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RESEARCH PAPER

Endoglucanase Produced by *Bacillus subtilis* Strain CBS31: Biochemical Characterization, Thermodynamic Study, Enzymatic Hydrolysis, and Bio-industrial Applications

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Abstract Microbial cellulases have become the mainstream biocatalysts due to their complex nature and widespread industrial applications. Here, homogeneous endoglucanase GluCB31 from *Bacillus subtilis subsp. inaquosorum* CBS31 was studied. GluCB31 was purified to 17.68-fold with an 8.33% yield and a specific activity of 1066.37 U/mg. Biochemical properties of GluCB31 were performed and the results are as follows; molecular mass of 35 kDa with an optimum pH at 7.5 and temperature at 50°C. GluCB31 was immobilized in calcium alginate gel and it exhibited the highest activity at 10°C higher temperature than soluble enzyme, as the entrapment in alginate gel made GluCB31 more stable. Kinetic studies showed the V_{max} of 1293.33 ± 2.51 U/mg and K_m of 0.0183 mg/mL. Enzymatic activity was activated by Tween-20 (106.7%), Tween-80 (111.6%), Triton X-100 (142.3%), SDS (135.5%), Mg^{++} (185.7%), Cu^{++} (167.6%), Zn^{++} (153.7%), Mn^{++} (106.3%), Ba^{++} (181.9%), Ni^{++} (107.2%) while inhibited by Fe^{++} (15.8%), β-mercaptoethanol (46.8%), EDTA (54.5%). Enthalpy, free energy, and entropy of activation were calculated to be 38.526 $kJmol^{-1}$, 44.187 $kJmol^{-1}$, and -17.518 $Jmol^{-1}K^{-1}$ respectively. Also, $\Delta G_{E\text{-}S}$ and $\Delta G_{E\text{-}T}$ were found to be -10.75 $kJmol^{-1}$ and -45.92 $kJmol^{-1}$ respectively. A low ΔS, $\Delta G_{E\text{-}S}$, and $\Delta G_{E\text{-}T}$ values were signified enzyme-catalyzed reaction occurs at a fast rate and the existence of the enzyme in its stable state. Cellobiose was the major end product of hydrolysis. These attributes of GluCB31 demonstrated the diversity of catalytic activities and serve in various biotechnological processes, thus deserve to be developed as a bio-industrial agent.

Keywords: endoglucanase, thermodynamic, bio-industrial, enzymatic hydrolysis

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1. Introduction

Cellulase is an enzyme which hydrolyzes 1, 4 glycosidic bonds in cellulose polymers to release glucose units [1]. Cellulolytic enzyme accounts for more the 20% of the enzyme market. Cellulose is the most plentiful and renewable biopolymer and ruling waste from agriculture [2]. Authentic application of renewable possessions imparts on hydrolysis of lignocellulosic waste thus fermentation of the following reducing sugars to yield anticipated metabolites. Cellulosic substrate and the physical characteristics of these substrates are important parameters in its enzymatic

hydrolysis. Mostly, the biomass of lignocellulosic is dissimilar from microbial action; hereafter suitable pretreatments are vital to interrupt lignin structure and raise accessibility of enzymes to accelerate the rate of its biodegradation [3].

Microbial degradation of lignocellulosic waste and downstream of the resultant reducing sugars and effective biological hydrolysis of cellulose into primary products glucose, cellobiose, and cello-oligosaccharides require the concerted action of several enzymes composed of endo-β-1,4-glucanase (randomly cleaving internal linkages, EG; EC 3.2.2.4), cellobiohydrolase (specifically hydrolyzing cellobiosyl units from non-reducing ends, CBH; EC 3.2.1.91), and β-D-glucosidase (hydrolyzing glucosyl units from cello-oligosaccharides, BG; EC 3.2.1.21) [4]. Carboxymethyl cellulose (CMC) stated as endoglucanases that are required to degrade it [5]. Cellulolytic microorganisms are mostly degraded carbohydrates and are usually incapable to employ proteins or lipids as their energy source for the growth and metabolism [3]. The ability to secrete huge amounts of extracellular proteins is a characteristic of some microorganisms and these are viewed as suitable for production of advanced levels of extracellular cellulases. Many *Bacillus* sp. have been used for cellulose production including *Bacillus brevis, B. pumilus* [6,7], *B. amyoliquefaciens* DL-3 [8], *B. subtilis* YJ1 [9], *B. cereus* [10], *B. licheniformis* C108 [11], and many others.

Cellulases are inducible enzymes and expensive aspects of industrial cellulase production. Commercial production of cellulases has been tried in many aspects such as by solid-state fermentation or submerged culture using batch, fed-batch, and continuous flow process. The production of cellulase to be used for hydrolysis of the raw materials need to be highly optimized to reduce the cost of production by providing inducers. Commercial-scale production of cellulase is induced either by growing the microorganisms on solid cellulose or culturing in the presence of disaccharides inducers such as lactose, CMC. However, on large scale production both methods of induction result in high cost. Consecutively, it may be probable to use cellulose comprising media for cellulase production but again the process is measured by the dynamics of induction and suppression. The main challenges in cellulase production comprise developing an appropriate bioprocess and media for cellulose fermentation. In this study, a determination has been made to screen endoglucanase-secreting *Bacillus* sp. from popular traditional Korean fermented food (Kimchi). Here, we isolate probable CMCase-secreting organisms by suitable methods. The main objective of this study was to purify endoglucanase and study its fundamental characteristics that can be employed for suitable bioprocess through cellulose fermentation. A study of this kind will improve our understanding of various enzymes used for biotechnological application.

2. Materials and Methods

2.1. Strain isolation and characterization

Approximately 10 g kimchi samples were collected from different provinces of Korea using a sterile sample bag. The kimchi samples were transported and stored at −20°C to the laboratory. Isolation was carried according to our previous methodology [12,13]. Briefly, 1 g of kimchi was mixed with 0.85% NaCl and incubated for 24 h at 37°C. Serial dilutions were performed up to 10^{-7} in Mueller-Hilton broth. From each dilution, streaking was done to find out the appropriate colony-forming unit (CFU). The appropriately diluted solution was stored as stock cultures in 20% glycerol at −70°C. About 100 bacterial specimens collected were spread onto the selective media containing 1.25% (w/v) carboxymethyl cellulose-Na and 0.1% (w/v) Congo red and cultured at 37°C for 24 h. After incubation, colonies showing clear hydrolysis zones were estimated as an endo-β-1, 4-glucanase producing bacterial strain (application of 0.1% Congo red solution for 30 min followed by flooding with 0.5% NaCl). Bacterial strain CBS31 showed the larger zone of hydrolysis and was taken for further study. Strain CBS31 was identified according to our previous methodology [14,15] and the procedures in Bergey's manual of systematic bacteriology. PCR amplification of the 16S rRNA gene sequences (accession no. AMXN01000021) was carried for molecular phylogeny of strain CBS31. 16S rRNA gene sequences were aligned manually with reference sequence retrieved from the GenBank database following BLAST searches. The Phylogenetic tree was constructed using the 16s rRNA gene sequences. Distance and clustering were based on the neighbor-joining and maximum-parsimony methods. The Topology of the neighbor-joining tree was done by bootstrap analysis by performing 1000 resampling. Bacterial strain CBS31 was placed to WDCM (World Data Centre for Microorganisms). Biochemical characterization, thermodynamic study, and bio-industrial applications have been shown for further characterization.

