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Purification and Biochemical Characterization of Alkalophilic Cellulase from the Symbiotic *Bacillus subtilis* BC1 of the Leopard Moth, *Zeuzera pyrina* (L.) (Lepidoptera: Cossidae)

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Abstract
In the current study, an extracellular cellulase belonging to symbiotic *Bacillus subtilis* Bc1 of the leopard moth is purified and characterized. The molecular mass of enzyme was 47.8 kDa using SDS-PAGE. The purified enzyme had optimum activity in temperature and pH around 60 °C and 8, respectively. The purified cellulase was introduced as a stable enzyme in a wide variety of temperature (20–80 °C) and pH (4–10) and remained active to more than 74% at 80 °C for 1 h. Moreover, the cellulase extremely was stabled in the presence of metal ions and organic solvents and its activity was increased by acetone (20% v/v), $CaCl_2$ and $CoCl_2$ and inhibited by $MnCl_2$ and $NiCl_2$. The values of enzyme's K_m and V_{max} were found to be 1.243 mg/mL and 271.3 µg/mL/min, respectively. The purified cellulase hydrolyzed cellulose, avicel and carboxymethyl cellulose (CMC) and the final product of CMC hydrolysis was cellobiose using thin-layer chromatography analysis. Consequently, owing to exo/endoglucanase activity and organic solvent, temperature and pH stability of the purified cellulase belong to *B. subtilis* BC1, it can be properly employed for various industrial purposes.

Keywords Cellulase · *Zeuzera pyrina* · Cellulolytic bacteria · *Bacillus subtilis*

Abbreviation

CMC	Carboxymethyl cellulose
CTAB	Cetyl trimethylammonium bromide
DNS	Dinitrosalicylic acid
NCBI	National center for biotechnology information
PCR	Polymerase chain reaction
SDS-PAGE	Sodium dodecyl sulfate–polyacrylamide gel
TLC	Thin-layer chromatography

Introduction

Lignocellulose is the most numerous organic matter of woody plants and dead plant materials containing lignin, cellulose and hemicellulose in its structure [1]. Due to abundance of cellulose and hemicellulose in lignocellulosic biomass, it can be an appropriate candidate to produce bioethanol [2]. Moreover, lignocellulose is known as a major renewable natural resource which cannot be completely decomposed in the environment because of its complex structure [3]. In general, microbial communities are the main producers of enzymes which can efficiently decompose lignocellulosic biomass [4].

Cellulase is one of the most important commercial enzymes that is being largely applied in different areas of industry including: biofuel, food, feed, beverages, paper, textile, pharmaceutical and agriculture [5, 6]. Hence, enzyme characterization and purification of cellulase producing bacteria from different sources with high catalytic activity and stability will be an important objective for many researchers due to importance of this enzyme in industry [7].

In general, xylophagous insects are capable of decomposing wood by producing their endogenous digestive enzymes and their association with fungi, protozoa and other microorganisms. It has been shown that both insect and microorganisms enzymes participate in lignocellulose degradation [8, 9]. Moreover, the genetic diversity of cellulase encoding gene in insect genome is less than that of symbiotic microorganisms. Thus, it does not allow complete

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cellulose degradation. It seems that microorganism's cellulase plays an important role in providing energy for xylophagous insects [10]. As a result, it seems that insect gut microbiome's enzymes, especially cellulase, are sources of novel enzymes with potential use in biotechnology [11].

The leopard moth, *Zeuzera pyrina* L. (Lepidoptera: Cossidae), is one of the most destructive wood borer pests. It is widely distributed all over the world and it can affect many tree and shrub species. Mature insects usually oviposit in young tree shoots. However, newly hatched larvae penetrate branches and feed on the wood. Then they gradually attack the larger branches and the trunk and feed themselves for two or three years until they reach maturity [12]. The extreme alkaline pH of the leopard moth midgut (pH range of 10–11) causes severely harsh conditions that leads special microorganism to harbor in it [11]. This way it will establish a new source of cellulolytic bacteria. The present study is designed to isolate cellulolytic bacteria from leopard moth gut and obtain the biochemical characterization of the cellulase enzyme.

