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Production and Characterization of Organic Solvent-Tolerant Cellulase from *Bacillus amyloliquefaciens* AK9 Isolated from Hot Spring

Muhammad Irfan[1] · **Ammara Tayyab**[1] · **Fariha Hasan**[1] · **Samiullah Khan**[1] · **Malik Badshah**[1] · **Aamer Ali Shah**[1]

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Abstract A cellulase-producing bacterium, designated as strain AK9, was isolated from a hot spring of Tatta Pani, Azad Kashmir, Pakistan. The bacterium was identified as *Bacillus amyloliquefaciens* through 16S rRNA sequencing. Cellulase from strain AK9 was able to liberate glucose from soluble cellulose and carboxymethyl cellulose (CMC). Enzyme was purified through size exclusion chromatography and a single band of ~47 kDa was observed on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The enzyme was purified with recovery of 35.5%, 3.6-fold purity with specific activity of 31 U mg^{-1}. The purified cellulase retained its activity over a wide range of temperature (50–70 °C) and pH (3–7) with maximum stability at 60 °C and pH 5.0. The activity inhibited by ethylenediaminetetraacetic acid (EDTA), suggested that it was metalloenzyme. Diethyl pyrocarbonate (DEPC) and β-mercaptoethanol significantly inhibited cellulase activity that revealed the essentiality of histidine residues and disulfide bonds for its catalytic function. It was stable in non-ionic surfactants, in the presence of various metal ions, and in water-insoluble organic solvents. Approximately 9.1% of reducing sugar was released after enzymatic saccharification of DAP-pretreated agro-residue, compared to a very low percentage by autohydrolysis treatment. Hence, it is concluded that cellulase from *B. amyloliquefaciens* AK9 can potentially be used in bioconversion of lignocellulosic biomass to fermentable sugars.

Keywords *Bacillus amyloliquefaciens* AK9 · Cellulase · SDS-Page · Purification · Characterization · Surfactants · Organic solvents

✉ Aamer Ali Shah
alishah@qau.edu.pk

[1] Department of Microbiology, Faculty of Biological Sciences, Quaid-i-Azam University, Islamabad 45320, Pakistan

Introduction

Cellulose, the most abundant renewable bioresource, encompasses a linear polymer of D-glucose concomitant with β-1,4 linkages which can be broken down into soluble sugars by the help of a class of enzymes called cellulases [1]. The degradation of cellulose involves multi-component cellulases including exoglucanase, endoglucanase, and β-glucosidase that are grouped into glycoside hydrolases [2]. Various cellulosic substrates are degraded by enzyme complex that leads to synthesis of a variety of products such as organic acids, ethanol, and some important chemicals. Cellulases from fungal strains have already been reported previously. Their profuse secretion in the extracellular medium has made its extraction and purification easier. Bacteria present an attractive potential for the exploitation of cellulases due to their rapid growth rate, enzyme complexity, and extreme habitat variability [1]. Cellulases from thermophilic bacteria offer many advantages such as high reaction rate, stability, low possibility of microbial contamination, longer half-life, and required in less amount [3]. Kazue et al. suggested that natural habitats for thermophilic microorganisms are most commonly the geothermally heated hot spring [4]. Thermophilic cellulolytic microorganisms have also been isolated from diverse environments like wastewater [5], soils and composts [6], and deep surface gold mines [7].

In the present study, we report an organic solvent thermophilic cellulose-degrading bacterium *Bacillus amyloliquefaciens* AK9 isolated from Tatta Pani, Azad Kashmir, Pakistan and optimization of parameters for improving cellulase production, purification, and characterization of the enzyme. The unique property of solvent-thermostable property proves the potential candidature of *B. amyloliquefaciens* AK9 for biomass conversion into fuel and other industrial process.

Materials and Methods

Isolation of Cellulase-Producing Bacteria

Water samples from a hot spring in Tatta Pani, Azad Kashmir, Pakistan were screened for the presence of cellulose-degrading bacteria by streaking the isolated colonies on cellulose containing medium (g/L: carboxymethyl cellulose (CMC) 5, $NaNO_3$ 1, K_2HPO_4 1, KCl 1, $MgSO_4$ 0.5, yeast extract 0.5, agar 16) [8]. After 48 h of incubation, the agar plates were stained and then de-stained with 1% Congo red and 1 M NaCl, respectively, On the basis of maximum zone of hydrolysis, a bacterial isolate, AK9, was selected for further studies.

