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Full Length Article

Isolation of a Thermostable Alkaline Cellulase-producing Bacterium Strain from a Garbage Dump

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Abstract

Thermostable alkaline cellulases can resist high temperature and high pH, which make them widely applicable in the industries of food, detergent, paper making, and biomass utilization. To obtain favorite thermostable alkaline cellulase-producing bacterial strains, these bacteria were isolated from collected soil and plant putrefaction samples in long-term garbage dumps. One bacterium strain from lime-applied garbage dumps, named as SWU-27, was picked up since its crude enzyme showed stronger activities of cellubiohydrolase, endoglucanase, and β-glucosidase. All these activities could tolerate the treatment of high temperature (up to 80°C) and endoglucanase and β-glucosidase activities could even tolerate high pH (over 10). Taxonomic study showed that strain SWU-27 is another geographic strain of Gram-negative *Klebsiella pneumoniae*. In conclusion it is suggested that lime-applied dumps are preferable places to isolate novel thermostable alkaline cellulase-producing bacterial strains. © 2015 Friends Science Publishers

Keywords: Bacteria; Garbage dump; *Klebsiella pneumonia*; Lime; Thermostable alkaline cellulase

Introduction

Cellulose is the most abundant biomass on the earth for its annual output as high as 10^{11} tons that is produced by the plants' photosynthesis (Shuler, 1980). The biological degradation and conservation of cellulose constitute a significant component of the natural carbon cycle (Sleat *et al*., 1984). Cellulose is known as an important reproducible resource for its vast existence and potential applications. However, due to their complicated and insoluble structures, most of them cannot be directly used and are usually discarded, resulting in a serious environmental pollution.

More and more biotechnologist are focusing on the processes for the conversion of renewable lignocellulosic substrates to fermentable sugars that can be used for the production of ethanol, which happens to be one of the most important biofuel (Singh *et al*., 2008). The effective conversion can help to mitigate energy and food crisis (Lutzen *et al*., 1983). At present, cellulose microbial fermentation takes cellulose as a major source for industrial fuels and chemicals, which in turn reduces the accumulation of cellulosic wastes (Sleat *et al*., 1984). Cellulases produced by microorganisms play an important role in the biodegradation of cellulose. Even though the *Trichoderma* cellulolytic enzyme systems have been employed during most of the enzymatic conversion of cellulose to fermentable sugars, more effective enzyme complex can be produced from other microorganisms (Lutzen *et al*., 1983). Therefore, challenging as it is, seeking an available cellulose-decomposing organism bears significant meanings.

Cellulase is a complex enzyme system consisted of endoglucanase, cellubiohydrolase, and β-glucosidase. Because of their characteristics in tolerance of high temperature and high pH, thermostable alkaline cellulases can be widely used in various industries including food, detergent, paper making, and biomass utilization. Many microorganisms, such as *Trichoderma*, *Aspergillus* and *Clostridium* have been reported as the cellulase producing strains for their thermophilic degradation of cellulose (Gritzali and Brown, 1979; Sissons *et al*., 1987; Zhou *et al*., 2008). However, large-scale cultivation of fungi and actinomycetes are usually costly since they grow slow and coproduce high viscous polymers. Comparatively, bacteria

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are more suitable for large scale production of cellulases.

Thermostable alkaline cellulolytic enzyme-producing bacteria have been isolated from soil (Hakamada *et al*., 1997), and marine sediments (Annamalai *et al*., 2012). However, all these environments are not extremely hot or alkaline, so the chance to find a favorite strain is relatively low. In some places of China, people spray lime to the dump for sterilization, which makes the dump and the soil there very hot and alkaline. The sites of long-term stacking for discarded plants have a lot of putrefaction containing abundant cellulose for the growth of cellulose-utilizing strains. To speculate that some bacteria in these environments can produce thermostable alkaline cellulolytic enzymes, one primary objective these investigations was to explore and identify the strain of bacterium, which produces several kinds of thermostable alkaline cellulolytic enzymes.

