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BIOCATALYSIS

Purification and characterization of an organic-solvent-tolerant cellulase from a halotolerant isolate, *Bacillus* sp. L1

Xin Li · Hui-Ying Yu

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Abstract A halotolerant isolate *Bacillus* sp. L1 producing extracellular cellulase was isolated from Yuncheng, China. Production of the enzyme started from mid-exponential phase of bacterial growth and reached a maximum level during the post-stationary phase. The cellulase was purified to homogeneity with molecular mass of 45 kDa. Substrate specificity test indicated that it was an endoglucanase for soluble cellulose. Optimal enzyme activity was found to be at 60 °C, pH 8.0, and 7.5 % NaCl. Furthermore, it was highly active and stable over broad ranges of temperature (30–80 °C), pH (7.0–9.0), and NaCl concentration (2.5–15 %), thus showing its excellent thermostable, alkali-stable, and halotolerant nature. The cellulase activity was greatly inhibited by ethylenediaminetetraacetic acid, indicating that it was a metalloenzyme. Significant inhibition by phenylmethylsulfonyl fluoride and phenylarsine oxide revealed that serine and cysteine residues were essential for the enzyme catalysis. Moreover, the cellulase was highly active in the presence of surfactants, and it showed high stability in the presence of water-insoluble organic solvents with log P_{ow} at least 0.88. Results from this study indicate that the purified cellulase from isolate L1 may have considerable potential for industrial application owing to its useful properties.

Keywords Purification · Endoglucanase · *Bacillus* sp. L1 · Organic solvent tolerance

X. Li (✉) · H.-Y. Yu
Life Science College, Yuncheng University, Yuncheng 044000, China
e-mail: lixin-eva@163.com

Introduction

Cellulose is the most abundant renewable material in the natural environment and is commonly used as raw material for the production of soluble sugars, biofuels, and other industrially important chemicals [5]. Hence, it has become of considerable economic interest to develop processes for the effective treatment and utilization of cellulosic waste as an inexpensive carbon source. Cellulases provide a key opportunity for exploiting the tremendous benefits of biomass utilization [14]. During cellulose processing, lignocelluloses are usually pretreated with alkalis or acids to release cellulose [9]. The alkalis or acids are then removed with large amounts of water, or neutralized with acid/alkaline solutions, which produces large amounts of salt [28]. Therefore, many enzymes lose their activity rapidly under the high-salt or high osmotic pressure environments. Recently, extremophiles have received considerable interest as a valuable source of novel enzymes [1]. Among the extremophiles, microorganisms that are able to live in saline environments are called halophiles or halotolerant microorganisms. Exoenzymes from these organisms with polymer-degrading ability at low water activity are of interest for potential use in many harsh industrial processes where concentrated salt solutions would otherwise inhibit enzymatic conversions [15].

The ability of enzymes to remain active in the presence of organic solvents has received a great deal of attention over the past two decades. There are numerous advantages of using enzymes in organic solvents or aqueous solutions containing organic solvents rather than in water alone, such as increased solubility of nonpolar substrates and elimination of microbial contamination in the reaction mixture [19]. Therefore, enzymes that remain stable in the presence of organic solvents might be very useful for

biotechnological applications in which such solvents are used [4, 7]. Since salt reduces water activity, a feature in common with organic solvent systems, enzymes from halophiles or halotolerant microorganisms are thought to be valuable biocatalysts in the low-water-activity environments [16]. Investigation into the behavior of the enzymes in the presence of organic solvents may lead to new enzymes with organic-solvent-tolerant potential. So far, only a few natural cellulases which are organic solvent stable have been reported [24, 25]. However, to the best of our knowledge, there are no reports on organic-solvent-tolerant cellulases from halotolerant species. In this paper, we report the purification and characterization of a novel cellulase from *Bacillus* sp. L1, including the organic solvent tolerance of this enzyme.

Materials and methods

Bacterial isolation, identification, and cellulase production

The strain L1 was isolated from saline soil from Yuncheng, China, and cultivated aerobically at 35 °C in complex medium (CM) with the following composition (g/l): casein peptone, 7.5; yeast extract, 10.0; sodium carboxymethyl cellulose (CMC-Na), 5.0; sodium citrate, 3.0; $MgSO_4 \cdot 7H_2O$, 20.0; KCl, 2.0; $FeSO_4 \cdot 7H_2O$, 0.01; NaCl, 50.0; and pH 7.5. Extracellular cellulase activity of the isolate was screened using Lugol's iodine solution as described by Kasana et al. [8].