Identification of Cellulase Producing Bacterium

Isolate AK9 having cellulase-producing ability was identified both conventionally as well as through 16S ribosomal RNA (rRNA) gene sequencing using forward (27F′) and reverse (1494R′) primers [9]. The purified PCR products were resolved on an Applied Bio-Systems Model 3100 automated DNA sequencing system (Applied Bio-Systems, USA) using BigDye terminator cycle sequencing kit v.3.1 (Applied Bio-Systems, USA) at the Macrogen, Inc., Seoul, Korea. Finally, obtained 16S rRNA gene sequence was submitted to NCBI GenBank,

and phylogenetic tree was constructed using neighbor-joining method (CLUSTAL software in MEGA 6).

Enzyme Activity Assays

Cellulase activity was determined by estimating the reducing sugars formed by the method given by Ghose [10], with slight modifications. About 0.5 ml of culture supernatant was mixed with 0.5 ml of 1% CMC in 0.05 M sodium citrate buffer (pH 4.8). The assay tubes were incubated in a water bath at 60 °C for 30 min. After incubation, 3 ml of dinitrosalicylic acid (DNS) reagent was added to all the tubes and then placed in vigorously boiling water for 10 min. Later on, the tubes were cooled at room temperature for considerable time period. Finally, the color developed in case of each assay tube was read against 540 nm wavelength. Concentration of glucose formed was determined using glucose standard curve. One unit of CMCase activity was expressed as 1 μmol of glucose released per milliliter enzyme per minute. Protein was estimated in each sample by Lowry's method [11]; BSA was used as a standard, whereas specific activity (U/mg) was calculated by dividing activity (U/ml) over protein content (mg/ml).

Production of Cellulase and Bacterial Growth

Various growth factors were considered for maximum production of cellulase enzyme, that include wide range of temperature (40–60 °C), pH (4.0–9.0), substrate (CMC) concentrations (0.5–3.0%), NaCl (1–10%), and inoculum size (1–6%). CMC-minimal salt broth [12] was inoculated with isolate AK9 suspended in saline (2.0 O.D at 600 nm) for each parameter studied. The cultures were incubated for 120 h with a constant shaking at 150 rpm. Samples were aseptically drawn after every 24 h. Cells were harvested by centrifugation at 10,000 rpm for 10 min, and then supernatant was processed for enzyme assay, protein estimation and finally specific activity was calculated.

Purification of Cellulase

A total of 6 ml of 48 h old culture of isolate AK9 was added to 94 ml of minimal salt broth (pH 5.0) containing 2.5% CMC in an Erlenmeyer flask. The flask was then placed into shaker incubator at 55 °C for 48 h and 150 rpm. The culture broth was centrifuged after incubation at 8000×*g* for 10 min at 4 °C (Kokusan Model H-2600), and cell free supernatant was collected for protein precipitation. Solid ammonium sulfate $[(NH_4)_2SO_4]$ was then added with gentle stirring at 4 °C until the solution reached 40% saturation. The solution was centrifuged at 8000 rpm for 10 min, and the supernatant was collected. Then, solid $(NH_4)_2SO_4$ was added to the supernatant until the solution reached 80% saturation. The solution was centrifuged, and precipitates were dissolved in 6 ml of 50 mM sodium citrate buffer (pH 4.8). Excessive salt was removed from crude protein through dialysis tubing and then stored in freezer at −80 °C.

About 2 ml of the crude enzyme extract was loaded on Sephadex G-75 column (10/50 mm, GE Healthcare UK). The enzyme was eluted using 50 mM sodium citrate buffer (pH 4.8) at a flow rate of 0.6 ml/min. The active fractions were pooled and then concentrated by ultrafiltration (Millipore, USA). The molecular mass of cellulase was calculated using SDS-PAGE [13].

