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A pH-stable alkaline pectate lyase produced by the newly identified strain *Bacillus altitudinis* CAS-WZS-08

Abstract

The stability of pectate lyase, including its thermal stability and pH stability, is extremely important for its application. In this study, a pH-stable pectate lyase with good thermal stability was purified and identified. The pectate lyase showed an excellent broad pH stability between pH 4.0 and 10.0 when it was stored at 4°C and room temperature, maintaining 100% relative enzyme activity after 24 h. The optimal pH was 10.0, showing that it was an alkaline pectate lyase. The optimal temperature was 60°C, while this pectate lyase was stable at 30~45°C. The enzyme was produced by a new isolated strain, *Bacillus altitudinis* CAS-WZS-08, identified by 16S rRNA sequencing and characteristics. Fermentation parameters for pectate lyase production were optimized utilizing a single variable optimization. The highest enzyme activity was 0.71±0.001 U/mL under the optimal fermentation parameters (4 g/L pectin, 20 g/L yeast extract, 2% inoculum size, pH 7.0, 33°C). The enzyme was activated by Mn^{2+}, Cu^{2+}, Co^{2+}, Ca^{2+} but was inhibited by Fe^{3+}, Ba^{2+}, and Mg^{2+}. Through ammonium sulfate precipitation, dialysis, cation exchange column, and Sephadex G-75, the electrophoretic pure protein was acquired. The results of liquid chromatography tandem-mass spectrometry (LC/MS-MS) indicated that the protein was pectate lyase with a molecular weight of ~37 kDa. This study provides an excellent pH-stable pectate lyase with good thermal stability that is a potential candidate for industrial applications.

Keywords: pH-stable Pectate Lyase, Isolation, Thermal Stability, *Bacillus altitudinis*

1.Introduction

Enzymes, catalyzing substances to products specifically and in an environmentally friendly manner, have an extensive range of applications (Uzuner 2019). Among them, pectinase is one of the major categories, including polygalacturonases (PG), pectate lyases (PL), and pectinesterase (PE) (Rehman 2021). Pectate lyase plays a significant role in the current industries, such as in paper making, wastewater treatment, oil extraction, textile processing, fibre degumming, wine clarification, animal feed industry, and coffee and tea fermentation (Yadav et al. 2009; Hugouvieux-Cotte-Pattat et al. 2014; Wu et al. 2020). The catalytic attributes and stability of pectate lyase in various physio-chemical environments are significant for their commercialization (Wang et al. 2018; Xiang et al. 2019; Xu et al. 2021). In particular, the temperature stability and pH stability of pectate lyase are extremely vital for its application. For example, the degumming process is usually executed at a temperature from 40°C to 70°C and in alkaline pH condition (8–11) (Bekli et al. 2019; Wu et al. 2020). Therefore, it is desirable for enzymes to remain stable and active under thermal and alkaline conditions for industrial application.

Many studies on the stability of pectate lyase have been reported, including studies on pH stability. Immobilization of enzymes was shown to be useful for improving the stability of the enzyme. Ran *et al*. (2017) immobilized alkaline polygalacturonate lyase to the surface of bacterial polyhydroxyalkanoate nanogranules, enhancing the thermostability and pH stability under certain conditions. Rational protein design and directed enzyme evolution were also efficient for improving the stability of pectate lyase (Xu et al. 2021; Zhou and Wang, 2021). Screening new strains for obtaining special enzyme is another efficient method due to the abundant microbial resources. Zhou (2017b) reported that pectate lyase from *Bacillus subtilis* PB1 had a broad pH stability from 5 to 11, maintaining 80% relative enzyme activity after 2 h. Sasaki (2015) screened a strain of *Georgenia muralis* JAM 3H7-3 that could produce the pectate lyase with an optimal pH of 10.0 and was stable at pH 6.5-11.0. The pectate lyase from *Bacillus tequilensis* SV11 was stable at pH 11.0, and the residual enzyme activity was 75% after 24 h (Chiliveri and Linga 2014). Although pectate lyases with relatively good stability were provided in the above reports, their stability still needs to be further improved. Screening pectate lyase with better stability is of great significance for the practical application of enzymes.