The strain was identified on the basis of typical cultural, morphological, and biochemical characteristics, and 16S rRNA gene sequencing. The 16S rRNA gene sequence was submitted to GenBank with the accession number JF796144.

The kinetics of bacterial growth and extracellular cellulase production were determined at different time intervals. The isolate L1 was inoculated in CM broth and incubated with shaking at 35 °C. Samples were withdrawn aseptically every 2 h and bacterial growth, along with enzyme activity, was measured by a spectrophotometric method (Shimadzu model UV-160A).

Purification of cellulase

Cell-free supernatant was harvested by centrifugation at 12,000*g* for 15 min at 4 °C and used for enzyme purification. Ammonium sulfate was added to the supernatant up to 75–80 % concentration with continuous overnight stirring. The precipitate collected by centrifugation (12,000*g* for 15 min) was dissolved in a minimum volume of buffer A (20 mM Tris–HCl containing 7.5 % NaCl, pH 8.0) and dialyzed against the same buffer overnight. The concentrated sample was loaded on a Q-Sepharose HP column (1.6 cm × 14 cm) pre-equilibrated with buffer A. Bound proteins were eluted by applying a linear gradient of 0.1–0.5 M NaCl in Tris–HCl buffer (20 mM, pH 8.0). Fractions exhibiting cellulase activity were pooled and concentrated by freeze-drying. The resulting concentrate was dissolved in buffer A and then loaded on a Sephadex G-100 column (2.0 cm × 75 cm). The sample was eluted with the same buffer at a flow rate of 0.5 ml/min. Active fractions were pooled, concentrated by freeze-drying, and used for further biochemical characterization.

SDS-PAGE and zymogram analysis

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) was performed with 12 % polyacrylamide gel by the method of Matsuoka et al. [17]. After electrophoresis, the gel was divided into two pieces; one was stained with 0.05 % Coomassie brilliant blue R-250. For activity staining, the other piece was incubated in 20 mM Tris–HCl buffer (pH 8.0, containing 5 % isopropanol) for 24 h. After several washings with 20 mM Tris–HCl buffer (pH 8.0), the slab gel was laid on an agar sheet containing 0.1 % CMC and 7.5 % NaCl and left for 2 h at 60 °C and pH 8.0. The cellulase band was visualized by staining the agarose gel with 0.5 % (w/v) Congo red.

Cellulase activity assay

Cellulase activity was evaluated by measuring the reducing sugars using CMC-Na as the substrate. Reaction mixtures contained 0.5 ml of CMC-Na (5.0 g/l) in 20 mM Tris–HCl buffer (pH 8.0) and 0.1 ml of enzyme solution. The reaction mixture was incubated in a water bath at 60 °C for 20 min. The amount of reducing sugar that was liberated in the reaction mixture was measured using the 3,5-dinitrosalicylate acid (DNS) method [18]. The absorbance of the reference sample (substrate solution incubated without enzyme) was subtracted from those of the test samples. One unit (U) of cellulase activity was defined as the amount of enzyme capable of releasing 1 μmol of glucose per minute under the assay conditions. The specific activity was expressed as units per milligram of proteins. Protein concentration was determined by the Bradford method [2], using bovine serum albumin as standard.

Effects of temperature, pH and NaCl on the cellulase activity and stability

The effect of pH on cellulase activity was studied over a pH range of 4.0–11.0. The pH stability of the enzyme was determined by incubation with different buffer systems at 30 °C for 24 h. The following buffer systems (0.1 M) were

used: sodium phosphate buffer, pH 4.0–7.0; glycine-NaOH buffer, pH 8.0–11.0.

To investigate the effect of temperature, the assay was conducted under different temperatures from 20 to 90 °C. The thermostability of the cellulase was determined by preincubating the enzyme at different temperatures for 24 h, and residual activity was measured using the standard assay.

The activity of the purified cellulase was measured in enzyme reactions containing 0–20 % NaCl. Salt stability of the enzyme was determined by incubating the enzyme with different concentrations of NaCl at 30 °C for 24 h, and residual activity was determined under the standard assay conditions.

