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Purification and identification of novel alkaline pectinase PNs31 from *Bacillus subtilis* CBS31 and its immobilization for bioindustrial applications

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Abstract−A robust alkaline pectinase PNs31 was produced from *Bacillus subtilis* CBS31 that was isolated from kimchi (a traditional Korean fermented food). The isolated strain CBS31 was identified as having 99.93% similarity to *Bacillus subtilis* subsp. The PNs31 was purified to homogeneity using two-step column purification, with an estimated 13.6% pectinase yield and 29.2-fold purity. The molecular mass (~35 kDa) and the N-terminal sequence residues of PNs31 established it as a novel pectinolytic enzyme. PNs31 was stable in the alkaline pH range (9-14) and up to 70 °C and had an optimum pH and temperature were 12.2 and 60 °C, respectively. PNs31 had definite V_{max} and K_m values of 220.43 mmol/min and 0.42 mg/mL, respectively, when different concentrations (5 to 50 mg/mL) of pectin were used as substrate. To develop the functional stability and reusability of the pectinase enzyme, PNs31 was immobilized in calcium alginate beads. The result was that the immobilized enzyme maintained approximately 83% and 65% relative activity in the second and third cycles of reusability experiments, respectively.

Keywords: Alkaline Pectinase, *Bacillus subtilis*, Biochemical Characterization, Immobilization, Purification

INTRODUCTION

Microorganisms are well known for their capability to produce an array of bioactive proteins, including commercially beneficial enzymes. Enzymes can be used as biocatalysts in biological processes, such as in biorefineries, and may contribute to the production of various bioproducts from biomass [1-4]. Pectinase is an essential enzyme with tremendous industrial potential that catalyzes the decomposition of pectic substances. Pectin is a hydrated gel that surrounds the cellulose-hemicellulose network found in plant cell walls. It is a high molecular weight polysaccharide polymer based on galacturonic acid. Pectinases are vital enzymes in the industrial sector, particularly in the juice and food company, besides widely in the fruit pulp and various paper industries. The requirement for more stable and substrate-specific pectinase is expanding rapidly in these industries [5,6]. Thus, it is essential to find pectinases that exhibit optimal activity under varying physicochemical conditions to meet various industrial requirements.

Pectinase is mostly produced using *Aspergillus niger* strains, and solid-state fermentation is known method for producing enzymes with high activity. This system offers several advantages over submerged fermentation, such as higher product concentration, higher productivity, simple fermentation equipment, and less effluent generation. In particular, fermented products can be used directly in the process as crude enzymes without purification. However, commercial bulk proteins are mass-produced through submerged culture system as scale-up is difficult and requires a lot of labor [7].

Despite the superior catalytic activity of pectinase, the use of free enzymes constantly presents the following problems for industrial applications: additional product recovery steps, low stability of the enzyme, and limited reusability [8]. These can be overcome through enzyme immobilization, which can improve catalyst stability and help in continuous reuse of expensive catalysts. We can use bound protein for further process with repeated use as well as it can be released by treatment. Thus, industrial application of the produced enzyme can be enhanced [9-11].

In this study, an active pectinase (PNs31) was purified from a *Bacillus* strain, and its biochemical characteristics were investigated. In addition, the immobilization of PNs31 with calcium alginate beads was carried out to the design of economic processes in industrial applications.

MATERIALS AND METHODS

1. Materials

Pectin, polygalacturonate, trigalacturonic acid, digalacturonic acid, and monogalacturonic acid were purchased from Sigma-Aldrich (MO, St. Louis, USA). The thin-layer chromatography (TLC) silica gel was obtained from the supplier, Merck (Germany). Chromatography resin, Sepharose CL 6B, and DEAE Sepharose Fast Flow were procured from GE Healthcare (Uppsala, Sweden). An

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experimental grade of all reagents was employed.

2. Screening, Isolation, and Identification of Bacterial Strain for Pectinase Production

Traditional Korean food, kimchi, was collected from different provinces in Korea for this study. For bacterial isolation, 0.80% NaCl was mixed with 1 g kimchi and incubated for 24 h at 37 °C. Thus, serial dilutions (till 10^{-7}) were streaked in MH (Mueller-Hinton) agar plate to get appropriate CFUs (colony forming units). Isolated bacterial strains were appropriately diluted and stocked for the future in 20% glycerol at −70 °C by diluting properly. Hereafter, In the preliminary screening process, 78 bacterial strain stocks were thoroughly streaked onto agar plates containing pectin (1%) with yeast extract (0.5%), tryptone (1%), K_2HPO_4 (0.7%), KH_2HPO_4 (0.3%), $MgSO_4 \cdot 7H_2O$ (0.01%), and agar (2%) and then kept for 36 h at 37 °C. After colonies had reached 2-3 mm, iodine-potassium iodide solution composed of 330 mL distilled water, 1 g iodine, and 5 g potassium iodide was added to plates and incubated for 20 minutes and rinsed with 1 M NaCl two to three times to identify pectin hydrolysis clearance zones. Further screening was carried out in broth culture with the same medium. The primarily selected strains were fermented in 500 mL Erlenmeyer flasks of 100 mL media for 84 h at 37 °C. Pectinases activity was checked each 12 h. The designated strain CBS31 constructed on taxonomical characteristics was identified corresponding to the Bergey's Manual of Systemic Bacteriology and followed by 16S rRNA gene sequence analysis [12].