Materials and Methods

Bacteria Isolation from Digestive Tract of Leopard Moth

Early larval ages of *Zeuzera pyrina* were collected from the infected branches of walnut orchards located in the Saman County of the Chaharmahal and Bakhtiari Provinces in the West of Iran. Whole digestive tracts were isolated in sterile conditions under a UV laminar flow hood, and individual gut was transferred to 200 µl sterile PBS (50 mM) buffer pH 7.

Screening of Cellulolytic Bacteria

The isolated guts were homogenized and spread on nutrient agar and LB agar plates. The inoculated plates were incubated for 7 days at 27 °C and 37 °C. For screening of cellulolytic bacteria, isolated strains were cultured on solid M-II medium which contained 0.5% (w/v) of CMC [13]. Cellulase activity zones were observed by plate staining with 0.1% (v/v) solution of Congo red for 15 min and de-stained in 1 M NaCl for 20 min [14]. The strains with high clearance zone of cellulase activity were used to determine the biochemical reactions of bacteria including gram staining [15], catalase and oxidase activity tests [16], Tween 80 hydrolysis test, carbohydrate fermentation test (lactose, glucose and maltose) and hydrogen sulfide (H_2S) production test [17]. For cellulase activity assay, 0.150 mL of culture broth supernatant was added to 0.250 mL of 50 mM PBS buffer (pH 7.0) containing 1% (w/v) of CMC as substrate. The resulting mixture was incubated at 35 °C for 1 h. The amount of reducing sugar that released during the enzyme activity was determined by the dinitrosalicylic acid (DNS) method [18]. One unit of cellulase activity has been defined as the amount of enzyme which can release 1 µmol of reducing sugar per min under assay conditions.

Identification of Isolated Bacteria

Selected strain's Genomic DNA was extracted by DNA extraction kit (CinnaGen`, Tehran) based on the manufacturer's instructions. Partial 16S rRNA genes were amplified from genomic DNA using the general primers 27F and 1492R in a Bio-Rad Mycycler Thermal cycler (Hercules, USA) [19]. DNA sequencing was performed at the Bioneer Biotechnology Company (Seoul, South Korea).

Optimization of Bacterial Culture Medium

In this experiment, M-II was used as a basal culture medium and optimization was performed using the single-valued method such that each factor was separately examined in various levels of each test. The factors included nitrogen sources (1% w/v): ammonium chloride, peptone, ammonium sulfate, urea and yeast extract; carbon sources (1% w/v): maltose, lactose, fructose, glucose and sucrose; different values of CMC (0–2% w/v); temperature (20–40 °C); various pH (5.0–10.0); and several amount of inoculums (1–5% v/v).

Purification of Enzyme

The bacteria was grown under optimal conditions for cellulase enzyme production and culture broth was centrifuged at 12,000×*g* for 20 min at 4 °C. The supernatant was precipitated with 85% (w/v) ammonium sulfate. The overnight resulting precipitations were collected by centrifugation for 20 min at 12,000×*g* (4 °C). Then, the produced pellet was dissolved in a slight amount of 20 mM Tris–HCl buffer (pH 8.0) and dialyzed against huge amount of the same buffer. For the final purification step of the enzyme, protein loading on a Q-sepharose column which was pre-equilibrated with 20 mM Tris–HCl buffer (pH 8.0). Fractions were collected by washing the column with linear gradient of NaCl (0.1—1 M) in Tris–HCl buffer (pH 8). Then, all of fraction was monitored for protein concentration at 280 nm and cellulase activity. Protein concentration was measured according to the Bradford method using bovine serum albumin as standard protein [20].