Characterization of Purified Cellulase

Effect of Temperature and pH on Enzyme Activity and Stability

The effect of temperature on enzyme activity was studied over a temperature range of 30–90 °C. Thermostability of enzyme was determined by preincubating the purified enzyme for several hours at wide range of temperatures, and then activity was determined under standard assay conditions. Similarly, the activity of enzyme was monitored at different pH ranges (2.0–10.0). Also, the stability was checked by incubating the purified enzyme with various buffer systems for several hrs at 50 °C. The buffer systems (50 mM) used in this study were as follows: sodium citrate (pH 3.0–6.0), sodium phosphate (pH 7.0), Tris-HCl (pH 8.0), and glycine NaOH (pH 9.0–10).

Effect of Metal Ions, Chemicals, and Surfactant

The behavior of purified enzyme in the presence of various additives, such as metal ions (10 mM) in SO_4 and Cl_2 salt, chemical reagents, and surfactant (0.5%) was studied. For this purpose, the enzyme was incubated for 30 min with these additives, and then the activity was checked under optimal assay conditions. The activity was considered 100% in the absence of any additive.

Effect of Organic Solvents on Cellulase Activity

Purified enzyme having maximum cellulase activity was incubated with 30% (*v*/*v*) of different organic solvent (*n*-dodecane, *n*-decane, xylene, iso-octane, *n*-butanol, *n*-hexane, cyclohexane, toluene, acetone, benzene, methanol, ethanol, and propanol for 1 week in screw crapped tubes at optimum conditions. The residual cellulase activity was estimated against the control, in which solvent was not present.

Substrate Specificity and Shelf Life

In order to determine its specificity, the hydrolytic potential of purified cellulase was investigated against a variety of substrates such as starch, xylan, avicel, and filter paper. About 1% of each substrate was incubated with enzyme solution (pH 5.0) at 50 °C for 30 min, and then activity was determined. The shelf life of enzyme at refrigerator as well as room temperature was also determined by measuring activity in samples withdrawn at different intervals up to the maximum of 10 weeks.

Application of Cellulase

Enzyme Treatment of Agro-residues

The agro-residue biomass was air dried for 2–3 days. In order to evaluate the effectiveness of enzymatic saccharification, autohydrolyzed and dilute acid pretreated (DAP) samples were used. During autohydrolysis, the solid to liquid ratio was set to 1:5 (500 mg biomass in 02.5 ml water) and subjected to autoclaving at 121 °C (15 psi) for 15 min, and DAP was carried out by using 0.5% H_2SO_4, under same conditions used for

autohydrolysis After pretreatments, all the samples were further subjected to enzymatic hydrolysis. The pH of all the pretreated samples was adjusted to 5.0 prior to enzymatic saccharification. Purified enzyme from *B. amyloliquefaciens* AK9 at an equivalent of 7.5 IU was added, and the final volume of the solution was made to 9.5 ml using citrate buffer (pH 5.0). Samples were incubated at 50 °C for 16 h. Reducing sugars were determined before and after enzymatic hydrolysis using DNS reagent.

Statistical Analysis

All experiments were carried out in triplicates and the data was evaluated by the method as previously described [14]. The means and standard errors of means (mean ± S.E) were considered for each factor, and one-way ANOVA was applied on results obtained [15]. p Value of 0.05 was set as a level of significance; the result was significant in case of p value less than 0.05.

Results

Isolation and Screening of Thermophilic Cellulolytic Bacteria

A total of 28 bacterial strains were isolated from hot spring on nutrient agar medium at 50 °C after 48 h of incubation. Among them, 10 isolates were found to grow exponentially and hydrolyse CMC on CMC-supplemented agar medium at 50 °C within 48 h of incubation. A bacterial isolate AK9 formed maximum size of zone of hydrolysis (4.2 cm) as compared to others, hence selected for further study.

Identification of Bacterial Isolate AK9

Isolate AK9 was aerobic, gram-positive, rod-shaped, motile, and spore-forming bacterium. The bacterium was found negative for indole production, methyl red, and urease test, whereas positive to starch and casein hydrolysis, Voges Proskauer, oxidase, catalase, nitrate reduction, and citrate utilization. A sequence of 1531 nucleotides of 16S rRNA was submitted to NCBI GenBank and aligned with reference sequences. Isolate AK9 was considered to be the member of genus *Bacillus* with maximum similarity (99%) to *B. amyloliquefaciens* (KP100335) (Fig. 1). The nucleotide sequence of isolate AK9 is submitted in NCBI GenBank under the accession number KR154350.