3. Enzyme Assay and Protein Estimation

Pectinase activity was analyzed by sequentially adding 100 mL of enzyme (diluted to 10 mM KCl/NaOH buffer, pH 12.2) to 0.1 mL substrate solution (pectin or pectin from apple (0.75%), Sigma-Aldrich) for 50 min at 60 °C. In parallel, control samples, prepared as sample mixture, were incubated in cold water (4 °C). Further, the reducing sugars released by the enzyme were determined by adding up to 0.1 mL of 0.75% 3,5-dinitro salicylic acid (DNS) solution using the DNS method [13]. The protein concentration in the sample was assessed by the Bradford method utilizing standard protein that is albumin from bovine serum [14].

4. Enzyme Production and Purification

The strain CBS31 was fermented for pectinase production in 2 L Erlenmeyer flasks with 200 mL main medium (pectin (1%), yeast extract (0.5%), tryptone (1%), K_2HPO_4 (0.7%), KH_2HPO_4 (0.3%), $MgSO_4 \cdot 7H_2O$ (0.01%)) at 37 °C with continuous shaking at 110 rpm, and pectinase activity was measured each 12 h. The cell-free supernatant was then accumulated at 48 h and combined with 30-80% saturation with ammonium sulfate and kept on overnight in stirring at 4 °C. The protein-containing precipitation was recovered by refrigerated centrifugation at 4 °C for 40 min at 10,000 rpm and resolved in 10 mM KCl/NaOH buffer (pH 12.0). PNs31 saturated with 30-80% ammonium sulfate was ultra-filtered with a 30 kDa cut-off membrane to recover fractions above 30 kDa. The sample having molecular weight >30 kDa was employed to DEAE-Sepharose (Fast Flow) ion exchange column (2.5×90 cm) pre-stabilized with 10 mM KCl/NaOH buffer (pH 12.2). The same buffer was used to wash the column and eluted with a linear-gradient of KCl (0-1 M). The active fractions were collected, and the same buffer system was applied and loaded into the Sepharose CL-6B column (1.5×25 cm). The column loaded with a 2 mL sample was collected at a constant flow rate of 0.25 mL/min.

5. Determination of Molecular Weight

The molecular weight (MW) of protein was established by SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis). The method was explained by Laemmli, using a 5% stacking (w/v) and 12% polyacrylamide resolving gels (w/v) [15]. To make the protein visible, Coomassie Brilliant Blue R-250 was utilized for staining and forwarded by destaining mixture solution of methanol : glacial acetic acid : dH_2O (1 : 1 : 8 by vol). Additional verification of MW was determined by Zymogram as defined by De Sousa with minimal modifications [16].

6. N-terminal Amino Acids

PNs31 was exposed to the Edman degradation method to detect the N-terminal amino acid sequence utilizing a protein sequencer machine Procise Model 492 (CA, Applied Bio, USA).

7. Effects of Temperature and pH on Enzyme Activity

Relative pectinase activity was established at different pH levels using 100 mM buffers (pH 5.2-14.0). The pH stability was determined by the incubating solution of enzyme samples at 0-4 °C for 24 h with proper pH maintained buffers ranging from pH 5.2 to 14.0. Residual enzyme activity was then defined under standard assay conditions. To determine the optimal temperature for this enzyme, enzyme solutions were incubated at 20-100 °C for 60 min, and for 4 h at 50, 60, 70, 80, 90, and 100 °C to determine the thermal stability. Solutions of samples were removed at every 10, 20, 30, 60, 90, and 120 min according to the previous standard assay protocol, and the residual enzyme activity was assessed.

8. Immobilization of Pectinase

Enzyme immobilization was executed by mixing an equivalent volume of 3% sodium alginate and the produced pectinase. The drop-wise addition of the mixture to 0.2 M $CaCl_2$ solution allowed for the development of calcium alginate beads at 4 °C.

9. Effects of Sodium Alginate and $CaCl_2$ on Immobilization

The impact of significant strengths of sodium alginate and $CaCl_2$ on immobilization was studied by changing their concentrations at differing times to obtain steady calcium alginate beads (a mixture of sodium alginate from 1.0-3.0% and calcium chloride from 0.05-0.5 M). The reusability of the immobilized pectinase was determined in line with the procedure described by Rehman et al. [17].

10. Effect of Metal Ions and Kinetic Parameters

The impact of metal ions on purified pectinase PNs31 activity was analyzed at 60 °C. The pure enzyme was incubated at a 5 mM last concentration of various metal ions. The relative activity was evaluated by determining the control devoid of metal ions. Furthermore, maximum reaction rate (V_{max}), Michaelis constant (K_m), and the kinetic constant of the purified pectinase were determined. It was established using the Lineweaver-Burk plot comparison after incubation with KCl/NaOH buffer (10 mM, pH 12.2) containing 1-10 mg/mL of pectinase at 60 °C for 40 min.