In this study, an excellent pH-stable pectate lyase with good thermal stability was found. The enzyme was produced by the *Bacillus altitudinis* CAS-WZS-08 preserved in China General Microbiological

Culture Collection Center (CGMCC NO. 22763). The culture medium and conditions were optimized using single-factor analysis. The enzyme was purified and properties were characterized, including the optimal temperature and pH, temperature and pH stability, and the affects of metal ions on pectate lyase activity. Finally, the purification and molecular weight of the enzyme were measured using the ammonium sulfate precipitation, dialysis, cation exchange column, Sephadex G-75, sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), and liquid chromatography tandem-mass spectrometry (LC-MS/MS).

2. Materials and methods

2.1 Media and reagents

Isolation medium: pectin (galacturonic acid≥74.0%, Shanghai Macklin Biochemical Co., Ltd.) 2 g/L, $(NH_4)_2SO_4$ 10 g/L, NaCl 2 g/L, KH_2PO_4 0.3 g/L, K_2HPO_4 1.0 g/L, $MgSO_4$ 0.3 g/L, $FeSO_4$ 0.1 g/L, Agar 15 g/L, pH was adjusted to 7.0 with NaOH, sterilized at 115℃ for 30 min. LB (Luria-Bertani) medium: yeast extract (Oxoid) 5 g/L, tryptone (Oxoid) 10 g/L, NaCl 10 g/L, sterilized at 121℃ for 20 min. Primary fermentation medium: pectin2 g/L, $(NH_4)_2SO_4$ 10 g/L, NaCl 2 g/L, KH_2PO_4 0.3 g/L, K_2HPO_4 1.0 g/L, $MgSO_4$ 0.3 g/L, $FeSO_4$ 0.1 g/L, pH was adjusted to 7.0 with NaOH, sterilized at 115℃ for 30 min. were purchased from Oxoid, England, and other regents were purchased from Sinopharm Chemical Reagent Co., Ltd. Shanghai, China.

2.2 Isolation and identification of strain

2.2.1 Screening for the enzyme

Samples were gathered from the tobacco leaf which was conserved in the laboratory, and rotting leaves were acquired from the park of Qingdao Institute of Bioenergy and Process Biotechnology of Chinese Academic of Science. Samples were diluted at 1:9 with distilled water, incubated at 37℃ for 30 min in a vibration culture oven. Strains were isolated using the isolation medium as mentioned above. All the monoclonal strain was transferred to new LB medium until pure culture. The ability of pectate lyase was measured using congo red and NaCl (Zhang et al. 2019; Prajapati et al. 2021). The Hc value indicates the ability of pectate lyase, which was the ratio of the diameter of clear zones and strains Each measurement was carried in triplicate.

2.2.2 Identification of strain

The strain was identified according to biochemical characteristics and the 16S ribosomal RNA (rRNA)

gene sequence. The biochemical characteristics were tested with microbial biochemical identification tubes (Qingdao Hope Bio-Technology Co., Ltd., China) according to the instruction. These tests included the Voges-Proskauer, citrate, propionate, D-xylose, L-arabinose, D-mannose, gelatin liquefaction, 7% NaCl, nitrate reduction, amylolysis, resistance to antibiotics (Amp 100 ug/mL; Cm 34 ug/mL; Kana 35 ug/mL; Spe 50 ug/mL), and gram staining. The temperature and pH range of strain growth was studied using LB medium under diverse temperature and pH. The 16S rRNA gene fragment was obtained using polymerase chain reaction (PCR) amplification with universal primers (BGI Life Tech Co., Ltd., Qingdao, China): 27F-primer (5'-3'): AGAGTTTGATCCTGGCTCAG and 1492R-primer (5'-3'): ACGGCTACCTTGTTACGACTT. And the DNS Polymerase was PrimerSTAR Max DNA Polymerase (2×). Subsequently, the product of PCR was sequenced by Tsingke Biological (Tsingke Biotechnology Co. Ltd.). In the end, the sequence was blast to NCBI database and deposited to GenBank. The phylogenetic tree was constructed through MEGA 7.0 (Kumar et al. 2016) using the neighbor-joining method, and the bootstrap analysis was based on 1000.

2.3 Determination of pectate lyase activity

Enzyme activity was measured using the 3, 5-dinitrosalicylic acid (DNS) method (Zhou et al. 2017b; Xu et al. 2021). For the enzyme assay, 1.0 ml of freshly grown culture was centrifuged at 10, 000 rpm for 10 min. The supernatant was used for enzyme activity determination. The substrate was prepared by mixing 0.1% (w/v) pectin in glycine-NaOH buffer (50 mmol/L). The control experiments were prepared using a deactivated enzyme instead of the normal pectate lyase. The absorbance of the samples and control were detected at 540 nm.

