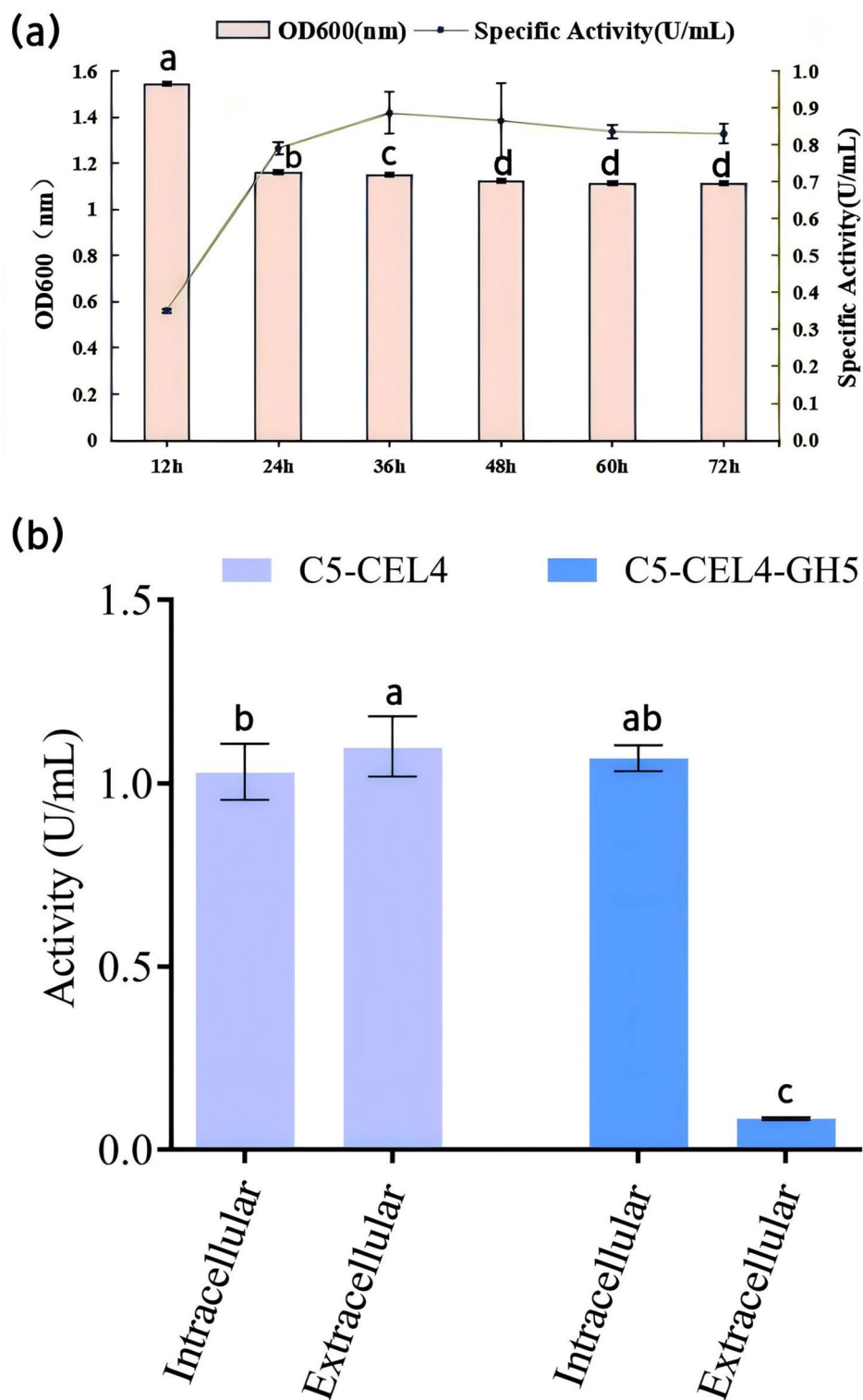


Fig. 3. Monitoring of extracellular crude enzyme activity of C5-CEL4 heterologous expression in *Escherichia coli*(a), and comparison of intracellular and extracellular cellulase activities of C5-CEL4 and C5-CEL4-GH5 (b). The error line represents \pm SEM ($n=3$). Different letters (a, b, c, d) indicate significant differences between groups ($p < 0.05$, it was derived through multiple comparative analyses conducted using the Duncan method in SPSS.). The same letters indicate no significant differences between groups.