Materials and Methods

Isolation of Bacterial Strains

Putrefying soil samples were collected from long-term stacking dumps around Southwest University, China. All these dumps had been applied with lime for sterilization purpose. Cellulose-producing bacterial strains were isolated from these samples following classical method. Briefly, 1 g of the sample was resuspended before settled in 100 mL deionized water, and the upper suspension fluid was serially diluted, 100 µL of each dilution were subcultured on a modified sodium carboxymethycellulose (CMCNa) screening medium consisting of 10 g CMCNa, 1 g tryptone, 4 g $(NH_4)_2SO_4$, 2 g KH_2PO_4, 0.5 g $MgSO_4 \cdot 7H_2O$ in 1000 mL ultrapure water (pH 7.0) (Mandels and Reese, 1957). Cultures were incubated at 37ºC under aerobic conditions until clear colonies appeared on the plate. Then Congo red solution (1 mg/mL) was added to the plate, and the colonies surrounded by transparent zone were regarded as cellulase-producing bacteria and were subsequently purified by repeated streaking onto CMCNa medium for three times. The ability of these colonies to produce β-glucosidase was further examined on a plate based on the esculin screening medium containing 1 g tryptone, 0.5 g yeast extraction, 1 g NaCl, 0.25 g Ammonium iron (III) critrate together with 0.1 g esculin in 100 mL distilled water (Kwon *et al*., 1994), and the activity was showed by a black circle surrounding the colonies.

Cellulolytic Enzyme Assay

CMCNa, Whatman No. 1 filter paper and Esculin, were applied to measure the activities of endoglucanase, exoglucanase, and β-glucosidase, respectively. The organism was incubated (1% v/v) aerobically in 500 mL conical flask containing CMCNa liquid medium with additional 1.5% (g/v) bran at 37°C on a reciprocal shaker. After 4 days of cultivation, cell-free supernatant was collected by centrifugation and was used for the enzyme assay. 3, 5-dinitrosalicylic acid (DNS) method (Miller, 1959) quantified by spectrometric determination of reducing sugars was used to measure the crude cellulase activity following a modified procedure (Trivedi *et al*., 2011). Briefly, after incubation at 35°C for 30 min, the reaction mixture that contained supernatants (1 mL), substrate solution (2 mL) in 2 mL 20 mM Citric acid sodium buffer (pH 7.0), was replenished of 2 mL DNS reagent and heated to 99°C for 10 min in a water bath system. An absorbance at 546nm exhibited the amount of released reducing sugar in the reaction mixture. One unit of enzyme activity was defined as the amount of enzyme liberated 1 µmol reducing sugar. The substrate solutions used were 1% (w/v) CMCNa, 4.0 mg/mL Esculin and 1% (w/v) Whatman No. 1 filter paper.

Taxonomic Study

Biochemical and morphological analysis of the strain were performed following Bergey's Manual (Garrity *et al.*, 2005). Total genomic DNA was extracted according to the methods by Huber *et al*. (Huber *et al*., 2002). Universal primers 8F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1512R (5'-ACGGCTACCTTGTTACGACTT-3') were employed to amplify the 16S rDNA by polymerase chain reaction. The PCR mixture was composed of 2.0 µL of 2.5 mM dNTPs, 2.0 µL of 2.5 mM $MgCl_2$, 2.5 µL of 10×PCR buffer (Mg^{2+} free), 1.0 µL of each primer (10 mM), 0.25 µL of r*Taq* DNA polymerase (2.5 U/µL) (TaKaRa, Dalian), 1.0 µL of template DNA, in a final volume of 25 µL. PCR amplification was carried out as follows: 94°C for 4 min; 35 cycles at 94°C for 20 s, 55°C for 30 s, and 72°C for 80 s; 72°C for 10 min. The 16S rDNA gene was cloned into pMD19-T vector. Nucleotide sequence of 16S rDNA was sequenced and identified through the online BLAST program. Multiple alignment was carried out with the CLUSTALX and a neighbor-joining phylogenetic tree was reconstructed using the MEGA program version 4.0 (Rastogi *et al*., 2009).