The standard curve was established using D-galacturonic acid (Beijing Solarbio Science and Technology co., Ltd.) as the reducing sugar (Prajapati et al. 2021). The enzyme activity (U/mL) is defined as the amount of enzyme required to catalyze the substrate to form 1 μmol of galacturonic acid per minute under the given conditions. The enzyme activity was calculated as follows.

$$\text{Enzyme activity (U/ml)} = (\Delta A \times 1000 \times N)/(K \times M \times T)$$

ΔA: the difference between the sample and control; N: dilution factor; K: the coefficient of the standard curve; M: the molecular weight of D-galacturonic acid T: time (min)

2.4 Effects of fermentation conditions

Pectinase, a member of the hydrolases family of enzymes, holds a leading position among the commercially produced industrial enzymes (Fawole and Odunfa 2003; Amin et al. 2019; Sharma et al.

2019). In fermentation condition research, the pectin concentration, nitrogen types and concentration, the initial pH of the media, inoculum size, and culture temperature were determined step by step using single-factor analysis at one time. The pectin concentration was designed for 2, 4, 6, 8, and 10 g/L, and the nitrogen types, including urea, NH_4Cl, $(NH_4)_2SO_4$, yeast extract, tryptone, and beef powder at 10 g/L were measured. In nitrogen concentration studies, seven concentrations were designed following the results of nitrogen type, including 4 g/L, 8 g/L, 12 g/L, 16 g/L, 20 g/L, and 24 g/L. The initial pH of the media were set to 5, 6, 7, 8, 9, and 10 for determination of optimal fermentation of pectate lyase. Moreover, the inoculum size, including 1%(v/v), 2% (v/v), 3% (v/v), 4% (v/v), 5% (v/v), 6% (v/v), and 7% (v/v), was studied for the strain in pectate lyase fermentation. Experiments were performed in triplicate (n=3).

2.7 Characterization of the enzyme properties

Enzymes work depending on the temperature, pH, the type of metal ions, buffer concentration, and so on (Gummadi and Panda 2003; Mei et al. 2013). Research on enzyme properties will be beneficial for effective application. Regarding the enzyme properties, the optimum temperature and stability in temperature, the optimum reaction pH and pH stability, the effects of metal ions on enzyme activity, and effects of buffer concentration were organized.

The optimum temperature was determined from 45 to 60°C (tested in interval of 5°C) in glycine-NaOH (50 mmol/L, pH 9.0). To determine the temperature stability of the enzyme, the supernatant crude enzyme was preincubated at 30°C, 35°C, 40°C, 45°C, 50°C, 55°C, and 60°C. The crude enzyme liquids were taken from the preincubated system to determine the remaining pectate lyase activity.

The optimum pH was determined at 60°C for 30 min in different buffers (50 mmol/L) with pH from 3.0 to 10.5 (Jadhav and Pathak 2019). The buffer incorporates Na_2HPO_4-Citric acid (pH 3.0, 4.0, 5.0, 6.0), KH_2PO_4-NaOH (pH 6.0, 7.0, 8.0), Tris-HCl (pH 8.0, 9.0), and glycine-NaOH (pH 9.0, 9.5, 10.0, 10.5). The pH stability of the enzyme was determined by measuring the relative enzyme activity (Jalil and Ibrahim 2021) after the partially purified enzyme incubation at 4°C and room temperature (25°C) at pH from 3.0 to 10.0. The enzyme activity without preincubation was defined as 100%.

The effects of metal ions were calculated through 1 mM K^+, Ca^{2+}, Mg^{2+}, Zn^{2+}, Mn^{2+}, Cu^{2+}, Ni^{2+}, Fe^{2+}, Fe^{3+}, Ba^{2+}, Co^{2+} in the reaction mixture. The mixture was incubated for 30 min at 60°C to evaluate the effect of metal ions. The effects of buffer concentrations of 0 mmol/L, 50 mmol/L, 100 mmol/L, 200

mmol/L were studied for enzyme activity.