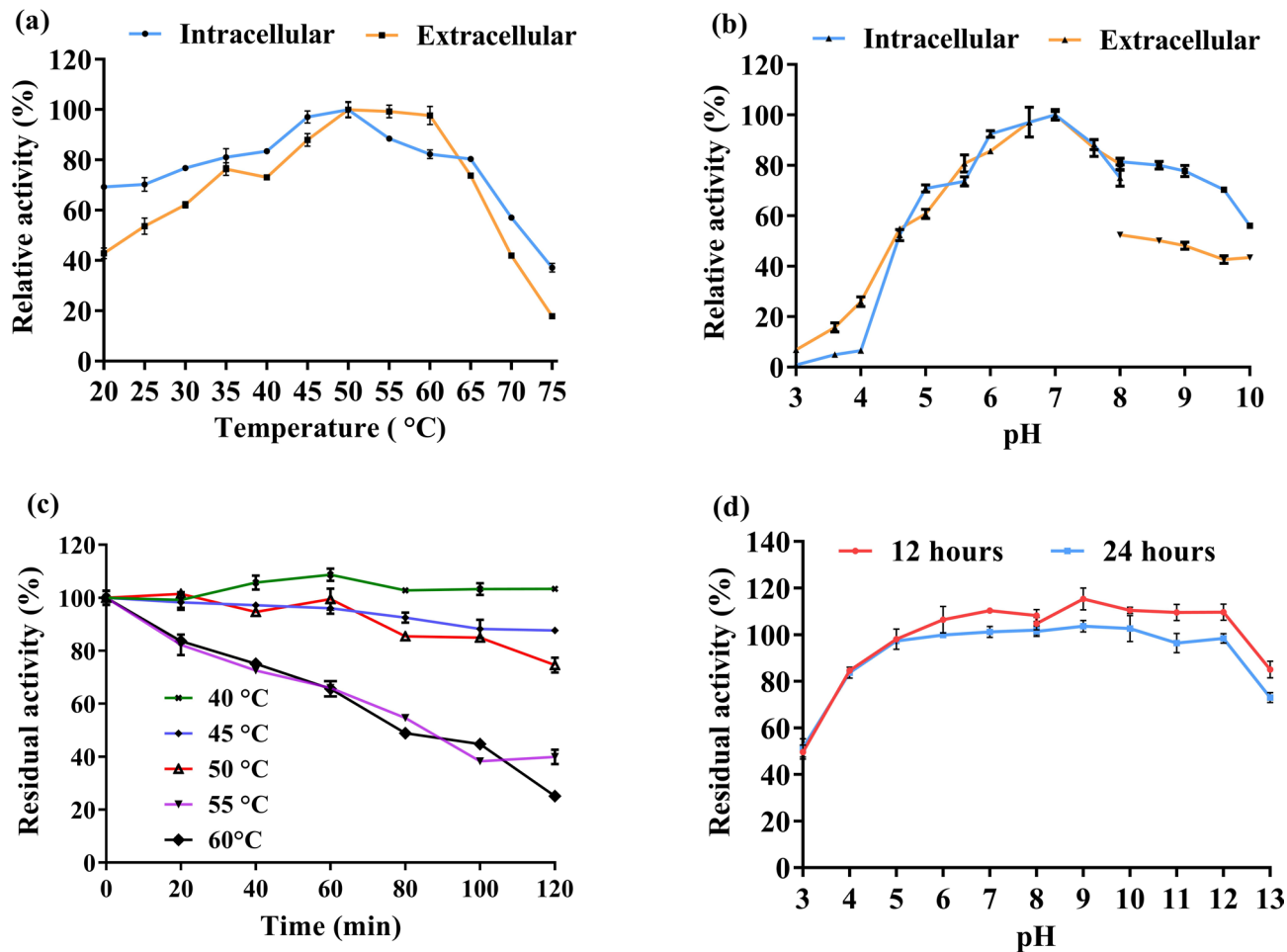


Fig. 4. Effect of temperature and pH on the activity and stability of recombinant cellulase C5-CEL4. (a), effect of temperature on the activity of C5-CEL4. (b), effect of pH on the activity of C5-CEL4. (c), effect of temperature on the stability of intracellular enzyme C5-CEL4. (d), effect of pH on the stability of intracellular enzyme C5-CEL4. Initial activity was 100%, and each value in the graphs represents the mean \pm SEM ($n = 3$), 100% of extracellular enzyme = 24.36 ± 0.23 U/mg and 100% of intracellular enzyme = 19.6 ± 3.47 U/mg.