11. Enzymatic Hydrolysis

Soluble sugars (enzymatic hydrolysis) were analyzed using the TLC method as described by Ninawe et al. [18] with minimal alterations [18]. Briefly, purified PNs31 (0.8 mg/mL) was incubated with solution of pectin (7.5 mg/mL) in an appropriate buffer (pH 12.2; 50 mM KCl/NaOH) at 40 °C. Controls (enzyme only [devoid of

substrate], substrate only [devoid of enzyme], and enzyme-substrate combination at 4 °C) were also carried out accompanied by the reaction. Every 60 min, 100 µL aliquots were taken out up to 180 min. The aliquots were then boiled for 15 min to stop the reaction and were identified on silica gel plate 60F 254 (Germany, E.Merck). The solvent system for running TLC was composed of deionized water/acetic acid/butanol (7 : 3 : 7). Spots of soluble sugar were visualized by spraying the mixture solution of methanol and sulfuric acid (95 : 5) on the plate. Different oligosaccharides were used as a standard at a concentration of 5 mg/mL (X1, monogalacturonic acid; X2, digalacturonic acid; X3, trigalacturonic acid).

12. Apple Juice Extraction

Five grams of apple pulp were incubated with 1 mL of partially pure PNs31 for 2 h at 40 °C, followed by incubation for 5 min at 100 °C to disable the enzyme. Here, juice was acquired by centrifugation at 3,000 rpm for 30 min. After cooling at room temperature, the supernatant was filtered and the volume of those filtrates and the dry weight of residue acquired were assessed.

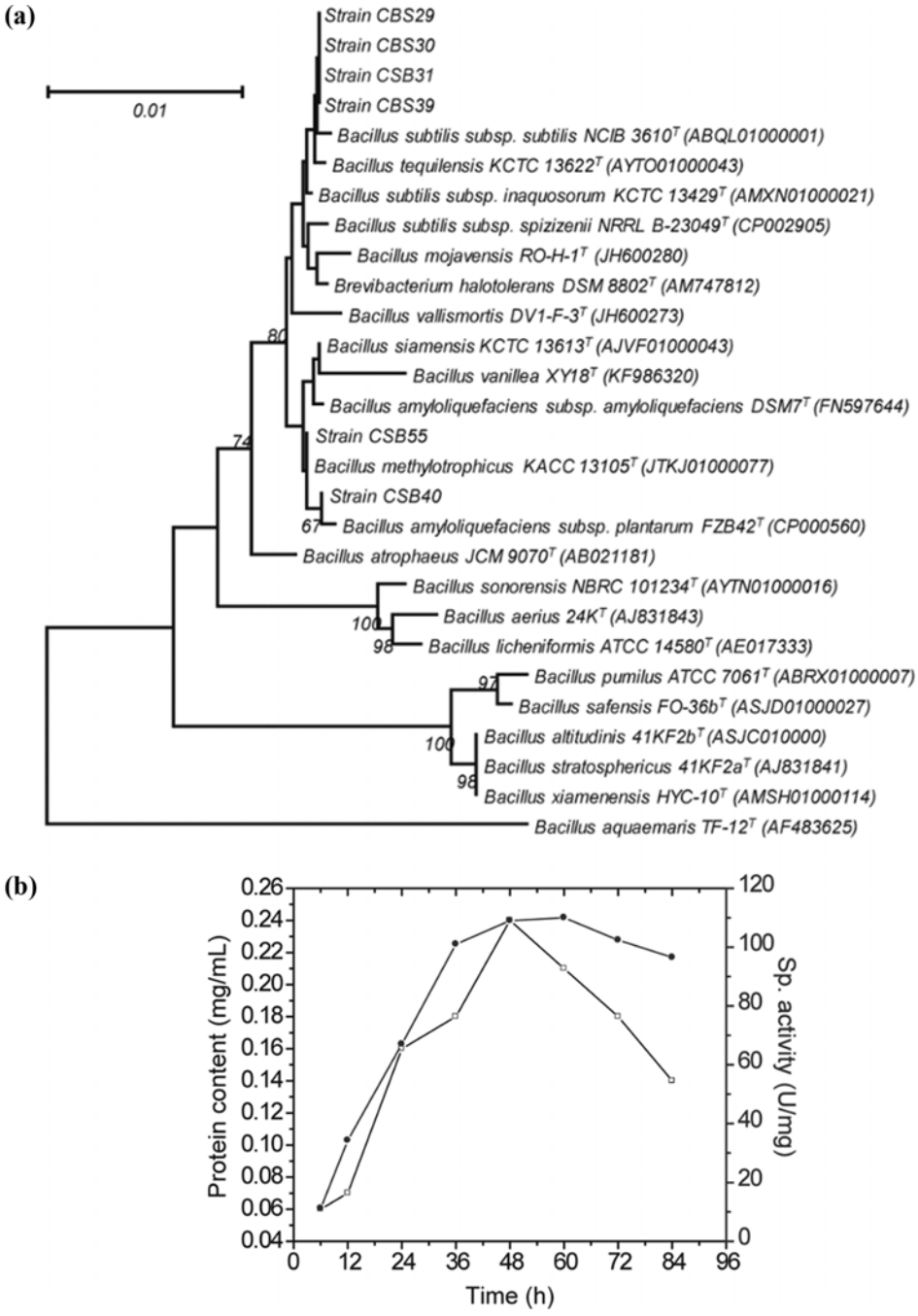

Fig. 1. Neighbor-joining phylogenetic tree established on 16S rRNA gene sequence relationships between CBS31 and closely related taxa of the genus *Bacillus* (a). The pectinase (PNs31) production and specific activity. The protein content per mL (black line) production increased hourly and specific pectinase activity U/mg (blue line) is maximum at 48-60 h (b).