2.2. Induction of endoglucanase

The bacterial strain CBS31 was seeded using overnight grown bacterial suspension on beef extract 0.4%, yeast extract 0.1%, peptone 0.4%, and NaCl 0.25%. The submerged culture was run under continuous shaking that inoculated in a culture media containing carboxymethyl cellulose-Na 1.25%, tryptone 1%, yeast extract 0.5%, beef

extract 0.5%, K_2HPO_4 0.03%, KH_2PO_4 0.07%, $MgSO_4.7H_2O$ 0.04%, NaCl 0.05%, $CaCl_2$ 0.02%, and cultured at 37°C for 72 h at 110 rpm. A 1000 mL supernatant culture was collected after centrifugation at 10,000 x g at 4°C for 30 min. The collected supernatant was subjected to 40-80% ammonium sulfate saturation overnight. The precipitate obtained after centrifugation was dissolved in tris-HCl buffer (pH 7.5, 10 mM). This sample was desalted by dialyzing against distilled water of pH 6.5 and applied to an anion exchange chromatography (DEAE sepharose fast flow) pre-equilibrated with tris-HCl buffer (10 mM, pH 7.5). Buffers used during anion exchange chromatography were tris-HCl buffer (10 mM, pH 7.5) (A) and tris-HCl buffer (10 mM, pH 7.5), and 1 M KCl (B). Enzyme was eluted with a linear gradient of buffer A for 60 min, AB for 90 min, B for 30 min, BA for 60 min, and A for 30 min with a flow rate of 0.35 mL/min. Bound protein was eluted with a lined gradient of KCl (0 to 1.0 M, pH 7.5). Active fractions were collected, concentrated and applied to gel filtration chromatography (Sephadex G-50) pre-equilibrated with tris- HCl (10 mM, pH 7.5), and eluted at the rate of 0.4 mL/min. Fractions showing activity were collected, dialyzed, and assayed for enzyme activity. The purified enzyme evaluated by 12.5% of sodium-dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and biological activity was carried by zymography. Zymography was carried in a 12.5% (w/v) polyacrylamide gel contained 0.25% (w/v) of the substrate by electrophoresing the enzyme [14,15]. After electrophoresis, the gel was washed with distilled water, soaked in 25% (v/v) isopropanol. Further treatment was done on the gel by incubating at 37°C for 60 min and stained with 0.1% (w/v) Congo red solution followed by destaining with 1 M NaCl.

2.3. Protein sequencing by Edman degradation method

The N-terminal amino acid sequence of purified GluCB31 was determined by Edman degradation method using the Procise 491 HT protein sequencer (Applied Biosystems, USA). The N-terminal sequence comprising of 13 amino acids of the purified enzyme was blasted in the National Center for Biotechnology Information (NCBI) GenBank (BLAST: http://blast.ncbi.nlm.nih.gov/Blast.cgi).

2.4. Preparation of calcium-alginate beads

Calcium alginate beads were made by external gel formation. The calcium alginate gel beads were formed by dropping a 2% [w/v] sodium alginate solution where a suitable amount of enzyme was diluted in 0.15 M calcium chloride solution with continuous stirring. The beads were submersed with enzyme in $CaCl_2$ for 1 h. Afterwards, washed many times with a 40 mM $CaCl_2$ solution until no enzymatic activity was demonstrated in the final washing. The final preparation was stored at 4°C prior to use. The carboxymethyl cellulase (CMCase) activity was considered as a control at every washing.

2.5. Characteristics of the enzyme GluCB31

Endo-β-1, 4-glucanase activity was measured according to the method which was described by Miller [16]. The reaction mixture containing 100 µL of 1% substrate and 100 µL of the suitably diluted enzyme solution (10 mM, tris-HCl, pH 7.5) was incubated for 60 min. Along with the reaction mixture, a control solution was assayed. The release of reducing sugar was assayed at 50ºC by 3, 5-dinitrosalicylic acid (DNS) method at 540 nm using calibration curve for D-glucose. One-unit endo-β-1, 4-glucanase activity was distinct as the amount enzyme which produces one µmol of reducing sugar (equivalent to glucose) per min under assay conditions. Protein estimation was done spectrophotometrically at 595 nm according to the Bradford method [17] using the bovine serum albumin (BSA) (10 mg/mL) as a standard during each step of experiment. All the enzyme assays were performed in triplicate.

The effect of the pH on the endoglucanase activity was determined at different pH between 2.0 to 13.5 and assaying the activity of enzymes under standard experimental conditions. Buffers include; citric acid-sodium phosphate (pH 2–6.8), Tris-HCl (pH 7.0–9.2), others like sodium bicarbonate-NaOH (pH 9.5–11), and KCl-NaOH (pH 11.2–13.5). pH stability was evaluated by addition of these buffers to GluCB31, incubating at 4ºC for 24 h. Residual enzyme activity was investigated under standard experimental conditions.

The optimum temperature for the hydrolysis of the substrate (CMC) was investigated by incubating the reaction mixture at various temperatures, ranging from 30 to 80ºC. Enzyme stability was assayed after incubation at different temperatures for 60 min. Residual enzymatic was determined by 3, 5-dinitrosalicylic acid (DNS) method at 540 nm.

The Michaelis-Menten kinetic constants (K_m and V_{max}) for purified enzyme were analyzed under the different concentration of 0.6-10 mg/mL of carboxymethyl cellulose, and enzyme concentration of 0.01 mg. The apparent kinetic constants such as, K_m and V_{max} were determined by DNS method at 50°C. Enzymatic reactions were carried out in tris-HCl buffer (10 mM, pH 7.5). Enzyme assay was analyzed under standard experimental conditions. The Michaelis-Menten constant (K_m), on the other hand maximum velocity (V_{max}) both were evaluated from Lineweaver-Burk Plot.

Substrate specificities of the enzyme GluCB31 were

determined using different types of substrate including, chitin from shrimp shells, beech wood xylan, gum arabic from acacia tree, pectin from apple, locust bean gum, sigma cell cellulose type 20, carboxymethyl cellulose, avicel, paranitrophenyl D- cellobioside (pNPC), and paranitrophenyl-β-D-glycopyranoside (pNPG).

2.6. Various metal ions and chemicals on the activity of GluCB31

The effects of various activating, inhibitory compounds, metals ions, and chemicals depend on the presence of the enzyme-substrate complex (E-S). The presence of temporary E-S structures plays a role to measure the activity of any enzyme-catalyzed reaction. An enzyme would not be the same as those designated to determine the concentration of its substrate. There are various parameters that affect the rate at which enzymatic reactions is carried out such as pH, temperature, substrate concentration, enzyme concentration, and presence of any inhibitors or activators. The enzymatic activity of endoglucanase was studied in 10 mM Tris-HCl buffer (pH 7.5) at 50°C.

Influence of chemicals (0.25%; v/v) such as β-mercaptoethanol and ethylene diamine tetra-acetic acid (EDTA) on enzymatic activity of GluCB31 was estimated according to our standard experimental assay.