Effects of organic solvents on cellulase activity and stability

The effect of organic solvents with different log P_{ow} values at 15 % (v/v) concentration on the purified cellulase was determined by incubating the enzyme solution in different organic solvents at 25 °C with constant shaking at 150 rpm. After incubation for appropriate periods of time, aliquots were withdrawn and remaining activity was measured under enzyme assay conditions. If residual activity was more than 50 % after 10 days, the half-life was taken to be more than 10 days; whereas, if the activity was less than 50 % after 1 day, the half-life was taken to be less than 1 day.

Effects of metal ions and chemical reagents

Effects of different metal ions and chemical reagents [ethylenediaminetetraacetic acid (EDTA), phenylmethylsulfonyl fluoride (PMSF), phenylarsine oxide (PAO), diethyl pyrocarbonate (DEPC), β-mercaptoethanol, SDS, Sarcosyl, Triton X-100, Tween 20, and Tween 80] on the cellulase activity were examined after the enzyme had been preincubated with them at 30 °C for 24 h, individually. Residual activity was determined under optimal enzyme assay conditions. Activity assayed in the absence of any additives was taken as 100 %.

Substrate specificity

The substrate specificity of the purified cellulase was determined by performing the assay with different substrates: Avicel (microcrystalline cellulose), carboxymethyl cellulose (CMC), cellobiose, *p*-nitrophenyl-β-D-glucopyranoside (PNPG), and CMC-Na. To determine the cellulase activity towards Avicel, CMC, and CMC-Na, the reaction mixture consisted of 20 mM Tris–HCl buffer (pH 8.0) containing 0.5 % (w/v) of each substrate and 0.1 ml enzyme solution (2.0 μg/ml). After incubation at 60 °C for 30 min, the amount of reducing sugar released was measured by the DNS method as described above. For the substrate PNPG, the cellulase activity was determined by hydrolysis of PNPG resulting in the release of *p*-nitrophenol, a pigment substance which can be measured spectrophotometrically at 400 nm. High-performance liquid chromatography (HPLC) was used to determine the enzyme activity towards cellobiose. The hydrolyzed product was detected using a refractive index detector. Glucose (Sigma) was used as the standard.

Results

Strain identification and production of extracellular cellulase

On the basis of the morphological, physiological, and biochemical characteristics, the isolate L1 is a Gram-positive, motile, spore-forming, rod-shaped, aerobic bacterium. Colonies are circular, slightly irregular, and light yellow on CM agar plate. It was able to grow in medium containing 0–20 % (w/v) NaCl and grew optimally at 5 % (w/v) NaCl. Thus this bacterium is considered to be a moderately halotolerant microorganism [10]. Optimal bacterial growth was observed at 35–37 °C and pH 7.0. H_2S production, nitrate reduction, methyl red, catalase, indole production, and gelatin hydrolysis were positive, whereas Voges–Proskauer test, oxidase, and Tween 20 hydrolysis were negative. Acid is produced from glucose, maltose, and inositol. Phylogenetic analysis based on 16S rRNA gene sequence comparisons revealed that the isolate L1 belonged to *Bacillus* species and was most closely related to *Bacillus sonorensis* NRRL B-23154T (98.7 % 16S rRNA gene sequence similarity) (Fig. 1).

As shown in Fig. 2, the cellulase was produced from the mid-exponential phase of bacterial growth (12 h), and reached a maximum level during the post-stationary phase (48 h). No cellulase activity was detected during the early-exponential growth phase.

Cellulase purification

The results of the cellulase purification are summarized in Table 1. The enzyme was well purified by ammonium sulfate precipitation, Q-Sepharose ion exchange, and Sephadex G-100 gel filtration chromatography. It was purified 5.5-fold with recovery of 17.1 % and specific activity of 75.1 units/mg protein. The purified enzyme showed a single protein band on SDS-PAGE with an estimated molecular mass of 45 kDa (Fig. 3, lane 2). Also, zymographic activity staining revealed the activity band for the cellulase at the corresponding position on SDS-PAGE (Fig. 3, lane 3).

