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Halostable cellulase with organic solvent tolerance from *Haloarcula* sp. LLSG7 and its application in bioethanol fermentation using agricultural wastes

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Abstract A haloarchaeal strain LLSG7 with cellulolytic activity was isolated from the saline soil of Yuncheng Salt Lake, China. Biochemical and physiological characterization along with 16S rRNA gene sequence analysis placed the isolate in the genus *Haloarcula*. Cellulase production was strongly influenced by the salinity of the culture medium with the maximum obtained in the presence of 25 % NaCl. Substrate specificity tests showed that the crude cellulase was a multicomponent enzyme system, and zymogram analysis revealed that five different endoglucanases were secreted by strain LLSG7. Optimal cellulase activity was at 50 °C, pH 8.0, and 20 % NaCl. In addition, it was highly active and stable over broad ranges of temperature (40–80 °C), pH (7.0–11.0), and NaCl concentration (17.5–30 %). The cellulase displayed remarkable stability in the presence of non-polar organic solvents with log $P_{ow} \geq 1.97$. The crude cellulase secreted by strain LLSG7 was further applied to hydrolyze alkali-pretreated rice straw and the enzymatic hydrolysate was used as the substrate for bioethanol fermentation by *Saccharomyces cerevisiae*. The yield of ethanol was 0.177 g per gram of pretreated rice straw, suggesting that it might be potentially useful for bioethanol production.

Keywords *Haloarcula* · Cellulase · Organic solvent tolerance · Halostable · Bioethanol

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Introduction

Cellulose as the major component of plant tissues is the most abundant renewable material in the natural environment. Without appropriate treatment, a mass of cellulosic wastes may lead to the risk of environmental pollution [12]. Since cellulose is a linear polymer of glucose monomers linked by β-1,4-glucosidic bonds, it is commonly used as a raw material for the production of soluble sugars, bioethanol, and other important industrial chemicals [36]. Various processes have been developed for cellulose degradation, and the use of cellulase is very effective for achieving tremendous benefits of biomass utilization. Interest in cellulases has grown considerably during recent years, largely because they can hydrolyze cellulosic materials to sugars, which can in turn be fermented to some commodities, such as bioethanol and other bio-based products.

The concerns about the diminishing of fossil fuels have resulted in an increasing focus on production of bioethanol from lignocellulosics, because the bioethanol is a renewable alternative to diminishing petroleum-based liquid fuels [31]. Therefore, screening of powerful cellulase-producing microorganisms and using them in the bioethanol production process are always the focus in bioenergy research. Most of the cellulases utilized in biotechnology are mainly derived from mesophilic bacteria and fungi [12, 16, 17], and some of them have found potential utilization in the bioethanol production process [21, 32]. However, research on cellulase has addressed enzymes from halophiles only to a very limited extent.

The haloarchaea are characterized as extremophiles that can grow in the presence of NaCl concentration ranging from around 8 % to approximately 36 % [20]. Because of their specific living conditions, enzymes from haloarchaea

have evolved to function optimally under extreme conditions such as high temperatures, salinities, and pH or in the presence of organic solvents, making them robust biocatalysts with potential applications in harsh industrial processes [20]. Although cellulose-degrading enzymes from halophilic or halotolerant bacteria are well studied [17, 18, 33], there is only limited information concerning haloarchaea as cellulase producers [27]. Since salt reduces water activity, a feature in common with organic solvent systems, enzymes from haloarchaea may possess organic solvent tolerance [23]. Organic-solvent-tolerant enzymes have received a great deal of attention because of several advantages that apply to biocatalysis in organic media, such as high solubility of hydrophobic species and reduced microbial contamination [5]. To date, studies on organic solvent tolerance of haloarchaeal enzymes are limited to proteases from *Halobacterium salinarum* [11] and *Natrialba magadii* [28], and α-amylase from *Haloarcula* sp. strain S-1 [7]. However, no reports have been published about organic-solvent-tolerant cellulases from haloarchaea.

In this paper, an efficient cellulase-production haloarchaeal strain LLSG7 was isolated and identified. The characteristics of its crude cellulase, especially the stability in the presence of organic solvents, were reported. Moreover, evaluation of biomass saccharification was performed with pretreated rice straw, and potential for ethanol production was also evaluated by fermenting rice straw hydrolysates with *Saccharomyces cerevisiae*.