Optimization of Culture Conditions for Cellulase Production

Various parameters were optimized for production of cellulase from *B. amyloliquefaciens* AK9. Maximum activity was recorded after 48 h of incubation at 55 °C, pH 5.0, 2.5% substrate concentration, and 6% inoculum. Enzyme production decreased in the presence of NaCl, as indicated by maximum activity after 96 h of incubation. The statistical analysis carried out by one-way ANOVA for the optimum enzyme production under various culture conditions was statistically significant ($p < 0.05$).

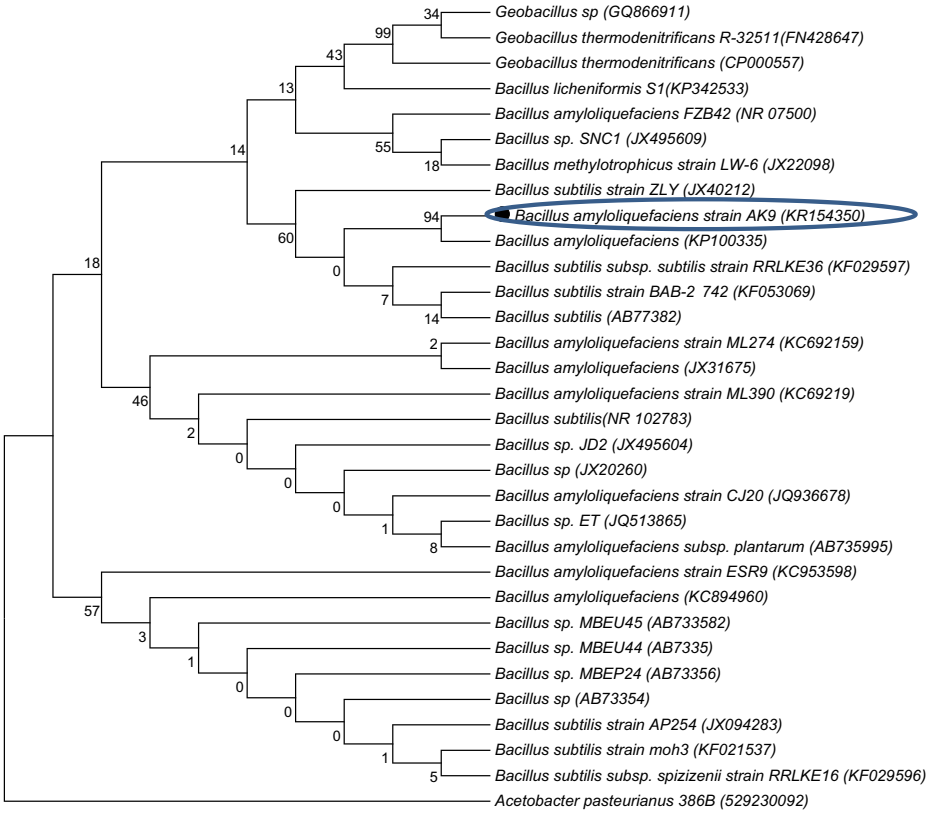

Fig. 1 Neighbor joining phylogenetic tree showing the position of isolate AK9 to other strains of *Bacillus*. Accession numbers of the sequences used in this study are shown in *parentheses* after the strain designation. *Numbers at nodes* are percentage bootstrap values based on 1000 replications. Evolutionary analysis was conducted using MEGA6

Purification of Cellulase

Table 1 shows the steps followed during purification of cellulase from *B. amyloliquefaciens* AK9. Protein peak fractions were eluted using 50 mM sodium citrate buffer (pH 4.8) at a flow rate of 0.6 ml/min by gel filtration column chromatography (Sephadex G-75 column (10/50 mm, GE Healthcare UK) (Fig. 2). Fraction 12 was collected having a single band and activity of 44.12 (U/ml). The molecular size of purified enzyme was determined by SDS-PAGE

Table 1 Purification summary of cellulase from *Bacillus amyloliquefaciens* strain AK9

Purification steps	Volume (ml)	Enzyme activity (U/ml)	Protein content (mg/ml)	Specific activity (U/mg)	Purification fold	% Yield
Crude enzyme	500	124.40	14.49	8.584	1.00	100
Ammonium sulfate precipitation	20.00	97.15	5.02	19.36	2.26	78
Sephadex G-75 chromatography	12	44.12	1.421	31.05	3.62	35.50