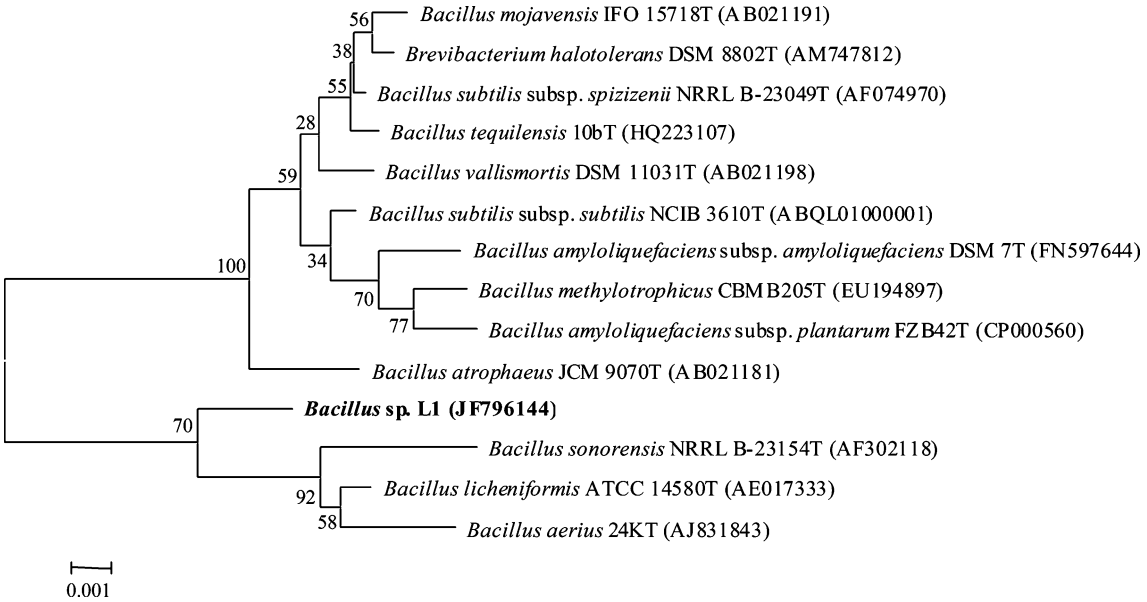

Fig. 1 Neighbor-joining phylogenetic tree showing the position of isolate L1 relative to other members of the genus *Bacillus*. Accession numbers of the sequences used in this study are shown in *parentheses* after the strain designation. *Numbers* at nodes are percentage bootstrap values based on 1,000 replications. *Bar* 0.001 substitutions per nucleotide position

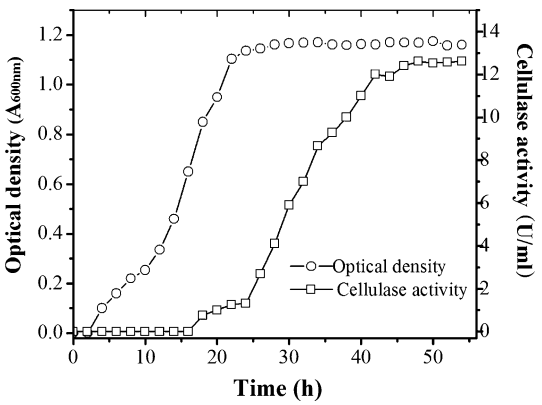

Fig. 2 Kinetics of bacterial growth and extracellular cellulase production of stain L1 in CM broth containing 5 % (w/v) NaCl at 35 °C

Properties of the purified cellulase

The cellulase had higher activity toward soluble cellulosic substrate (CMC-Na) than insoluble cellulosic substrates (Avicel and CMC) (Table 2). In addition, it cannot hydrolyze PNPG or cellobiose to produce glucose. These results suggest that the enzyme has no exoglucanase, β-glucosidase, and transglycosidase activity. Thus, it was a true endoglucanase for soluble cellulose.

The enzyme showed high activity over a wide temperature range (30–80 °C), pH range (7.0–9.0), and NaCl concentrations (2.5–15.0 %), with an optimum at 60 °C, pH 9.0, and 7.5 % NaCl (Fig. 4). The thermal stability profile indicated that it was highly stable at temperatures below 80 °C after 24 h incubation, after which it retained more than 70 % activity. However, after incubation at 90 °C for 24 h, its activity disappeared (Fig. 4a). Also, the cellulase showed good pH stability in the pH range 7.0–9.0, but only 20 % activity was retained at pH 5.0 and 11.0 (Fig. 4b). Furthermore, high halotolerant stability of the enzyme was observed, e.g., it retained more than 80 % activity over a broad range of NaCl concentrations (2.5–20 %) (Fig. 4c).