Materials and methods

Strain isolation and identification

The soil sample was obtained from the salt lake of Yuncheng, China. Aliquots of the soil (5 g) were added to 100 ml of complex medium (CM) containing (g/l) casein peptone, 7.5; yeast extract, 10; sodium citrate, 3; $MgSO_4 \cdot 7H_2O$, 20; KCl, 2; $FeSO_4 \cdot 7H_2O$, 0.01; NaCl, 250; and pH 7.0. Incubation was carried out at 37 °C with constant shaking under aerobic conditions. After 2 days of incubation, the enrichment cultures were spread on CM agar plates (2 % agar, w/v). Pure cultures were isolated and screened for extracellular hydrolytic activity, and the isolate LLSG7 was chosen for further study because it appeared to be the best producer of extracellular cellulase.

The strain was identified on the basis of morphological and biochemical characteristics, along with 16S rRNA gene sequencing, according to Yang et al. [34]. The strain is deposited in the China Center of Industrial Culture Collection, China. The 16S rRNA gene sequence of strain LLSG7 has been deposited in GenBank with the accession number JF796144.

Extracellular cellulase production and effect of different salts

Production of extracellular cellulase was carried out in the CM medium containing 1 % (w/v) sodium carboxymethyl cellulose (CMC-Na salt) at 37 °C with shaking at 180 rpm. The preculture was prepared by inoculating 0.5 ml of the strain suspension containing 1.0×10^8 cfu/ml in 20 ml of the culture medium and incubated at 37 °C on a rotary shaker at 180 rpm for 72 h. One milliliter of the preculture was inoculated in a 500-ml conical flask containing 150 ml of the culture medium. The culture was incubated under the same conditions as the preculture medium. Samples were withdrawn aseptically every 4 h. Strain growth was determined at 600 nm using a UV-160A spectrophotometer (Shimadzu), while cellulase activity were measured by the 3,5-dinitrosalicylic acid (DNS) method [24].

The effect of salts on cellulase production was tested using CM broth with different concentrations of KCl, NaCl, $NaNO_3$, Na_2SO_4, and sodium citrate, respectively. Sodium chloride in the medium was replaced by the aforementioned salts. After incubation at 37 °C for 120 h, the culture broths were centrifuged at 10,000*g* for 15 min and cell-free supernatant was assayed for cellulase activity under the optimal conditions (50 °C, pH 8.0, and 20 % NaCl).

Crude cellulase preparation

The strain LLSG7 was incubated in a 500-ml Erlenmeyer flask containing 150 ml CM broth at 37 °C with shaking at 150 rpm for 120 h. Culture broth was centrifuged at 10,000*g* for 15 min, and then the cell-free supernatant was used as the crude enzyme sample. Concentration of the enzymes was done by precipitation with 70 % saturated ammonium sulfate with continuous overnight stirring. The precipitates obtained were recovered by centrifugation at 10,000*g* for 12 min at 4 °C and were resuspended in Tris–HCl buffer (20 mM, pH 8.0) containing 20 % NaCl. These preparations were stored at 4 °C and used for further characterization.

SDS-PAGE and zymogram analysis

SDS-PAGE was performed in a 10 % (w/v) polyacrylamide gel in accordance with the method described by Laemmli [15] with some modifications. The crude enzyme samples mixed with the same volume of loading buffer were boiled at 100 °C for 5 min and subjected to SDS-PAGE stained with Coomassie Brilliant Blue R-250. The molecular masses of the crude cellulase were estimated using a molecular mass marker (D532S, Takara). Zymogram analysis was performed by using 0.1 % CMC-Na (w/v)

incorporated into the polyacrylamide as described by Liu et al. [21] with some modifications, and enzyme samples were denatured with 3 % SDS in Tris–HCl buffer (50 mM, pH 8.0). The CMC-Na was premixed with polyacrylamide during gel preparation. Following SDS-PAGE, the gel was washed four times at 4 °C for 30 min in renaturation buffer (50 mM KH_2PO_4/NaOH, pH 8.0). The first two washes contained 25 % (v/v) 2-propanol, and then the gel was left for 1 h at 50 °C and pH 8.0. The cellulase band was visualized on the agarose gel after staining with 0.5 % (w/v) Congo Red.