RESULTS AND DISCUSSION

1. Strain Identification and Enzyme Production

Among the 78 cultured strains screened, strain CBS31 indicated high activity and was, therefore, considered for additional studies. The identification of CBS31, named *Bacillus subtilis* CBS31, was 99.93% identical to *Bacillus subtilis* subsp. (Accession: AMXN01000021). The nucleotide sequence was consigned to GenBank under accession no. KY305677 (ncbi.nlm.nih.gov/GenBank) and a phylogenetic tree was prepared from the 16S rRNA sequencing study (Fig. 1(a)). The strain *Bacillus subtilis* subsp. CBS31 has been presented at the Korean Collection for Type Culture (KCTC) fit into the World Data Centre for Microorganisms (WDCM). Here, the Accession number was designated as KCTC18676P. Furthermore, the highest pectinase activity was exhibited by the bacterial strain CBS31 and selected for the present study. The maximum manufacture of PNS31 was attained in optimized culture media at 37 °C at 100 rpm for 46 h (Fig. 1(b)). The PNs31 pectinase specific activity (U/mg) lasted for 48-60 h, and up to 109 U/mg of specific activity was observed.

2. Purification of PNs31

PNs31 was produced in pectinase media. The purification of PNs31 from the supernatant of the 48 h culture samples (30-80% saturation; ammonium sulfate) of CBS31 is condensed in Table 1. A two-step harmony process was presented to purify PNs31 (Fig. 2(a)-(b)), causing a 29.2-fold purification and 13.6% activity healing. Polyacrylamide gel electrophoresis was utilized to define the molecular mass. The molecular weight of PNs31 indicated by a single band was estimated to be 35 kDa, and the purified enzyme consistent with the SDS-PAGE result was confirmed as pectinase by showing a bright band from Zymography (Fig. 2(c)). Our results were supported by several previous reports indicating the molecular weight (MW) of alkaline pectinase from *Aspergillus terricola* MTCC 7588 as approximately 35±01 kDa using SDS-PAGE [19]. Likewise, another report showed the molecular weight (MW) of pectinase from *Acrophialophora nainiana* as about 35.5 kDa and

Table 1. Purification summary

Purification step	Total vol. (mL)	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Yield (%)	Purity fold
Crude broth	853	203.52	22,194.16	109.1	100.0	1.0
$(NH_4)_2SO_4$	15	25.63	16,467.34	642.5	74.2	5.9
DEAE Sepharose Fast Flow	16	1.49	4,280.77	2,873.0	19.3	26.3
Sepharose CL 6B	11	0.95	3,028.84	3,188.3	13.6	29.2

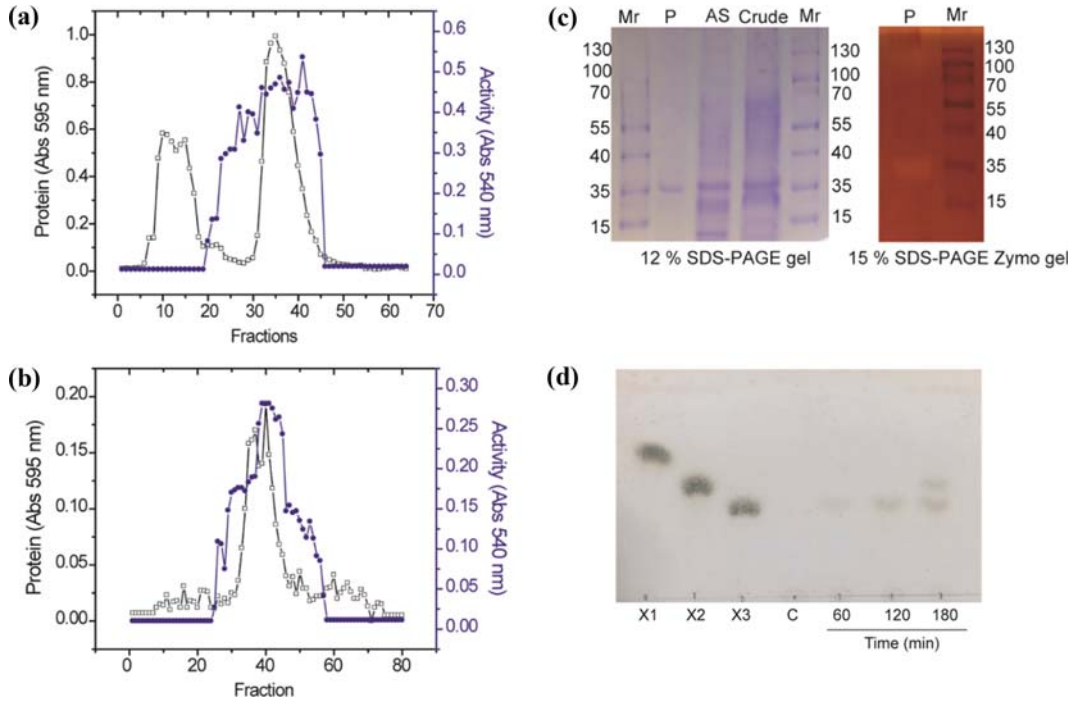

Fig. 2. The elution profile of PNs31 from a DEAE Sepharose ion exchange column (a) and a Sepharose CL 6B permeation column (1.5× 26 cm) (b). SDS-PAGE and Zymogram analysis. Lane 1, protein marker; Lane 2, Sepharose CL 6B pooled purified PNs31; lane 3, 30-80% ammonium sulfate saturated sample; lane 4, crude or broth sample; and lane 5, protein marker. Zymogram is assay shown in the right panel (c). (d), Reaction products hydrolyzed from the polygalacturonic acid by PNs31 of *Bacillus subtilis* CBS31. The reaction mixture was contained 1.0 mg of each substrate in 100 μL of 10 mM KCl/NaOH (pH 12.2) and 0.1 mL of the PNS31 (0.1 U). 10 mL was used for TLC analysis. X1, monogalacturonicacid; X2, digalacturonicacid; X3, trigalacturonicacid.