Polyacrylamide Gel Electrophoresis and Native Page

The concentrated cellulase fraction was loaded on 12% and 5% (w/v) separating and stacking sodium dodecyl sulfate–polyacrylamide gel (SDS-PAGE), respectively, to

measure the molecular weight of the purified enzyme. For zymogram analysis, non-denaturing polyacrylamide gel electrophoresis was done on 12% (w/v) separating gel containing 1% CMC. Electrophoresis resulting gel was washed with Triton 100-X and floated in 20 mM Tris–HCl buffer (pH 8) at 30 °C for 4 h. The cellulase activity band was seen as a clear zone against background by staining the gel with 0.1% Congo red and de-staining it with 1 M NaCl.

pH and Temperature Effect on Cellulase Activity and Stability

Broad-range buffering systems containing 50 mM of each buffer were used: acetate (pH 4.0–6.0), sodium phosphate (pH 7.0–8.0), Tris/HCl (pH 8.0–9.0) and glycine–NaOH (pH 9.0–10.0) to determine the optimum pH for cellulase activity. Moreover, the enzyme reaction mixture without substrates was incubated at different pH buffers systems as above before at 4 °C for 1 h to determine pH stability. Subsequently, enzyme's residual activity was measured at standard conditions.

Optimal temperature of cellulase activity was calculated at different temperatures ranged from 20 to 80 °C, with 10 degree intervals. Thermal stability was studied by incubating the enzyme without substrates at different temperatures from 20 to 80 °C for 1 h. Subsequently, the residual activity of the purified enzyme was determined at standard conditions.

The Effect of Organic Solvents, Metal Ions and Inhibitors on Cellulase Activity

The effect of 2.5, 5, and 10 mM concentration of metal ions/organic solvents/inhibitors which include, $FeCl_3$, $CaCl_2$, $MgCl_2$, KCl, $MnCl_2$, $BaCl_2$, NaCl, $CoCl_2$, $ZnCl_2$, $NiCl_2$, beta mercaptoethanol and ammonium persulfate, 10 and 20% (v/v) concentration of acetone, n-butanol, toluene, diethyl ether, triton, isopropanol, ethanol, methanol and hexane and 0.5 and 1% (v/v) concentration of tween was determined. The effect of sodium dodecyl sulfate (SDS), hydrogen peroxide (H_2O_2), cetyl trimethylammonium bromide (CTAB) was also measured by adding them to the purified enzyme reaction mixture at 4 °C for 1 h. Subsequently, residual activity was measured in standard enzyme assay conditions. Cellulase activity in the absence of any additives was considered as 100% activity.

Kinetic Parameters

The kinetic parameters (V_{max} and K_m) of the purified cellulase for CMC as a substrate were measured using the Michaelis–Menten equation. The kinetic constants of cellulase were measured using the GraphPad Prism 6 software.

Substrate Specificity

In this case, 0.250 ml of each substrate solution (1% w/v) including cellulose, Avicel and CMC in 50 mM Tris–HCl buffer (pH 8) was mixed with 0.150 ml of the purified enzyme solution. Then the mixture was incubated at 60 °C for 60 min and finally enzyme activity was determined by the DNS method.

Thin-Layer Chromatography of Enzyme Hydrolysis Products

Enzymatic action of cellulase on CMC substrate at times 6, 12, and 24 h in standard enzyme assay conditions was analyzed using thin-layer chromatography (TLC) with a slight modification [21].

Results

According to the results of the cellulase screening approach on CMC plates, only 4 strains including Ac4, Bc1, Bc8 and Dc4 showed cellulolytic activity from among all purified isolates. The biochemical characteristics and cellulase activity of the isolated strains are shown in Table 1. Strain Bc_1 with 0.42 (U/ml) catalytic activity showed the highest cellulase activity and was selected for further analysis.

Table 1 Biochemical characteristics and cellulase activity of the isolated strains (values of the selected strain were presented in bold)

Strain	Gram staining	Catalase activity	Oxidase activity	Fermentation of glucose	Fermentation of maltose	Fermentation of lactose	H_2S production	Tween 80 hydrolysis	Enzyme activity (U/mL)
Ac4	−	+	−	+	+	−	+	+	0.30 ± 0.004
Bc1	+	+	−	+	+	−	−	+	**0.42 ± 0.002**
Bc8	+	+	−	+	−	−	+	−	0.31 ± 0.005
Dc4	−	+	−	+	+	+	+	−	0.26 ± 0.001