Enzyme activity assay

Reaction mixtures contained 0.5 ml of CMC-Na (5.0 g/l) in Tris–HCl buffer (20 mM, pH 8.0) and 0.1 ml of crude enzyme solution, and these were incubated at 50 °C for 30 min. The amount of reducing sugar released from the enzymatic reaction was measured using the DNS method [24] with glucose as the standard. The absorbance of the reference sample (substrate solution incubated with the thermal inactivated enzyme solution) was subtracted from those of the test samples. One unit of cellulase activity was defined as the amount of enzyme capable of releasing 1 μmol of glucose per minute under the assay conditions. Bradford's method was used to determine protein content [2].

Effects of temperature, pH, and NaCl on the activity and stability of the crude enzyme

The effect of temperature on cellulase activity was examined in 20 mM Tris–HCl buffer (pH 8.0) containing 20 % NaCl under different temperatures from 20 to 90 °C. To evaluate the stability, the crude enzyme solution (1 ml) was incubated at various temperatures for 72 h, and residual activity was determined as described above. The effect of pH on crude cellulase activity was studied over a pH range of 5.0–11.0 at 50 °C and 20 % NaCl. The pH stability test was performed by preincubating the crude enzyme in different buffer systems at 50 °C for 72 h, and then residual activity was determined under optimal assay conditions (50 °C, pH 8.0 and 20 % NaCl). Three buffer systems (20 mM) were used: sodium phosphate (pH 5.0–7.0); Tris–HCl (pH 8.0–9.0); glycine-NaOH (pH 9.5–12.0). The reaction salinity that maximized crude enzyme activity was measured in Tris–HCl buffer (20 mM, pH 8.0) that contained various concentrations of NaCl (0–30 %, w/v) at 50 °C. Salt stability was determined by incubating the crude enzyme with different NaCl concentrations at 50 °C for 72 h, and residual activity was determined.

Substrate specificity

To determine the hydrolytic ability against rice straw, wheat straw, corn stover, cotton straw, CM-cellulose (C4146, Sigma), cellobiose (Sigma), *p*-nitrophenyl-β-D-glucopyranoside (pNPG, Sigma), and sodium carboxymethyl cellulose (CMC-Na, Sigma), the reaction mixture consisted of 50 mM Tris–HCl buffer (pH 8.0) containing 1 % (w/v) of each substrate, 20 % (w/v) NaCl, and 0.02 ml of enzyme solution. The reaction mixture was incubated at 50 °C for 30 min to evaluate the substrate specificity of the crude cellulase as described previously [16]. The agriculture wastes, such as rice straw, wheat straw, corn stover, and cotton straw, were firstly dried, cut into small pieces, then ball-milled and passed through a 40-mesh sieve before use. The pretreated substrate was used immediately for enzyme hydrolysis experiments.

Cellulase activity and stability in different organic solvents

The effect of organic solvents with different log P_{ow} values at 25 % (v/v) concentration on the purified cellulase was determined by incubating the enzyme solution in different organic solvents (Tianjin, China) at 30 °C with constant shaking for 10 min, and then the residual activity was determined under optimal enzyme reaction conditions. To determine the cellulase stability in organic solvents, aliquots were withdrawn at different time intervals, and residue activity was measured using the DNS method under optimal assay conditions. If residual activity was more than 50 % after incubation with organic solvents for 5 days, the half-life was taken as ">5 days". If the activity was less than 50 % after 1 h, the half-life was taken as "<1 h".

Biomass saccharification and bioethanol fermentation

The rice straw pretreatment was performed as described by Sukumaran et al. [32]. Briefly, it was firstly milled to reduce the size, and then was pretreated with 0.1 M NaOH for 1 h at 120 °C in an autoclave. After cooling, the samples were washed several times in tap water to neutralize the solution followed by a final rinse in distilled water, after which they were air-dried by spreading on paper. The pretreated rice straw was used immediately for hydrolysis experiments. Enzymatic saccharification of the biomass was done as described previously with some modifications [21]. The reaction mixture contained 2 g of pretreated rice straw (dry weight), 47 ml of Tris–HCl buffer (0.05 M, pH 8.0) containing 20 % NaCl, 1 ml 1 % (w/v) NaN_3, and 2 ml crude enzyme solution (about 100 units/ml). The incubation was done at 50 °C for 56 h with shaking at 180 rpm. Samples (0.5 ml) were taken at regular intervals

and centrifuged at 10,000*g* for 12 min. The supernatants were assayed for content of total reducing sugars.