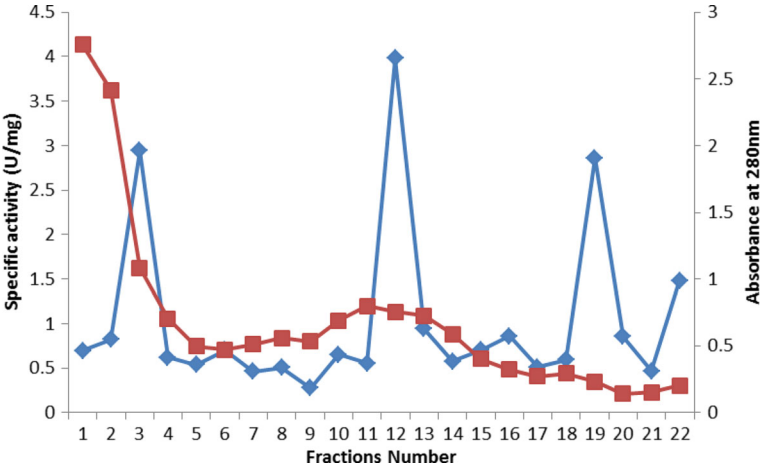

Fig. 2 Fractionation by gel filtration column chromatography (Sephadex G-75 column (10/50 mm, GE Healthcare UK). Protein peak fractions were eluted using 50 mM sodium citrate buffer (pH 4.8) at a flow rate of 0.6 ml/min. (i) Absorbance at 280 nm (*red line*). (ii) Specific activity (U/mg) (*blue line*)

using 15% polyacrylamide gel. The molecular size of the enzyme was measured approximately 47 kDa with specific activity of 31 U mg^{-1}, recovery rate 35.5%, and 3.6-fold purity (Fig. 3).

Characterization of Purified Cellulase

Effects of pH and Temperature on Stability of Cellulase

The cellulase activity was measured at different pH (2.0–10.0) and temperature (10 °C–90 °C) values. The purified enzyme was found stable at wide range of temperature (50–70 °C) ($p < 0.05$) and pH (3.0–7.0) ($p < 0.05$) with maximum stability at 60 °C and pH 5.0 (Fig. 4).

Effects of Metal Ions, Surfactant, and Chelating Agent on the Cellulase Activity

Fe^{+2} and Hg^{+2} ions considerably inhibited the activity of purified cellulase, whereas slightly affected by the rest of monovalent and divalent cations ($p < 0.05$) (Table 2). The enzyme activity significantly decreased in the presence of EDTA and β-mercaptoethanol whereas PMSF and phenylarsine oxide partially inhibited the activity ($p < 0.05$) (Table 2). The enzyme lost more than 80% of its activity in the presence of SDS and sarkosyl, while remained stable in the presence of non-ionic surfactants ($p < 0.05$) (Table 2). The activity was 138% enhanced by dimethylformamide (DMF) ($p < 0.05$) (Table 2).

Effect of Organic Solvents on Cellulase Stability

The enzyme considerably retained its activity in the presence of some of organic solvents.

The effect of various organic solvents (30%, *v*/*v*) on cellulase activity and stability was investigated for 1 week, and the residual activity was calculated under optimum assay conditions. After incubation with *n*-dodecane, *n*-decane, hexane, iso-octane, methanol,