2.8 Purification of pectate lyase

Pectate lyase has potential application in industries such as paper making, fertilizer, beverage, coffee fermentation, wastewater treatment, and ramie degumming (Yadav et al. 2009; Hugouvieux-Cotte-Pattat et al. 2014; Wu et al. 2020). To obtain the purified enzyme from fermentation broth for its application, several purification methods were evaluated in this study. First, the ammonium sulfate precipitation method (SB 2009) was deployed with supernatant after the centrifugation at 10, 000 rpm for 10 min. The ammonium sulfate fractional precipitation was applied to determine the saturation of ammonium sulfate, containing 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, and 100%. The activities of the enzyme in the supernatant and precipitate were measured to select the saturation of ammonium sulfate. Dialysis was carried out to exclude low-molecular-weight substances and other ions that may interfere with enzyme activity. Subsequently, the ion exchanger column (HiTrap SP HP, 5 mL) was applied to obtain purified pectate lyase using low-pressure liquid chromatography (2001-A-I, Shanghai Jiapeng Technology Co., Ltd). The buffer was Na_2HPO_4-Citric (pH 6.0, 50 mmol/L). Na_2HPO_4-Citric equilibration buffer (50 mmol/L, pH 6.0) with 0 M NaCl and 1 M NaCl were prepared. Then, liner gradient elution was carried out at 0.8 ml/min, and collection was carried out using a separator. Each tube was 1.2 ml. After that, the sample was purified through Sephadex G-75. 1 M NaCl as elution at pH 7.2 (Tris-HCl, 50 mmol/L) at 0.3 ml/min. Each tube was collected after 10 min.

The protein content in purified enzyme were measured by a BCA (bovine serum albumin) standard curve (Beijing Solaibao Technology Co., Ltd) according to the manufacture's instruction. The weight and purity of pectate lyase were measured using SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) (Zheng et al. 2020) under the 12% separation gel and 5% stacking gel according to the protein marker.

2.9 LC-MS/MS identification of pectate lyase

The purpose of signal bond was cut off from the SDS-PAGE gel and subjected to LC-MS/MS (liquid chromatographic tandem-mass spectrometric) (Vogeser and Parhofer 2007) analysis by Beijing BGI Technology Co., Ltd. The protein was processed utilizing trypsin for 12 h at 37℃ after the protein was digested, discolored, and dehydrated. According to the traditional approach, mobile phase A (0.1% formic acid) and B (0.1% formic acid in 84% acetonitrile) were transported by utilizing ultimate 3000

(Dionex). The mass spectrometer was operated in MS/MS mode with a scanning range of 350-2, 000 Da. The top 23 charge ions from each scan were selected for MS/MS analysis. Subsequently, MASCOT 2.2 (Matrix Science, London, UK) was applied to search the MS/MS spectrum and identify the protein by searching the Uniport (https://www.uniprot.org/).

3. Results

3.1 Screening of strains for pectate lyase

The Hc value was the ratio of the diameter of the transparent strain to the growth diameter of the strain. Through the primary and secondary screening, five strains were obtained, including CAS-WZS-08, MEI-2-27, MEI-2-29, CAS-MEI-2-33, and CAS-07. The strain of CAS-MEI-2-33 was used as the control to ferment tobacco stalk to generate alkaline pectinase in a previous study (Zhang et al. 2019). The results were shown in Figure 1. The CAS-WZS-08 strain had a higher Hc value than the others and was selected for further studies.

3.2 Phylogenetic tree and characteristics of the strain

Through the 16S rDNA sequence of CAS-WZS-08, a phylogenetic tree was constructed utilizing MEGA 7.0 (Kumar et al. 2016). The result was shown in Figure 2. CAS-WZS-08 had 96% similarity to *Bacillus altitudinis* 41KF2b T. 23 MN543854.1:1-1423. The physiological and biochemical characteristics of CAS-WZS-08 were shown in Table 1. The sequence of the 16S rDNA was deposited in GenBank, and the accession number was SUB11024406 CAS-WZS-08 OM462373. The results of L-arabinose, D-mannose, Gelatin liquefaction, Nitrate reduction, Gram staining were positive, and the *Bacillus altitudinis* CAS-WZS-08 strain could grow under 7% NaCl conditions. Other tests were negative, including V-P (Voges-Proskauer), Citrate, Propionate, D-xylose, and amylolysis. It was demonstrated that the *Bacillus altitudinis* CAS-WZS-08 strain was susceptible to Amp (100 ug/ml), Cm (34 ug/mL), Kana (35 ug/mL), and Spe (50 ug/mL) from the results of the antibiotic resistance test. These results were consistent with the past physiological and biochemical identification results of *B. altitudinis* (Shivaji et al. 2006; Vijay Kumar et al. 2011; Madhuri et al. 2012; Thite and Nerurkar 2018).

Table 1. Physiological and biochemical characteristics of *Bacillus altitudinis* CAS-WZS-08.