Table 2 Optimizing the culture medium to cellulase production using a one factor at the time (OFAT) approach (optimum levels presented in bold)

Runs	Various levels/cellulase activity U/ml				
Carbon sources (1% w/v)	Glucose ($0.176^{b} \pm 0.002$)	**Maltose ($0.182^{a} \pm 0.002$)**	Lactose ($0.177^{ab} \pm 0.001$)	Saccharose ($0.178^{ab} \pm 0.005$)	Fructose ($0.174^{b} \pm 0.004$)
Nitrogen sources (1% w/v)	$(NH_4)_2SO_4$ ($0.075^{d} \pm 0.003$)	$(NH_4)_2Cl$ ($0.077^{d} \pm 0.003$)	Urea ($0.086^{c} \pm 0.005$)	Peptone ($0.210^{b} \pm 0.001$)	**Yeast extract ($0.219^{a} \pm 0.002$)**
CMC (% v/v)	0 ($0.167^{d} \pm 0.003$)	0.5 ($0.192^{c} \pm 0.004$)	1 ($0.214^{b} \pm 0.005$)	**1.5 ($0.234^{a} \pm 0.0005$)**	2 ($0.197^{c} \pm 0.01$)
Inoculum size (% v/v)	1 ($0.166^{b} \pm 0.005$)	**2 ($0.189^{a} \pm 0.004$)**	3 ($0.168^{b} \pm 0.002$)	4 ($0.164^{b} \pm 0.003$)	5 ($0.162^{b} \pm 0.005$)
Temperature (°C)	20 ($0.098^{e} \pm 0.0005$)	25 ($0.112^{d} \pm 0.005$)	30 ($0.150^{b} \pm 0.005$)	**35 ($0.173^{a} \pm 0.005$)**	40 ($0.142^{c} \pm 0.006$)
pH	6 ($0.157^{d} \pm 0.002$)	7 ($0.174^{b} \pm 0.003$)	**8 ($0.197^{a} \pm 0.003$)**	9 ($0.161^{c} \pm 0.0005$)	10 ($0.129^{e} \pm 0.006$)

The means followed by different letters in the same row are significantly different ($p < 0.05$, Tukey)

Table 3 Purification steps for the cellulase isolated from *Bacillus subtilis* Bc1

Purification steps	Activity (U/ml)	Protein (mg/ml)	Total activity (units)	Total protein (mg)	Specific activity (units/mg)	Purification (fold)	Yield (%)
Cell-free supernatant	6.26	1.56	1877.04	469.83	4.0	1.00	100
Ammonium sulfate precipitation	2.81	1.30	84.28	19.65	4.29	1.07	4.5
Q-Sepharose	1.43	0.33	3.58	0.33	10.86	2.72	0.2

Sequence Analysis

Partial 16S rRNA sequence of isolated strain was deposited in NCBI GenBank database under MN658389 accession number. Based on 16S rRNA sequence analysis and biochemical tests, strain BC1 was identified as *Bacillus subtilis* with more than 98% homology with *Bacillus subtilis subsp. spizizenii*.

Culture Optimization for Cellulase Production

Based on the results, it was revealed that 1% (w/v) of maltose, 1% (w/v) of yeast extract, 1.5% (w/v) of CMC, pH 8, 2% (v/v) of inoculums size and temperature of 35 °C are the most effective factors for cellulase producing by *B. subtilis* BC1 (Table 2).

Enzyme Purification and Molecular Weight Determination

The optimal broth culture supernatant had 6.25 U/mL activity, 1.56 mg/mL protein, 1877.04 U/mL total activity, 469.83 mg total protein and 4 U/mg specific activity. The whole culture medium (500 mL) was precipitated using 85% ammonium sulfate salt and this improved enzyme specific activity to 4.29 U/mg. Subsequently, enzyme specific activity was increased to 10.86 U/mg with 2.72-fold purification in the final purification step of enzyme using Q-sepharose ion-exchange chromatography (Table 3). The molecular weight of purified enzyme on SDS-PAGE is about 47.8 kDa as shown in Fig. 1. Moreover, zymography results of β-1,4-glucanase show a single activity band on native PAGE.