Analysis of sugar production from agricultural straw hydrolyzed by C5-CEL4

As depicted in Fig. 8, C5-CEL4 hydrolyzed wheat bran and cornstalk crude substrates. The hydrolysis of wheat bran was more effective, with reducing sugar concentration increasing to approximately $1.2 \mu\text{mol/mL}$ at 2 h and stabilizing at $3 \mu\text{mol/mL}$ after 10 h. In contrast, the hydrolysis of cornstalks was less efficient, with a maximum sugar concentration of about $0.2 \mu\text{mol/mL}$ at 4 h before ceasing entirely. Hydrolysis of wheat bran and cornstalk by C5-CEL4 showed its ability to degrade both cellulose and hemicellulose polymers. It means that C5-CEL4 may be an excellent candidate for hydrolysis of agricultural residues.

Discussion

Endoglucanases from the GH5 family, integral to hybrid enzymes for biomass conversion, necessitate considerable thermal stability²¹. Current research identifies optimal reaction temperatures for most GH5 enzymes at 50°C and above (Table 2). Notably, C5-CEL4 exhibits robust enzymatic activity across a broad temperature spectrum, with an optimal reaction temperature of 50°C , indicating its potential as a thermostable cellulase. This higher thermal stability is crucial for industrial, animal husbandry, and biomass conversion applications. Enhancing the economic feasibility of industrial processes hinges on the optimal temperature and thermal stability of potent endoglucanases²². Our findings reveal that, unlike most GH5 endoglucanases which exhibit low alkaline tolerance and pH stability, C5-CEL4 maintains high activity at a higher optimal pH range of 5.0–9.6 (Table 2). The pH stability of C5-CEL4 is on par with the salt-tolerant AgCMCase from *Aspergillus glaucus* CCHA, both retaining over 40% residual activity from pH 4.0 to 10.0. Consequently, C5-CEL4 is an excellent candidate for industries in alkaline conditions, such as textiles, paper, and detergents. In summary, the moderate thermal stability and extensive pH tolerance of C5-CEL4 present significant potential in biomass conversion applications.

C5-CEL4 also exhibits superior salt tolerance relative to other GH5 family endoglucanases (Table 2). Structural analyses suggest an abundance of acidic amino acids on the protein surface, imparting a negative charge that enhances solubility in high-salt solutions by forming hydrated ion networks with cations and

