

Screening, Cloning, Enzymatic Properties of A Novel Thermostable Cellulase Enzyme and Its Potential Application on Water Hyacinth Utilization

Xiaoshen Zhao (✉ 1170001807@qq.com)

Guangdong Pharmaceutical University

Liyang Liu

Guangdong Pharmaceutical University

Zujun Deng

Guangdong Pharmaceutical University

Shan Liu

Guangzhou Basic Clean cosmetics Manufacturing Co. Ltd.

Jeonyun Yun

Guangzhou Basic Clean Cosmetics Manufacturing Co. Ltd.

Xiong Xiao

Guangzhou Basic Clean Cosmetics Manufacturing Co. Ltd.

He Li

Guangdong Pharmaceutical University

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Abstract

Cellulose is the cheapest, natural, renewable organic substance that is used as a carbon source in various fields. Water hyacinth, an aquatic plant rich in cellulose, is often used as a raw material in fuel production. However, natural cellulase can be hardly used in industrial production on account of its low thermal stability and activity. In this study, a metagenomic library was constructed. Then a new cellulase gene, *cel1029*, was screened by Congo red staining and expressed in the prokaryotic system. Enzymatic properties of *Cel1029* were explored, including optimum temperature and pH, thermal and pH stability, and tolerance against organic solvents, metal ions and salt solutions. Finally, its ability of degrading water hyacinth was identified and evaluated. *Cel1029* displayed high homology with endoglucanase in the glycoside hydrolase family 5 (GH5) and had high stability across a broad temperature range. More than 86% of its enzymatic activities were retained between 4 and 60°C after 24 h of incubation. Single-factor analysis and orthogonal design were further conducted to determine the optimal conditions for the highest reducing sugar yield of water hyacinth. Interestingly, *Cel1029* efficiently transformed water hyacinth with a reducing sugar yield of 430.39 mg/g in 22 h. These findings may open the door for significant industrial applications of a novel GH5 cellulase (NCBI Reference Sequence: MK051001, *Cel1029*) and help identify more efficient methods to degrade cellulose-rich plants.

Introduction

Cellulase, including endoglucanase (endo-1,4-D-glucanase, EC3.2.1.4), exoglucanase (exo-1,4-D-glucanase, EC3.2.1.91) and glucosidase (1,4-D-glucosidase, EC3.2.1.21), is a type of enzymes that can hydrolyze the β -1,4-glycosidic bond between cellulose molecules (Lin et al. 2016). Endocellulase mainly catalyzes degradation of the amorphous region in cellulose, providing the starting site for further catalysis of exocellulase, so it plays an important role in efficient degradation of cellulose. Cellulase is distributed in many families, and the endoglucanases belonging to the glycoside hydrolase family 5 (GH5) have high similarity in both amino acid sequence and two strict conserved catalytic residues of glutamic acid (Yuan et al. 2019; Matsuyama et al. 1999). The two residues are considered as catalytic proton donors and active site nucleophiles, respectively. They can maintain the structure of the substrate isocarbon of the enzyme (Valérie et al. 1995). Cellulase is widely used in many fields, such as biofuel production, papermaking, textiles, food processing, brewing, and extraction of active ingredients of traditional Chinese medicine (Garg et al. 2016; Chia et al. 2016; Samkelo et al. 2020). Endocellulase has attracted attention because of its catalytic efficiency. So far, although various cellulase has been isolated from bacteria, fungi (Okada 1976), plants and higher animals, thermostable cellulase with excellent properties still needs to be discovered (Morteza et al. 2020; Zheng et al. 2018).

Ferula asafoetida is a rare medicinal plant resource distributed in Xinjiang in China. The distribution area of *Ferula asafoetida* in Shihezi on the southern edge of Junggar Basin experiences much dramatic temperature changes (e.g. long time sunshine, large difference of daily temperature) and high content of salt-alkali. Therefore, microbes and enzymes in the soils of this area are characteristic of salt alkali resistance and high thermal stability. Hence, the soils here are a good sample for screening new enzymes

with industrial application potential. However, most of the microorganisms in the environment cannot be cultured with the existing techniques or be used for biotechnology or basic research (Amann et al. 1995; Schmitz et al. 2004). For example, only 1% or even less of prokaryotes in the soils are readily cultivatable (Griffiths et al. 1996). Metagenomic libraries containing DNA extracted directly from environmental samples provide genomic sequences, and phylogenetic and functional information (Jo et al. 2002). Thus, metagenomics, the genomic analysis of collective genomes in an assemblage of organisms, is suitable for screening new functional biocatalysts and molecules with industrial application potential from soils (Handelsman et al. 2004). Many new enzymes have been discovered by using metagenomics, such as lipases (Tirawongsaroj et al. 2008; Bayer et al. 2009), amylases (Yun et al. 2004), and cellulase (Nimchua et al. 2012; Ko et al. 2013; Amitha et al. 2013). To our knowledge, no study has reported any highly thermostable cellulase that is found through metagenomic screening from the soils of *Ferula asafoetida* distribution area.

Burning fossil fuels can cause environmental pollution (Bao et al. 2011). Cellulose is the most abundant component of lignocellulose in the biosphere, and the cheapest renewable and natural organic substance (Liu et al. 2011). Cellulosic ethanol is the best alternative to fossil fuels (Raj et al. 2018; Costa et al. 2018). However, many intermolecular, intramolecular hydrogen bonds and other intermolecular interactions result in the long-lasting chain conformation and tight chain filling of cellulose, which cannot be dissolved by ordinary solvents (Medronho et al. 2012). Moreover, the hydrolysis of cellulose by chemical methods to produce cellulosic ethanol is complicated and expensive (Salinas et al. 2011). The utilization of lignocellulosic biomass lacks effective low-cost means (Lynd et al. 2002).

Water hyacinth (*Eichhornia crassipes*) has abundant utilizable lignocellulosic biomass (Ismail et al. 1995). It can tolerate seasonal changes in flow rate, water level, pH, nutrient availability, temperature, and toxic substances (Asrofi et al. 2018; Sumrith and Dangtungee 2019). Water hyacinth can multiply quickly at suitable temperature (between 10 and 35°C) in a nutrition balanced environment (Mayo and Hanai 2017). The infestation of water hyacinth affects water transport and ecological balance, and causes secondary pollution of water bodies. These limitations are great challenges to the ecosystem. To solve these problems and make reasonable use of water hyacinth resources, some researchers have studied enzymatic degradation of water hyacinth. The reactions of 170 U/g cellulase from *Trichoderma reesei* with alkali-treated water hyacinth for 36 h (Ganguly et al. 2013), 30 FPU/g cellulase from *Aspergillus fumigatus* with acid-treated water hyacinth for 24 h (Das et al. 2013, 2016), and 10 FPU/g cellulase from *T. atroviride* with acid-treated water hyacinth for 70 h (Rajesh et al., 2014) lead to the maximum sugar yields of 299.13, 425.6 and 380.97 mg/g respectively. In this work, after 121.25 U/g *Cel1029* (the enzyme loading of 1 g of water hyacinth is 121.25 U) reacts with acid-treated water hyacinth for 22 h, the reducing sugar yield is 430.39 mg/g (sugar yield per gram of water hyacinth).

We metagenomically screened a novel type of cellulase through from the soils of *Ferula asafoetida* distribution area. The cellulase shows excellent thermal stability, tolerance against metal ions, organic solvents, and salt solutions, and high ability of degrading water hyacinth, which together prove its great potential for industrial application.

Materials And Methods

Strains, Materials, and Chemicals

Escherichia coli BL21 (DE3) and *E. coli* DH5a (both from TSINGKE Biological Technology, Guangzhou, China) were used as the expression host and cloning host, respectively. The pET-32a (+) (Novagen) was used for protein expression. T4 DNA Ligases, restriction endonucleases and pUC118/*Bam*HI (BAP) were purchased from TaKaRa (Dalian, China). DNA extraction kit, plasmid extraction kit and His-tag protein purification kit (Novagen) were provided by OMEGA (San Diego, CA, USA). Ampicillin (Amp), 5-Bromo-4-chloro-3-indolyl- β -D-galactopyranoside (X-Gal), and isopropyl- β -D-thiogalactopyranoside (IPTG) were bought from Sigma. All other chemicals and reagents were produced by RUI SHU Biological Technology (Guangzhou, China) unless otherwise stated. Water hyacinth was gathered locally. Molecular biology reagents from TaKaRa were used according to the manufacturer's instructions.

Preparation of Water Hyacinth Biomass (WHB)

Water hyacinth was collected from the lake of Guangdong Pharmaceutical University. Then the water hyacinth was chopped, dried at 105°C, and finally ground into 0.1-1 mm powder (Amriani et al. 2016). A certain amount of WHB was added with dilute H₂SO₄ (2%) at the ratio of 1:10 (g/ml) under soaking for 1 h at 40°C. Then the dilute H₂SO₄ was drained, and WHB was washed to neutralize the pH with distilled water.