Results

Microorganism and Cellulolytic Activity

From the collected samples, more than 100 strains of cellulase-producing bacteria were isolated. One stain, designated as SWU-27, was selected for further study because it exhibited stronger cellulase activity. This strain showed a transparent zone on CMCNa agar plate (diameter of clearance zone: colony/3.78:1) and black hydrolysis circle on Esculin plate (diameter of black hydrolysis circle: colony/2.87:1), indicating its potential activities of CM Case and β-glucosidase (Fig. 1).

To test the cellulolytic activity, SWU-27 strain was cultured in CMCNa medium and the cell-free supernatant

Table 1: Biochemical and physiological tests of strain SWU-27

Test items	Result
Oxygen	facultative anaerobic
Acetyl methyl methanol (V.P.)	+
Gelatin	-
Citrate	+
Nitrate reduction test	+
Oxidase test	-
Lactose	+
Inositol	+
Sucrose	+
Methyl red	-
Phenylalanine deamination test	-
Contact enzymatic reaction	+

+ Positive, - Negative

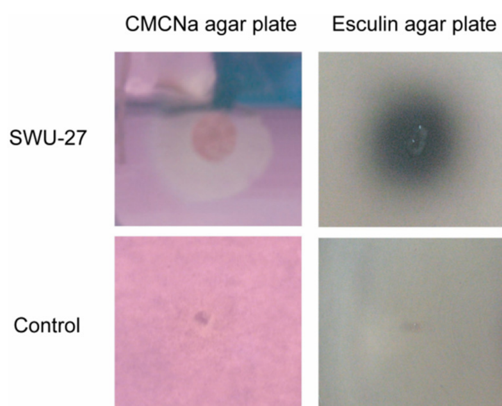

Fig. 1: The screening plate for cellulase-producing strain SWU-27. Strain SWU-27 was inoculated onto CMCNa plate, and was cultured at 37ºC for about 24 h. Congo red was then added to show cellulase activity. A clearance zone after Congo red stain on CMCNa plate indicated a CMCase activity and a black hydrolysis circle around the strain on Esculin plate showed the capacity of hydrolyzing oligosaccharide. *E. coli* strain DH5a was used as a negative control

was collected as crude enzyme for the activity assay. Results showed the activity of endoglucanase, cellubiohydrolase and β-glucosidase were 10.7 U/mL, 15.91 U/mL and 20.1 U/mL, respectively. These activities were stronger than those of *Clostridium* and *Bacillus flexus* (Benoit *et al*., 1992; Trivedi *et al*., 2011). Strain SWU-27 was stored at the State Key Laboratory of Silkworm Genome Biology, Southwest University, Chongqing, China.

Effects of pH and Temperature on the Cellulase Activities of Strain SWU-27

Usually, enzymatic activities are sensitive to pH and temperature changes. To characterize the cellulolytic enzymes of strain SWU-27, the activity profile of the crude enzyme was analyzed. The optimum activities of all the cellulases were around pH 6.0-7.0 (Fig. 2a). Interestingly, the activities of all these three cellulase components were extremely active in the pH range from 5.0 to 10.0. As shown in Fig. 2b, their optimum temperatures were about 40-50°C. Notably, both the endoglucanase and cellubiohydrolase could effectively hydrolyze their substrates in the temperature range from 20 to 90°C. In contrast, most previously reported bacteria did not functionally work under the wide range of temperature and pH (Sleat *et al*., 1984; Tai *et al*., 2004; Taya *et al*., 1988; Trivedi *et al*., 2011).