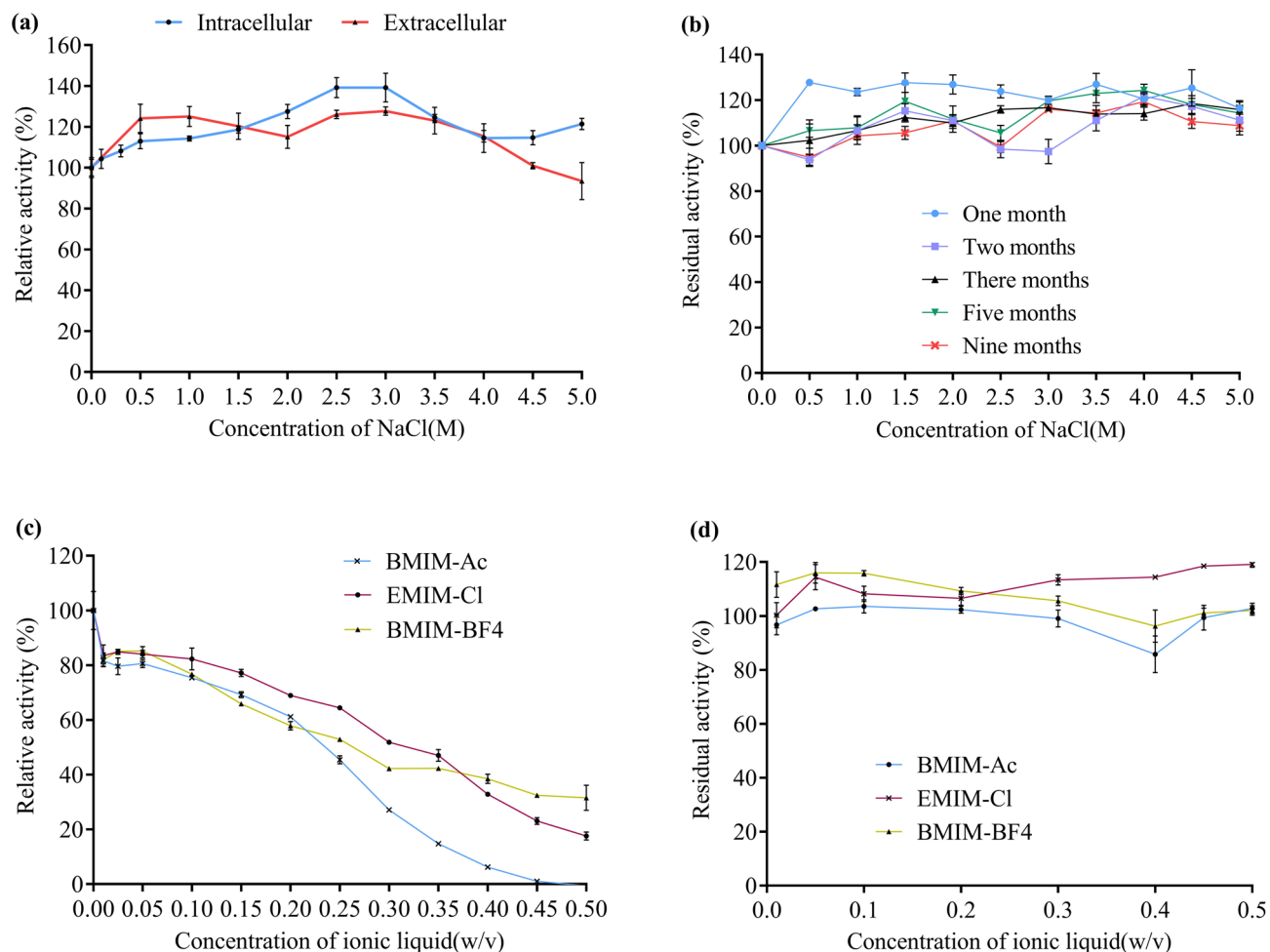


Fig. 5. Impact of NaCl and ionic liquids on C5-CEL4 activity. **(a)**, influence of NaCl on the catalytic performance of intracellular and extracellular C5-CEL4 enzymes. **(b)**, stability assessment of intracellular C5-CEL4 enzyme under varying NaCl concentrations after a 9-month incubation period. **(c)**, effect of ionic liquid solutions on the enzymatic activity of intracellular C5-CEL4. **(d)**, stability evaluation of intracellular C5-CEL4 enzyme in ionic liquid solutions following 24 h of incubation at 4 °C. The initial enzymatic activity was standardized to 100%, with each graphical data point representing the mean \pm SEM, where 100% corresponds to 19.6 ± 3.47 U/mg.

preventing protein aggregation through surface electrostatic repulsion. The salt tolerance mechanisms of most of the salt-tolerant cellulases reported so far are associated with a higher distribution of negative charges on their protein surfaces (Table S3)^{27–30}. Lignocellulosic biomass in saline soils is challenging for microbial degradation due to high salt concentrations. However, salt-tolerant cellulases maintain high activity and stable catalytic performance in such environments³¹. The application of salt-tolerant cellulases can facilitate the degradation of saline lignocellulosic biomass. Acid or alkali pretreatment of lignocellulosic biomass generates significant salt during pH adjustment, impairing the efficiency of salt-intolerant cellulases³².

Therefore, salt-tolerant cellulases are preferable for degrading pretreated lignocellulosic biomass, offering greater application potential than conventional cellulases. The high salt tolerance of C5-CEL4 suggests its utility in the feed, textile, and biofuel industries. It has been postulated that common structural motifs within the GH5 family contribute to the relative resistance of these enzymes to ionic liquids³³. C5-CEL4 retained over 90% activity after 24 h in 50% BMIM-Ac, EMIM-Cl, and BMIM-BF4. In contrast, GH5 family Cel5A activity dropped to 50% after 5 h in 2.5 M BMIM-Cl at room temperature³⁴. Cellulase from *Trichoderma reesei* showed a decline to 31% activity after 24 h in 30% EMIM-Ac³⁵. Additionally, *Aspergillus fumigatus* endoglucanase retained only about 80% activity after 12 h in 10% EMIM-Ac³⁶. Compared to these studies, C5-CEL4 demonstrates exceptional ionic liquid tolerance, underscoring its potential for biomass conversion applications.

Some metal ions protect enzymes from thermal denaturation and can be relied upon to promote their activity and stability at higher temperatures³⁷. Therefore, it is clear that Mn^{2+} and Co^{2+} protected C5-CEL4 from thermal denaturation during the reaction at 50 °C, instead promoting its catalytic activity. It has been shown that Mn^{2+} has a protective effect against thermal inactivation of the enzyme isolated from *Escherichia coli*^{38,39}. Previous studies have demonstrated that Co^{2+} and Mn^{2+} augment the enzymatic activity of various enzymes, including GH5 family endoglucanases such as EG5C⁴⁰, TrepCel3/TrepCel4⁴¹, CEL-5A⁴², Cel776Sc⁴³, and CelRH5⁴⁴. This highlights