Effect of detergents (0.25%; v/v) on enzymatic activity of GluCB31 was determined with non-ionic compounds such as Tween-20, Triton X-100, Tween-80, anionic compounds such as deoxycholic acid, sodium dodecyl sulfate (SDS), and zwitter-ionic compound such as CHAPS.

Influences of different metal ions (1 mM) such as K^+, Na^+, Ca^{2+}, Mg^{2+}, Co^{2+}, Cu^{2+}, Ni^{2+}, Zn^{2+}, Mn^{2+}, Fe^{2+}, Ba^{2+} on enzymatic activity of GluCB31 were estimated as per the standard experimental assay conditions.

The effects of urea on GluCB31 enzyme activity were determined by incubating the enzyme with different concentrations of urea (0–10%, w/v) at 37°C for 60 min. The effects of NaCl (0–15%, w/v) on the purified GluCB31 activity were determined at pH 7.5 and 50°C.

The relative enzymatic activities were determined at 540 nm under standard assay conditions. The control (without any additives) was regarded as baseline activity (100% activity). For enzymatic assay, three independent replicates were evaluated using 3, 5-dinitrosalicylic acid method.

2.7. Thermodynamic study of substrate hydrolysis

Thermodynamic parameters for substrate CMC hydrolysis were calculated by rearranging the Eyring absolute rate equation obtained from the transition state theory [18].

$$\text{Turn over number, } k_{cat} = \frac{k_b T}{h} e^{(-\Delta H|RT)} e^{(-\Delta S|RT)} \quad (1)$$

where, k_b is Boltzmann's constant (1.38×10^{-23} JK^{-1}), T is absolute temperature (K), and R is gas constant (8.314 J K^{-1} mol^{-1}), h is the Planck's constant (6.626×10^{-34} J s).

$$\text{Enthalpy of activation, } \Delta H = E_a - RT \quad (2)$$

$$\text{Free energy of activation, } \Delta G = -RT\, ln \frac{k_{cat} h}{K_b T} \quad (3)$$

$$\text{Entropy of activation, } \Delta S = \frac{\Delta H - \Delta G}{T} \quad (4)$$

The free energy of substrate binding (ΔG_{E-S} and free energy of transition state formation (ΔG_{E-T}) were determined using the following equations:

$$\Delta G_{E-S} = -RT \ln K_a \quad (5)$$

where, $K_a = 1/K_m$

$$\Delta G_{E-T} = -RTln \frac{k_{cat}}{K_m} \quad (6)$$

The outcome of temperature on the rate of reaction was measured in terms of the temperature quotient (Q_{10}), which is the distinct factor by which the rate increases due to an increase in the temperature of 10°C. Q_{10} was calculated by rearranging the equation of Dixon and Webb [19]:

$$Q_{10} = antilog \frac{E_a \times 10}{RT^2} \quad (7)$$

2.8. Wheat bran pre-formulation and enzymatic hydrolysis

Wheat bran used in our study was subjected to chemical and physical pre-treatments. At first, 1 g wheat bran was treated with 50 mL 1% [w/v] NaOH for 1 h at 121°C (105 kPa). Then, washed with distilled water several times to maintained neutral pH and dried at 110°C. Enzymatic hydrolysis of wheat bran was done in 250 mL Erlenmeyer flask. The prepared lignocellulolytic mass in buffer solution was sterilized to prevent any microbial contamination at 121°C, 105 kPa for 15 min. Finally, wheat bran was distributed to a volume of 100 mL at a concentration of 0.5% (dry basis).

GluCB31 was added at a concentration of 0.1 mg/mL and the reaction was carried out on a shaking incubator at 160 rpm at 30°C for 2 days. At every 8 h, 2 mL of sample was withdrawn and suspended in 10 mL of 10 mM Tris/HCl buffer (pH 7.5), and vortexed thoroughly. The properly vortexed sample was centrifuged at 10,000 *g* at 6°C for 30 min in order to remove the residue after enzymatic hydrolysis. The reducing sugars of obtained supernatant were assayed using 3, 5-dinitrosalicylic acid method. A

parallel hydrolysis was carried for non NaOH treated wheat bran along with control hydrolysis (without addition of enzyme).

2.9. Cello-oligosaccharide production

Thin layer chromatographic technique was employed for the determination of GluCB31 hydrolysis. The cello-oligosaccharides production was carried on time course hydrolysis ranging from 0, 10, 30, 60, and 90 min. Thin layer chromatography was analyzed as described previously [14]. Briefly, purified 0.4 mg/mL GluCB31 was incubated with 1% (w/v) carboxymethyl cellulose in tris-HCl buffer (10 mM, pH 7.5) at 37°C. The reaction was stopped by boiling and then samples were dotted on the silica gel plates (E. Merck, Germany, 60F 254). Mixture of chloroform, acetic acid, and water (6: 10: 2, v: v: v) was taken as the developing solvent system. After dried, plate was sprayed with a methanol: sulfuric acid (mixture, 95:5, v: v), and heated for few min at 140°C. Here, a mixture of cello-oligosaccharides consisting of G1-G3 (10 mg/mL) was used as standard.

3. Results

3.1. Induction of *Bacillus* strain CBS31

Eighty-three bacterial strains were isolated from the Korean tradition fermented food (Kimchi) on the screening plates and among them, only twenty-two bacterial isolates displayed 1, 4-β-glucanase activity on the basis of their halo zone according to Congo red assay. The isolate CBS31, exhibiting largest zone of clearance was selected for further experiments. The morphological, physiological, and biochemical properties of isolate CBS31 according to Bergey's manual of systematic bacteriology revealed the parent species to be *Bacillus*. Molecular characteristics carried by PCR amplification of the 16S rRNA gene demonstrated 99.93% homology with *Bacillus subtilis* subsp. *inaquosorum*- KCTC 13429(T) (accession no. AMXN01000021) and *B. tequilensis*- KCTC 13622(T) (accession no. AYTO01000043), 99.86% homology with *B. subtilis* subsp. *subtilis*- NCIB 3610(T) (accession no. ABQL01000001), and 99.80% homology with *B. subtilis* subsp. *spizizenii*- NRRL B-23049(T) (accession no. CP002905). 16S rRNA gene