Stability Analysis of the Cellulolytic Enzyme Activity

Many enzymes are very sensitive to high temperature and high pH. However, for the application in industry, cellulases are usually required to endure high temperature and high pH. To detect the stability of the crude enzyme of strain SWU-27, firstly the crude enzyme was pre-treated under different temperatures or pH conditions for 30 min, and then the residual enzyme activities were determined. Results showed that the endoglucanase and β-glucosidase were very stable under a wide range of pH, and more than 60% activities remained after the treatment under pH 5-12. While the cellubiohydrolase was more sensitive to pH variation, and only 20% activity remained after the treatment under pH value below 5 or over 9 (Fig. 2c). Interestingly, all these enzyme activities showed resistance against high temperature. After the treatment for 30 min, more than 90%, 70%, and 60% activities remained in the β-glucosidase, cellubiohydrolase, and endoglucanase, respectively (Fig. 2d). Compared with those from *Bacillus*, *Geobacillus*, and *Trichoderma* (Annamalai *et al*., 2012; Bhikhabhai *et al*., 1984; Hakamada *et al*., 1997; Tai *et al*., 2004), the cellulolytic enzymes are more stable against high temperature.

Taxonomic Classification of Strain SWU-27

Strain SWU-27 was Gram-negative, motile, and rod-shaped (0.6-0.9 by 1.2-2.1 µm) without spore or flagellum (Fig. 3). It could utilize lactose, inositol and sucrose as the carbon source. The contact enzymatic reaction exhibited a positive result. Other biochemical properties were listed in Table 1. All the properties were in accordance with the standard identification of *Klebsiell*a (Garrity *et al.*, 2005).

Partial 16S rDNA sequence of strain SWU-27 was determined and the BLAST results showed that its share of more than 99% identifies it with those of *Klebsiella* at nucleic acid level. Based on multiple alignments of all related 16S rDNA sequences comprised of ten sequences derived from seven *Klebsiella* and four elements from other species, the NJ phylogenetic tree was constructed for further identification. Strain SWU-27 is the member of clade comprised of various *Klebsiella pneumoniae* strains (Fig. 4). All these results demonstrate that SWU-27 belongs to *Klebsiella*, termed as *K. pneumoniae* strain SWU-27. The

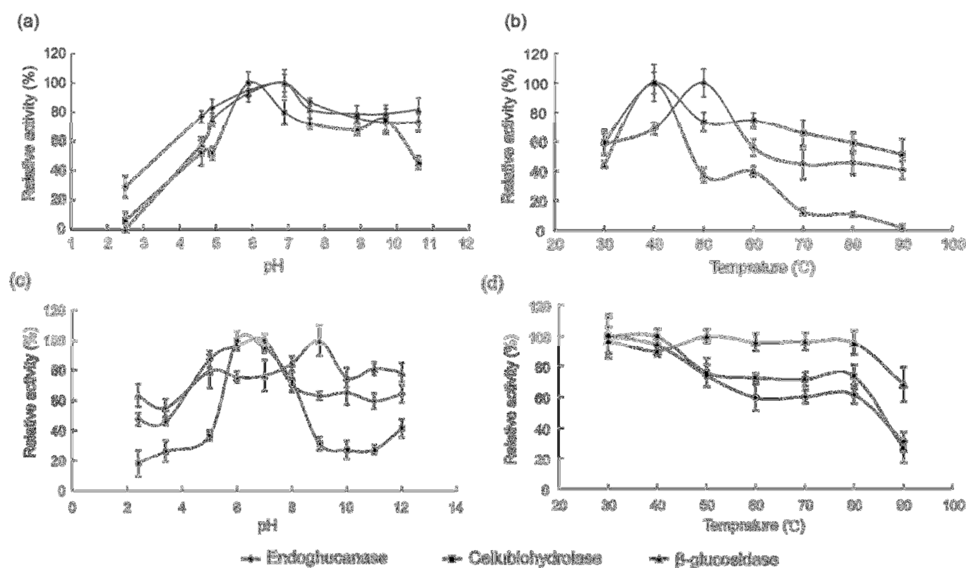

Fig. 2: Analysis of the cellulase activities of strain SWU-27. After 4 days of cultivation, cell-free supernatant of strain SWU-27 was collected for the enzyme assay. The DNS method was used to measure the crude cellulase activity. The optimum pH (a) and temperature (b) were determined by measuring the enzyme activity under different pH at 40ºC or various temperatures under pH 7.0. The stability of the celluloytic enzyme under different pH (c) was determined by incubating the enzyme in different buffers (20 mM sodium phosphate, pH 5-7; 20 mM Tris/HCl, pH 7-9; 20 mM carbonate, pH 9-11) for 30min and the residual enzyme activity was assayed. The stability of the celluloytic enzyme under various temperature (d) was determined by incubating the enzyme in 20 mM Tris/HCl buffer (pH 7.0) for 30 min at different temperatures (30-90ºC)