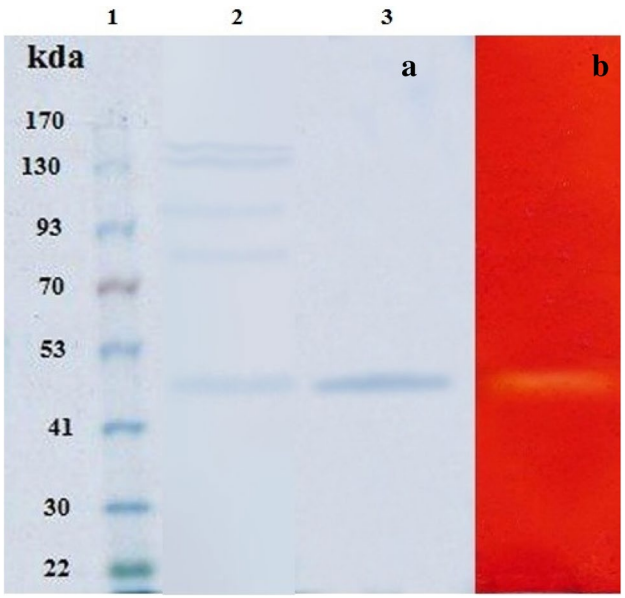

Fig. 1 SDS-PAGE (**a**) and zymogram analyses (**b**) of purified cellulase by *Bacillus subtilis* BC1. In (a), lane 1, protein ladder; lane 2, the crude enzyme extract; lane 3, the purified sample

pH and Temperature Effect on Cellulase Activity and Stability

The effect of pH value (4–10) on activity and stability of purified enzyme is shown in Fig. 2a. The results indicated that the highest enzyme activity is observed at pH 8. The results also indicate that enzyme has a broad range of pH stability from 4 to 10 and after treatment at pH values ranging from 4 to 10 it maintains 75% of its own activity.

The optimal temperature for purified cellulase activity was 60 °C as shown in Fig. 2b. The results demonstrated that enzyme was very stable at temperatures between 20 and 60 °C since it maintained 100% of its activity after 60 min of incubation. Moreover, more than 74% of cellulase activity remained at temperatures between 60 and 80 °C.

The Effect of Various Metal Ions, Organic Solvents and Inhibitors on the Enzyme Activity

Purified cellulase enzyme can be completely active in all concentrations of organic solvents as shown in Fig. 3a. The results showed that in the presence of 20% (v/v) of acetone, enzyme activity was increased. Among the metal ions which were evaluated, $MnCl_2$ and $NiCl_2$ decreased enzyme activity, whereas $CaCl_2$ and $CoCl_2$ increased enzyme activity. In addition, $FeCl_3$, KCl, $MgCl_2$, $BaCl_2$, NaCl and $ZnCl_2$ had no significant effect on enzyme activity (Fig. 3b). CTAB and H_2O_2 completely inhibited enzyme activity as shown in Fig. 3c.

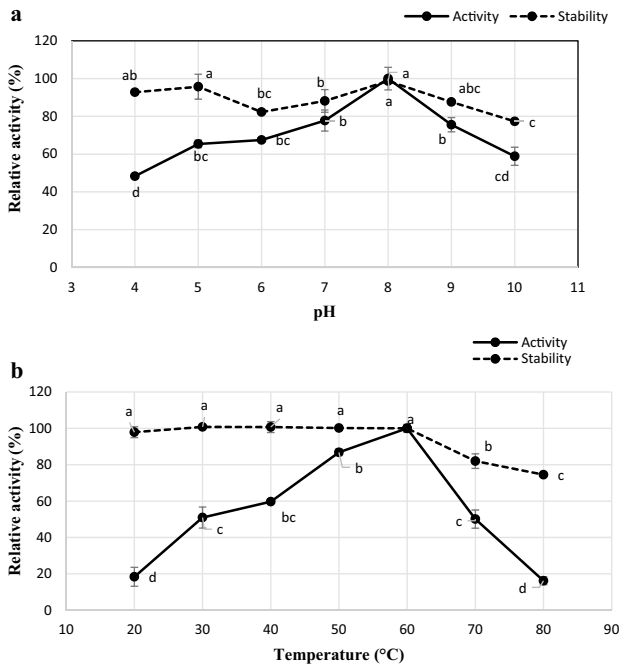

Fig. 2 Effect of pH (**a**) and temperature (**b**) on cellulase activity and stability produced by *Bacillus subtilis* BC1