DNA Extraction, Metagenomic Library Construction, and Cellulase Gene Screening

A soil sample was collected from the wild *Ferula asafoetida* distribution area in Shihezi on the southern edge of Junggar Basin in Xinjiang. The sample was taken from 5–10 cm under the surface, sealed in a sterile bag, and preserved at -20°C until DNA extraction. Total genomic DNA from the soil sample was extracted according to a reported method (Zhou et al. 1996). The amount of the DNA extraction buffer was increased from 2.7 to 3.5 mL/g, and the time of vortex shocking was prolonged from 30 to 45 min. Genomic DNA was partially digested with *Eco*RI/*Pst*I/*Bam*HI/*Sau*3A I. After determination of the optimal endonuclease, the enzyme digestion time of the soil DNA was optimized.

The DNA fragments obtained by the above method were purified using the OMEGA gel extraction kit. Then the purified DNA fragments were ligated with a pUC118/*Bam*HI vector overnight at 16°C for 12 h with T4 DNA ligase. This recombinant plasmid was converted into *E. coli* DH5a and cultivated in a solid medium containing 50 μ g/mL Amp, 20 μ g/mL IPTG, and X-Gal (50 μ g/mL) at 37°C overnight.

The white colonies were expanded and cultured at 37°C and 220 rpm for 14 h. White colonies were randomly picked for the cellulase screening culture (5 mg/ml CMC, 50 μ g/mL Amp) at 37°C for 24 h. Then the hydrolysis circle of CMC was observed by Congo red staining. Firstly, 15 mL of a Congo red dyeing solution (1 mg/mL) was added for 1 h of dyeing, and after the waste liquid was discarded, and 15 mL of NaCl (1 mol/L) was added for 1 h of decolorization. An obvious hydrolysis circle demonstrating a

potential for cellulase production was identified, as shown on the plates of solid CMC stained with Congo red (1 mg/mL). Colonies were purified by repeated streaking. Finally, the DNA of positive colonies was sequenced by TSINGKE Biological Technology (Guangzhou, China) and analyzed using bioinformatics programs, such as ORFfinder, BLAST and MEGA-X.

Construction of Recombinant Plasmid and Transformation

During PCR amplification, pUC118-*cel1029* plasmid as the template, and *cel1029*-F and *cel1029*-R as primers were used. The amplified product and vector, pET-32a (+), was digested with *Bam*HI and *Hind* III, and then linked by T4 DNA Ligase. Finally, the constructed vector, pET32a-*cel1029*, was transferred into *E. coli* BL21 (DE3). PCR primers for *cel1029* amplification were as follows: *cel1029*-F, 5'-CGGGATCCATGGGATCCGAACATCATCATCATC-3'; *cel1029*-R, 5'-CCAAGCTTTGCGCCC TTTTCCTTGTCGTTCA-3' (the *Bam*HI and *Hind* III restriction sites were italicized). The PCR process was as follows: denaturation 2 min at 98°C; 30 cycles of 10 sec at 98°C, 5 sec at 64°C, and 5 sec at 72°C; elongation for 8 min at 72°C. The expression vector pET-32a (+) was digested using *Bam*HI and *Hind* III at 37°C for 20 min and then ligated to the PCR products, which were treated with the same restriction endonuclease. The double-digested pET-32a (+) and the PCR products were ligated by TaKaRa T4 DNA Ligase at 16°C for 14 h. This recombinant plasmid was transformed into *E. coli* BL21 (DE3) by a heat shock method.

Expression, Protein Purification, and Electrophoresis

The protein was expressed by growing the *E. coli* BL21(DE3) cells in an LB medium (50 µg/mL, Amp). For purification of *Cel1029* expressed by *E. coli*, the sediment was collected by centrifugation at 8000 rpm and 4°C for 10 minutes. The sediment was washed with sterile water twice and broken using an ultrasonicator with an amplitude set at 30 W for 15 minutes. Recombinant cellulase *Cel1029* was purified using the His-tag protein purification kit. The molecular weight of denatured protein was determined by SDS-PAGE. The molecular weight of the expressed protein was determined based on the protein markers (TaKaRa) as the standards. According to the predicted molecular weight of protein and its relationship with different concentrations of polyacrylamide gel, separation and concentration gels with the contents of 12% and 5% respectively were selected. The protein was stained with Coomassie brilliant blue G-250 for 10–12 h, and then decolorized with eluent. For the CMC zymogram analysis, 2.75 ml of a 1% CMC solution was added into 12% separation gel. Then 5% concentrated gel was prepared, and the purified *Cel1029* was loaded. After electrophoresis, the *Cel1029* was fixed with glacial acetic acid for 1 min, then reacted for 3 h, dyed with a 1 mg/ml Congo red solution for 1 h, and finally decolorized with 1 mol/L NaCl for 1 h.

Cellulase Activity Assay

Cellulase activity was measured using CMC as the substrate. Cellulase hydrolyzed CMC into reducing sugar. The absorption peak of the reducing sugar was 540 nm after oxidation with dinitrosalicylic acid (DNS) (Miller et al. 1959) in a boiling water bath. The absorption was linearly related with the content of reducing sugar. A standard curve was drawn using glucose concentration as the abscissa (X) axis, and

A_{540} as the ordinate (Y) axis. Reducing sugar content can be detected from the standard curve after the A_{540} of the solution was measured. One unit was defined as the amount of enzyme that released 1 μmol of reducing sugar per minute under the optimal conditions (Kang et al. 2007). All assays were performed in triplicate.

Evaluation of Enzymatic Properties

Cellulase activity was determined using 1% (w/v) CMC as the substrate, as previously mentioned. At 50°C, the enzyme activity was measured within pH 2–10. Into 90 μL of 1% (w/v) CMC prepared with the 40 mM BR buffer of different pHs, 10 μL of purified enzyme solution was added for 30 min of reaction. Then 150 μL of the DNS solution was added to react in boiling water for 10 min, and the supernatant was collected to determine the A_{540} and thereby the optimal pH. The optimal temperature was investigated from 4 to 80°C at the optimal pH. In brief, 2 mL of a crude enzyme solution was prepared with BR buffer solution of different pHs, and stored at 4°C for 24 h. Then 10 μL of the crude enzyme solution was taken every 2 h, and the residual enzyme activity was determined under the optimum temperature and pH. The maximum enzyme activity was set as 100%. The purified enzyme solution was placed at different temperatures (4–80°C) for 24 h, and sampled every 2 h for measurement of residual enzyme activity at the optimum temperature and pH according to the method above.

The effects of various metal ions (K^+ , Na^+ , Fe^{2+} , Mg^{2+} , Mn^{2+} , Ca^{2+} , Cu^{2+} , Zn^{2+} , Ni^{2+} , and Co^{2+}) and chemicals (SDS, EDTA, urea, imidazole, methanol, ethanol, isopropanol, DMSO and Triton X-100) on *Cel1029* activity were investigated by preincubating the enzyme with the reagents for 20 min at the optimum temperature and pH. The enzyme activity without any reagent was set as 100%. NaCl was used to study the salt tolerance of the recombinant enzyme. The activity of the enzyme was determined in the optimal pH buffer with a 0 to 3 mM salt solution at the optimal temperature and pH. The enzyme without adding agent was used as a control.

CMC solutions in concentrations from 0.0 to 5.0 mg/mL were prepared with the buffer solution of the optimal pH. Then 90 μL of the CMC solution at each concentration was added with 10 μL of the 0.649 mg/ml enzyme solution, and reacted for 30 minutes under the optimal temperature. After that, the reaction solution was taken out and added with 150 μL of the DNS solution for 10 min of reaction in boiling water, followed by measurement of A_{540} . The absorbance at 0.0 mM was taken as a negative control, and the concentration of glucose was determined. According to the substrate concentration [S] and the measured initial reaction rate V, The Linear Weaver Burk double reciprocal plot ($V = V_{\text{max}} [S]/(K_m + [S])$) was plotted for $1/V$, and the enzymatic kinetic parameters K_m and V_{max} of cellulase were calculated.

Enzymatic Hydrolysis of WHB

The optimal conditions were tested with WHB. The water hyacinth contained high amounts of cellulose that was converted to a reducing sugar by cellulase. First, single-factor analysis was used to study the WHB degradation by changing the pH, temperature, enzyme dosage, and enzymatic hydrolysis time. Then, an L9(3⁴) orthogonal design (Table 1) was used to determine an optimal degradation combination.

The reaction mixtures contained the WHB (0.2 g), Britton-Robinson buffer (40 mM), and cellulase in a 20-mL system at 200 rpm. Enzymatic extraction of the WHB was determined using spectrophotometry. The supernatant was centrifuged to measure the absorbance at 540 nm. The same amount of an inactivated enzyme solution was used as a negative control. All reactions were performed in triplicate.

Table 1
Orthogonal tests with three levels of each factor from low to high (1, 2 and 3).

Factor/Level	1	2	3
A-Temperature/°C	45	50	55
B-Enzyme amount /ml	2	4	6
C-pH	4.0	5.0	6.0
D-Time/h	20	22	24

Nucleotide Sequence Accession Number

This nucleotide sequence was declared to the GenBank at the registration number of MK051001.