Ethanol production using the enzymatic hydrolysates of rice straw was evaluated as described by Liu et al. [21]. The hydrolysates were concentrated by evaporation to reduce the water content and make the sugar content 5 %. About 60 ml of the enzymatic hydrolysates were sterilized by ultrafiltration and inoculated with 10 % (v/v) of a 12-h-old seed culture (LB medium) of *S. cerevisiae* (CICC 1001, China Center of Industrial Culture Collection). About 66 ml of the reaction mixture was incubated at 30 °C in a stoppered flask (250 ml) without agitation. Samples were collected at regular intervals and centrifuged at 10,000*g* for 12 min at 4 °C. The supernatant was filtered using 0.22-mm filters and the ethanol content was determined by gas chromatography (7890A GC System, Agilent) using a ZBWax column at 200 °C, FID detector at 180 °C, and nitrogen with a 20-psi pressure carrier.

Results and discussion

Identification of strain LLSG7

The strain LLSG7 is a Gram-negative, strictly aerobic, and motile rod. Colonies are red-pigmented on CM agar plates. The strain is able to grow in media containing 12–30 % (w/v) NaCl with optimum growth at 25 % NaCl. No growth was observed in the absence of NaCl. Thus this strain was considered to be an extremely halophilic archaea [14]. Optimal growth was observed at 37–39 °C and pH 7–8. H_2S production, nitrate reduction, methyl red, catalase, indole production, and gelatin hydrolysis were positive, whereas Voges–Proskauer test, oxidase, Tween 60, starch and casein hydrolysis were negative. Acid was produced from glucose, sucrose, galactose, mannitol and fructose. As shown in Fig. 1, phylogenetic analysis based on 16S rRNA gene sequence comparisons revealed that the strain LLSG7 belonged to the genus of *Haloarcula*, and was closest to *Haloarcula amylolytica* BD-3T (96.82 % 16S rRNA gene sequence similarity).

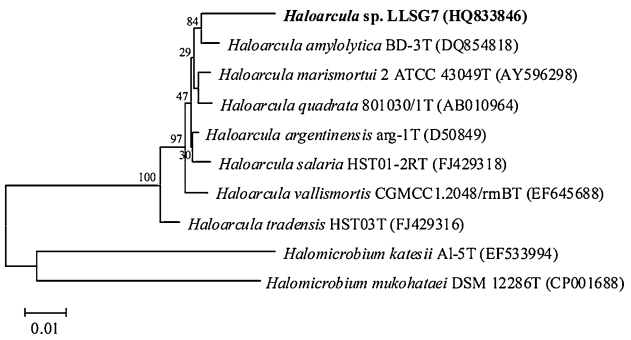

Fig. 1 Phylogenetic tree based on 16S rRNA gene sequences, showing the position of isolate LLSG7 to other members of the genus *Haloarcula*. Accession numbers of the sequences used in this study are shown in parentheses after the strain designation. Numbers at nodes are percentage bootstrap values based on 1,000 replications. *Bar* 0.01 substitutions per nucleotide position

Cellulase production and effect of different salts

The strain LLSG7 showed cellulase activity at the early exponential phase (24 h) and reached a plateau during the stationary phase of growth (100 h). Furthermore, inductive production of the cellulase was shown by CMC-Na and no enzyme activity was detected in the absence of CMC-Na. All these characteristics were similar to other cellulases produced by *Thalassobacillus* sp. LY18 [17] and *Bacillus* sp. L1 [18].

It has been widely reported that the salinity of the culture medium strongly influenced the extracellular enzyme production of halophiles [9, 10]. As shown in Table 1, the effect of different salts on the haloarchaeal growth and cellulase production of strain LLSG7 were evaluated. Optimal cellulase production (about 41.1 units/ml) occurred when 25 % (w/v) NaCl was added. In addition, the strain was able to produce considerable cellulase in the presence of KCl. No growth was found in the medium containing $NaNO_3$, whereas 20 and 25 % of sodium citrate or Na_2SO_4 provided some haloarchaeal growth and enzyme production. Together these results clearly revealed the halophilic nature of *Haloarcula* sp. LLSG7, for which the salt appeared to be a prerequisite for haloarchaeal growth and enzyme production.