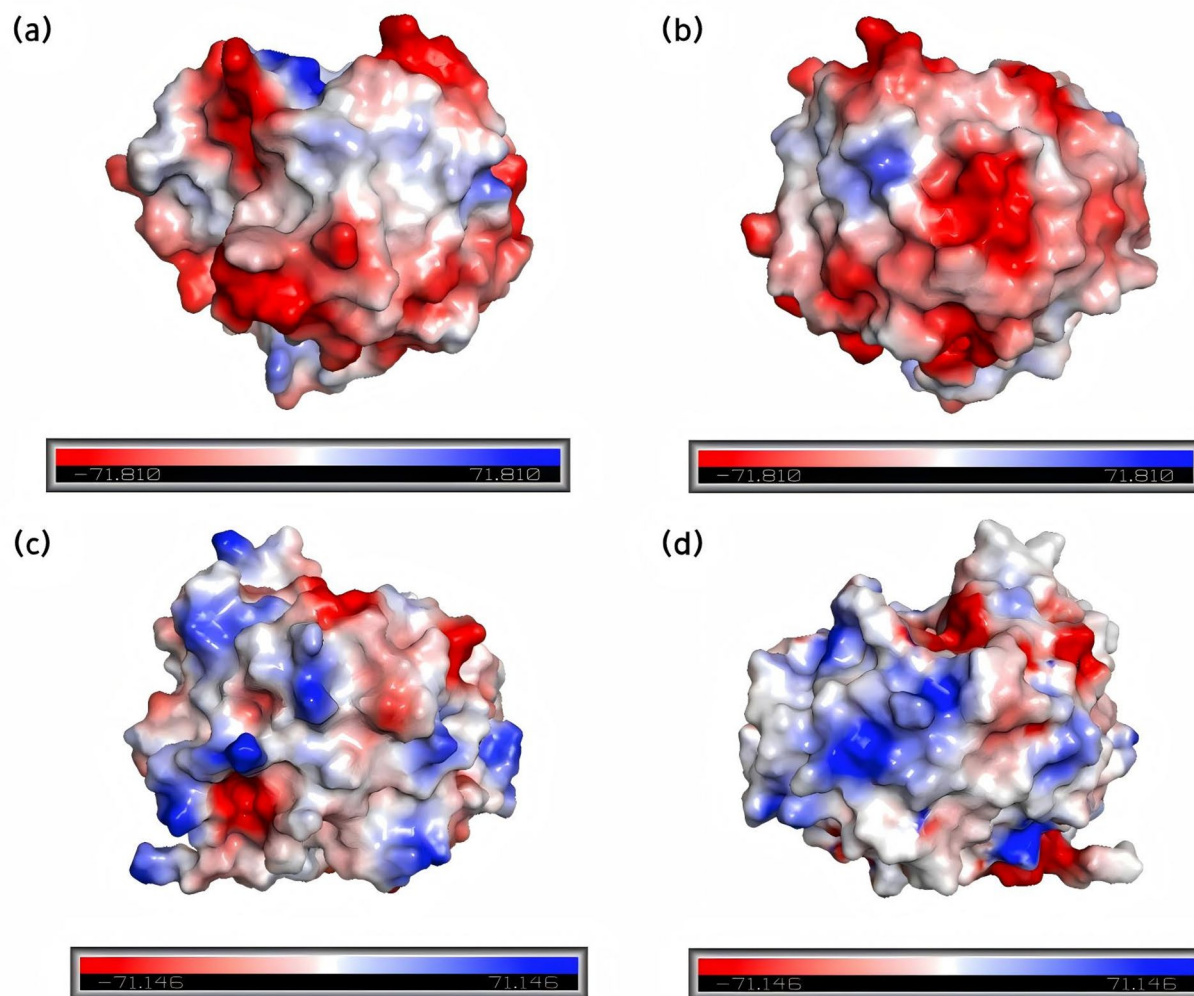


Fig. 6. Predicted surface electrostatic potentials of C5-CEL4 and GH5-family cellulase. **(a)** Surface electrostatic potential of C5-CEL4. **(b)** Surface electrostatic potential obtained by flipping figure a by 180° in Pymol 2.6. **(c)** Surface electrostatic potential of TfCel5A. **(d)** Surface electrostatic potential obtained by flipping figure c by 180° in Pymol 2.6. Negative and positive electrostatic potentials are shown in red and blue, respectively. Note: Fig. 6a was plotted in Pymol 2.6 from the structure prediction map of Fig. 1b. Figure 6c was plotted in Pymol 2.6 based on the structure of GH5_4 downloaded from the Protein Data Bank (<https://www.rcsb.org/>). GH5_4 (PDB code: 6XSU) is a GH5 family cellulase from *Ruminococcus flavefaciens*.