Results

Screening for Cellulase Using the Soil Metagenomic Library

Metagenomic strategies are applied to isolate and identify novel enzymes with new catalytic or secondary metabolites from different environmental samples (Tang et al. 2018; Fan et al. 2017). The method of carboxymethyl cellulose sodium (CMC)-Congo red staining (Teather and Wood 1982) is often used to screen active clones of endoglucanase. In this study, we screened 7, 200 positive clones from a metagenomic library, which was built by extracting the total DNA from soil microbes. Results showed the optimum endonuclease was *BamHI* and the optimum time was 80 min. The average fragment size of the total DNA was about 2-2.5 kb. Cellulases were screened from the metagenomic library by Congo red staining, and one putative cellulase clone was visually identified by a transparent hydrolytic circle with a diameter of 1.25 cm that appeared on the Congo red plate (Fig. 1).

The clone was sequenced and analyzed with ORFfinder of the National Center for Biotechnology Information (NCBI), which showed the full-length 1029 bp fragment contained a cellulase gene named *cel1029*. The *cel1029* had an open reading frame of 927 bp and encoded a 34.21 kDa protein consisting of 309 amino acids. Conserved domain analysis revealed that *Cel1029* had a domain belonging to GH5 (312–1013 residues). Through multiple sequence alignments and protein BLAST search by BioEdiet, ESPript 3.0 and NCBI, *Cel1029* was found to be similar with endo-1,4-D-glucanase (62.81%, NCBI accession: P07103.2), endo-1,4-D-glucanase (48.48%, P15704.1), endo-1,4-D-glucanase (41.06%, P10475.1), endo-1,4-D-glucanase (41.06%, P07983.2), endo-1,4-D-glucanase (40.07%, P23549.1), endo-1,4-D-glucanase (42.57%, P06565.1), endo-1,4-D-glucanase (48.07%, O85465.1), and endo-1,4-D-

glucanase (48.07%, P06566.1) (Fig. 2). These sequences were strictly conserved at two catalytic residue sites – Glu176 and Glu264. The two residues served as catalytic proton donors and active site nucleophiles, respectively, and can maintain the structure of the substrate isocarbon for the enzyme. A phylogenetic tree was constructed by neighbor joining to verify the evolutionary relationship of *Cel1029* with 14 known endoglucanases. Results show that *Cel1029* is closely related to three endocellulases from *Bacillus sp.* (Fig. 3). Multiple sequence alignments and phylogenetic tree analysis indicate that *Cel1029* is an endo-1,4-D-glucanase (EC3.2.1.4) belonging to GH5.

Cloning and Overexpression of Cellulase Gene in *E. coli* and Purification of Recombinant Protein

PCR was conducted by using plasmids pUC118-*cel1029* as the template and *cel1029*-F and *cel1029*-R as primers. The amplified fragment length was consistent with the predicted target DNA fragment length of 1029 bp. pET32a-*cel1029* was identified by the verified enzyme digestion (Fig. 4) and the confirmed DNA sequencing. *Cel1029* was expressed in *E. coli* BL21 (DE3). According to the optimal induction temperature (30°C) and time (14 h), the optimal induction concentration of IPTG was 0.9 mM. Expression strain was induced by IPTG and then broken by ultrasound. The crude enzyme was purified and then analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) (Cheeseman et al. 2001). The enzyme was purified by about 9-fold, with a specific activity of 883 ± 25 U/mg. Figure 5 shows the approximate target band (37.8 kDa), including the theoretically calculated the cellulose enzyme molecular weight (34.21 kDa) and protein molecular tag weight (3.63 kDa). The recombinant protein was highly soluble expressed in *E. coli*, which is also the premise and basis for industrial application. The results of CMC zymogram analysis show a translucent zone (Fig. 6) that indicates CMCase activity.

M, DL45, 00 DNA Marker; Lane 1, Recombinant plasmid; Lane 2, Recombinant plasmid digestion by *Bam*HI and *Hind*III. The target band is located between 800 and 1200 bp

Characterization of Recombinant Cel1029

According to the relationship between the concentration of the standard glucose solution and the absorbance at 540 nm, we obtained the regression equation of the standard curve ($Y = 0.7609 \cdot X + 0.00916$, $R^2 = 0.998$). *Cel1029* was active over a wide pH range, maintaining 40% of its optimum activity at pH 2.0 and stable between pH 5.0 to 7.0 (Fig. 7A). The optimal reaction temperature of *Cel1029* at the optimal reaction pH was measured with CMC as the substrate (Fig. 7B). The maximum enzyme activity was defined as 100%. *Cel1029* retained 85.3% of the maximum activity at 4°C and 68.5% at 80°C. Maximum activity occurred at 55°C, and more than 50% activity was retained at 4 – 80°C, indicating that *Cel1029* has high adaptability at a wide temperature range. More than 86% of maximum activity was retained at 4 – 60°C after 24 h (Fig. 7D), indicating that *Cel1029* has excellent thermal stability. Cellulase activity was obviously enhanced by the addition of Co^{2+} and slightly inhibited by Ni^{2+} (Fig. 8). The influence of Co^{2+} concentration on *Cel1029* was further studied, and results showed 10 mM Co^{2+} promoted enzyme activity to 156.18%.

Cel1029 was stable in some common organic and nonionic solvents, as more than 50% activity was retained in all tested solvents at different concentrations (Fig. 9). Especially, the residual enzyme activity of *Cel1029* was 95.3% when the SDS concentration was 1 mM, and still reached about 70% and 50% when the SDS concentration increased to 10 and 100 mM, respectively. *Cel1029* was highly resistant against methanol and isopropanol, and its activity increased in the 1% (v/v) concentration.

NaCl (0 to 3 M) was used to study the salt tolerance of the recombinant enzyme under the optimal temperature and pH. The enzyme activity was maintained above 80% when the salt concentration was 3.0 M (Fig. 10).

Determining K_m , the most important characteristic constant of enzymes, is an important method to study enzyme kinetics and reflects the magnitude of affinity between an enzyme and the substrate. In the past, Michaelis-Menten parameters were used to test the K_m and V_{max} of cellulase (King et al. 2009). Then Lineweaver and Burk used the equation derived from the Michaelis-Menten equation to determine K_m and V_{max} to be 0.535 mM and 1.588 mM/min, respectively.

Enzymatic Hydrolysis of Water Hyacinth

The content of reducing sugar was 17.3 ± 0.7 mg/g in the negative control, and was 59 ± 3.8 mg/g in WHB before elution, indicating a part of reducing sugar was lost during the pretreatment of water hyacinth with H_2SO_4 . The optimal conditions of enzymatic hydrolysis to WHB were studied by spectrophotometry (Fig. 11). The sugar yield (sugar production/WHB quality, mg/g) reached 331.99 mg/g at pH 5 (Fig. 11A) and decreased almost linearly with the increasing pH. The sugar yield was only 213.65 mg/g at pH 8, and minimized to 230.92 mg/g at 35°C. The sugar yield also increased with temperature rise and maximized to 383.57 mg/g at 50°C (Fig. 11B). However, the sugar yield decreased when the temperature exceeded 50°C. Therefore, the sugar yield of WHB was highest at 50°C. The maximum sugar yield reached 356.30 mg/g with 2 mL of enzyme (24.25U, Fig. 11C). Figure 11D shows the effect of enzymatic hydrolysis time on the sugar yield. The sugar yield of WHB maximized to 395.19 mg/g when the enzymatic hydrolysis time was 24 hours. Table 2 shows the results of the orthogonal test. The primary factor affecting the sugar yield of WHB is enzymatic hydrolysis time, followed by the secondary factors, including pH, addition amount and temperature. The optimal conditions for a peak sugar yield (430.39 mg/g) of WHB are as follows: 50°C, 4 mL of enzyme, pH 6.0, and 22 hours of enzymatic hydrolysis.

Table 2

Orthogonal test results and analysis. K_1 , K_2 and K_3 represented the average value of the corresponding factor in each column in three experiments at level 1, 2 and 3. R represented the difference between the maximum K value and the minimum K value in each column

Test No.	A-Temperature/°C	B-Enzyme amount/ml	C-pH	D-Time/h	Sugar yielded/mg/g
1	1	1	1	1	278.02
2	1	2	2	2	430.39
3	1	3	3	3	212.67
4	2	1	2	3	335.01
5	2	2	3	1	327.28
6	2	3	1	2	312.72
7	3	1	3	2	365.52
8	3	2	1	3	285.90
9	3	3	2	1	362.00
K_1	308.36	326.19	266.70	321.10	
K_2	337.81	346.52	375.80	369.54	
K_3	323.67	297.13	301.82	279.19	
R	29.45	49.39	109.10	89.75	

Discussion

Metagenomic strategies have been applied to isolate and identify novel enzymes with new catalytic or secondary metabolites from different environmental samples. A metagenomic library was built successfully using DNA extracted from crop residues of red rice with castor bean cake and a new phytase was screened (Farias et al. 2018). A novel β -glucosidase gene *bgl2238* was screened from the macrogenomic library by functional screening (Tang et al. 2018). A novel gene (*aii810*) encoding an N-acylhomoserine lactonase was isolated from the Mao-tofu metagenome for the first time (Fan et al. 2017). CMC-Congo red staining (Teather and Wood 1982) is often used to screen active clones of endoglucanase. The main limitation of metagenomic libraries is the need of suitable hosts to promote heterologous expression (Carlos et al. 2016). This limitation is mainly attributed to the difficulties in identifying regulatory elements and the existence of different codons (Wang et al. 2016). In an arid agricultural system, the dominant flora include spore-forming strains of *Bacillus* and *Paenibacillus*, while *Streptomyces* is more abundant in natural desert systems (Schrempf 2013). The codons of the actinomycete shine Dalgarno promoter region are different from those of *E. coli*, so heterologous

expression is difficult for the cellulase mainly from *Actinomycetes* screened in natural desert systems. In this study, the cellulase gene *cel1029* was successfully expressed in *E.coli*. Direct extraction of soil samples was adopted, and the amount of DNA extraction buffer and the time of vortex shock were increased to reduce microbial adsorption by soils and enhance the degree of DNA dissolution. In addition, a Congo red screening medium was used here, which can show a transparent hydrolysis circle, increase the recognition and reduce the probability of missing positive clones from the metagenomic library.