Biochemical Test	*Results*
V-P (Voges-Proskauer)	-
Citrate	-
Propionate	-
D-xylose	-

L-arabinose	+
D-mannose	+
Gelatin liquefaction	+
7% NaCl	+
Nitrate reduction	+
Amylolysis	-
Ampicillin（Amp, 100 ug/mL）	S
Kanamycin (Kana, 35 ug/mL)	S
Chloramphenicol (Cm, 34 ug/mL)	S
Spectinomycin (Spe, 50 ug/mL)	S
Gram staining	+
Shape	Rod

Note: The '+' means positive, '-' means negative. 'S' means sensitive.

3.4 Growth characteristic of B. altitudinis CAS-WZS-08

The pH and temperature of media were vital to the strain's fermentation. The influence of different pH conditions, from 2.0 to 11.0, in LB medium on CAS-WZS-08 growth was measured, as shown in Figure 3a. The strain could grow at pH 5.0 to pH 10.0. When the pH was 7.0, the absorbance at 600 nm (OD_{600}) was highest, with 4.75±0.12, while the growth of the strain was markedly inhibited at pH 10.0. The results of strain growth under diverse temperatures, from 27℃ to 47℃, were shown in Figure 3b. The OD_{600} at 33℃ was higher than that at the other temperatures. This study provides guideline for further research on *B. altitudinis* CAS-WZS-08.

3.5 Optimization of fermentation conditions

The fermentation parameters of *B. altitudinis* CAS-WZS-08 were optimized using one signal factor at one time (El-Ghomary et al. 2021). The results were shown in Figure 4, including the pectin concentration, nitrogen source, nitrogen concentration, medium pH, inoculum size, and culture temperature. The standard curve was Y = 13.375X - 0.1063, R^2 = 0.9996. The effect of pectin concentration on enzyme activity was shown in Figure 4a. When pectin concentration was 4 g/L, the pectate lyase activity was the highest (0.064 ± 0.002 U/mL) under the test conditions. Yeast extract was better than the tested media, and the enzyme activity was 0.263 ± 0.013 U/mL (Figure 4b). Organic nitrogen sources were better than inorganic nitrogen in the experiments. The enzyme activity was enhanced 4.1-fold compared to that under a 4 g/L pectin concentration. With a pectin concentration of 4 g/L, the effects of yeast extract concentration in the medium were tested, and with increasing yeast extract concentration from 4 g/L to 20 g/L, the pectate lyase activity increased gradually, and the highest enzyme activity was 0.54 ± 0.017 U/mL (Figure 4c). When the yeast extract was 24 g/L, the pectate lyase activity

was below 20 g/L. Under the optimal yeast extract concentration, the effect of pH of the medium was studied (Figure 4d). The enzyme activity presented a normal distribution, reaching 0.69 ± 0.040 U/mL at pH 7.0. Subsequently, the influence of inoculum size was determined from 1% (v/v) to 7% (v/v). When the inoculum size was higher than 4% (v/v), the enzyme activity declined. The highest enzyme activity obtained was 0.79 ± 0.001 U/mL at 2% (v/v). Finally, the influences of culture temperature from 27°C to 47°C were tested, and the results were shown in Figure 4f. There was little difference in enzyme activity between 27°C and 40°C. However, the enzyme activity significantly declined when the temperature exceeded 44°C. Combined with the results of strain growth under different temperatures, the highest temperature had little effect on the growth of the strain. The reduction in enzyme activity at high temperatures may be caused by pectate lyase denaturation at higher temperature (Peterson *et al.*, 2007).

3.6 Enzyme properties

The stability of the enzyme and maintaining its activities in extreme temperature, harsh pH environments, and organic solvents, are critical for its commercialization (Wu et al. 2020). The enzyme properties of *B. altitudinis* CAS-WZS-08 were shown in Figure 5. The optimal temperature was shown in Figure 5a. Under the experiments, the pectate lyase increased gradually with increasing temperature until 60°C and then decreased. The collision between enzyme and substrate was encouraged due to the temperature increased, increasing enzyme activity. however, when a certain limitation was reached, the tertiary structure of the protein was damaged, and the enzyme activity was destroyed. The effects of different pH buffers on pectate lyase activity were shown in Figure 5b. The isolated enzyme was an alkaline pectate lyase, and the highest enzyme activity occurred when pH was 10.0 (Glycine-NaOH, 50 mmol/L). There was no enzyme activity under acid conditions. The activity of this enzyme was also affected by the category of the buffer. The Tris-HCl (pH 7.0, pH8.0) buffer was more suitable for pectate lyase activity than the KH_2PO_4-NaOH (pH 7.0) and glycine-NaOH (pH 8.0). Among the different roles that metal ions can play in the catalytic event, the most common is their ability to orient the substrate correctly for the reaction, to exchange electrons in redox reactions, and to stabilize negative charges (Prejanò et al. 2020). The influences of metal ions on enzyme activity were shown in Figure 5c. Enzyme activity was activated by Mn^{2+}, Cu^{2+}, Co^{2+}, Ca^{2+}. Especially in the Mn^{2+} condition, the pectate lyase activity was 2.11-fold that of the control. Bacterial pel enzyme works predominantly requires Ca^{2+} ions, which could acidify the C5 proton of galacturonic acid binding to the +1 subsite of PGLs (Bekli et al.