30.8 kDa as estimated by SDS-PAGE and followed by mass spectrometry, individually [20]. However, molecular weights (MW) of 30 kDa, 38 kDa, 18 kDa, and 50 kDa were discovered for hybrids *of A. flavipe* and *A. niveus* CH-Y-1043, *F. oxysporum f.* sp. *radicis-lycopersici, Penicillium oxalicum*, and *A. flavus* MTCC 7589, respectively [21-24]. Of the total enzyme, nearly 74.2% of it was precipitated within 30-80% saturation of ammonium sulfate, and the specific activity improved to 642.5 U/mg. After the column DEAE Sepharose Fast Flow run for the process, the enzyme activity appeared abundantly in the previous protein peak. Hereafter, the specific activity exhibited equal to 2,873 U/mg and then removed by 19.3%. The chromatographic separation was continued through gel filtration chromatography, Sepharose CL 6B, and most of the activity appeared likely in the previous protein peak. However, the main alkaline PNs31 was purified to apparent consistency. This was authenticated by SDS-PAGE (Fig. 2(c)). The purified enzyme PNS31 demonstrated a specific activity of 3,188.3 U/mg, with a yield of 13.6% (Table 1). The values of these process may not inevitably relate to pectinolytic enzymes from different sources. It happened due to high interlaboratory adaptability as well as the various mechanisms of alkaline pectinase enzymes [20,25]. The major alkaline pectinase enzymes were established as alkaline endo-pectinolytic enzymes as they exhibited not only alkaline pectinase activity but then again revealed alkaline endo-pectinolytic activity. These outcomes are very comparable to those stated for alkaline pectinolytic enzymes from *Fusarium oxysporum* sp. [24].

3. Amino Acid Sequencing Determination

The N-terminal amino acid sequence of PNs31 was determined to be Ala-Glu-Leu-Val-Asp-Gly-Gln-Ile-Tyr-Ala-Asn-Tyr-Phe-Tyr-X. After undergoing BLAST sequence analyses against GenBank, PNs31 showed an entirely novel amino acid sequence.

4. Effect of Temperature, pH, and Metal Ions on PNs31

There were noteworthy effects of temperature and pH on PNs31 activity. The effect of temperature and pH on the actions and constancies of PNs31 related to another pectinolytic enzymes from *Bacillus* are displayed in Table 2 [5,27-29]. PNs31 had a maximum comparative activity at 60 °C and retained ~80% constancy up to 70 °C (Fig. 3(a)-(b)). The impact of pH on PNs31 activity was assessed at 40 °C in an optimized buffer of 10 mM KCl/NaOH. PNs31 remained active in a wide range of pH, and displayed more than 50% of the activity from 9.0 to 14, with an optimum pH of 12.2 (Fig. 3(c)-(d)). This result corresponded with values stated for *F. oxysporum* sp. (pH 9.5) [24], although not with those described for hybrids of *A. terricola* MTCC 7588 (pH 8.0), *A. flavipes*, *Penicillium adametzii* (pH 8.0), *A. flavus* MTCC 7589 (pH 8.0), *A. nainiana* (pH 8.0), and *P. oxalicum* (pH 8.0), or *A. niveus* CH-Y-1043 (pH 8.0) [19-23]. Pectinases that remain stable and effective beneath alkaline conditions are commercially vital for textile industries and paper. However, the consumption of alkaline pectinase has just recently attracted interest [23,30,31].

Table 3. Metal ion effects

Metal ions	Concentration (mM)	Relative activity (%)[a]
Ca^{2+}	5	84.65±1.01
Mg^{2+}	5	106.24±0.54
Cu^{2+}	5	87.42±1.07
Ni^{2+}	5	105.40±1.23
Zn^{2+}	5	104.30±0.86
K^{+}	5	105.31±0.66
Mn^{2+}	5	104.57±1.06
Na^{+}	5	89.14±0.91
None	-	100.00±0.01

[a]Averages of three separate determinations (n=3), and ±standard deviation.