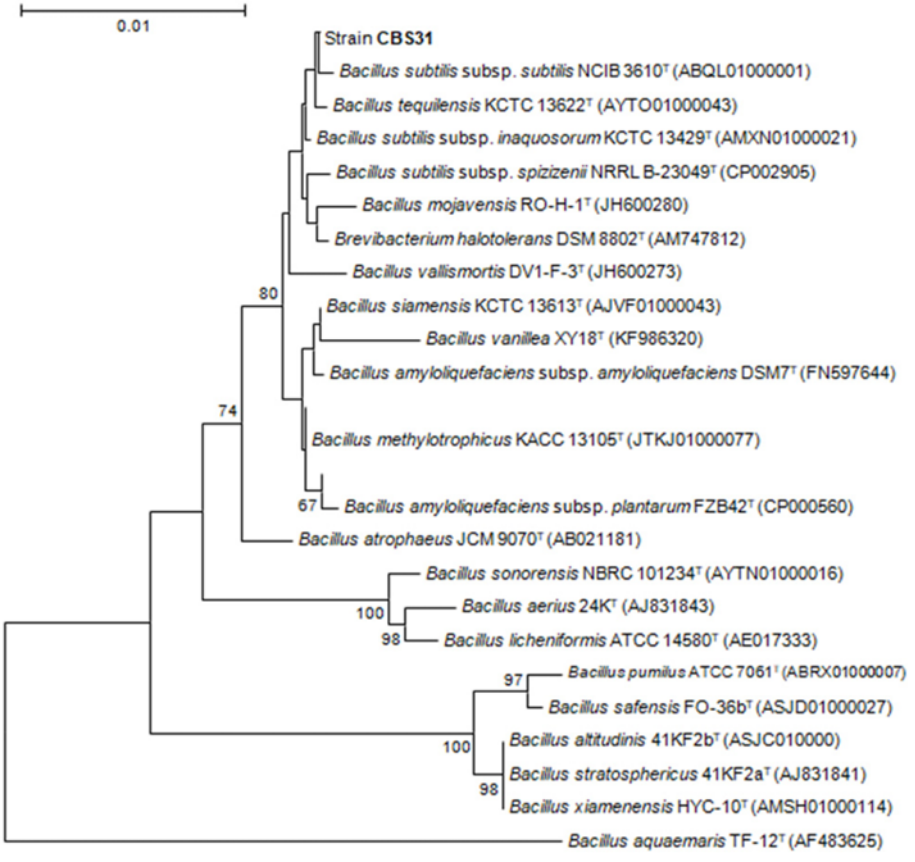

Fig. 1. Phylogenetic tree based on complete 16S rRNA gene sequence showing relationships between strain CBS31 and some closely related taxa of the genus *Bacillus*. Reference sequences were retrieved from GenBank under the accession number indicated in parentheses after the strain name. Numbers of nodes are percentage bootstrap values based on 1000 replications; only value greater than 65% are shown. Bar, 0.01 substitutions per nucleotide position.

sequences were aligned manually with reference sequences retrieved from Genbank database following BLAST searches. Phylogenetic tree was constructed by neighbor-joining and maximum parsimony methods using the MEGA7 software. The phylogenetic tree of the 16S rRNA gene sequence of strain CBS31 is shown in Fig. 1. On the basis of polyphasic taxonomic data presented, bacterial isolate CBS31 was identified as *B. subtilis* subsp. *inaquosorum* CBS31 [15]. *B. subtilis subsp. inaquosorum* CBS31 (Accession no. KCTC18676P) was placed for culture collection at KCTC (Korean collection for type culture) to the World Data Centre for Microorganisms (WDCM).

3.2. Induction of Endo-1, 4-β-glucanase

The culture supernatant of *B. subtilis* subsp. *inaquosorum* CBS31 having total enzyme activity of 13569.42 U and specific activity of 60.32 U/mg was carried for purification by ammonium sulfate precipitation, anion-exchange chromatography and gel permeation chromatography. The precipitation fraction of 40-80% salting out of ammonium sulfate was detected as containing 1, 4-β-glucanase activity with 1.47-fold purification. The bacterial extracellular 1, 4-β-glucanase was further purified to 4.98-fold by DEAE sepharose fast flow and specific activity of 300.67 U/mg. Final step of purification to homogeneity by Sephadex G-50 column chromatography resulted to 17.68-purity fold with 8.33% yield and specific activity of 1066.37 U/mg. Electrophoretic homogeneity was carried for the elution peak with 1, 4-β-glucanase through SDS-PAGE of the aliquots. The SDS-PAGE of the purified aliquot showed a single band with a molecular weight corresponding to the PageRuler Prestained Protein Ladder of ~ 35 kDa mass (Fig. 2). The zymogram of the purified GluCB31 confirmed the successful purification of the functional protein (Fig. 2). After staining with the solution 0.1% (w/v) Congo red and 1 M NaCl, the clear zone of zymogram indicated to the in-gel digestion of carboxymethyl cellulose by the purified GluCB31.

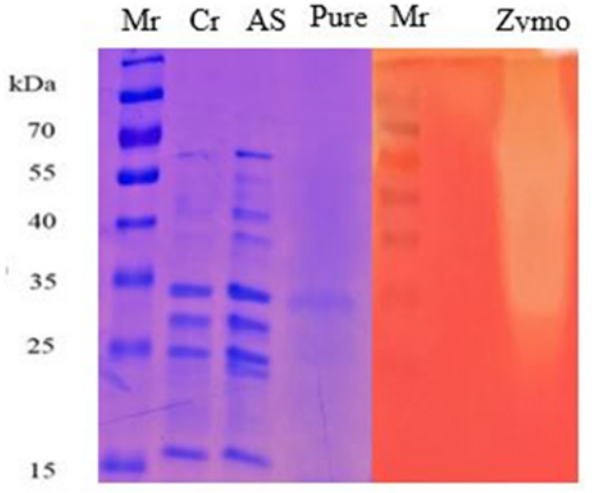

Fig. 2. 12.5% (w/v) SDS-PAGE (sodium-dodecyl sulfate polyacrylamide gel electrophoresis) and Zymography of purified endoglucanase from *Bacillus subtilis* CBS31. Lanes Mr, protein molecular weight marker (Fermentas); lane Cr., crude extract; lane AS, 40-80% ammonium sulfate precipitation fraction; lane pure, purified GluCB31; lane zymo, zymogram of GluCB31.

3.3. N-terminal amino acid sequence of GluCB31

The Edman degradation method applied for the detection of amino acid sequence of purified GluCB31 showed Phe-Pro-Gly-Val-Met-Ala-Leu-Val-Met-Ala-Ile-X-Ala. GluCB31 showed some degree of similarity with different proteins from *B. subtilis*. No full query was covered during protein BLAST search. The alignment of N-terminal amino acid sequence of GluCB31 and related protein from *B. subtilis* is shown in Table 1.

3.4. Biochemical characteristics of GluCB31

The endoglucanase activity of GluCB31 estimated using CMC as substrate at 50°C in the pH range of 2.5-13.0 showed that pH 7.5 in Tris-HCl buffer was optimum for the enzyme activity. The enzyme exhibited more than 60% of the activity in the range of pH 6.5-10.0, but its activity dropped remarkably (approximately 30% activity) at pH 4.6 and 11.0 conditions. Immobilized GluCB31 hydrolyses with CMC solution in pH range 2.5-13.0, within 30 min, 50°C revealed broad range of pH 4.6-10.0 with optimum at pH 7.5. The correlation of free enzyme and immobilized enzyme is shown in Fig. 3A. The 100% stability of GluCB31 in the range of pH 6.5-10.0 was observed up to 6-24 h of incubation (Fig. 3B).