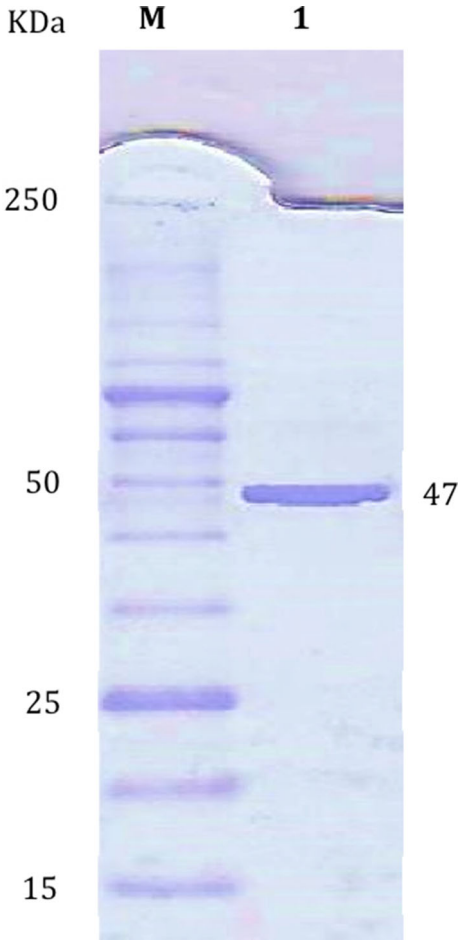

Fig. 3 SDS-PAGE showing purified enzyme obtained from chromatography. *M*, SDS-PAGE molecular mass standards 10–250 kDa New England Biolab; *1*, purified fraction

toluene, and *n*-butanol, the cellulase activity increased to 141 (48 h), 132 (48 h), 111 (48 h), 129 (24 h), 117 (48 h), 113 (24 h), and 119% (48 h), respectively. The presence of benzene, xylene, propanol, and ethanol marginally reduced the cellulase with residual activities of 81, 85, 79, and 82%, respectively (Table 3).

Effects of Different Substrates on the Activity of Cellulase

Specificity of enzyme to β-1,4-linkages is evident from its activity against CMC, avicel, filter paper, and polymeric substrates such as xylan and β-glucan ($p < 0.05$) (Table 4).

Shelf Life Determination Cellulase

The enzyme retained its activity for the maximum up to 5 and 10 weeks when stored at room temperature and under refrigeration, respectively; a gradual decrease was observed after this period ($p < 0.05$).

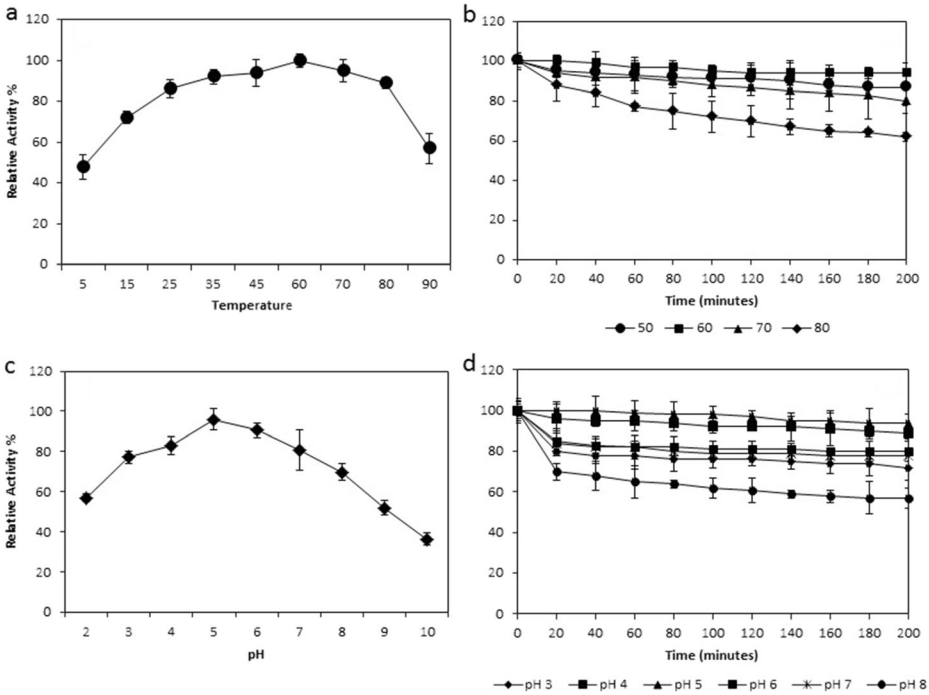

Fig. 4 Effect of temperature (**a**, **b**) and pH (**c**, **d**) on activity (**a**, **c**) and stability (**b**, **d**) of the purified cellulase, respectively. Relative activity was defined as the percentage of activity detected with respect to the maximum enzyme activity. For determining the stability, the activity of the enzyme without any treatment was taken as 100%. Data are the average of three independent experiments

Table 2 Effects of metal ions and chemical reagents on the activity of purified cellulase from *Bacillus amyloliquefaciens* strain AK9