The impact of numerous metal ions on PNs31 activity is presented in Table 3. PNs31 was activated by Mg^{2+} (106.26%), Ni^{2+} (105.40%), K^{+} (105.31%), and Mn^{2+} (104.57%). Furthermore, its activity was constrained for the most part of the evaluated metal-ions, Zn^{2+} (84.65%), followed by Cu^{2+} (87.65%) and Na^{+} (89.14%). This has also been reported between various ions and alkaline pectinolytic enzyme sources. For instance, the activity of alkaline pectinase from *A. flavus* MTCC 7589 was constrained by Ag^{+}, Ca^{2+}, Cu^{2+}, Hg^{2+}, K^{+}, Mg^{2+}, and Zn^{2+} ions and a little recommended by Co^{2+}. Mn^{2+} had no comparative effect [6]. The alkaline pectino-

Table 2. Comparison of PNs31 to similar pectinase from *Bacillus*

Strains	Enzyme type	MW	pH	Temp	Ref.
Bacillus pumilis	Pectic Acid Trans-Eliminase	20	8.0-8.5	60	[1,22-25]
Bacillus stearothermophillus	Pectic Acid Trans-Eliminase	24	9	70	
Bacillus sp. DT7	Pectin Lyase	106	8	60	
Bacillus subtilis	Pectate Lyase	33	8.5	60-65	
Bacillus sp. KSM-P410	Exopolygalacturonate Lyase	45	7	60	
Bacillus licheniformis	Exopolygalacturonate Lyase	38	11	69	
Bacillus macerans	Polygalacturonate Lyase	35	9	60	
Bacillus sp.	Polygalacturonate Lyase	38	11	69	
Bacillus sp. TS44	Polygalacturonate Lyase	50	8	70	
Bacillus clausii KSM-K16	Alkaline Pectin Lyase	35	10.5	50-55	
Bacillus subtilis PEL168	Alkaline Pectate Lyase PEL168	46	9.5	50	
Bacillus sp. MBRL 576	Acidic Pectate Lyase MBRL576	66	4	45	
Bacillus subtilis CBS31	Alkaline Pectinase PNs31	35	12.2	60	This study

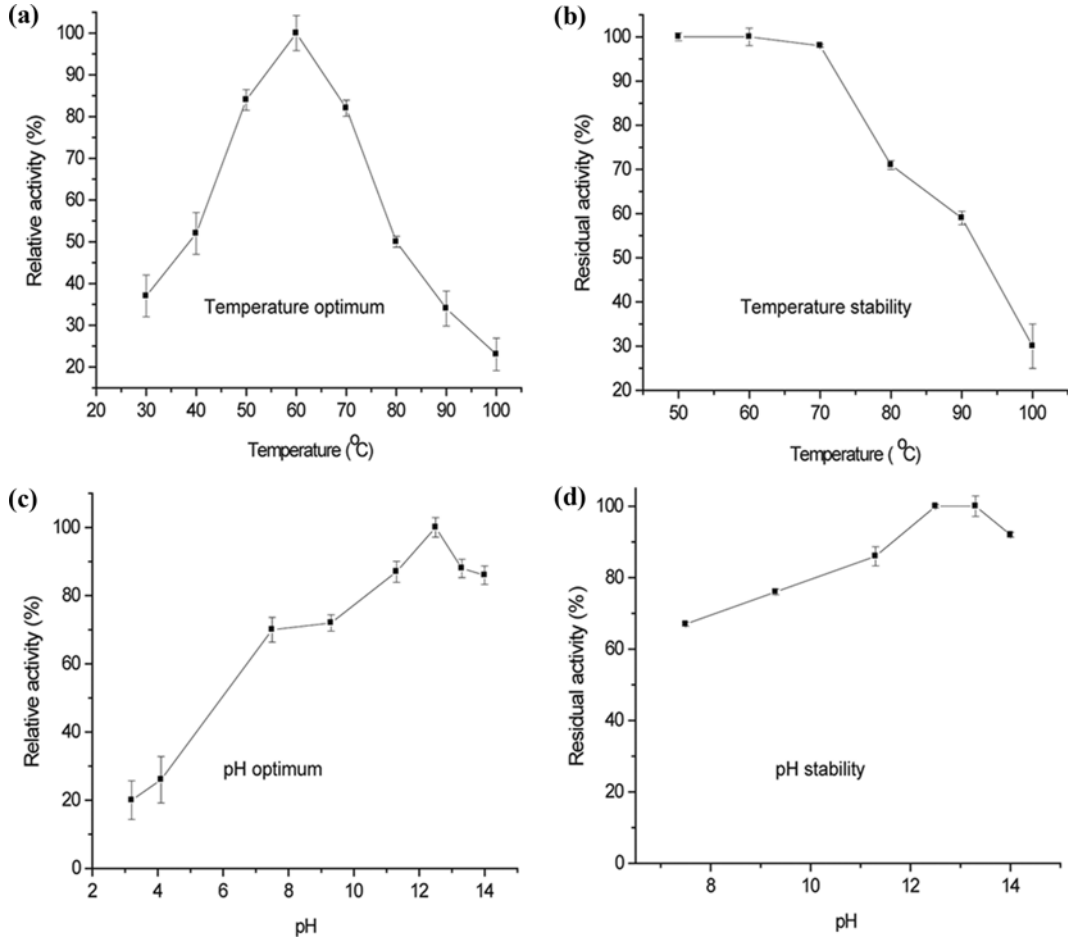

Fig. 3. Optimal temperature (a), thermal stability (b) of pectinase PNs31 from *Bacillus subtilis* CBS31. Different temperatures at optimum pH (12.2) were used to measure optimum temperature, whereas to determine pectinase stability, purified PNs31 was stored at different temperatures for 1 h. Activities were assayed under the standard assay conditions. Optimal pH (c), pH stability (d) of pectinase PNs31 from *Bacillus subtilis* CBS31. To determine pH optimum, the activity of pectinase was determined at 60 °C using different pH buffers. To examine pectinase stability, pectinases were incubated with different pH buffers (2.0-13.6) at 4 °C for 24 h and relative pectinase activity was evaluated under standard assay conditions. Each point is represented as a mean (n=3).