The analysis of the GluCB31 activity at pH 7.5 in the temperature range of 20-80°C showed that the optimum temperature for the free enzyme activity was at 50°C and

Table 1. Alignment of N-terminal amino acid sequence of GluCB31 and related protein from *Bacillus subtilis*

SN	Protein	Amino acid sequence	Identity (%)	NCBI Reference Sequence
1	GluCB31	**FPGVMALVMAIXA**	100	Current study
2	Beta-hexosaminidase lipoprotein	**F**GQED**AVVMAI**K**A**	53.84	WP_003241470.1
3	Hypothetical protein	YI**GVLALI**M**VIAA**	53.84	WP_003240290.1
4	Glutaminase	SG**GI**M**ALV**PPSAR	38.46	WP_003241345.1

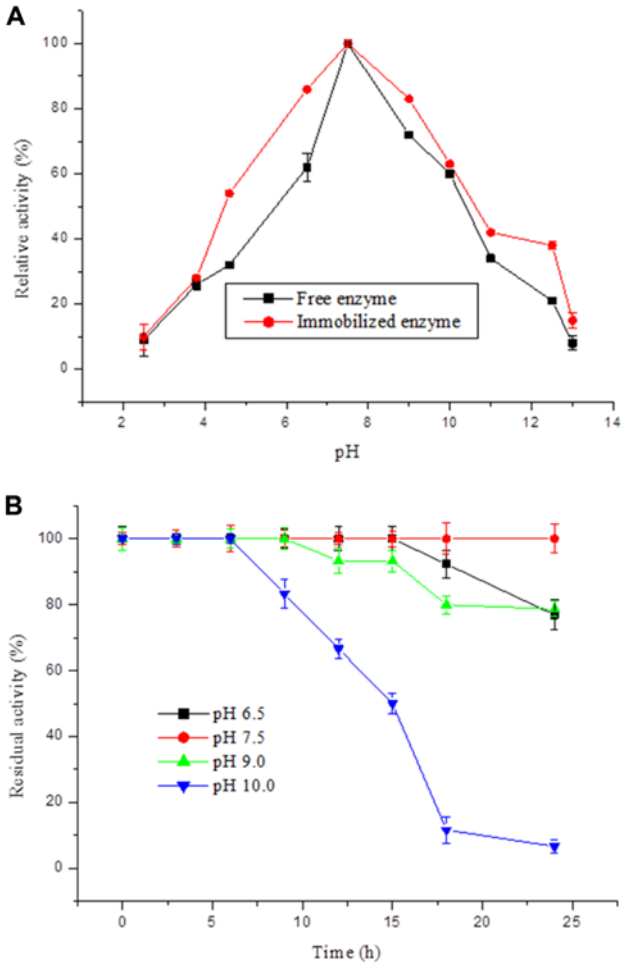

Fig. 3. The pH correlation of free enzyme and immobilized enzyme (A) and stability of GluCB31 in the range of pH 6.5-10.0 up to 6-24 h of incubation (B).

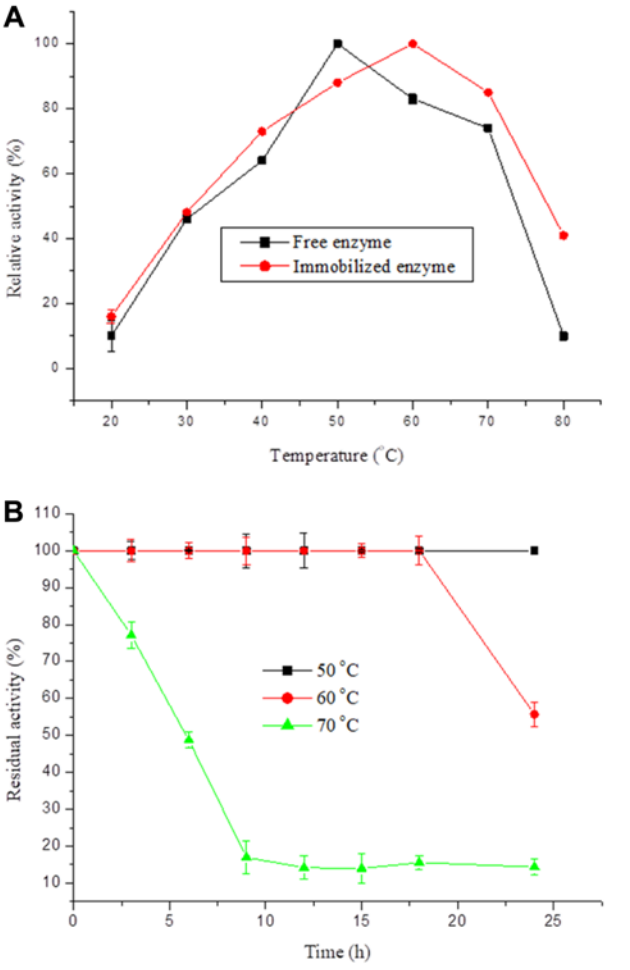

Fig. 4. The temperature correlation of free enzyme and immobilized enzyme (A) and temperature stability of GluCB31 (B).

for immobilized enzyme at 60°C. The enzyme showed more 60% activity at 40-70°C for both free enzyme and immobilized but 85% correlation activity was observed at 70°C for immobilized GluCB31 (Fig. 4A). The temperature stability was estimated as high as 70°C, the enzyme activity dropped to about 14.4% and finally stopped at 70°C after more 24 h of incubation (Fig. 4B).

In order to study the effect of metal ions on the GluCB31, the activity assays were carried in the presence of various metal ions (Table 2). The results showed that Mg^{++} and Ba^{++} at 1 mM concentrations could significantly enhance the enzyme activity by almost 2 times. Also, Cu^{++} and Zn^{++} increased the enzyme activity by about 1.5 times. In contrast, Fe^{++} severely decreased the activity to 15.8% and Ca^{++} and Co^{++} slightly decreased the enzymatic activity while slightly increased activity was seen with Mn^{++} and Ni^{++}. There was no effect on enzymatic activity when treated with K^{+} and Na^{+}.

The exposure of GluCB31 to various denaturing agents at 0.25% concentrations showed that deoxycholic acid and CHAPS decreased the GluCB31 activity by 17.5% and 11.9% respectively. However, the enzyme exhibits a slightly enhance activity in the presence of tween 20 and tween 80, while enzymatic activity was significantly increased by Triton X-100 and SDS at 0.25% concentration. Overall, GluCB31 proved and also showed tolerant against tested denaturants to be used in detergent industry and various biotechnological applications.

The reducing agent (β-mercaptoethanol) tested in this study demonstrated partially inactivated GluCB31 at 0.25% concentration and chelating agent (EDTA) decreased the enzyme activity by 45.5% at 0.25% concentration. In contrast, GluCB31 was significantly tolerant against urea exhibiting more than 50% activity at various urea concentrations up to 7%. The enzyme could also tolerate or even be enhanced with the high concentrations of NaCl. Effect of urea and NaCl on the GluCB31 activity is shown in Fig. 5.