Substrate specificity

Cellulose can be biodegraded by three types of cellulases according to their substrate specificities and the mode of hydrolysis, i.e., endoglucanase (EC 3.2.1.4), cellobiohydrolase (EC 3.2.191), and β-glucosidase (EC 3.2.1.21). The efficient degradation of lignocellulosic biomass usually requires the synergistic action of different cellulolytic enzymes [8, 25].

As shown in Table 2, the crude cellulase from *Haloarcula* sp. LLSG7 was active towards all the substrate tested, showing broad substrate specificity. Maximal activity (361.2 units/g protein) was obtained with soluble cellulosic substrate (CMC-Na salt), indicating that the crude enzyme had endoglucanase activity. This behavior was similar to other cellulases from *Thalassobacillus* sp. LY18 [17] and *Bacillus* sp. L1 [18]. For the agricultural wastes, such as rice straw, wheat straw, corn stover, and cotton straw, a high level of cellulase activity was obtained (>200 units/g protein). Moreover, the crude cellulase showed activity

Table 1 Effect of different salts on strain growth and cellulase production by *Haloarcula* sp. LLSG7

Salt	Cell growth (OD_{600nm})	Activity (units/ml)
None	0	0
NaCl (%)		
<12	0	0
12	0.1	0
15	0.5	15.7 ± 0.9
20	1.2	28.2 ± 1.4
25	1.8	41.1 ± 1.1
30	1.6	22.2 ± 1.3
KCl (%)		
<13	0	0
13	0.08	1.2 ± 0.2
15	0.8	9.0 ± 0.3
20	1.5	24.8 ± 1.2
25	1.0	14.4 ± 1.2
30	0	0
Na_2SO_4 (%)		
<16	0	0
16	0.07	0
20	0.4	7.1 ± 0.4
25	0.5	8.9 ± 0.3
30	0	0
$NaNO_3$ (%)		
(5–30)	0	0
Sodium citrate (%)		
5–15	0	0
20	0.4	11.7 ± 0.5
25	0.2	4.8 ± 0.2
30	0	0

Enzyme activity is expressed as the average of three independent experiments ± standard deviation

Table 2 Substrate specificity of the crude cellulase from *Haloarcula* sp. LLSG7

Substrates	Specific activity (units/g protein)
CMC-Na salt	361.2 ± 3.1
CM-cellulose	327.2 ± 1.2
Rice straw	350.1 ± 1.7
Wheat straw	254.1 ± 2.1
Corn stover	225.2 ± 2.2
Cotton straw	231.6 ± 1.9
Cellobiose	116.8 ± 1.3
pNPG	213.2 ± 2.0

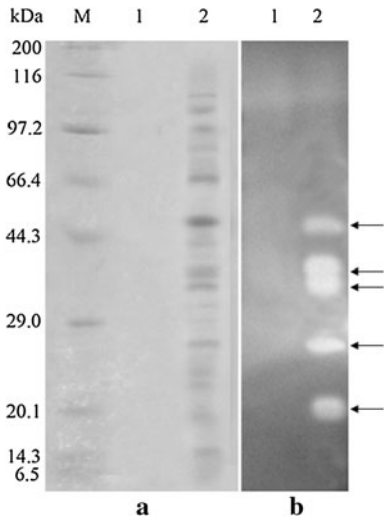

Fig. 2 SDS-PAGE and zymogram analysis of the crude cellulase. **a** *M* molecular mass markers, *lane 1* blank sample, *lane 2* crude cellulase (6 µl); **b** *lane 1* blank sample, *lane 2* zymogram analysis of the crude enzyme (10 µl)

against pNPG and cellobiose, indicating it had exoglucanase and β-glucosidase activities. Together these results indicated that the crude cellulase from *Haloarcula* sp. LLSG7 was a multicomponent enzyme system, which might be due to the presence of cellulosomes, multienzyme complexes which contribute to the efficient degradation of cellulose [1].