Cel1029 has high adaptability at wide ranges of temperature and pH, and excellent thermal and pH stability. Several reports on thermostable cellulase show similar catalytic activity at high temperature. The optimal temperature of *Cel1029* is slightly higher than that of *Lp-egl-1* from *Lyrodus pedicellatus* (Hiroshi et al. 2008) and *PersiCel1* from camel rumen metagenome (Morteza et al. 2020). On the basis of hot activity, *Cel1029* outperforms *PersiCel2* with about 10% activity at 80°C (Morteza et al. 2020), but is worse than *R63* from *Thermoanaerobacterium sp* with about 80% activity at 75°C, (Harnvoravongchai et al. 2020) and *PW2* from *Bacillus sp.* with 100% activity at 80°C (Divyanshi et al. 2019). The residual activity at 70°C is 48.1% after incubation for 24 h, which is better than that of *MaCel* (Tong et al. 2020). In terms of pH adaptability, the optimum pH of *PersiCel1* with good pH adaptability is 5 (Morteza et al. 2020), and the relative enzyme activity is about 40% at pH 4 and pH 10. The optimum pH of *ZFYN184* from soil metagenomic library is 4 (Chai et al. 2020), and the relative enzyme activity is lower than 10% at pH 3 and pH 6. The optimum pH of *Cel1029* is 6, and the relative enzyme activity is slightly higher than 40% at pH 2, and above 45% at pH 10. In conclusion, *Cel1029* has obvious advantages in temperature and pH adaptability.

Activity of *Cel1029* is obviously enhanced by the addition of Co^{2+} and slightly inhibited by Ni^{2+} . Similar phenomena have been reported, in which 0.5 mM Co^{2+} double-increased the enzyme activity of *MaCel* (Tong et al. 2020) and 1 mM Co^{2+} promoted the activity of AgCMCase (Kumar et al. 2015). The results suggest that *Cel1029* is resistant against most metal ions. The enzyme activity can be increased by adding appropriate metal ions in industrial production. To determine the usefulness of *Cel1029* in industrial applications, it is necessary to study the effects of organic and nonionic solvents on its activity and stability. Interestingly, when the SDS concentration increases to 10 and 100 mM, the residual enzyme activity is still about 70% and 50% respectively. *BC1*, an alkalophilic cellulase from the symbiotic *Bacillus subtilis*, retains about 30% at the presence of 1% SDS (Dehghanikhah et al. 2020). *GAC 16.2* from *Acinetobacter junii* reserves 66.1% activity with the presence of 5 mM SDS (Sandipan et al. 2020). *Cel1029* has obvious advantages in tolerance against SDS. On the one hand, SDS increases the surface negative charge of cellulase and the substrate, increasing the electrostatic repulsion between cellulase and the substrate. Finally, the binding ability between cellulase and the substrate and the hydrolysis ability of cellulase are decreased. On the other hand, the ineffective adsorption between cellulase and the substrate makes the enzyme irreversibly adsorbed on the substrate surface (Palonen et al. 2004), which results in the change of protein conformation, protein denaturation, and decrease of cellulase activity (Norde and Favier 1992). SDS is adsorbed onto the surface of the substrate through hydrogen bonding (Borjesson et al. 2007) and hydrophobic interaction, which significantly reduce the ineffective adsorption

of the substrate and enzyme and enhances the cellulase activity in the liquid phase (Bálint et al. 2011). The relative activity of *Cel1029* is still about 50% with the presence of 100 mM SDS, indicating the negative charge of SDS has a weak effect on the binding degree of *Cel1029* and the substrate, which may be affected by the charge properties of *Cel1029*. SDS may largely reduce the ineffective adsorption of *Cel1029* onto the substrate. *Cel1029* can well resist methanol and isopropanol, and its activity is increased at the concentration of 1% (v/v). Similar performances have been reported. The activity of *HSC7* from *Bacillus sonorensis* increases to 166% in the presence of methanol (Azadian et al. 2017). The activity of *Est906* (Xiaolin et al. 2020) from paper mill waste water sediments is enhanced to 119.8% in the presence of isopropanol. These results suggest that adding appropriate organic or nonionic solvents into industrial production may strengthen the enzyme activity. Recombinant *Cel1029* reserves above 80% activity when the salt concentration is 3.0 M, and thus is more stable than *SR22* from *Bacillus sp.* strain (Santos et al. 2018), indicating that *Cel1029* has high tolerance against salt solutions. The K_m and V_{max} of cellulase from *Aspergillus fumigatus* are 10.052 mM and 1.38 mM/min, respectively (Prabhpreet et al. 2020). *NTU-05* from *Salinivibrio sp.* has K_m of 3.03 mg/ml (12.5 mM) and V_{max} of 0.14 mM/min when tested using CMC (Chungyi et al. 2009). The K_m and V_{max} of *Cel1029* are 0.535 mM and 1.588 mM/min, respectively, suggesting that *Cel1029* has higher affinity to CMC as the substrate.

Water hyacinth is rich in cellulose, which is hydrolyzed into cellobiose or glucose by enzymes such as cellulase. Cellulase can degrade cellulose into cellulosic ethanol, and then produce bio-fuels by microbial fermentation, which can optimize the use of WHB as a resource (Ma et al. 2010). In this study, the degradation of water hyacinth by cellulase *Cel1029* was studied preliminarily. The reducing sugar yield of WHB through biotransformation of *Cel1029* under optimal conditions is 84.36% of the theoretical maximum reducing sugar yield, which is about 510.2 mg/g (Klass et al. 1981; Nigam et al. 2002; Kumar et al. 2009). So far, it has not been reported that the cellulase degrades water hyacinth with such high efficiency.

Conclusions

A soil metagenomic library was constructed and a new cellulase gene, *cel1029*, was cloned by using an activity-based functional method. *Cel1029* was successfully expressed in the prokaryotic system to explore its stability, enzymatic properties, and ability to degrade water hyacinth. It showed high activity in a wide pH range, good thermostability, and excellent tolerance against organic solvents and salts. The recombinant enzyme was purified and characterized. The selected new cellulase was used to degrade and transform water hyacinth, and directly damaged the plant cell walls. Cellulase directly converted water hyacinth into glucose during the short test period, indicating a potential application of water hyacinth for use in energy production. We present new cellulase from the soils of *Ferula asafoetida* distribution area, enrich the source of cellulase, and make full use of cellulase. Meanwhile, this study provides a research basis for improving the utilization rate of cellulose-rich plants.

Declarations

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Compliance with ethical standards

Conflicts of Interest

Xiaoshen Zhao, Liyang Liu, Zujun Deng, Shan Liu, Jeonyun Yun, Xiong Xiao and He Li declare that they have no conflict of interest.

Consent to participate

(Not applicable)

Consent for publication

(Not applicable)

Availability of data and material

(Not applicable)

Code availability

(Not applicable)

Authors' contributions

Conceptualization, X.S.Z.; methodology, J.Y.; software, Z.J.D.; validation, X.S.Z., S.L. and J.Y.; formal analysis, X.S.Z.; investigation, Z.J.D.; resources, Z.J.D.; data curation, X.X.; writing—original draft preparation, X.S.Z.; writing—review and editing, L.Y.L.; visualization, X.X.; supervision, H.L.; project administration, H.L.; funding acquisition, S.L.. All authors have read and agreed to the published version of the manuscript.