	Extracellular enzyme			
	Vmax (μmol/min/mg)	Km (mg/mL)	Kcat(s ⁻¹)	Kcat/Km
CMC-Na	158.73	45.03	98.20	2.18
Xylan from bagasse	15.55	16.05	12.32	0.77
	Intracellular enzyme			
	Vmax (μmol/min/mg)	Km (mg/mL)	Kcat(s ⁻¹)	Kcat/Km
CMC-Na	357.14	101.71	220.95	2.17

Table 1. Kinetic parameters of C5-CEL4.

the activating influence of Co²⁺ and Mn²⁺ on most GH5 family endoglucanases. Conversely, 0.1% EDTA, PMSF, and DTT did not impact C5-CEL4 enzyme activity, whereas SDS exhibited a slight inhibitory effect. C5-CEL4 was marginally inactivated by 1% EDTA, PMSF, and SDS, but showed slight activation with 1% DTT. Enzyme activity increased by 1.3-fold with 0.1% Tween 80, known to enhance cellulase stability⁴⁵. The enzyme's activity remained unaffected by methanol, ethanol, and isopropanol, with moderate ethanol concentrations shown to

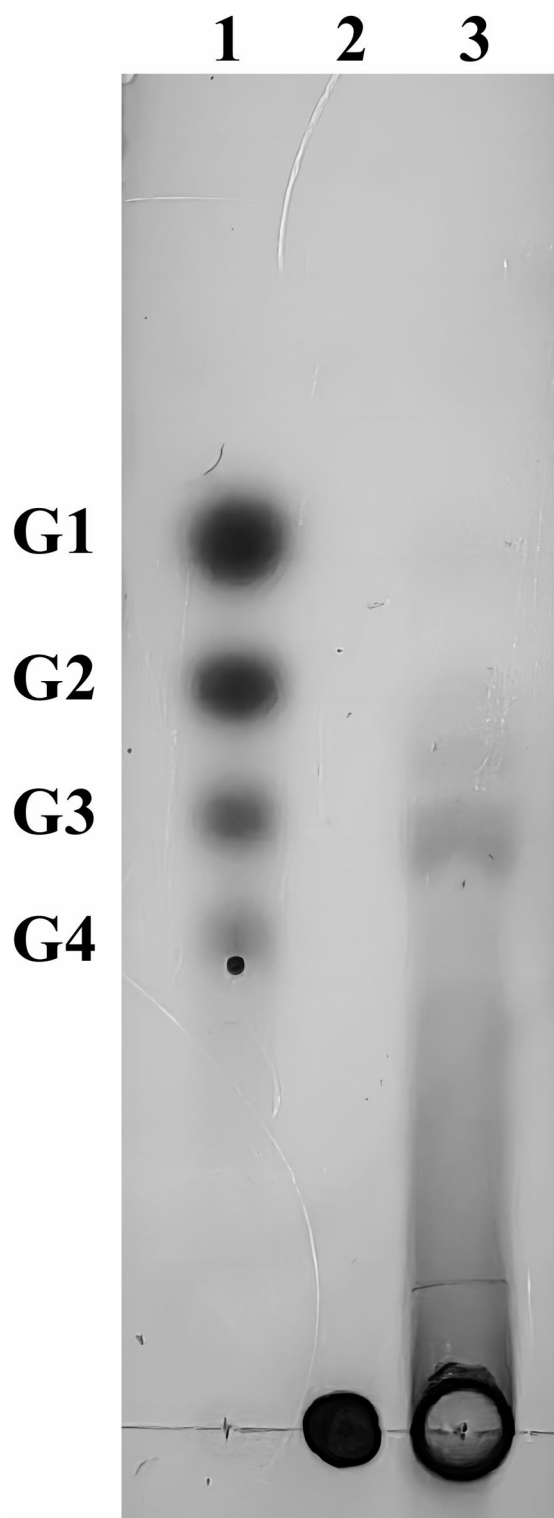


Fig. 7. TLC analysis of CMC-Na hydrolyzed by C5-CEL4. Lane 1, standards: glucose (G1), cellobiose (G2), cellotriose (G3), and cellotetraose (G4). Lane 2, CMC-Na without enzyme solution. Lane 3, hydrolysis of CMC-Na by purified C5-CEL4.

promote cellulase activity⁴⁶. C5-CEL4's performance in the presence of divalent cations, inhibitors, and alcohols common in industrial detergents indicates its robust potential for industrial applications.