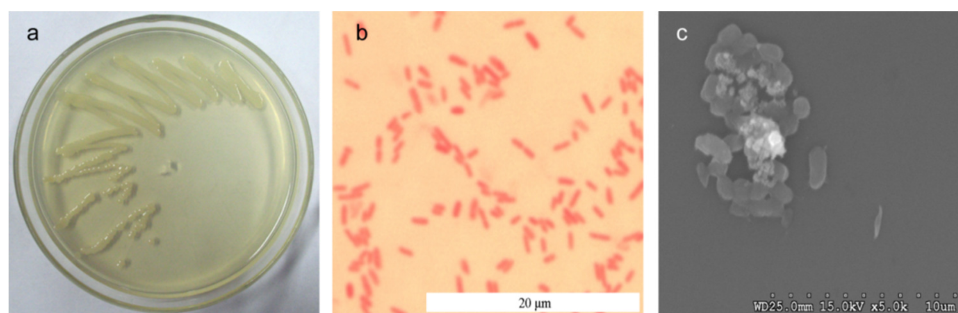

Fig. 3: The morphological observation of strain SWU-27. (a) colonies on nutrient LB agar medium,(b) the result of Gram method staining,(c) observation under electron microscope (×5000)

16S rDNA sequence of strain SWU-27 has been deposited in the GenBank database under the accession No. EU128493.

Discussion

More and more fungi, such as *Trichoderma* and *Aspergillus*, have been isolated for highly producing cellulase enzyme complex and employed for enzymatic conversion of cellulose to fermentable sugars (Bansal *et al*., 2014; Lutzen *et al*., 1983; Raghuwanshi *et al*., 2014). However, large-scale cultivation of fungi was usually costly since they grow slow and coproduce high viscous polymers (Kuhad *et al*., 2011). Recently, bacteria has become an ideal candidate to produce large scale cellulases for their rapid reproduction and low requirement of culture medium. For the application in industry, cellulases are usually required to endure high temperature and high pH. Therefore, challenging as it is, seeking an available cellulose-decomposing organism bears significant meanings. Up to date, cellulolytic enzyme-producing bacteria have been isolated from soil (Hakamada *et al*., 1997), and marine sediments (Annamalai *et al*., 2012). But most previously reported bacteria did not functionally work under the wide range of temperature and pH (Sleat *et al*., 1984; Taya *et al*., 1988; Trivedi *et al*., 2011; Tai *et al*., 2004). This seriously limited their wide application in industrial fermentation. More studies were focused on seeking for thermostable alkaline cellulase-producing