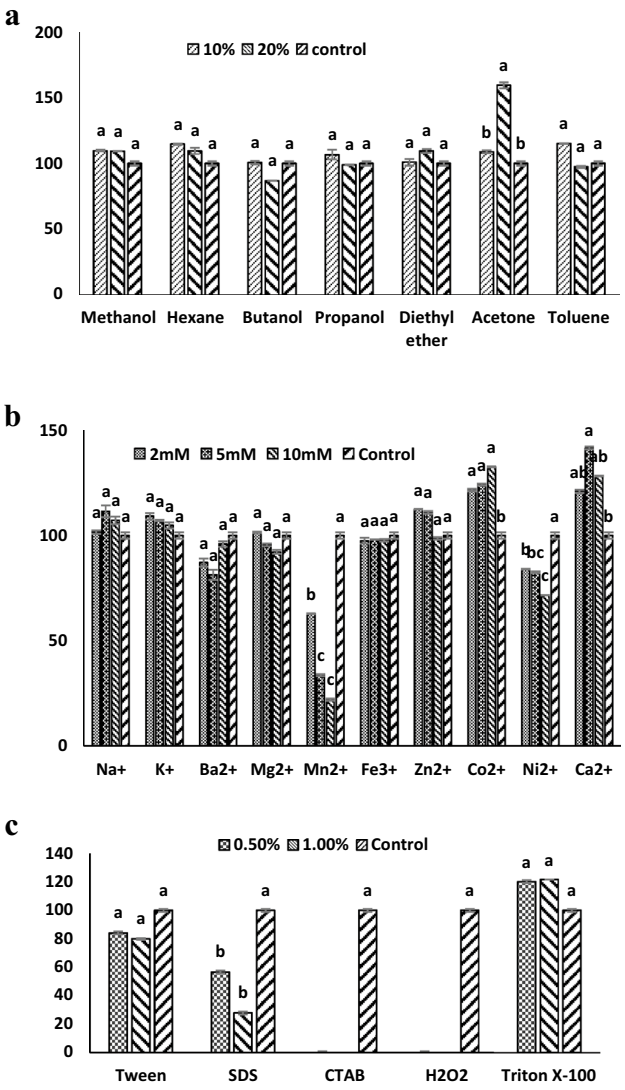

Fig. 3 Effect of organic solvents (**a**), metal ions (**b**) and inhibitors (**c**) on the activity of cellulase of *B. subtilis* BC1

Enzyme Kinetics

Kinetic parameters of the purified cellulase toward CMC were determined according to the Michaelis–Menten plots. K_m and V_{max} values of the enzyme were found to be 1.243 mg/mL and 271.3 µg/mL/min, respectively.

Substrate Specificity and Thin-Layer Chromatography

The purified cellulase showed the highest activity of $0.77^a \pm 0.001$ (U/mg) in the presence of cellulose as

substrate, whereas the enzyme activities toward avicel and CMC were $0.72^{b} \pm 0.001$ and $0.69^{c} \pm 0.002$ (U/mg), respectively. Thin-layer chromatography (TLC) results after 12 h and 24 h incubation revealed that cellobiose was the final hydrolytic product of the purified cellulase toward a CMC as substrate (Fig. 4). This indicates exo and endoglucanase activities of the *B.subtilis* BC1 cellulase.