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Figures

transparent hydrolysis circle

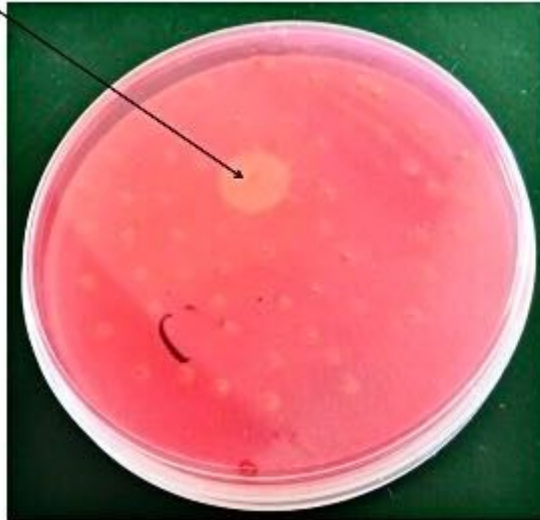


Figure 1

Screening of the positive clones with cellulase activity. The arrow points to a 1.25 cm transparent hydrolytic circle

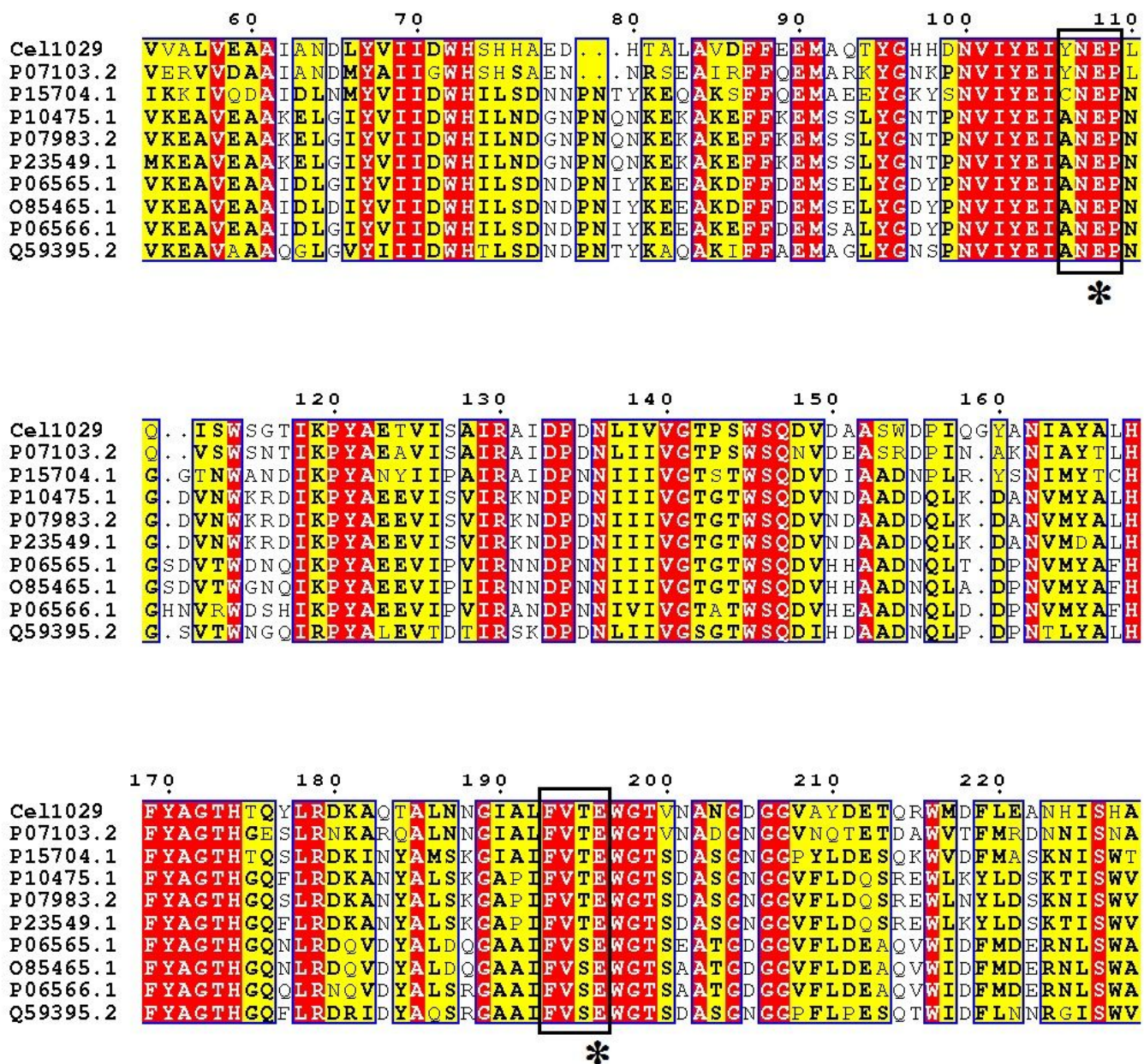


Figure 2

Multiple amino acid sequence alignment of Cel1029 with its closest homologues. Sequences of all closest homologues were obtained from NCBI. Similar sequences were indicated by colored backgrounds. The two catalytic residue sites Glu176 and Glu264 labeled as asterisks(*) were strictly conserved in GH5. P07103.2, P15704.1, O85465.1, Q59395, P10475 & P07983 & P23549.1, and P06565 & P06566.1: endo-1,4-D-glucanases from *Dickeya dadantii* 3937, *Clostridium saccharobutylicu*, *Salipaludibacillus agaradhaerens*, *Pectobacterium parmentieri*, *Bacillus subtilis* and *B. cellulosilyticus* respectively.

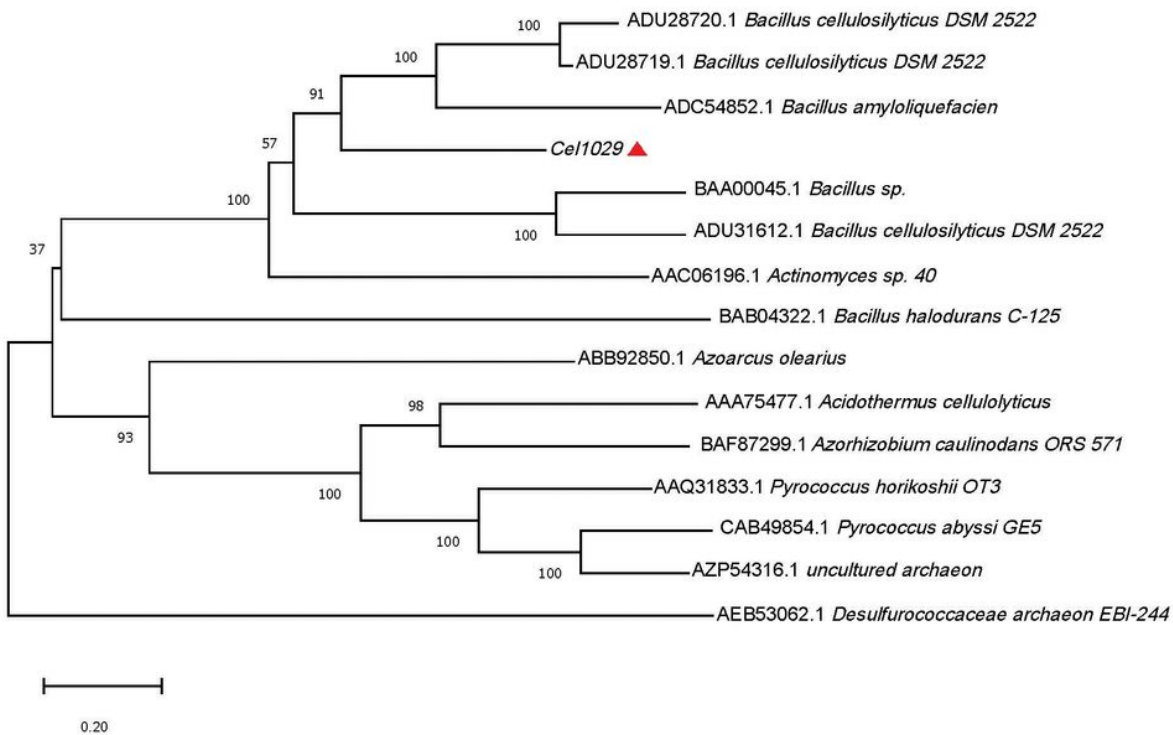


Figure 3

Phylogenetic tree of Cel1029 and 14 known endoglucanases built by neighbor-joining. Evolutionary history was inferred using neighbor-joining by MEGA-X. Sequences of all endoglucanases were cited from the CAZy. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches

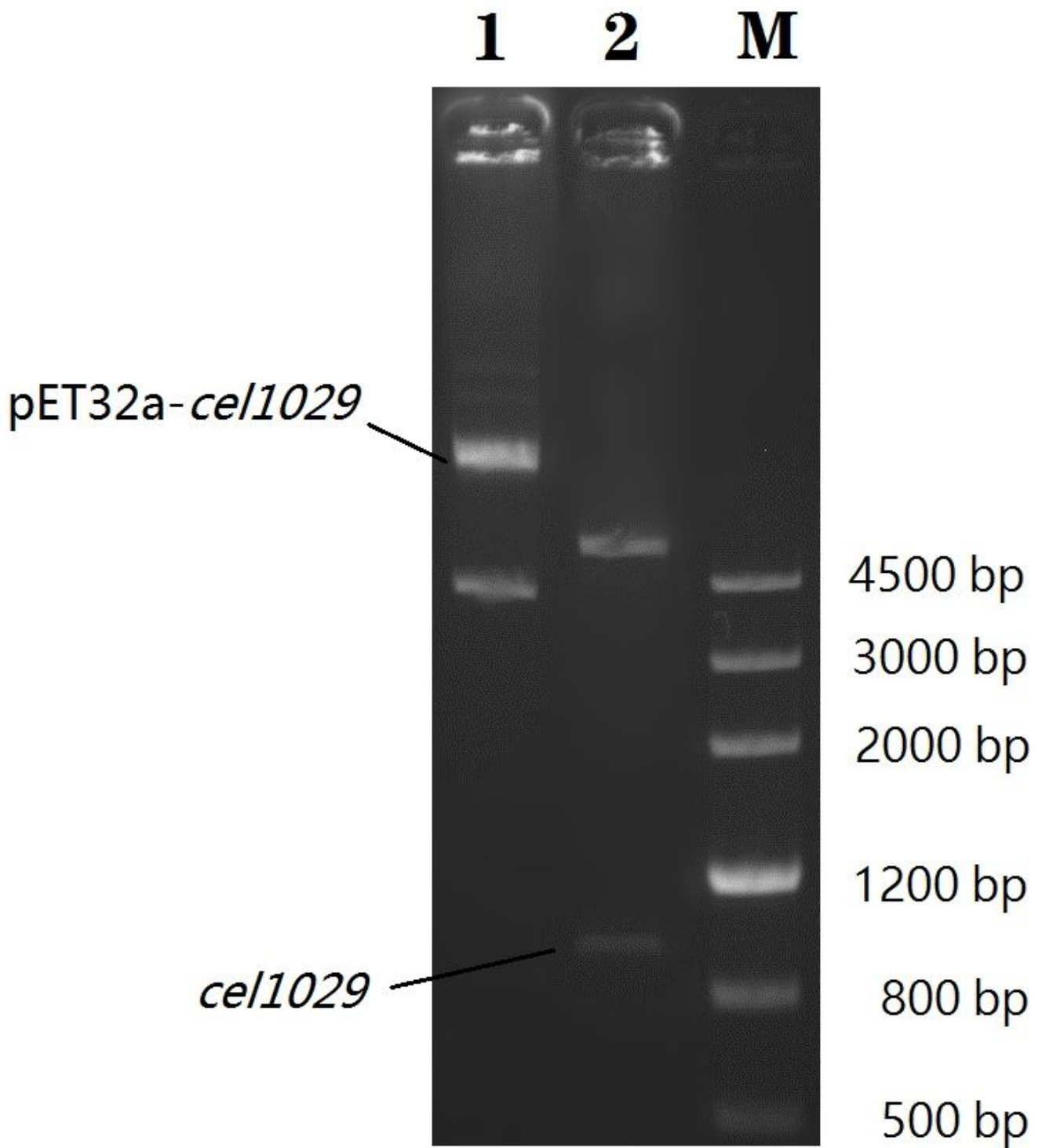


Figure 4

Electrophoresis identification of recombinant plasmid by BamH I and Hind III M, DL45, 00 DNA Marker; Lane 1, Recombinant plasmid; Lane 2, Recombinant plasmid digestion by BamH I and Hind III. The target band is located between 800 and 1200 bp

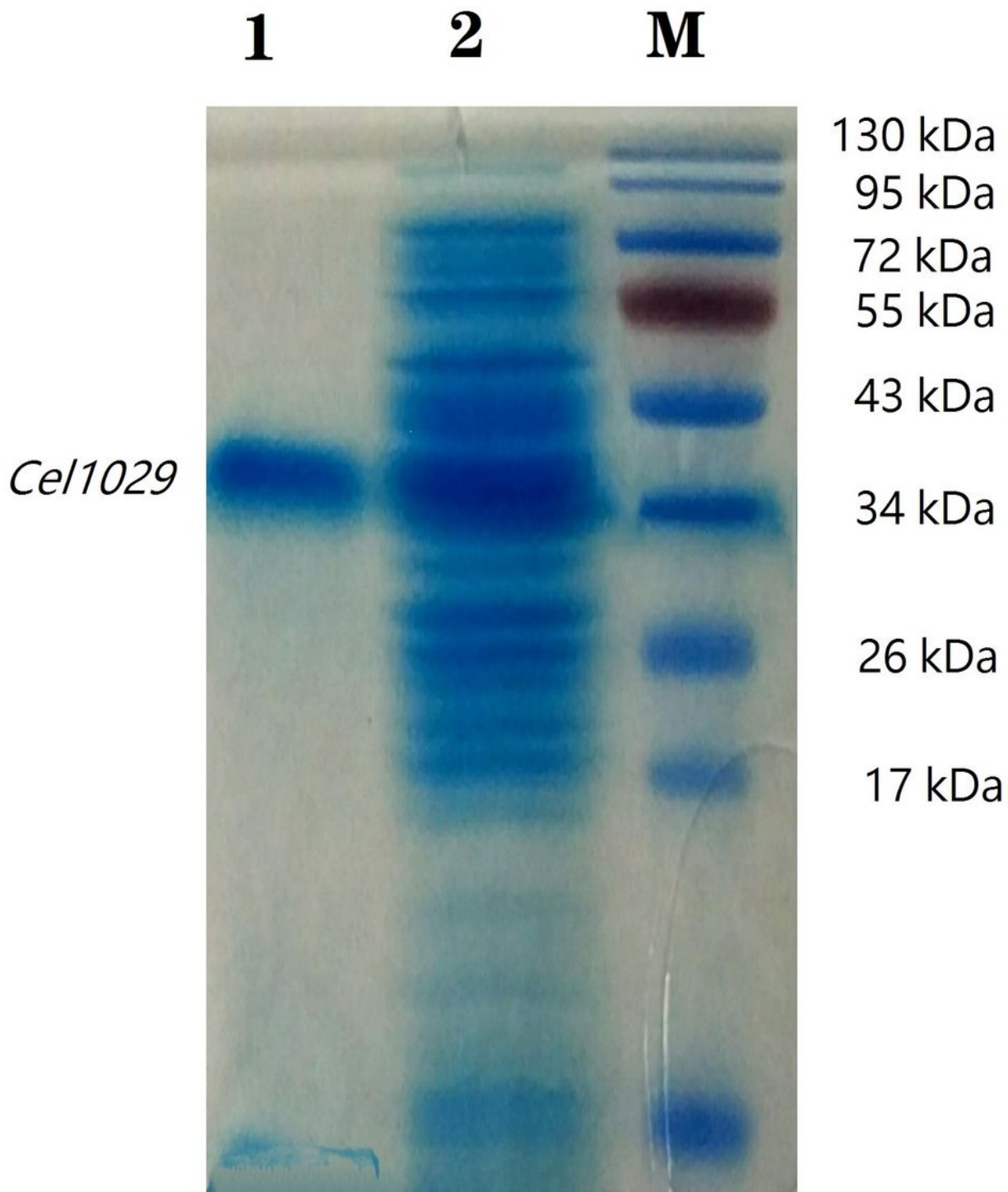


Figure 5

SDS-PAGE of recombinant Cel1029. M, Protein MW Marker; Lane 1, purified recombinant Cel1029; Lane 2, unpurified recombinant Cel1029. The approximate target band (37.8 kDa) includes the theoretically calculated cellulose enzyme molecular weight (34.21 kDa) and protein molecular tag weight (3.63 kDa)

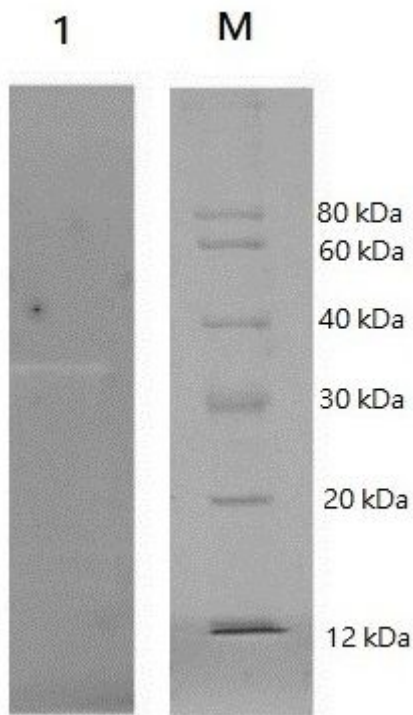


Figure 6

CMC zymogram analysis of purified Cel1029. The translucent zone at about 37.8 kDa indicates that Cel1029 has CMCase activity