lytic enzyme activity of *Penicillium citrinum* MTCC 8897 was prevented by Ag^{+}, Cu^{2+}, Hg^{2+}, K^{+}, Mg^{2+}, Mn^{2+}, Na^{+}, and Zn^{2+}. Furthermore, it was slightly stimulated by Ca^{2+} and Co^{2+} ions [32]. The activity of alkaline pectinolytic enzyme from *A. nainiana* was reduced by Ca^{2+}, Cu^{2+}, Mn^{2+}, and Zn^{2+} ions, while no effect was observed by Mg^{2+} [20]. Our outcomes are, thus, relatively consistent with those observed in the current article. Alkaline pectinase enzymes are in great demand in the textile industry, where they are utilized for retting plant fibers such as sunn hemp, ramie, flax, jute, and hemp [19,23,31].

5. Kinetics Parameter

The Lineweaver-Burk plot was utilized to establish the kinetic constants of the purified PNs31. The kinetic constants K_m and V_{max} of PNs31 were 0.42±2.2 mg/mL and 220.48±1.60 mmol/min mg, respectively, utilizing various concentrations of pectin (5-50 mg/mL). The K_m of the alkaline pectinase from *B. clausii* S-4 is greater than that of the pectinolytic enzyme from *A. flavus* (0.6 mg/mL) [23]. The optimum pH (10.2) of pectinase from *B. clausii* S-4 is, likewise, greater than that of pectinase from *A. flavus* (8.2). The lower K_m value indicated that the enzyme (PNs31) has a higher affinity for its substrate.

6. Pectinolytic Acid Hydrolysis

The finely purified enzyme PNs31 hydrolyzed. The response products from polygalacturonic acid are shown on the TLC plate in Fig. 2(d) Original monogalacturonic acid (X1), digalacturonic acid (X2), and trigalacturonic acid (X3) rose in sequence corresponding to the molecular weight. As the reaction proceeded, oligogalacturonide emerged from the mixture, trigalacturonicacid (X3) was generated from polygalacturonic (PGA) acid along with ultimately transferred to digalacturonicacid (X2) in 180 min. X2 was not found to be hydrolyzed to monogalacturonicacid (X1) after additional incubation. These results reveal that the purified pectinase enzyme could be categorized under endopolygalacturonase (EC. 3. 2. 1. 15, poly-alpha-1,4-galacturonide glycanohydrolase).

7. Immobilization of PNs31 Using Calcium Alginate as a Support

A mechanically stable alginate gel network is formed through the ionic bond of the carboxyl group of sodium alginate with Ca^{2+}

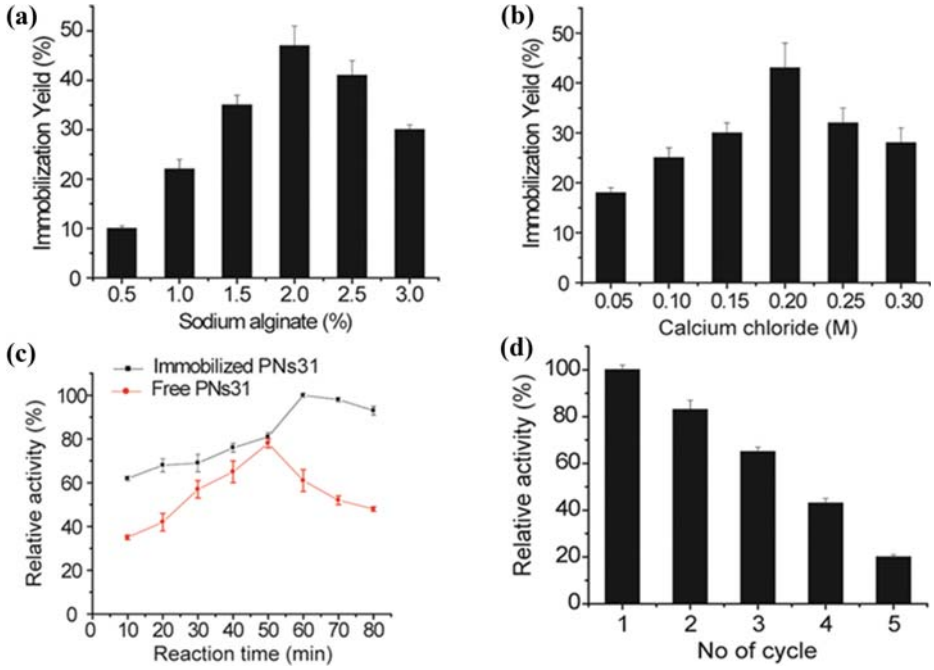

Fig. 4. Effect of different concentrations of 0.5-3% sodium alginate (a) and 0.05-0.30 M $CaCl_2$ (b) on the immobilization yield. Effect of different ranges of reaction times (10-80 min) on immobilized and free enzyme (c). Reusability of immobilized enzyme in batch reactions (d). Each point is represented as a mean (n=3).

ions [33]. Consequently, the effects of $CaCl_2$ concentration and alginate on immobilization were first examined to achieve steady beads with great immobilization yield. Primarily, sodium alginate concentration was diverted from 0.5-3%, while keeping $CaCl_2$ concentration the same (0.2 M). The greatest immobilization yield was found at 2% sodium alginate complex concentration (Fig. 4(a)). An additional increase in the intensity of sodium alginate with a concentration away from 2% reduced the immobilization yield.