Discussion

Insects are the most tolerant group of multicellular organisms that live in various conditions along with many microorganisms present in their gut [22]. Among all microorganisms, bacterial population which live in insect guts play a fundamental role in efficient digestion of woody material by producing various cellulolytic and ligninolytic enzymes [11]. Research studies have shown that Proteobacteria and Firmicutes are common phyla in insect gut [23]. Manfredi et al. indicated that Firmicutes and especially *Bacillus* genus is the prevalent bacteria that degrades cellulose in many insect families in Northwest of Argentina [24]. In the present study, cellulolytic strain *B. subtilis* BC1 was isolated from the gut of leopard moth and subsequently cellulase enzyme was purified and characterized for the first time. *Bacillus sp*. belonging to the gram-positive groups firmicutes with cellulolytic activity have also been reported in other lepidopteran insects previously [25, 26]. Furthermore, *B. subtilis* is a common cellulolytic strain in xylophagous termite gut [27, 28].

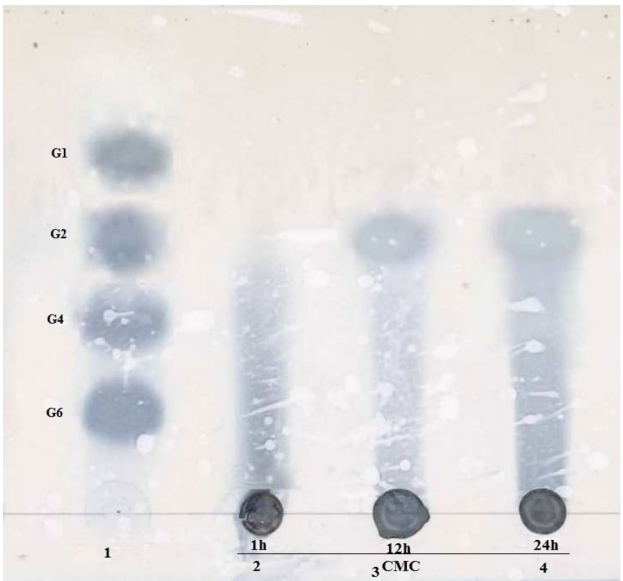

Fig. 4 TLC analysis of hydrolysis products using CMC as substrate. Lane 1, sugar standards of G1: glucose, G2: cellobiose, G4: cellotetrose and G6: cellohexose. Lane 2, 3 and 4, enzyme product after 1, 12 and 24 h

The 16S sequence of this strain was highly similar (more than 98% identity) to *Bacillus subtilis* subsp. spizizenii strain Cpl13 (accession number: MN960275) isolated from *Calotropis procera* leaf and also with *Bacillus halotolerans* strain af-M9 (accession number: MN512294) which has been isolated from honey bee gut. On the base of biochemical tests and morphological characters, this strain was identified as *Bacillus subtilis*.

The results of this study showed that the molecular mass of purified cellulase is 47.8 kDa that is close to the molecular mass of another cellulase reported from *B. subtilis* strain that is 46 kDa [29]. *B. subtilis* YJ1 and *B. subtilis* UMC7 have shown different hydrolysis bands corresponding to 32.5 kDa and 56 kDa [27, 30].

In the current study, the results of optimal pH for purified cellulase activity (pH 8) was completely similar to β1,4-glucanases isolated from gut bacteria of other insects.[26, 31, 32]. It should be mentioned that optimal pH for enzyme activity of some strains such as *B. pumilus* MGB05 and *B. subtilis* UMC7 isolated from silk worm midgut and termite *Macrotermes malaccensis* gut, respectively, is less than 8 [25, 27]. Since purified cellulase activity can be recovered after exposure to a wide range of pH values (4 to 10), this enzyme can possibly be applied in different industrial applications.

The results showed that cellulase of *B. subtilis* isolated from gut of leopard moth has an optimum temperature activity at 60 °C that is similar to those reported for other cellulase from *B. subtilis* [27, 30, 33]. As reported, the purified cellulase was stable in a wide range of temperatures. Thereby, this enzyme can be employed as a heat-resistant enzyme in many industrial applications, particularly in bioethanol production.