SDS-PAGE and zymogram analysis

The components of the crude cellulase produced by *Haloarcula* sp. LLSG7 were detected by SDS-PAGE. About eleven major and seven minor bands were detected on the gel after staining with Coomassie Brilliant Blue R-250 (Fig. 2a, lane 2). Protein bands were examined for the ability to hydrolyze CMC-Na incorporated into the gel. Zymogram analysis indicated five protein bands (52, 38, 35, 27, and 20 kDa) showing activity against CMC-Na (Fig. 2b, lane 2), which revealed that five different endoglucanases were produced by *Haloarcula* sp. LLSG7. These results further confirmed that the crude cellulase was a multicomponent enzyme system. Similarly, Zhou et al. [37] studied the main components of cellulases from a mutant strain of *Trichoderma viride* T 100-14, and zymogram analysis indicated that three different endoglucanases were secreted by the strain. A basidiomycete species strain H2 produced 5, 4, and 3 cellulase-active bands under aerobic, microaerophilic, and anaerobic conditions, respectively [6].

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

As shown in Fig. 3, optimal enzyme activity was found at 50 °C, pH 8.0, and 20 % NaCl. Meanwhile, it was highly active over a broad temperature range (40–80 °C), pH

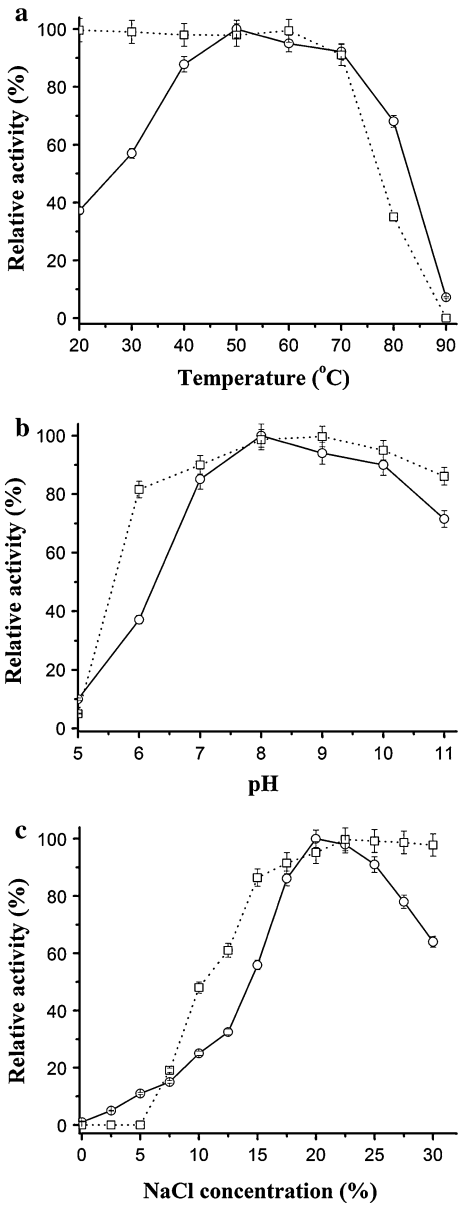

Fig. 3 Effect of temperature (**a**), pH (**b**), and NaCl concentration (**c**) on activity (*solid lines*) and stability (*dotted lines*) of the crude cellulase. Relative activity was defined as the percentage of activity detected with respect to the maximum enzyme activity. For determining the stability, the activity of the crude enzyme without any treatment was taken as 100 %. *Error bars* represent the standard deviation

range (7.0–11.0), and NaCl concentrations (17.5–30 %). The thermal stability profile showed it was stable at temperatures below 70 °C after 72-h incubation, after which it retained more than 90 % activity. A similar range of optimal temperatures has been reported for other cellulases from *Thalassobacillus* sp. LY18 [17] and *Bacillus* sp. L1 [18]. However, after incubation at 90 °C for 72 h, the crude cellulase completely lost activity (Fig. 3a).

Many researchers all over the world are now trying to exploit microbes for the isolation of alkaline enzymes because of their tremendous potential application in industry [22]. The optimal pH for the crude cellulase activity and stability ranged from 7.0 to 11.0, but only 5 % activity was retained at pH 5.0 (Fig. 3b), clearly indicating its alkali-stable nature. As shown in Fig. 3c, more than 80 % activity was retained after incubation with 17.5–30 % NaCl for 72 h, indicating the halostable nature of the cellulase under high salinity. With the exception of recent reports on cellulase activity with extreme halotolerance (20 % NaCl) [18, 33], to the best of our knowledge, no investigation has reported a cellulase with the tolerance for 25 % NaCl or higher salinities. No activity was detected in the absence of NaCl (Fig. 3c), showing the halophilic nature of the crude cellulase.