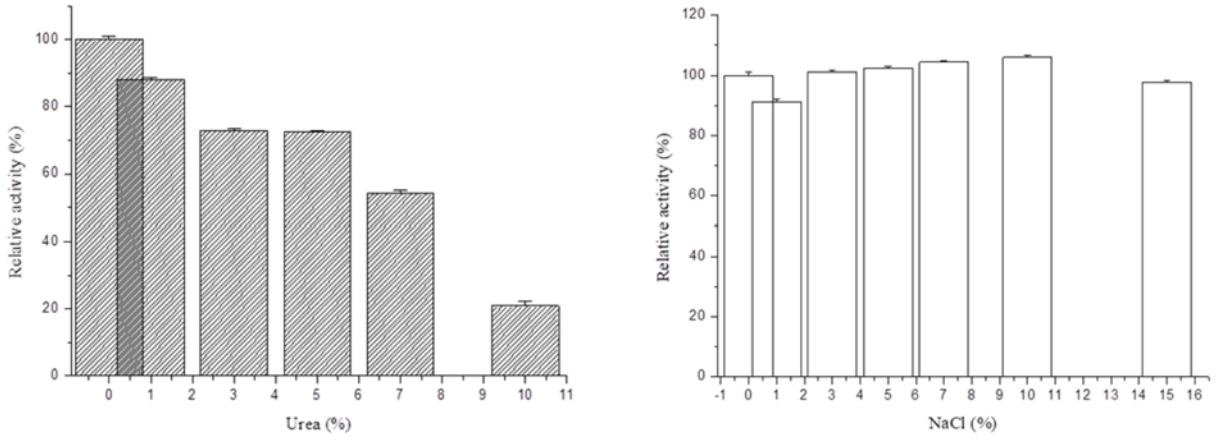

Fig. 5. Effect of urea (0-10%, w/v) and NaCl (0-15%, w/v) on the GluCB31 activity.

Table 2. Effect of various activators and inhibitory agents

Reagents	Concentration	Relative activity (%)
β-mercaptoethanol	0.25%	46.8 ± 1.59
EDTA	0.25%	54.5 ± 1.30
Tween-20	0.25%	106.7 ± 0.66
Tween-80	0.25%	111.6 ± 0.67
Triton X-100	0.25%	142.3 ± 0.59
Deoxycholic Acid	0.25%	82.5 ± 2.27
SDS	0.25%	135.5 ± 0.62
CHAPS	0.25%	88.1 ± 1.46
Ca^{++}	1 mM	76.5 ± 0.92
Mg^{++}	1 mM	185.7 ± 1.07
Fe^{++}	1 mM	15.8 ± 2.24
Cu^{++}	1 mM	167.6 ± 0.57
Zn^{++}	1 mM	153.7 ± 1.21
Mn^{++}	1 mM	106.3 ± 0.70
Ba^{++}	1 mM	181.9 ± 0.78
K^{+}	1 mM	95.8 ± 0.64
Na^{+}	1 mM	97.6 ± 1.09
Ni^{++}	1 mM	107.2 ± 1.44
Co^{++}	1 mM	84.2 ± 0.55

3.5. Enzyme kinetics and substrate specificity of GluCB31

The substrate specificity of GluCB31 was studied on chitin from shrimp shells, beech wood xylan, gum arabic from acacia tree, pectin from apple, locust bean gum, sigma cell cellulose type 20, carboxymethyl cellulose (CMC), avicel, paranitrophenyl D- cellobioside (pNPC), and paranitrophenyl-β-D-glycopyranoside (pNPG) at 1% concentration. The enzyme was able to hydrolyze CMC (100%) whereas gum arabic from acacia tree (52.36%) and locust bean gum (56.59%) but very little activity was detected on other tested substrates. The K_m and V_{max} of the GluCB31 were calculated to be 0.0183 mg/mL and 1293.33 ± 2.51 U/mg for CMC hydrolysis respectively.

Table 3. Kinetic and thermodynamic parameters of GluCB31

Parameters	Properties
Mol. Wt.	~35 kDa
K_m	0.0183 mg/mL
V_{max}	1,293.33 ± 2.51 U/mg
E_a	40.212 kJ/mol
ΔH	38.526 kJ/mol
K_{cat}	484,998.75 /sec
ΔG	44.187 kJ/mol
ΔS	- 17.518 J/mol/K
ΔG_{E-S}	- 10.75 kJ/mol
ΔG_{E-T}	- 45.922 kJ/mol
Q_{10}	1.607

3.6. Thermodynamic study

Thermostability is the measure to describe thermodynamic as well as kinetic stabilities of any enzyme. The low value of activation energy of GluCB31 (40.212 $kJmol^{-1}$) might demonstrate the availability of right conformation on the active site for the formation of enzyme-substrate complex explaining less energy requirement for catalysis. The Q_{10} value for CMC hydrolysis was 1.607. Q_{10} is the effect of temperature on rate of reaction. Q_{10} determines whether or not the metabolic reactions carried are controlled by temperature parameter or by other means. Enthalpy of activation (ΔH), Gibbs free energy of activation (ΔG), and entropy of activation (ΔS) for CMC hydrolysis by using GluCB31 were calculated to be 38.526 $kJmol^{-1}$, 44.187 $kJmol^{-1}$, and -17.518 $Jmol^{-1}K^{-1}$ respectively. Change in Gibbs free energy for the conversion of the enzyme-substrate complex into product. Free energy for substrate binding (ΔG_{E-S}) and formation of activated complex (ΔG_{E-T}) is the measure to determine the feasibility and scope of an enzyme-catalyzed reaction. In our study, ΔG_{E-S} and ΔG_{E-T} were found to be -10.75 $kJmol^{-1}$ and -45.92 $kJmol^{-1}$

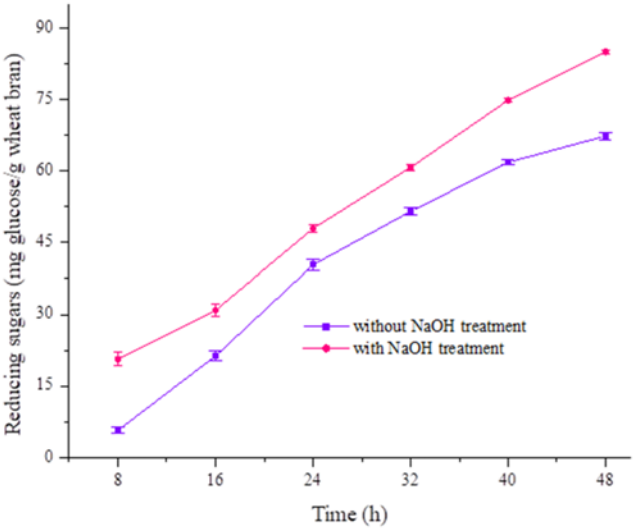

Fig. 6. Graph showing the result obtained when the native and pretreated wheat brans were saccharified with GluCB31.

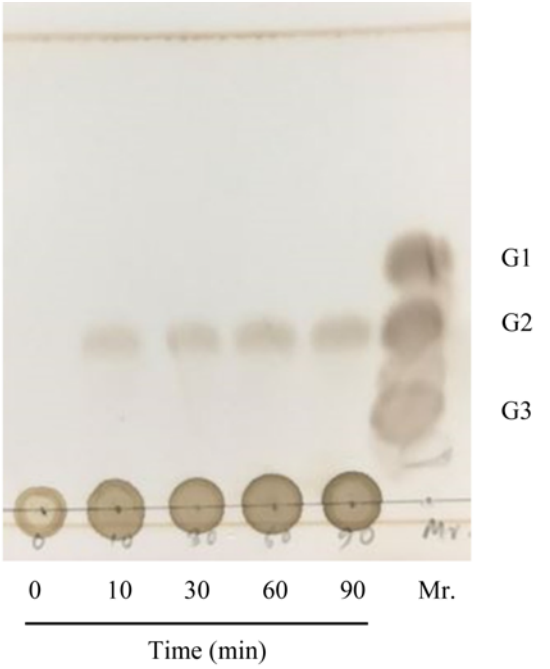

Fig. 7. Plot showing time course hydrolysis of substrate at 50°C, pH 7.5 with GluCB31 from *Bacillus subtilis* CBS31. Lane Mr, mixture of glucose (G1), cellobiose (G2), and cellotriose (G3).

respectively. Table 3 shows the thermodynamic parameter of GluCB31. Our results confirm the high affinity of GluCB31 for carboxymethyl cellulose.