Chemicals	Residual activity (%)
Control	100 ± 0.3
Ca^{+2}	79 ± 0.2
Hg^{+2}	45 ± 1.0
Mg^{+2}	88 ± 0.8
Fe^{+2}	55 ± 1.0
Zn^{+}	77 ± 0.3
β-Mercaptoethanol	21 ± 0.47
Phenylarsine oxide	90 ± 0.8
EDTA	25 ± 1.0
PMSF	95 ± 0.5
Diethyl pyrocarbonate	16 ± 0.51
SDS	18 ± 0.2
Tween 20	90 ± 2.5
Tween 80	92 ± 1.5
Triton X-100	88 ± 3.0
Sarkosyl	85 ± 0.6
Dimethylformamide	138 ± 1
DMSO	77 ± 0.41

Table 3 Effect of various organic solvents on activity of purified cellulase from *Bacillus amyloliquefaciens* strain AK9

Organic solvents	Residual activity % (h)						
	1	24	48	96	120	144	168
Ethanol	99	103	103	101	94	87	82
Iso-propanol	98	99	95	91	86	83	79
Butanol	101	109	119	117	110	102	95
Benzene	100	102	104	98	91	84	81
Acetone	99	98	95	93	90	87	82
Cyclohexane	98	103	111	116	103	91	85
Toluene	102	113	110	105	101	97	90
Xylene	99	98	96	93	90	87	85
Methanol	100	109	117	113	109	99	94
Iso-octane	105	129	123	119	113	103	98
Hexane	109	117	111	108	113	105	98
n-decane	101	111	132	119	121	107	98
n-dodecane	105	117	141	133	129	104	94

Enzyme was preincubated with different organic solvents at a concentration of 30% (*v*/*v*) at optimum conditions for different time period and assayed as standard assay method. The enzyme activity without incubation with organic solvent was taken as 100%

Enzyme Treatment of Agro Residues

It was observed that the autohydrolyzed samples released 1.1 and 8.1% of the reducing sugar before and after enzymatic hydrolysis, respectively. Similarly, 1.13% reducing sugar was obtained from DAP-pretreated sample prior to enzymatic hydrolysis. However, maximum of 9.1% of reducing sugar was obtained after enzymatic saccharification (strain AK9 cellulase) of DAP-pretreated agro-residue (Table 5), which was comparatively better than autohydrolysis treatment.

Discussion

We have reported here a newly isolated *B. amyloliquefaciens* AK9 from hot spring having high cellulolytic activity on CMC-supplemented agar medium at 50 °C within 48 h of incubation. Although a number of microorganisms (fungi, bacteria, or actinomycetes) could produce cellulases, however, a thermophilic bacterium such as *B. amyloliquefaciens* AK9 exhibits several advantages such as high growth rate in fermentation medium with high temperature

Table 4 Specificity of purified cellulase from *Bacillus amyloliquefaciens* strain AK9 for various substrates

Substrate	Specific activity (U/mg)
CMC	32 ± 1.0
Filter paper	21 ± 2.0
β-Glucan	23 ± 0.5
Xylan	14 ± 0.2
Starch	8 ± 0.6
Avicel	23 ± 0.3

Table 5 Conversion of agro-residues into reducing sugars

No.	Pretreatments	Reducing sugar before enzymatic saccharification	Reducing sugar after enzymatic saccharification
1	Autohydrolysis	1.1%	8.1%
2	Dilute acid pretreatment	1.13%	9.1%

and pH. A thermoacidophile, *Bacillus subtilis* strain BY-3, has recently been reported with maximum CMCase activity of crude cellulase enzyme at 60 °C and pH 5.5 [16]. Rawat and Tewari reported a similar cellulase that could function well at high temperature (60 °C) and low pH (5.0) [17].

An extracellular cellulase enzyme was purified from *B. amyloliquefaciens* AK9 with estimated MW to be about 47 kDa, close to that produced by *Streptomyces* strain C188 42 [18]. It was observed that substrate (CMC) addition to the medium induced its production. Cellulase of different molecular sizes has been purified from various bacterial and actinomycete species [19, 20, 18]. The temperature and pH have a crucial role in activity of enzyme; any change beyond the optimum limit leads to enzyme inactivation [21]. Cellulase from *B. amyloliquefaciens* AK9 was stable at wide temperature and pH ranges for several hours, with maximum activity at 60 °C and pH 5.0. Vijayaraghavan and Vincent reported a cellulase enzyme from *Bacillus* sp. that remained stable at 50 °C and pH 5–7 for 30 min [22]. Rastogi et al. purified a thermostable cellulase from *Bacillus* strain DUSELR13 with maximum stability at 60 °C [23].