The extracellular cellulase activity of C5-CEL4 secreted by *Escherichia coli* was measured at 0.886 U/mL (Fig. 3a). It has been reported that signal peptides enhance the secretion and expression of cellulase in *Escherichia coli*. When compared to the crude enzyme activity in the fermentation broth, which ranges from 0.81 to 1.33 U/

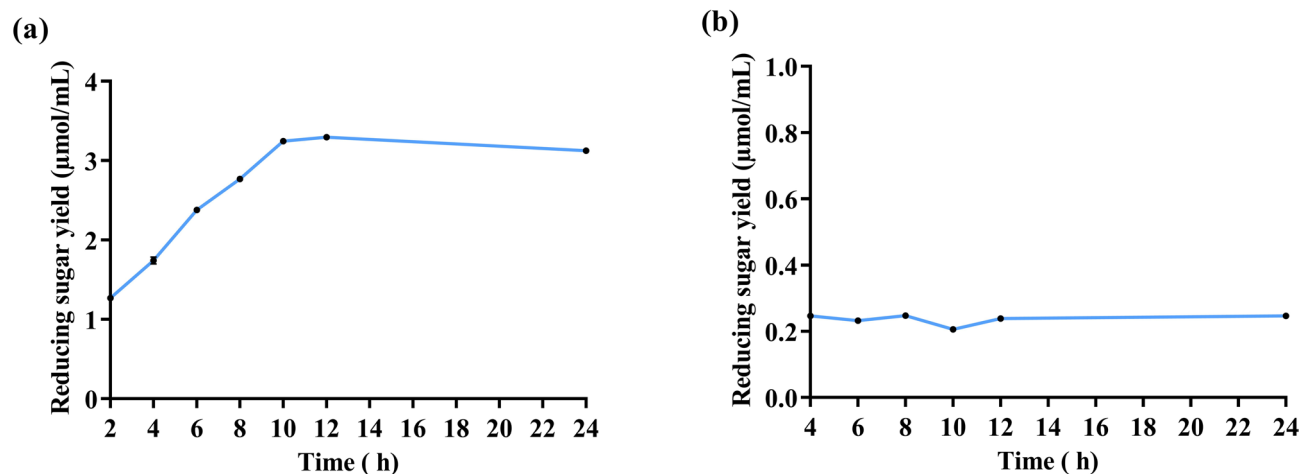


Fig. 8. Comparative analysis of sugar yield from C5-CEL4 hydrolyzed wheat bran (a) and corn stalks (b). Data points on the graph represent the mean values \pm SEM.

Enzyme	Source	Temperature (°C)	pH	Thermal stability	pH Stability	Salt resistance	References
C5-CEL4	Ebinur Lake in Xinjiang	50	7.0	74% (50°C, 2 h)	73% (pH 13.0, 24 h) > 70% (pH 4.0–12.0, 24 h)	> 108% (5 M NaCl, Nine months)	This text
Cel5R	Soil metagenomes	58	6.0	> 90% (55°C, 75 h)	> 80% (pH 9.0, Room temperature, 7 d)	80% (4 M NaCl, 30 d)	12
CelDZ1	Icelandic Hot Springs	70	5.0	> 70% (70°C, 24 h)	< 60% (pH 5.0–6.6)	100% (5 M NaCl, 20 d)	6
Cel7482	Beer lees	70	5.5	87% (70°C, 1 h)	> 80% (pH 4.5–6.5)	50% (2 M NaCl, 96 h)	23
AgCMCase	Salt-tolerant fungi	55	5.0	> 60% (70°C, 4 h)	> 40% (pH 4.0–10.0, 48 h)	> 95% (4 M NaCl, 4 h)	24
Cel5A	Roots of the saline plant <i>Rosa rugosa</i>	50	4	40% (55°C, 3 h)	> 50% (pH 4.0–6.0, 24 h)	50% (17.5% NaCl)	25
cel28a	Metagenomic library of goat rumen microorganisms	50	5.0	> 80% (50°C, 1 h)	> 70% (pH 4.0–6.0, 24 h)	—	26

Table 2. Comparison of enzymatic properties of C5-CEL4 with different sources of GH5 family endoglucanases.

mL, this activity is slightly lower⁴⁷. Unlike some GH5 family endoglucanases, the C5-CEL4 extracellular enzyme exhibited a low K_m and high V_{max} , indicating a higher affinity for CMC-Na and requiring less substrate to reach V_{max} (Table 1). The V_{max} of C5-CEL4 was significantly higher than those of CelC307 (62.58 U/mg)⁴⁸, Cel-5 M (27.1 U/mg)⁴⁹, StCel5A (194 U/mg)⁵⁰, and BaGH5-WT (6.0 U/mg)⁵¹, suggesting C5-CEL4's superior catalytic capability. Additionally, V_{max} is directly proportional to enzyme concentration, thus increasing enzyme concentration enhances reaction rates under sufficient substrate conditions. Furthermore, C5-CEL4 displayed activity toward CMC-Na and xylan from various sources, indicating its multifunctional nature. Multifunctional cellulases are vital for advancing the renewable bioeconomy by aiding in the conversion of agricultural residues into feed for ruminants. The synergistic action of multifunctional enzymes can pre-treat the complex structure of fibers, improving the utilization of agricultural residues by ruminants⁴¹. Consequently, C5-CEL4 may serve as an effective enzyme in feed additives.