As shown in Table 3, the cellulase activity was greatly enhanced in the presence of Mg^{2+} (131.5 %), but was inhibited by Hg^{2+} with 66.9 % activity lost. Other metal ions tested had no significant effect. Among the specific enzyme inhibitors, the chelating agent EDTA inhibited the enzyme activity by 90.9 %. Meanwhile, significant inhibition of the cellulase was shown by PMSF and PAO, whereas β-mercaptoethanol and DEPC had only weak effects.

Cellulase activity was also studied in the presence of some surfactants. The enzyme retained more than 91 % activity toward Sarcosyl, Triton X-100, Tween 20, and Tween 80. However, it lost about 41.1 % activity in the presence of SDS (Table 3).

Effects of organic solvents on the cellulase activity and stability

As shown in Table 4, no complete inactivation of the cellulase was observed in the presence of the organic solvents tested. More than 90 % activity was retained after

Table 1 Purification steps for the cellulase isolated from *Bacillus* sp. L1

Purification steps	Total activity (units)	Total protein (mg)	Specific activity (units/mg)	Purification (fold)	Yield (%)
Culture supernatant	920.9	67.8	13.6	1	100
$(NH_4)_2SO_4$ precipitation	637.6	25.1	25.4	1.9	69.2
Q-Sepharose	312.4	8.3	37.6	2.8	33.9
Sephadex G-100	157.8	2.1	75.1	5.5	17.1

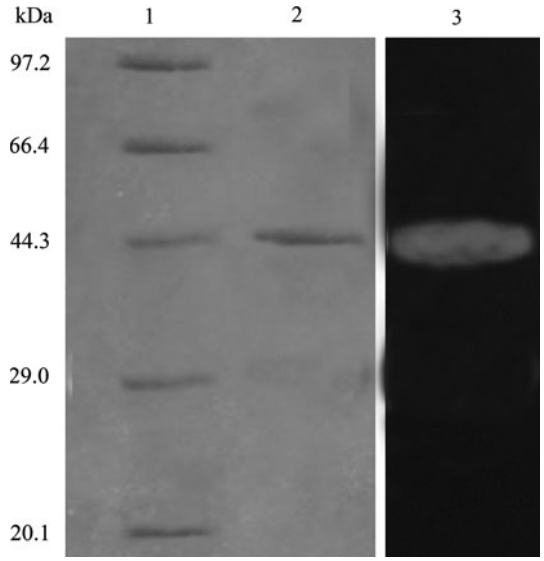

Fig. 3 SDS-PAGE and zymogram analysis of the purified cellulase. *Lane 1* molecular mass markers; *lane 2* cellulase in 12 % SDS-PAGE; *lane 3* zymogram analysis of cellulase activity

Table 2 Specific activity of the purified cellulase toward different substrates at pH 8.0 and 60 °C

Substrates	Specific activity (U/mg protein)
CMC-Na salt	37.5 ± 0.2
CMC	5.9 ± 0.1
Avicel	0
Cellobiose	0
PNPG	0

incubation with dimethyl sulfoxide (DMSO), *N*,*N*-dimethylformamide (DMF), and acetone. Interestingly, DMF and acetone even enhanced the enzyme activity to 118.4 and 127.3 %, respectively. The half-lives of the enzyme were drastically decreased in the presence of organic solvents with log P_{ow} less than 0.88, but in the presence of organic solvents with higher log P_{ow}, the half-lives were much longer (more than 10 days) than in the absence of the solvents (4 days).

Discussion

Being intrinsically stable and active at high salt concentrations, enzymes from halophiles or halotolerant microorganisms have important potential biotechnological applications. Previously, we reported some moderately halophilic bacteria with hydrolytic activities, which were isolated from Yuncheng Salt Lake [11, 12]. In this investigation, an organic-solvent-tolerant cellulase from a halotolerant isolate *Bacillus* sp. L1 was studied.