During cellulose processing, lignocelluloses are usually pretreated with alkalis or acids to release cellulose [13]. The alkalis or acids are then removed with large amounts of water, or neutralized with acid/alkaline solutions, which produces large amounts of salt and neutral pH [13]. Sukumaran et al. [32] reported that alkali treatment of agricultural waste could result in a better yield of reducing sugars from cellulose compared to acid treatment. Thus, the pretreatment of the cellulose might lead to extreme conditions with high salinity or pH. Considering the halostable and alkali-stable properties of the crude cellulase from strain LLSG7, it may find potential application in biotechnological processes for cellulose hydrolysis.

Effect of organic solvents on the cellulase activity and stability

The effect of organic solvents on the activity and stability of the crude cellulase is shown in Table 3. More than 80 % activity was retained after incubation with toluene, benzene, 1-decanol, and *n*-hexane. Significant reduction of the cellulase activity was found in the presence of organic solvents with log $P_{ow} \leq 0.88$, such as DMSO, methanol, ethanol, and acetone, which was considered to be due to the stripping off of the crucial bound-water monolayer from the enzyme molecule essential for its activity [26]. Similar results were obtained from some lipases, which were very tolerant towards non-polar organic solvents with significant instability in polar solvents [4, 19]. Interestingly, benzene and *n*-hexane even enhanced the enzyme activity to 127.9 and 126.6 %, respectively. Such behavior might be due to the residues of carried-over non-polar solvent providing an interface, thereby keeping the enzyme in an open conformation and thus resulting in the observed activation [35, 37].

After incubation with polar organic solvents (log $P_{ow} \leq -0.3$), the cellulase stability was drastically reduced (<1 h). However, in the presence of solvents with

Table 3 Activity and stability of the cellulase in different organic solvents

Organic solvents	Log P_{ow}^{a}	Residual activity (%)
Control[b]	–	100 (3 days)[c]
DMSO	−1.35	41.2 (<1 h)
DMF	−1.0	58.1 (<1 h)
Methanol	−0.76	38.1 (<1 h)
Acetonitrile	−0.34	51.8 (<1 h)
Ethanol	−0.3	35.2 (<1 h)
Acetone	−0.24	47.3 (2 days)
1-Butanol	0.88	56.1 (1 days)
Chloroform	1.97	72.5 (3 days)
Benzene	2.13	127.9 (5 days)
Toluene	2.73	84.1 (>5 days)
Cyclohexane	3.3	75.0 (>5 days)
n-Hexane	3.5	126.1 (>5 days)
1-Decanol	4.1	81.8 (>5 days)
Isooctane	4.7	79.9 (>5 days)

[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 activity of the crude cellulase in the absence of organic solvents was taken as control (100 %)

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

higher log P_{ow} values (≥1.97), its half-lives were much longer (>5 days) (Table 3). The enzyme appeared to be more stable in the presence of non-polar organic solvents. Together these results indicated that, in contrast to the organic solvent stability of some proteases and lipases [30], which had no relationship with the polarity of the organic solvents, the stability of the cellulase was probably dependent on the polarity of the solvents, which increased only in non-polar organic solvents with higher log P_{ow} values. The halophilic cellulases from *Thalassobacillus* sp. LY18 [17] and *Bacillus* sp. L1 [18] also showed similar behavior in organic solvent stability.

Biomass saccharification and bioethanol fermentation

The rice straw was used as feed stock for the production of reducing sugars. Figure 4 shows the yield of reducing sugars from alkali-pretreated rice straw after 56 h of incubation. The content of reducing sugars increased quickly at the beginning stage (20–40 h), and the highest concentration (32.1 g/l) was obtained after 44 h of reaction. The total amount of reducing sugars released from 1 g of dry feed stock is 0.803 g g^{-1} dry substrate and this was a considerable yield. Deswal et al. [3] isolated a brown rot fungus *Fomitopsis* sp. RCK2010 and used the crude enzymes for saccharification of the alkali-pretreated rice straw. The yield of reducing sugar was 0.157 g g^{-1} dry substrate, and a lower yield was obtained when the substrate was treated with acid. Interestingly, the saccharification yield using the crude cellulase from strain LLSG7 was higher than that of some commercial enzymes, such as Celluclast, and Viscostar 150 l (0.565 g g^{-1} dry substrate) [29], and this may be because these commercial enzymes contained various pure enzymes from different strains [3].