Owing to sodium alginate at minimal concentrations showing unstable, fragile, and soft beads, pectinase might leak from beads during immobilization [34]. A similar observation was reported by Rehman et al. [17]. In contrast, higher concentrations of 3% sodium alginate decreased the dimensions of the pores of calcium alginate. This might affect the constraint of substrate penetration into the beads and the effective site of the ensnared enzyme [35]. Fig. 4(b) shows the various concentrations (0.05-0.3 M) of $CaCl_2$ utilized to investigate the effects of $CaCl_2$, and it was found that 0.2 M $CaCl_2$ reserved the highest immobilization yield.

The reaction time (10-80 min) and relative activity of available and immobilized pectinase (PNs31) are exhibited in Fig. 4(c). An increase in the intensity of reaction time was because of the distribution of substrate fragments into calcium alginate beads, which needed a proper time for the substrate to attach to the site of the immobilized enzyme [36]. Immobilized enzyme PNs31 had a prolonged reaction time and relative activity compared to free enzymes.

The recurrent use of the immobilized enzyme is extremely valuable since it is crucial excellence for the commercial viability of bioprocesses using immobilized enzyme systems [37]. The recurrent usability of the immobilized pectinolytic enzyme was carried out at 60 °C in batch effects, and the same response conditions were utilized for all batches. The initial immobilized pectinase PNs31 was considered as 100% relative activity and on its second cycle, 83%, followed by the third (65%), fourth (43%), and fifth cycle (20%), as demonstrated in Fig. 4(d). Our result suggested that the immobilized enzymes can undergo repetitive use though the available enzyme could simply be used one time. The immobilized enzymes could be retrieved from the response combination devoid of any denaturation.

8. Apple Juice Extraction

Pectin, one of the vital elements of the plant cell wall, is discovered in several fruits. Pectinolytic enzymes are commonly used to break down pectin in the process of preparing fruit juice. PNs31 treated the fruit (apple) from *Bacillus subtilis* CBS31, and the enzyme treatment developed the yield. The results showed that pulp treated with PNs31 exhibited better pressing characteristics (Table 4 and Fig. 5). The juice achieved via enzymatic treatment, was less sticky contrasted to the unprocessed sample, probably the reason for breaking down of pectin. The dehydrated weight of the solid scum reduced considerably after enzyme therapy of apple pulp as contrasted with controlling. This result strongly suggests that PNs31 has excellent juice extraction ability.

Table 4. Extraction of juice from treated and untreated apple pulp

	Apple Juice extraction	
	Untreated	Treated by PNs31
Volume of pulp (mL)	7.5	7.5
Volume of juice (mL)	7.8	8.7
Dry weight of residue (g)	1.59	0.73

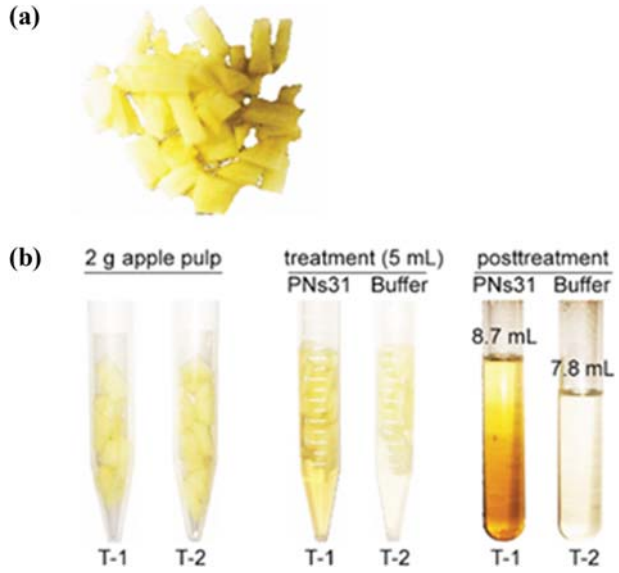

Fig. 5. Treatment of apple pulp by PNs31 from *Bacillus subtilis* CBS31. (a) Pieces of apple pulp (5 g). (b) Two different tubes; Tube-1 (T-1) and Tube-2 (T-2) were filled up with 2 g of apple pulp each. T-1 is received 5 mL PNs31 pectinase and T-2 was received buffer (5 mM Tris-HCl) as control.

CONCLUSIONS

The high yield of alkaline pectinase PNs31 with its temperature stability properties suggested that strain CBS31 would be a better pectinase producing bacteria than formerly described organisms. Immobilized enzymes have numerous applications rather than free enzymes for commercial processes. Owing to the multiple applications of pectinase in food processing and related bioindustries, pectinase from *Bacillus subtilis* CBS31 was immobilized in calcium alginate beads by entrapment, a simple and cost-effective technique for enzyme immobilization. To the best of our knowledge, PNs31 from *Bacillus subtilis* CBS31 with a unique amino acid sequence and bio-characteristics, as well as the approaches of a cost-effective method for enzyme immobilization has never been reported. Thus, PNs31 could be used at a commercial level for the retting of flax, degumming of ramie, and the pretreatment obtained from water from juice processing industries.

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