Moreover, it was observed that the cellulase of *B. subtilis* BC1 is completely stable in the presence of all organic solvents tested in the present research study and some organic solvents such as acetone (20% v/v) even enhance enzyme activity. Organic solvent tolerant cellulases have many industrial applications such as bioremediation of carbohydrate-polluted salt marshes and treatment of industrial waste water contaminated with organic solvents [34]. Enzyme activity enhanced by organic solvents might be due to residues of carried-over non-polar hydrophobic solvents which provide an interface and thus keep the enzyme in an open conformation which results in stimulated activation [35]. Thus, organic solvent stability of the purified cellulase indicates that this enzyme can be used in the industry under harsh conditions.

In current study, cellulase retained 79.9, 121.0, and 28.04% activity in the presence of Tween 20 (1%v/v), Triton X-100 (20% v/v) (non-ionic surfactant) and SDS (anionic

surfactant), respectively. Alkalophilic cellulase from *B. halodurans* CAS 1 in contrast to cellulase from *B.subtilis* BC1 of the leopard moth had more and low tolerance to SDS and Triton X-100 (84.33% and 68%), respectively [34]. It seems that purified cellulase is non-ionic surfactant tolerant enzyme and it can be used in detergent industry dealing with cellulases.

Purified cellulase activity has been stimulated with Ca^{2+} and most frequently similar results have been reported for *Bacillus* strains [34, 36–38]. The presence of Ca^{2+} ions may enhance the substrate binding affinity of the enzyme by stabilizing the conformation of the catalytic site [27]. Based on the results obtained in this study the purified cellulose activity is inhibited by Mn^{2+} and Ni^{2+} and stimulated with Co^{2+}. Gaur and Tiwari reported that cellulase activity from *Bacillus vallismortis* RG-07 was slightly inhibited by Ni^{2+}, whereas it was severely inhibited in the presence of Mn^{2+} [37]. Annamalai et al. indicated that Mn^{2+} can enhance but Co^{2+} inhibits cellulase activity from *Bacillus halodurans* CAS 1 [34]. Similarly, Co^{2+} had also inhibitory effect on cellulase activity from *Bacillus* sp. AC-1 [39].

The purified cellulase significantly exhibited high activity toward cellulose, avicel and CMC. Therefore, this enzyme was able to efficiently hydrolyze both amorphous cellulose (CMC and cellulose) and crystalline cellulose (avicel) by simultaneous assistance of exo and endoglucanases activity [40]. Annamalai et al. isolated cellulase which had only endoglucanase activity and did not hydrolyze cellulose and avicel, while cellulase from yak rumen metagenome and *Thermobifida halotolerans* YIM 90,462 exhibited both endo and exoglucanase activities and a broad substrate specificity [41, 42]. In general, the enzyme which was introduced in this study has high affinity toward crystalline cellulose that can be a very strong candidate for efficient saccharification of lignocellulosic biomass.

The results of TLC Analysis showed that cellobiose is a final hydrolyze product of purified cellulase on CMC as substrate. This confirms the exoglucanase activity of the purified enzyme. Exoglucanases has a key role in the solubilization of cellulose which results in crystalline cellulose degradation in both complexed and non-complexed cellulolytic systems [43]. Wu et al. showed that beta-1,4-glucanase from *B. subtilis* BS-5 produces a mixture of cellotetraose, cellotriose and cellobiose as the final products of CMC hydrolysis [43]. In other *Bacillus* strains isolated from rosaceae branch borer beetles and termites, the main product of CMC hydrolysis is cellobiose [13, 38].

In conclusion, a novel cellulase has been purified from *B. subtilis* BC1 of the leopard moth gut bacteria in this research study for the first time. This enzyme exhibited high thermostability, acidic and alkaline tolerance and organic solvent compatibility that make it an appropriate option for biotechnological processes and industrial applications. Since *B. subtilis* BC1 cellulase is an alkaline enzyme, it seems that future studies should be focused on studying amino acid sequence of the enzyme to better understand the activity mechanism of enzyme at high pH and also determination of 3D-structure of enzyme for further biotechnological approaches. Moreover, designing of a tailored-made enzyme to stable under extreme industrial conditions.

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