3.7. Enzymatic hydrolysis experiment

The reducing sugar content in wheat bran was evaluated and found to be 8.495% after pre-formulation with NaOH and enzymatic hydrolysis. From the experiment, 26.20% more reducing sugar was obtained when the wheat bran was pretreated with NaOH. The result of enzymatic degradation of wheat bran demonstrated that entrapment of cellulolytic enzymes could decrease the catalytic efficiency during a high-level enzymatic hydrolysis. Fig. 6 shows the result obtained when the native and pretreated wheat brans were saccharified with GluCB31.

3.8. Oligosaccharide production by time course hydrolysis

The cello-oligosaccharides production was carried on time course hydrolysis ranging from 0, 10, 30, 60, and 90 min, and demonstrated cellobiose as the end product of hydrolysis (Fig. 7). Oligosaccharides were released from the substrate by cumulative action of the enzyme [14]. The reaction was carried with solvent system of chloroform, acetic acid, and water (6:10:2, v: v: v) and reaction chromatography plate was sprayed with both mixture of methanol and sulfuric acid (95:5, v: v). A mixture of cello-oligosaccharides consisting of G1-G3 (10 mg/mL) was used as standard.

4. Discussion

Cellulases are inducible enzymes and expensive aspects of industrial cellulase production. Bioconversion of plant biomass to value-added products is an important application that has been hindered by the high cost of enzymatic hydrolysis of the biomass into fermentable sugars [20]. The discovery of novel enzymes with improved catalytic and biochemical characteristic is deemed as a solution to lower the costs of industrial cellulase production. Now days, neutral β-glucanases, with optimum pH ranges of 6–8, have been investigated to have useful applications in the various bio-industrial sectors. Cellulases successfully been used both in biological de-inking processes [21] and for improving the characteristics of recycled pulp [22]. As a result, there has been a substantial growth in demand for neutral β-glucanases, particularly those enzymes with specific biochemical properties [23]. Although many cellulolytic enzymes have been investigated from various organisms, particularly bacteria and fungi, just a few have been obtained from food-source *Bacillus* strains.

The computer-assisted protein blast and polyphasic taxonomic data indicated that the food-source bacterial isolate belongs to *B. subtilis* subsp. *inaquosorum* CBS31 was quite interesting in terms of 16S rRNA gene sequence which demonstrated 99.93% homology with the known *B. subtilis* subsp. *inaquosorum*- KCTC 13429 (T) (accession no. AMXN01000021). The GluCB31 production was highest during the duration of microbial growth with an increase in microbial biomass. Enzyme production increased

progressively at the growth phase and afterward the enzyme level increased slightly. Our study showed microbial growth and enzyme production develop a fine co-relation between endoglucanase production and microbial biomass at growth phase. GluCB31 was produced as a result of microbial fermentation by *B. subtilis* subsp. *inaquosorum* CBS31 in the production media containing carboxymethyl cellulose as a substrate. Maximum enzyme production hugely relies on the different types of microbial strains and their genetic constituents. It also depends upon the culture and environmental parameters employed during the growth of microorganism [24]. The molecular mass of monomeric GluCB31 was about 35 kDa as determined by SDS-PAGE and Sephadex G-50 column chromatography resulting to 17.68-purity fold with 8.33% yield and specific activity of 1066.37 U/mg. This result is inconsistent with low molecular weight endo-glucanase obtained from *Bacillus* strain (40 kDa) [25], *B. subtilis* YJ1 (32.5 kDa) [9], *B. licheniformis* (37 kDa) [26], *Chalara paradoxa* CH32 (35 kDa) [27]. The clear zone on zymogram SDS-PAGE gel produced might be due to in-gel digestion of carboxymethyl cellulose by the purified GluCB31. Our result signifies the high activity of GluCB31 inferred from the pattern of substrate hydrolysis extending over the entire lane of the enzyme movement during the zymogram SDS-PAGE.

The N-terminal amino acid sequence of purified *B. subtilis* GluCB31 showed 53.84% similarity with beta-hexosaminidase lipoprotein (NCBI reference sequence; WP_003241470.1), 53.84% with hypothetical protein (NCBI reference sequence; WP_003240290.1), and 38.46% with glutaminase (NCBI reference sequence; WP_003241345.1) from different *Bacillus* strains. Native and immobilized glucanase from *B. subtilis* CBS31 showed a pH optimum of 7.5. A study reported that the pH optimum of soluble and immobilized cellulase remained the same (pH-6.0) [28]. Next report showed that pH profile of immobilized endoglucanase was changed to alkaline portion in comparison to that of soluble endoglucanase [29]. Our finding suggested that the charge present on matrix had no effect on the active site of immobilized GluCB31, therefore, the results of the pH optimum of immobilized enzyme remains the same. The residual activity of immobilized GluCB31 shows higher activity with reference to the soluble enzyme in acidic conditions and remains unchanged in alkaline region. Hence, it can be said that the enzyme immobilization effectively protects its subunit dissociation from protonation and de-protonation. The optimum temperature for native and immobilized GluCB31 were 50 and 60°C, respectively. Since the immobilized enzyme exhibited highest activity at 10°C higher temperature than soluble enzyme, as the entrapment in alginate gel made GluCB31 more stable. Therefore, enzyme immobilization guides in enhancing the thermal stability by increasing configuration rigidity, which prevents the tertiary structure of a protein from various environmental conditions [30].