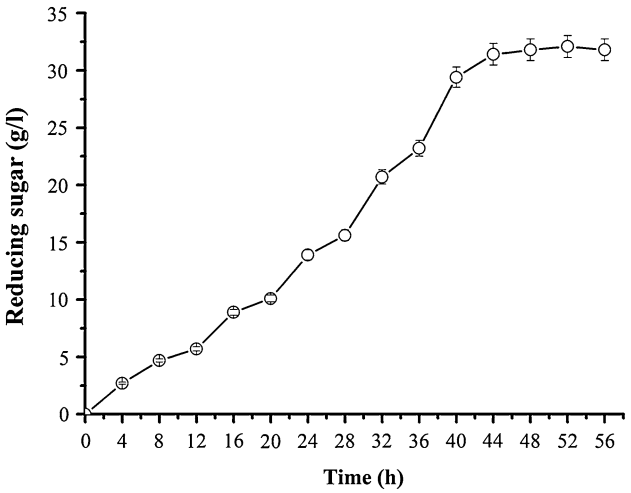

Fig. 4 Reducing sugar production from alkali-pretreated rice straw hydrolysis using the crude cellulase produced by strain LLSG7. Results are the average of three replicates. *Error bars* represent the standard deviation

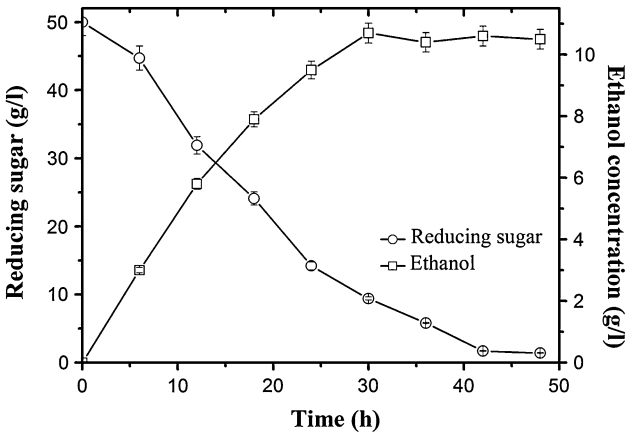

Fig. 5 Ethanol production using *S. cerevisiae* from rice straw hydrolysate (5 % initial reducing sugar concentration). Results are the average of three replicates. *Error bars* represent the standard deviation

The enzyme hydrolysates of rice straw were used for bioethanol fermentation by *S. cerevisiae*. As shown in Fig. 5, production of bioethanol increased along with the fermentation period and maximal yield (10.7 g/l) was obtained at 30 h. No increase in the ethanol production was detected after 30 h; however, the concentration of total

reducing sugars decreased with the incubation time and remained at about 1.4 g/l at the end of incubation. The ethanol yield obtained was about 0.177 g g^{-1} dry substrate, and the efficiency of reducing sugars conversion to ethanol was about 41.9 %. These values were much higher than those reported for other fungal cellulases, which were applied in bioethanol fermentation using *S. cerevisiae* [21, 32].

In the present study, a haloarchaeal strain *Haloarcula* sp. LLSG7 showing cellulolytic activity was isolated and identified. The crude cellulase produced by strain LLSG7 was a multicomponent enzyme system, which had endoglucanase, cellobiohydrolase, and β-glucosidase activities. It showed excellent thermostable, alkali-stable, halostable, and organic-solvent-tolerant properties. The rice straw hydrolysate obtained by application of the crude cellulase was applied for bioethanol fermentation. The yield of bioethanol was 0.185 g g^{-1} dry substrate, suggesting it might be potentially useful for bioethanol production. This study forms the basic trials conducted to test the feasibility of using enzymes produced by haloarchaea for biomass hydrolysis and subsequent bioethanol fermentation. Attempts will be made to increase the sugar content of the hydrolysates so as to obtain a better yield of ethanol.

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