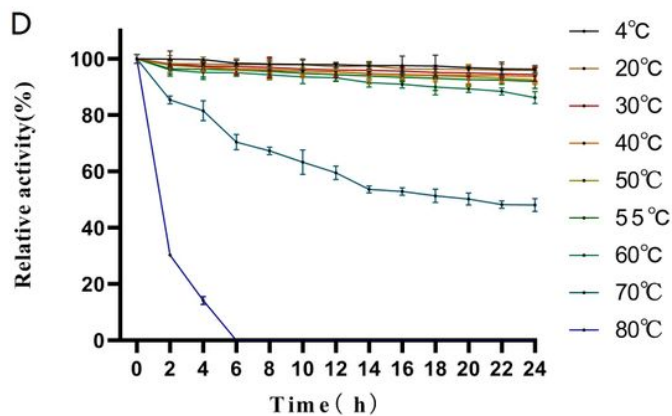
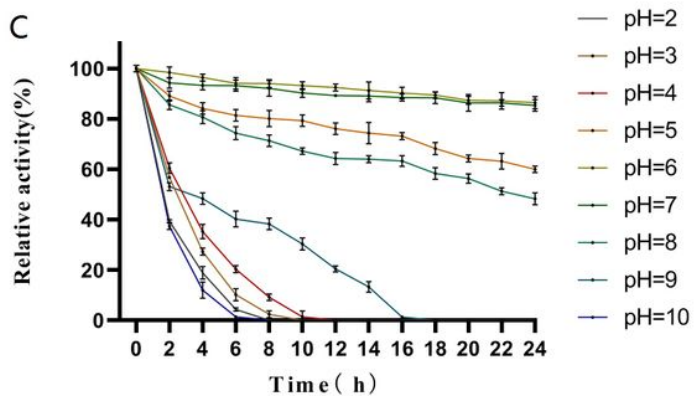
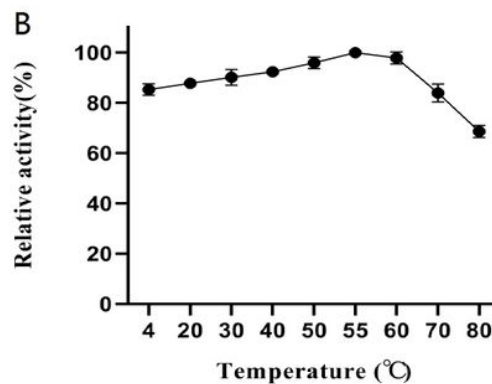
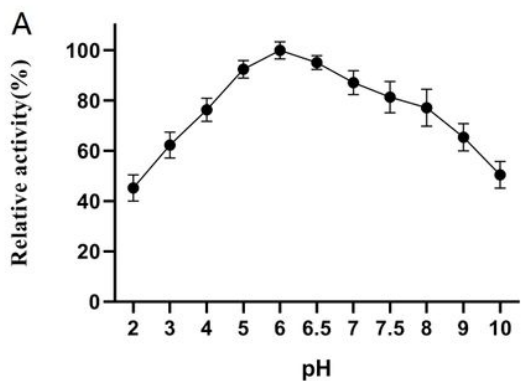


Figure 7

Effects of pH and temperatures on activity and stability of Cel1029 with CMC as the substrate. Data points and error bars represent mean and standard deviation respectively. (A) Effect of pH on activity of Cel1029 measured after the reaction in 90 μ L of 40 mM Britton-Robinson (B-R) buffer (pH 2.0-10.0) at 50 $^{\circ}$ C for 30 min. Enzyme activity at the optimum pH was defined as 100%. (B) Effect of temperature on activity of Cel1029 measured after the reaction in 90 μ L of 40 mM B-R buffer (pH 6.0) from 4 to 80 $^{\circ}$ C for 30 min. Enzyme activity at the optimum temperature was defined as 100%. (C) Effect of pH on stability of Cel1029 measured after the reaction in 90 μ L 40 mM B-R buffer (pH 6.0) at 55 $^{\circ}$ C for 30 min. Crude enzyme solution prepared with 2 mL 40 mM B-R buffer (pH 2.0-10.0) was stored for 24 h and taken every 2 h. (D) Effect of temperature on stability of Cel1029 measured after the reaction in 90 μ L of 40 mM B-R buffer (pH 6.0) at 55 $^{\circ}$ C for 30 min. Pure enzyme solution was stored from 4 to 80 $^{\circ}$ C for 24 h and taken every 2 h. Values are shown as percentage of maximal activity (100%).

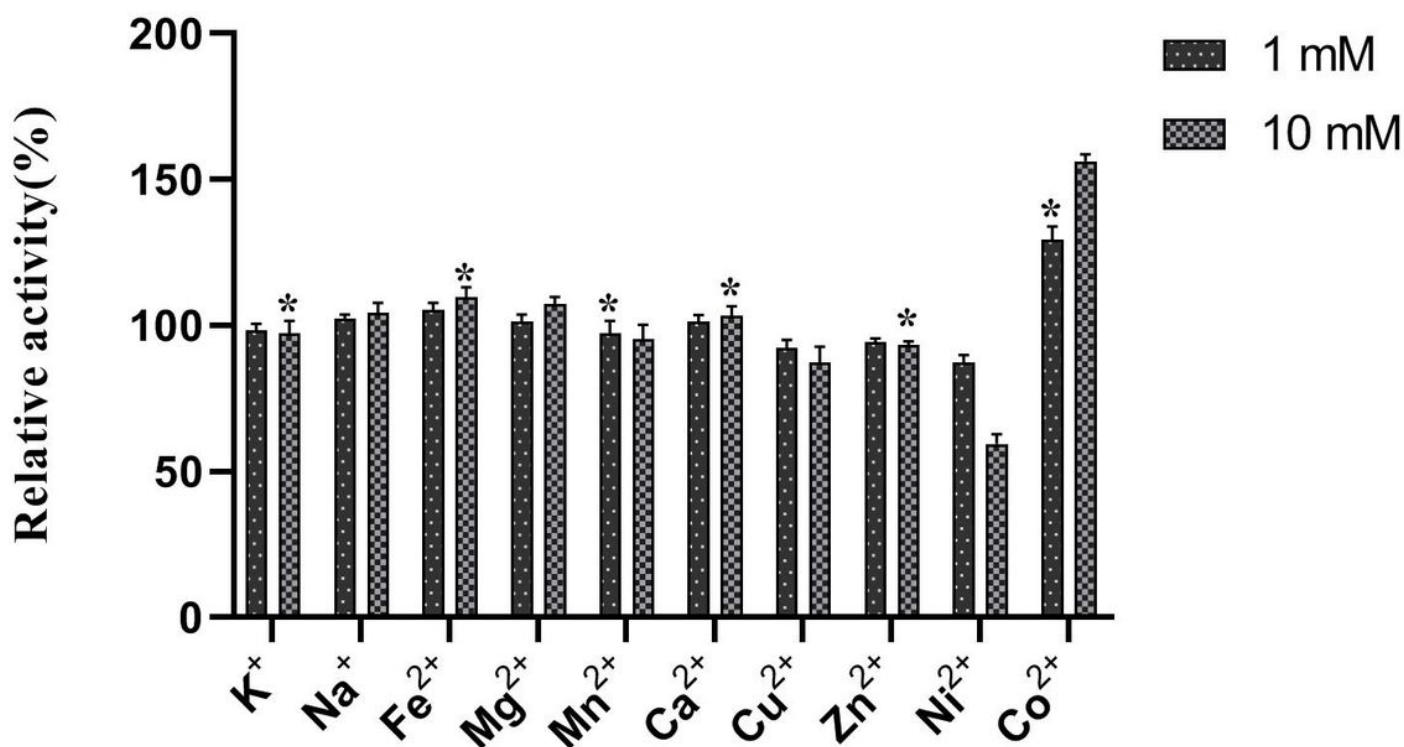


Figure 8

Effects of metal ions on activities of Cel1029 with CMC as the substrate. Enzyme activity was measured after the reaction in 90 μ L of 40 mM B-R buffer (pH 6.0) at 55 $^{\circ}$ C for 30 min. B-R buffer was added with different metal ions at 1 mM or 10 mM. Enzymatic activity without metal ions was defined as 100%

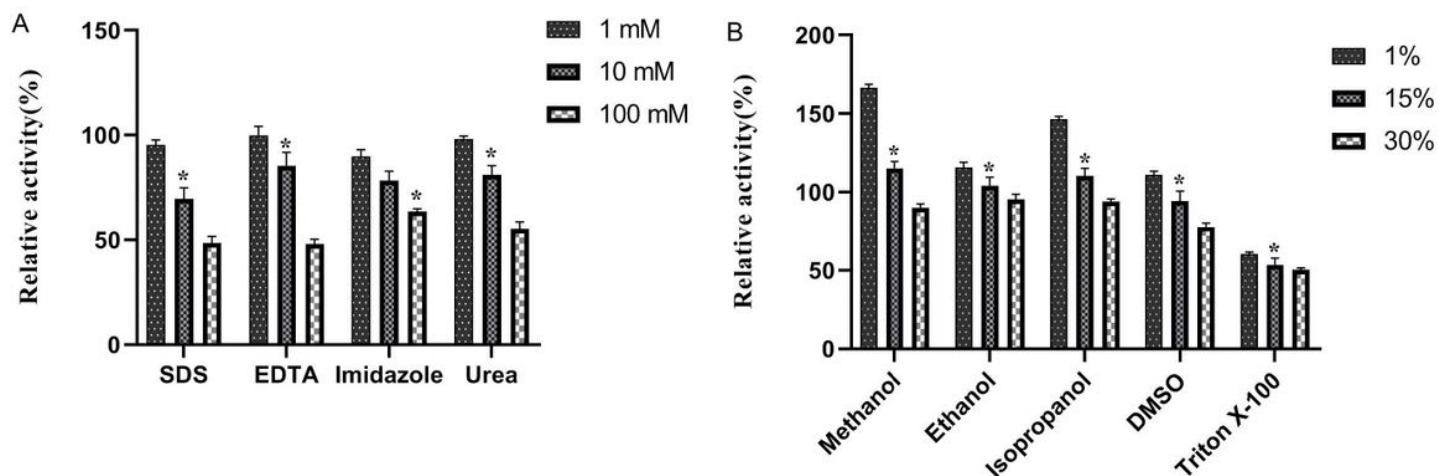


Figure 9

Effects of organic and nonionic solvents on activities of Cel1029 with CMC as the substrate. Enzyme activity was measured after the reaction in 90 μ L of 40 mM B-R buffer (pH 6.0) at 55 $^{\circ}$ C for 30 min. B-R buffer was added with the organic and nonionic solvents at different concentrations. Enzymatic activity without organic and nonionic solvents was defined as 100%