Searching glucanase of special bio-industrial characteristics receives interest. GluCB31 being neutral β-glucanase, and been produced from *Bacillus* strain possess useful applications in the various bio-industrial sectors [23]. Large number of bio-industrial processes are carried at elevated temperature and at controlled catalytic rate. The activity of GluCB31 was enhanced by Tween 20, Tween 80, Triton X-100, and SDS. Low concentration of detergents used did not destroy or even unfold the secondary structure of GluCB31 by increasing β-sheets configuration with a cleft crossing on one side where the substrate is bound. The short interaction time and low concentration of SDS (0.25%) improved 135.5% of GluCB31 activity, suggesting it may be used as an efficient additive in domestic and household detergents. The use of Mg^{++}, Ba^{++}, Cu^{++}, and Zn^{++} highly enhanced the enzyme activity of GluCB31. It was reported that the endoglucanase activity from *B. subtilis* YJ1 [9], *B. sphaericus* JS1 [31], and *Trichoderma harzianum* [32] could be activated by Mg^{++}. Slight increase in enzyme activity of GluCB31 was observed with Mn^{++} and Ni^{++}. A report of endoglucanase from *B. cereus* [10] showed enzymatic activity was slightly increased with Mn^{++} similar to our observation. Use of Ca^{++} and Co^{++} slightly decrease the enzymatic activity of endoglucanase from *B. cereus* [10], which is similar to our result. Also, endoglucanase from *B. subtilis* YJ1 [9] showed slight decrease in enzymatic activity with Ca^{++}. In our observation, Na^{+} and K^{+} shows no effect on the enzymatic activity of GluCB31 inconsistent with the reports of endoglucanase from *B. cereus* [10] and *Bacillus* sp. HSH-810 in reference to Kim [33]. Fe^{++} severely decreased the enzymatic activity of GluCB31 and a similar study from *Bacillus* sp. HSH-810 [33] also reported the inhibition of enzymatic activity on addition of Fe^{++}. The enhancement of enzyme activity of GluCB31 by Zn^{++} at low concentration might be due to its binding to substrate. This characteristic of binding to substrates promotes the right orientation of the substrate in the enzyme-catalyzed reaction. Reduction of enzymatic activity by EDTA and other metal ion like Ca^{++} indicated that GluCB31 is a metallo protein meaning some metal ions is required to initiate the enzyme catalyzed reaction. The effect of NaCl on GluCB31 showed that endoglucanase activity was increased at the concentration 3 to 10% NaCl and the maximal activity was observed in the presence of 10% NaCl (106.1%). GluCB31 activity was 91.3% at 1% NaCl concentration and about 97.7% enzyme activity was observed with 15% NaCl concentration, which was higher than that of 1% NaCl concentration. The halophilic enzymes are generally inactive at less than 2% NaCl or KCl concentration [34]. Halophilic stability and

adaptation of GluCB31 suggested that more acidic surface and less hydrophobic core is available [35]. GluCB31 was significantly tolerant against urea exhibiting more than 50% activity at urea concentrations up to 7%, suggesting the potential use as an additive to the detergents.

The purified GluCB31 showed the highest activity against CMC. There was almost no hydrolysis or little hydrolysis activity (< 60%) against various other substrates used. Therefore, it was regarded that the purified enzyme to be an endo-1, 4-glucanase accordingly. Enzyme kinetic parameters like, K_m and V_{max} of the GluCB31 were calculated and found to be 0.0183 mg/mL and 1293.33 ± 2.51 U/mg for CMC hydrolysis respectively. K_m is a representation of the apparent affinity of an enzyme towards its substrate. It is the measurement of the amount of substrate needed to attain half the maximal initial velocity [36]. Lower the K_m value signifies higher substrate affinity, which means small amount of substrate can attain the reaction rate half the maximum, V_{max}.

Higher thermostability of any enzyme can be achieved by stabilizing its native form by adding non-covalent bonds (such as; hydrogen bonds, hydrophobic-interactions, and salt-bridges) or by reducing the entropy of activation of unfolding [37,38]. Study of thermodynamic parameters is necessary to understand the behavior of molecules in different conditions. The thermal inactivation of enzymes occurs in two steps; $N \leftrightarrow U \rightarrow I$, (where N is native) form of enzyme (folded), U is the inactive enzyme (unfolded), which is reversibly refolded after cooling, and I is the inactive formation of enzyme formed after extensive contact to heat and thus cannot be obtained upon cooling also [39]. The thermal denaturation of enzymes may also result in the breakage of noncovalent bonds which also include hydrophobic interactions with concomitant increase in the enthalpy (ΔH) of activation for denaturation. The opening up of the enzyme configuration is carried by increase in the entropy of activation (ΔS) for denaturation [40]. Enthalpy of activation (ΔH), Gibbs free energy of activation (ΔG), and entropy of activation (ΔS) for CMC hydrolysis by using GluCB31 were calculated to be 38.526 $kJmol^{-1}$, 44.187 $kJmol^{-1}$, and -17.518 $Jmol^{-1}K^{-1}$ respectively. The negative value for ΔS in our study demonstrates that the entropy decreases in forming the transition state. A low ΔS value signifies the existence of the enzyme in its stable state [41], which is a major characteristic of an enzyme to be used as bio-industrial agent. The positive value of ΔS is symbol of increased randomness of the protein-solvent system upon denaturation of enzyme. The enthalpy is the amount of heat required to denature the enzyme. Here, a large and positive ΔH value for substrate hydrolysis by GluCB31 could be related to a more stable enzyme, meanwhile larger amount of energy is needed for the denaturation process to happen. The net stability of any protein is described as the difference in free energy between the native and that of denatured conformation. The higher the free energy, the more resistant the unfolding of its transition state. In our study, ΔG_{E-S} and ΔG_{E-T} were found to be -10.75 $kJmol^{-1}$ and -45.92 $kJmol^{-1}$ respectively. The low values of ΔG_{E-S} and ΔG_{E-T} are evidence that the binding of GluCB31 to the substrate and formation of the transition complex occur at a fast rate. The free energy relates the values of both enthalpy and entropy and hence it is regarded as a more consistent indicator of the enzyme stability [19]. Q_{10} for GluCB31 was 1.6. Q_{10} is defined as the rate of the enzymatic reaction due to an increase in temperature by 10°C, is an indicator of a measure of temperature sensitivity. Performance increases rapidly with rising temperatures (Q_{10} values of 2.0 - 4.0) to a maximum level. The thermodynamic parameters study supports the conversion of the reactant to the product is spontaneous with higher hydrolytic efficiency and the feasibility of the enzymatic reaction.

5. Conclusion

In our study, 26.20% more reducing sugar was obtained when the wheat bran was pretreated with NaOH which means the entrapment of cellulolytic enzymes could decrease the catalytic efficiency during a high-level enzymatic hydrolysis. In a report, about 47% more glucose yields were seen from enzymatic hydrolysis of 5% rice straw pretreated with organosolv method [42]. The observed differential effect of pretreatment on glucose release was probably due to the relative cellulose, hemicellulose, and lignin content of the substrate during treatment. Cello-oligosaccharide production of GluCB31 resulted as cellobiose as a product of enzymatic digestion by time course hydrolysis by thin-layer chromatography technique. Thermostable and halophilic endoglucanase from *B. licheniformis* C108 also showed majority of the hydrolyzed product was cellobiose [11]. Different industrial applications like biochemical characteristics and thermodynamic properties were assessed and found suitable for bio-industrial applications and development. Endoglucanase from *B. subtilis* CBS31 could be a choice for some biotechnological applications that require high halo stability and biochemical properties. The bio-industrial attributes of the purified endoglucanase GluCB31 were evaluated by thermostability, substrate specificity, enzyme kinetics, and thermodynamic study. Production of a bio-industrial applicable endoglucanase was considered as an industrially active enzyme for further process. GluCB31 could behave like a good biocatalyst for bio-industrial applications where enzyme-catalyzed reaction

is carried, and our results support the conversion of the reactant to the product is spontaneous along with higher hydrolytic efficiency and the feasibility of the enzymatic reaction.

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Conflict of Interest

The authors declare that further, they have no conflict of interest.

Ethical Statement

This article does not comprise any studies through human participants or animals performed by any of the authors.

Informed Consent

Here, Informed consent was attained from all individual participants included in the study.

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