Substrate specificity tests showed that the cellulase had endoglucanase activity. Similar findings were reported for other cellulases from *Bacillus* sp. BG-CS10 [28] and yeast *Cryptococcus* sp. S-2 [23]. Production of the enzyme started in the exponential growth phase and reached a maximum level during the stationary phase (Fig. 2). Furthermore, inductive production of the cellulase was shown by CMC-Na and there was no enzyme production in the absence of CMC-Na. This phenomenon was previously reported for other hydrolyses-producing halophiles [20, 21]. SDS-PAGE analysis revealed that the molecular mass of the cellulase was 45 kDa. This finding is consistent with those of monomeric cellulases (25–67 kDa) reported from *Bacillus* species [6, 13, 22].

The enzyme showed optimal activity at 60 °C. Excellent thermostability was observed under high temperatures (70–80 °C). Meanwhile, it was highly active and stable over broad concentrations of NaCl. About 40 % activity remained in the absence of NaCl, showing the halotolerant nature of the cellulase. This type of extreme halotolerance has also been observed in other extracellular enzymes from halophiles, such as *Halomonas meridiana* [3] and *Chromohalobacter* sp. TVSP 101 [20]. The optimal pH for cellulase activity and stability ranged from 7.0 to 9.0, which clearly indicated the alkali-stable nature of the purified enzyme. Several researchers all over the world are now trying to exploit microbes for the isolation of alkaline enzymes because of their tremendous industrial potential [15]. Therefore, the cellulase with these characteristics may have interesting applications in biotechnological processes which are carried out under harsh conditions, such as high temperature, pH, and salinity.

Organic-solvent-tolerant halophilic enzymes appear to be quite attractive for industrial applications such as

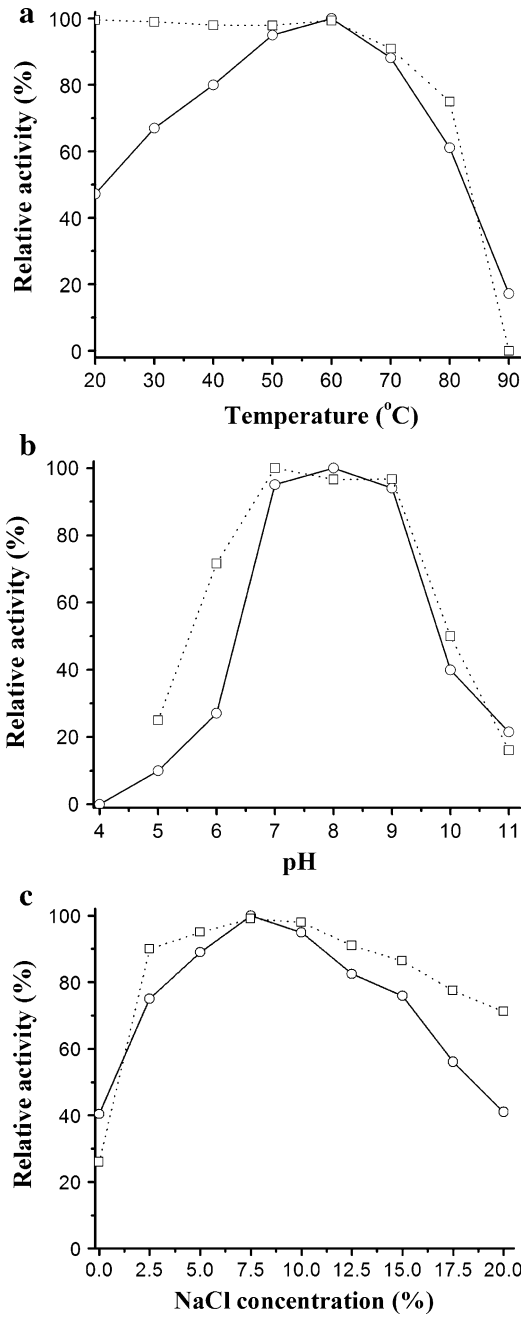

Fig. 4 Effect of temperature (**a**), pH (**b**), and NaCl concentration (**c**) on activity (*solid lines*) and stability (*dotted lines*) of the purified cellulase. Relative activity was defined as the percentage of activity detected with respect to the maximum enzyme activity. For determining the stability, the activity of the enzyme without any treatment was taken as 100 %

bioremediation of carbohydrate-polluted salt marshes and industrial wastewaters contaminated with organic solvents [21]. However, reports about halophilic/halotolerant enzymes with organic solvent tolerance are scarce. Thus, the behavior of the cellulase from strain L1 in the presence of organic solvents was studied. As shown in Table 2, the