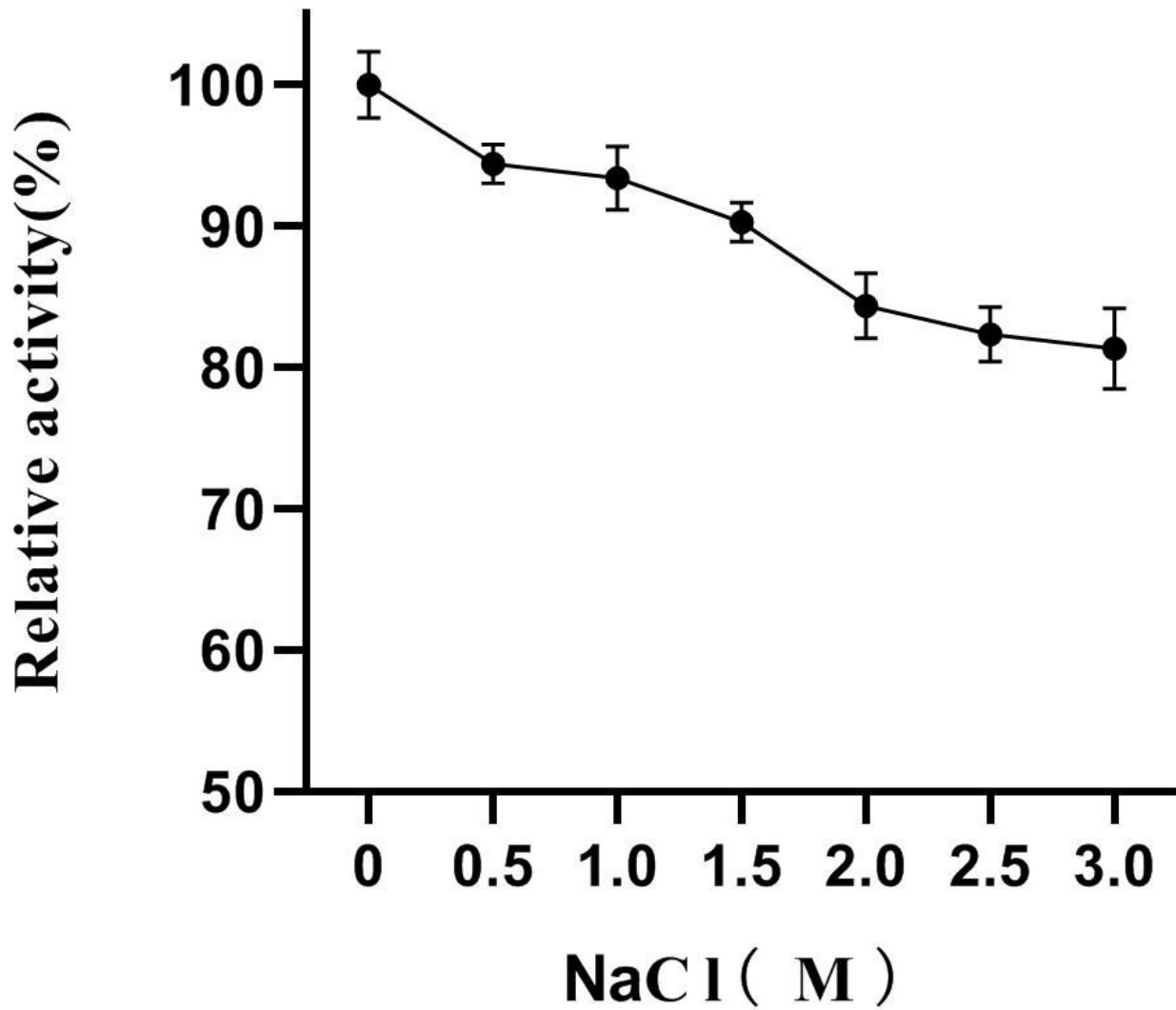


Figure 10

Effects of NaCl on activities of Cel1029 with CMC as the substrate. Enzyme activity was measured after the reaction in 90 μ L of 40 mM B-R buffer (pH 6.0) at 55 $^{\circ}$ C for 30 min. B-R buffer was added with NaCl at different concentrations. Enzymatic activity without NaCl was defined as 100%

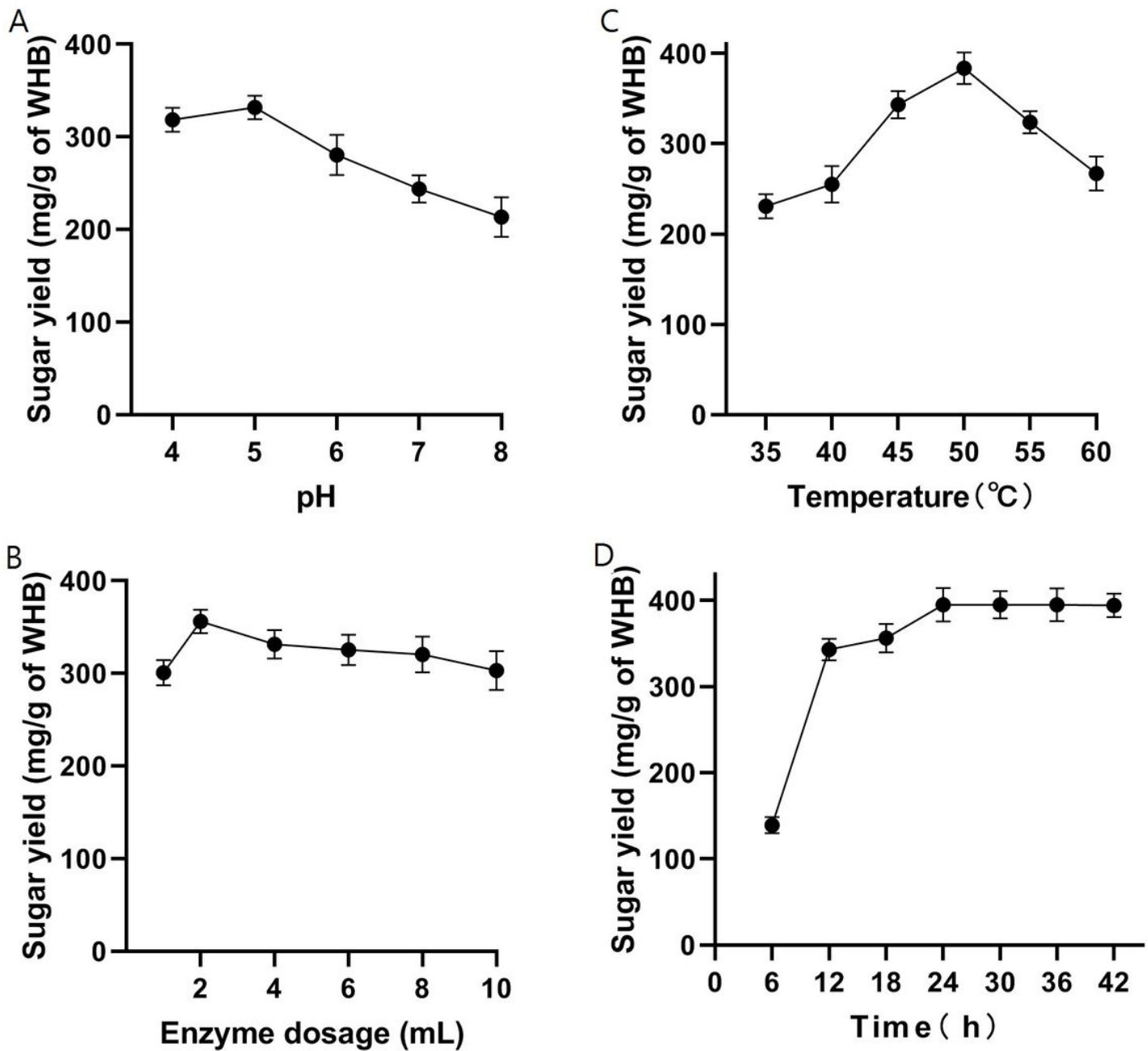


Figure 11

Effects of pH, temperature, enzyme amount and time on enzymatic saccharification of WHB. Data points and error bars represent mean and standard deviation respectively. (A) Effect of pH tested in 40 mM B-R buffer (pH 4.0-8.0) at 55 °C. (B) Effect of temperature tested in 40 mM B-R buffer (pH 5.0) from 35 to 60 °C. (C) Effect of enzyme amount tested in 40 mM B-R buffer (pH 5.0) at 50 °C. (D) Effect of time tested in 40 mM B-R buffer (pH 5.0) at 50 °C. Values are shown as reducing sugar yield/WHB quality in reaction systems. Each reaction involved 0.2 g of WHB and 24.25 U cellulase, and the same amount of an inactivated enzyme solution was used as a negative control