2019; Zheng et al. 2021). The enzyme activity was inhibited by Fe^{3+}, Ba^{2+}, and Mg^{2+}, especially Fe^{3+}, in which the pectate lyase activity was only 18% of the control. K^{+}, Zn^{2+}, Ni^{2+}, and Fe^{2+} have little effect on enzyme activity. The enzyme activity was also affected by buffer concentration, and the highest pectate lyase activity occurred at 50 mmol/L.

The temperature stability results were shown in Figure 5e. And the enzyme was stable at 30-50°C, especially since the enzyme activity was not affected at 30°C and 35°C after 180 min. When the temperature was 50°C, the residual enzyme activity was 65.39% of the initial after 180 min. When the enzyme was incubated at 55°C, the half-life was approximately 100 min. The half-life of pectate lyase was approximately 28 min at 60°C. Zhou *et al*. (Zhou et al. 2017b) retorted the pectate lyase produced by *B. subtilis* PB1 and it maintained 90% relative activity at 50°C after 2 h. And the pectate lyase was stable at 50°C after 24 h (Chiliveri and Linga 2014). In addition, rational design and site-directed mutation are expected to further improve the thermal stability of pectate lyase (Xu et al. 2021; Zhou and Wang 2021).

The pH stability of the enzyme was shown in Figure 5f. Pectate lyase has a wide range of stability from pH 4.0-10.0 at 4°C, maintaining 100% relative enzyme activity. At room temperature (25°C), the relative enzyme activity was higher than the initial activity at pH 6.0-8.0, and it was not affected at pH 4.0-5.0 and pH 9.0, in accordance with the initial enzyme activity. But the enzyme activity diminished at pH 10.0. To the best of our knowledge, the pH stability of the pectate lyase is better than that of former reporters. In particular, the enzyme activity could be improved at room temperature with a different buffer. It was not reported in the past. The pH-stable pectate lyase from *B. altitudinis* CAS-WZS-08 is expected to be used in industry. The studies on the stability of pectin lyase were summarized in Table 2, including isolation and heterogeneous expression.

Table 2 Pectate lyase properties with acid-base stability and thermal stability in previous studies.

	Organism	Optimal Tm	Temperature stability	Optimal pH	pH stability	Reference
Isolation	*Bacillus subtilis* PB1	50℃	50℃ ~ 2 h ~ 90%	9.5	pH 5.0-11.0 ~ 2 h ~ 80%	(Zhou et al. 2017b)
	Georgenia muralis JAM 3H7-3	50℃	/	10.0	pH 6.5-11.0 ~ 40℃ ~ 1h	(Sasaki et al. 2015)
	Bacillus tequilensis SV11	60℃	50℃ ~ 24 h ~ 50%	9.0	pH 11.0 ~ 24 h ~ 75%	(Chiliveri and Linga 2014)
	Bacillus pumilus BK2	70℃	30℃ ~ 75 h	8.5	/	(Klug-Santner et al.