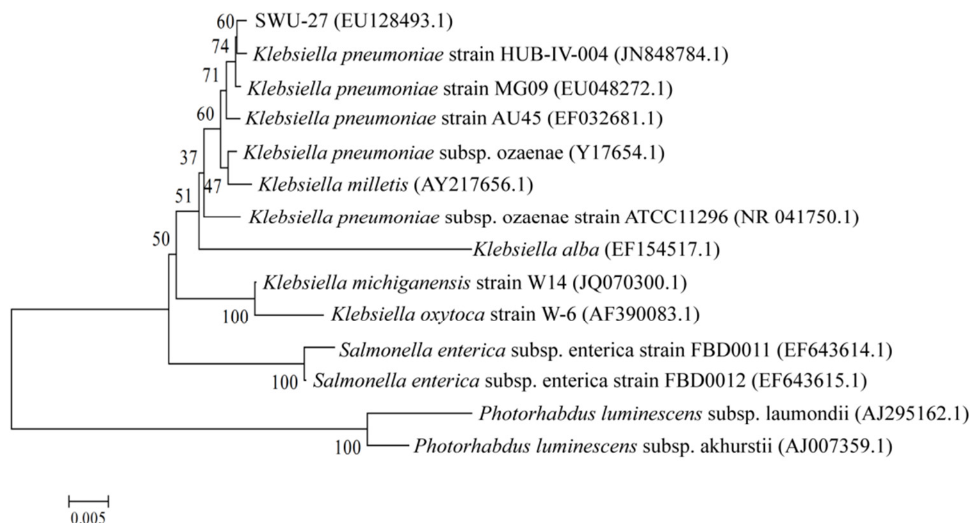

Fig. 4: Phylogenetic tree based on 16S rDNA gene sequences using the Neighborhood-joining method. Numbers at nodes indicate percentages of 1,500 bootstrap resamplings; Bar 0.005 sequence divergence; The codes after the names are the GenBank Accession numbers

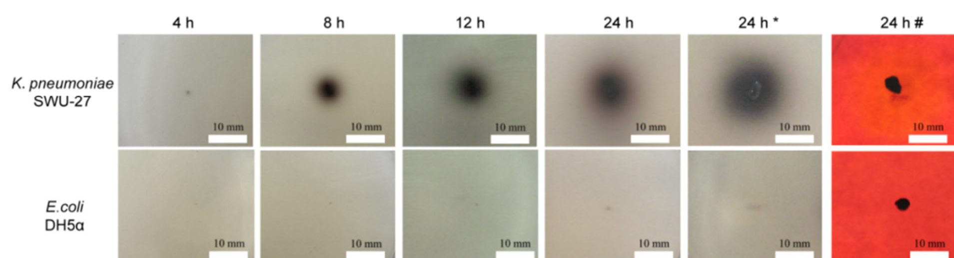

Fig. 5: Test on cellulolytic activities of strain SWU-27 under different time points after inoculation. The strain apparently showed black hydrolysis circle on Esculin plate and a transparent zone on CMCNa agar plate. *E. coli* strain DH5α was employed as a negative control. Asterisk and pound sign indicated tests on Esculin plate and CMCNa agar plate, respectively

bacteria.

In this study, a bacterium was screened as a high yield cellulolytic-producing strain, *K. pneumoniae* SWU-27. The 16S rDNA analysis proved that strain SWU-27 was a member of *K. pneumoniae* and the results of morphological, biochemical and physiological tests, obeying to the *Bergey's Manual of Systematic Bacteriology*, confirmed these results. In previous studies, *K. oxytoca* have the phenol-degrading activity and have been certified to produce ethanol and 2, 3-butanediol from xylose and glucose (Ji *et al*., 2009). The mechanism and improvement of producing 1,3-propanediol and extended spectrum beta-lactamases by *K. pneumoniae* had become the main focuses of the previous studies (Menzel *et al*., 1997; Pena *et al*., 1998). But no research before ours was found to describe *K. pneumoniae* as a source of extracellular alkali-thermotolerant cellulolytic enzyme.

Strain SWU-27 was isolated directly from the samples just because of the superior capacity of degrading CMC substrates, Filter paper. Meanwhile, the cellulase system of the strain, SWU-27, showed notably higher β-glucosidase, which was both significant for splitting the cellulose into monosaccharide, accompanied with a rapid production. Immediately after stabinoculation for 4 h, the black hydrolysis circle appeared as soon as the formation of the colony was initiated (Fig. 5). A clear black zone was larger and larger around strain SWU-27 as time passed. Meanwhile, a clear orange zone was also observed around the colony of SWU-27 after congo red stain, which suggested a CMCase activity. The rapid growth rate with timely production for cellulase of strain SWU-27 indicated the potential commercial value of this newly isolated bacterial strain.

Conclusion

The *K. pneumoniae* strain SWU-27 proved as a cellulase high-yielding organism. The characters of low nutritional requirement, rapid growth speed and highly stable enzyme system, which can endure high temperature and high pH, suggested a potential application during the industrial progress towards biomass utilization.

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