The enzyme from *B. amyloliquefaciens* AK9 specifically targeted C-3 and C-6 positions of the glucopyranosyl unit of cellulose as evident by its activity against β-glucan and xylan. However, it also degraded Avicel, CMC, and filter paper that referred to as cellulase rather than exoglucanase or endoglucanase alone. Asha et al. reported a cellulase from thermophilic *Paenibacillus barcinonensis* strain that showed similar pattern of substrate specificity for cellulose [24]. Although majority of the endoglucanases reported from *Bacillus* sp. that act upon CMC only, Ogawa et al. reported an endoglucanase from *Paenibacillus* strains with a carbohydrate binding domain [25]; therefore, it could also degrade CMC as well as crystalline cellulose. Han et al. [26] and Kim [27] reported endoglucanases from *Bacillus.* sp. D04 and *Bacillus circulans* with avicelase activity.

The effect of various additives on the purified cellulase was observed after incubation for several hrs. More than 50% of activity was inhibited in the presence of Hg^{+2}, while partially inhibited by various mono and divalent cations. Vijayaraghavan and Vincent [22] reported that the activity of purified cellulase from *Bacillus* sp. was inhibited by divalent cations indicating that it was a type of metalloenzyme. The activity was affected in the presence of EDTA also, which indicated the essentiality of divalent cations for enzyme activity. The enzyme showed high activity in the presence of non-ionic detergents such as Triton X-100, Tween 80, and Tween 20 as well as ionic detergent such as SDS. Non-ionic surfactants help to minimize the irreversible inactivation of cellulase, thereby modifying its surface property during hydrolysis and, therefore, highly advantageous for application in paper industry [24]. DEPC and β-mercaptoethanol significantly inhibited the enzyme activity that indicates the essentiality of histidine residues and disulfide bonds for its catalytic function [2].

Annamalai et al. reported an alkaline organic solvent stable cellulase from *Bacillus halodurans* CAS 1 strain with enhanced activity in the presence of organic solvents (25%, *v*/*v*) [28]. It is manifested from our study that cellulase of *B. amyloliquefaciens* AK9 is

remarkably stable in the presence of broad range hydrophilic as well as hydrophobic organic solvents used in this study. The cellulase of *B. amyloliquefaciens* AK9 is extraordinarily stable in the presence of organic solvents under this study. It was observed that except benzene, xylene, propanol, and ethanol, the presence of other solvents enhanced the cellulase activity. The thermophilic enzymes along with stability to organic solvents possess an attractive choice for the environmentalists for their application in areas such as bioremediation of organic solvents contaminated industrial wastewaters and carbohydrate-polluted marshes. Interestingly, the organic solvent stimulated the activity of enzyme from *B. amyloliquefaciens* AK9. This behavior might be due to the residues of carried over non-polar hydrophobic solvent providing an interface, thereby keeping the enzyme in an open conformation and thus resulting in the observed activation [29, 30].

The potential of cellulase enzyme was determined by enzymatic hydrolysis of the samples pretreated with autohydrolysis and DAP. It was observed that the autohydrolyzed samples released 1.1% of the reducing sugar before enzymatic hydrolysis; however, after the saccharification of biomass by the cellulase enzyme, these samples exhibited 8.1% reducing sugar. Similarly, 1.13% reducing sugar was obtained from DAP-pretreated sample prior to enzymatic hydrolysis. However, maximum of 9.1% reducing sugar was obtained from enzymatic saccharification (using *B. amyloliquefaciens* AK9 cellulase) of DAP-pretreated agro-residue, which was comparatively better than autohydrolysis treatment, thus proving the hydrolytic potential of the selected cellulase.

Conclusion

A cellulase from *B. amyloliquefaciens* AK9 showed excellent thermostable, acid-tolerant, and surfactant-stable properties. Considering activity and stability in the presence of organic solvents, with respect to increased cellulase activity in the presence of various solvents of greater hydrophobicity, therefore, the enzyme might be useful for practical applications in biotechnological processes.

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