			~ 50%			2006)
	Bacillus altitudinis CAS-WZS-08	60℃	50℃ ~ 3 h ~ 65.39%	10.0	pH 4.0-10.0 ~ 4C° ~ 24 h ~ 100%	This study
Heterogeneous expression	*E. coli* (*Paenibacillus* sp. 0602 G241A)	67.5℃	60℃ ~ 61.86 min ~ 50%	/	/	(Zhou and Wang 2021)
	E. coli (*Paenibacillus polymyxa* KF-1)	40℃	40℃ ~ 60 min > 50%	10.0	pH 5.00-11.0 ~ 25℃	(Yuan et al. 2019)
	E. coli (*Antarctic bacterium*)	30℃	40℃ ~ 10 min ~ 0%	10.0	pH 9-10.5 ~ 4℃ ~ 70%	(Tang et al. 2019)
	A. luchuensis var. saitoi	30℃	60℃ ~ 60 min ~ 71%	8.0	pH 6-12 ~ 4℃ ~ 24 h ~ 80%	(Kamijo et al. 2019)
	E. coli (*B. amyloliqu-efaciens* S6)	60℃	/	10.0	/	(Bekli et al. 2019)
	E. coli (*Paenibacillus polymyxa* KF-1)	50℃	< 50℃	9.0	pH 5.0-11.0 ~ 24 h ~ 50%	(Yan et al. 2018)
	E. coli (*Bacillus clausii*)	70℃	75℃ ~ 30 min ~ 140%	10.0	pH 6.5-11.5 ~ 30℃ ~ 6 h ~ 80%	(Zhou et al. 2017a)
	P. pastoris (*Volvariella volvacea*)	60℃	40℃ ~ 1 h ~ 60%	10.0	pH 4.0-11.0 ~ 4℃ ~ 24 h ~ 80%	(Shi et al. 2015)
	E. coli (*Bacillus pumilus* (ATCC 7061))	65℃	50℃ ~ 2.7 min ~ 50%	/	/	(Liang et al. 2015)
	E. coli (*Paenibacillus sp. 0602*)	65℃	50℃ ~ 9 h ~ 50%	9.8	pH 7.1-11.6 ~ 45℃ ~ 1 h ~ 80%	(Li et al. 2014)
	E. coli (*Streptomyces sp. S27*)	60℃	50℃	10.0	pH 7.0-12.0 ~ 37℃ ~ 1 h ~ 55%	(Yuan et al. 2012)
	B. subtilis WB600 (*Bacillus subtilis*)	50℃	50℃ ~ 2 h ~ 65%	9.0	pH 7.0-10.0 ~ 80%	(Liu et al. 2012)
	E. coli (*Thermotoga maritima*)	90℃	95℃ ~ 2 h ~ 50%	9.0	/	(Kluskens et al. 2003)

Note: "/" means not mentioned.

3.7 Purification of pectate lyase and characterization with LC/MS-MS

Ammonium sulfate fractional precipitation, dialysis, cation exchange column, and Sephadex G-75 were applied to obtain purified pectate lyase. The results were shown in Figure 6. The result of ammonium sulfate graded precipitation was shown in Figure 6a. The pectate lyase activity was markedly decreased when the ammonium sulfate saturation reached 50% in the supernatant, while the enzyme activity began to commence with precipitation. When the ammonium sulfate saturation reached 70%, the pectate lyase activity diminished to 0 in the supernatant, while the relative activity of pectate lyase in the corresponding precipitation reached 93.17%. Ultimately, the saturation of ammonium sulfate

precipitation was determined to be 50%-70%. Subsequently, the crude enzyme after desalting by dialysis was further purified by a cation exchange column at pH 6.0. The result was shown in Figure 6B. The collection tubes were detected through enzyme activity and SDS-PAGE. In the purification process, the enzyme activity and protein content were measured, and the specific enzyme activity and purification fold were calculated as shown in Table 3.

The fermentation liquid was obtained under optimal fermentation conditions. A 1.5 L of fermentation liquid was obtained, the total enzyme activity was 927.71 U and the protein content was 12936.72 μg. After ammonium sulfate precipitation, 50 mL dialysate was obtained with 221.41 U, the protein content was 245.30 μg, and the purification fold was 12.59. After purification with the cation exchange column, the total enzyme activity was 65.09 U, the protein content was 61.33 μg, and the purification factor was 14.80. After Sephadex G-75, the pectate lyase was obtained, as shown in Figure 6c. The purification steps were shown in Figure 6d with ~37 kDa as an example. The LC/MS-MS results showed that the molecular weight of the pectate lyase was 37 kDa, which was consistent with the SDS-PAGE results.

Table 3 Purification of extracellular alkaline protease from *Bacillus altitudinis* CAS-WZS-08.