Table 3 Effects of different metal ions and chemical reagents on the cellulase from *Bacillus* sp. L1

Substances	Concentration (mM)	Residual activity[a]
Control	–	100
Ca^{2+}	5	98.9 ± 1.1
Zn^{2+}	5	92.4 ± 1.2
Fe^{2+}	5	98.3 ± 2.2
Fe^{3+}	5	94.5 ± 1.5
Cu^{2+}	5	91.6 ± 1.1
Mn^{2+}	5	92.4 ± 1.2
Hg^{2+}	5	33.1 ± 0.8
Mg^{2+}	5	131.5 ± 1.4
EDTA	10	9.1 ± 0.2
PMSF	10	13.1 ± 0.9
DEPC	10	97.6 ± 1.3
PAO	10	21.9 ± 0.5
β-Mercaptoethanol	10	97.1 ± 1.5
SDS	10	58.9 ± 1.2
Sarcosyl	10	95.1 ± 1.1
Triton X-100	10	91.2 ± 1.3
Tween 20	10	91.5 ± 1.5
Tween 80	10	91.3 ± 1.3

[a] Residual activity was determined as described in "Materials and methods" and expressed as the percentage of the control value (without any additives). The average relative value ($N = 3$) and standard deviation (SD) are shown

enzyme showed high activity, and obvious stimulation by some organic solvents (DMF and acetone) was observed. Such behavior might be due to the residues of carried-over nonpolar hydrophobic solvent providing an interface, thereby keeping the enzyme in an open conformation and thus resulting in the observed activation [27]. Furthermore, half-lives of the cellulase were significantly decreased in the presence of organic solvents with log P_{ow} less than 0.88, but in the presence of organic solvents with higher log P_{ow}, their half-lives were much longer than in the absence of the solvents. These results indicated that the cellulase stability might be dependent on the polarity of the solvent and increased only in the presence of water-insoluble solvents with higher log P_{ow} values. Halophilic α-amylase from *Nesterenkonia* sp. strain F also showed similar behavior [21].

The cellulase activity was greatly enhanced by Mg^{2+}, but inhibited by EDTA, indicating that it was a metalloenzyme. Complete inhibition by PMSF (a serine modifier) and PAO (a cysteine modifier) revealed that serine and cysteine residues played an important role in its catalytic function. Similar structural characteristics of the enzyme active site have not been reported in other cellulases from halophiles [26, 28]. Furthermore, the enzyme was highly

Table 4 Activity and stability of the cellulase in different organic solvents

Organic solvents	Log P_{ow}^{a}	Residual activity (%)
Control[b]	–	100 (5)[c]
DMSO	−1.35	91.5 (3)
DMF	−1.0	118.4 (4)
Methanol	−0.76	68.6 (1)
Acetonitrile	−0.34	65.7 (3)
Ethanol	−0.3	67.4 (1)
Acetone	−0.24	127.3 (2)
1-Butanol	0.88	86.1 (4)
Chloroform	1.97	71.7 (6)
Benzene	2.13	77.9 (8)
Toluene	2.73	78.1 (>10)
Cyclohexane	3.3	71.1 (>10)
n-Hexane	3.5	63.6 (>10)
1-Decanol	4.1	51.2 (>10)
Isooctane	4.7	49.1 (>10)

The purified cellulase was incubated with various solvents (15 %, v/v) at 25 °C with shaking, and residual activity was determined under the standard assay conditions

[a] The log $P_{o/w}$ is the logarithm of the partition coefficient, *P*, of the solvent between *n*-octanol and water and is used as a quantitative measure of the solvent polarity

[b] The activities of the purified cellulase in the absence of organic solvents were taken as controls

[c] The numbers in brackets are the half-lives (in days) of the enzyme in different organic solvents

active in the presence of the surfactants tested, and may be useful in surfactant industries.

In this paper, the cellulase from *Bacillus* sp. L1 showed excellent thermostable, alkali-stable, halotolerant, and surfactant-stable properties. Also, in light of its high activity and stability in the presence of organic solvents, it could be potentially useful for practical applications in biotechnological processes with nonconventional media.

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