Fibre trisaccharides (G3) are regarded as potent prebiotic oligosaccharides⁵². Numerous studies^{53–55} have shown that oligosaccharides are crucial for promoting probiotic growth and maintaining gut flora stability. This implies the potential application of C5-CEL4 in prebiotic production.

Cellulases hydrolyze the β -1,4-glycosidic bonds of cellulose to form soluble oligosaccharides, cellobiose, and glucose. However, cross-linking of the xylan and cellulose regions can limit the attachment of cellulases, whereas the breakdown of the β -1,4-xyloside bonds by xylanases increases the accessibility of cellulose to hydrolyzing cellulases, thereby increasing the efficiency of the enzyme mixture in disrupting the cellulose structure⁵⁶. Therefore, a mixture of cellulases and xylanases has been shown to be a very efficient method for achieving hydrolysis of lignocellulosic material⁵⁷. However, it is costly if individual cellulase and xylanase blends are used for hydrolysis during application. If multifunctional enzymes are applied in biomass hydrolysis, the cost of

applying multiple enzymes can be reduced and the efficiency of hydrolysis can be improved. The cellulase and xylanase activities of C5-CEL4 improved its efficiency in hydrolyzing wheat bran and cornstalks, and it has a large potential for agricultural applications. Wheat bran and cornstalks, both rich in cellulose, are common agricultural waste materials. Hydrothermal pretreatment of these substrates offers several benefits over acid-base pretreatment, including significantly reduced environmental impact, lower capital investment, the avoidance of chemical use, and diminished by-product formation⁵⁸. This method enhances lignocellulose saccharification by disrupting the cell wall matrix, thereby improving cellulase accessibility to cellulose microfibrils⁵⁹. The enzyme C5-CEL4 has demonstrated the ability to hydrolyze hot water-treated wheat bran and maize stalks to produce reducing sugars, indicating that hydrothermal pretreatment enhances cellulase hydrolysis efficiency^{60,61}. These observations suggest that C5-CEL4 is promising for applications in biofuel production, biochemicals, the textile industry, the food industry, and environmental treatment¹¹.

Conclusion

In this study, a novel cellulase gene, *c5-cel4*, was identified from the metagenome of substrates in Ebinur Salt Lake, Xinjiang, cloned, and expressed in *Escherichia coli*. Structural analysis revealed that the C5-CEL4 protein has a high surface concentration of acidic amino acids, resulting in a negative electrostatic potential. This feature enhances the enzyme's surface hydrophilicity and water-binding capacity, thereby conferring salt resistance. C5-CEL4 exhibited hydrolytic activity against both CMC-Na and bagasse xylan, confirming its multifunctional nature and resistance to heat, alkali, salt, and ionic liquids. Additionally, the enzyme demonstrated tolerance to various metal ions, inhibitors, and alcohols, and was effective in hydrolyzing hot water-pretreated wheat bran and corn stalks to produce reducing sugars. These findings position C5-CEL4 as an ideal candidate for biomass conversion and industrial applications in washing and textiles, with significant potential in the feed, food, and bioenergy industries.

Data availability

Original contributions presented in the study are included in the article/Supplementary Materials. The nucleotide sequence of the C5-CEL4 gene was submitted to GenBank (<https://www.ncbi.nlm.nih.gov/nuccore/PV014881>). Further inquiries can be directed to the corresponding author (yinyirui@dali.edu.cn).

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Author contributions

L.J.W., L.Q.Y., and Y.R.Y. conceived the study. L.J.W. constructed the constitutive expression plasmid. M.H. and C.Q.H. cloned the gene and cultured strains. N.Y. and D.Z. constructed the three-dimensional structure of the protein. R.W. and M.L. purified the recombinant proteins. X.C.G. and Z.H.L. measured enzymatic activity. K.Q.X. and Z.F.Y. performed the data analysis and mapping. The manuscript was authored by L.J.W., M.H., L.Q.Y., and Y.R.Y. All authors contributed to the discussion of results and provided comments on the manuscript. All authors reviewed and approved the final manuscript.

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Declarations

Competing interests

The authors declare no competing interests.

Additional information

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