	Total activity (U)	Total protein(μg)	Specific activity (U/mg)	Purification fold
Fermentation liquid	927.7066	12936.72	71.711	1
Ammonium sulfate	221.4116	245.3008	902.613	12.58679
Cation exchange column (pH 6.0)	65.09151	61.33385	1061.266	14.79918
Sephadex G-75	18.54376	7.515395	2467.437	34.408

4. Discussion

The production capacity of the screened strains was an important direction parameter (Rebello et al. 2017). In this study, the fermentation conditions for optimal higher pectate lyase activity were determined, with activities ranging from 0.03 ± 0.01 U/mL to 0.79 ± 0.001 U/mL. Nevertheless, compared with previous studies, the fermentation parameters should be optimized for high production. An pectin-rich substrate, such as lemon peel (Gummadi and Kumar 2006b; Muslim et al. 2015), wheat bran, and orange bagasse (Ferreira et al. 2010), should be utilized instead of pure pectin for pectate lyase production. In addition, agricultural wastes or industrial wastes have attracted the attention of researchers for their utilization. Thite *et al*. (Thite et al. 2020) reported the ability of strains to generate the xylanase and pectinase with four different agro-waste biomasses. The pectinase activity was 220-280 units using the

citrus peel. Li *et al*. (2020) reported that the *Aspergillus niger* NRRL 322 could produce pectinase, and the highest yield was 9.5 U/mL using soybean hull. Aslam *et al*. (2020) provided a strain of *Bacillus licheniformis* KIBGE-IB3, in which the pectinase yield was higher using the date fruit wastes. Jadhav *et al*. (Jadhav and Pathak 2019) screened strain of *Bacillus licheniformis* UNP-1, and the highest pectinase activity was 55.2 U/mL under the optimal conditions. However, related studies on pectate lyase production using agricultural wastes or industrial wastes were limited. To obtain a relatively high fermentation yield, the method of response surface design was used, except for the single factor at one time. For example, Songpim *et al*. (Songpim et al. 2010) optimized the pectate lyase production using response surface methodology with an enzyme activity of 84.5 U/ml. The Plackett-Burman design and central composite design were carried out for optimization of pectate lyase production in *B. subtilis* PB1, and the pectate lyase activity was 19.50 ± 0.28 U/ml (Zhou et al. 2017b). The production of pectate lyase was 10.73 U/mL from *Debaryomyces nepalensis* through Placket-Burman design and response surface methodology (2006a). In the latter study, the higher pectate lyase activity from *B. altitudinis* CAS-WZS-08 might be researched using statistical methods.

The stability of pectate lyase, including its thermal stability and pH stability, is extremely important for its application. In this study, the pectate lyase has a wide range of stability from pH 4.0-10.0 at 4℃ and room temperature (25℃), was more stable than previous reports. Prajapati (2021) reported the pectate lyase produced by *B. subtilis* BK-3 was stable at pH 4.0-10.0, with 80% relative enzyme activity after 3 h. Zhou (2017b) isolated *B. subtilis* PB1 strain at pH 5~11 after 2 h. Temperature and the pH determine the changes in enzyme activity collectively. The acid-base property of the enzyme determines its application, and alkaline pectate lyase is generally applied in textile refining, wastewater treatment, and tea or coffee fermentation (Wu et al. 2020; Xu et al. 2021; Zheng et al. 2021). The stability of pectate lyase also determines the production cost of the industry. The pH stability of pectate lyase not only has a great advantage for storage but also has a wide selection of conditions in the purification steps. This result is also a basis for researching the mechanism of pH stability to further enhance the stability of industrial enzymes.

5. Conclusion

In this research, a pH-stable alkaline pectate lyase with good thermal stability was produce by *B. altitudinis* CAS-WZS-08. Subsequently, the production yield was optimized with the highest enzyme

activity of 0.71 ± 0.001 U/mL (4 g/L pectin, 20 g/L yeast extract, 2% inoculum size, pH 7.0, 33°C). The enzyme properties indicated that the pectate lyase has broad pH stability between pH 4.0 and pH 10.0. This enzyme was an alkaline pectate lyase and had the highest enzyme activity at 60°C under pH 10.0. In addition, the pectate lyase was activated by Mn^{2+}, Cu^{2+}, Co^{2+}, Ca^{2+}, but was inhibited by Fe^{3+}, Ba^{2+}, and Mg^{2+}. Through ammonium sulfate precipitation, dialysis, cation exchange column, and Sephadex G -75, the pectate lyase bond was purified to be of electrophoretic purity. The LC/MS-MS results indicated that the protein was pectate lyase, with a molecular weight of 37 kDa. This study provided a new strain that could generate a fairly pH-stable alkaline pectate lyase, and the enzyme has a good thermal stability. All the results indicated that this enzyme is not only a potential candidate for researching the mechanism of pH stability to further enhance the stability of industrial enzymes but also for industrial applications.

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