

## SHORT COMMUNICATION

# Characterization of a New Thermoalkalophilic Xylanase-Producing Bacterial Strain Isolated from Cimanggu Hot Spring, West Java, Indonesia

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Alkalophilic bacteria and their enzymes are important for industrial applications. Therefore, finding out new strains of alkalophilic bacteria from Indonesian microbial diversity is still required. In this study, a thermoalkalophilic bacterium was isolated from sediment of local hot spring, Cimanggu, West Java. The temperature of the spot was 60 °C and the pH was 8. The bacterium could live at pH 11 and temperature 55 °C, and produced xylanase that have optimal activity at alkaline pH 9 and high temperature 70 °C. Based on the analyses of 16S rRNA sequence similarity and biochemical characteristics, this strain was clustered into the same group of *Bacillus halodurans* species with 99% identity to *Bacillus halodurans* C-125. The isolate also showed other enzyme activities such as amylase, protease, and gelatinase, promising its potential use as an industrial enzymes producer.

**Key words:** 16S rRNA, *Bacillus halodurans*, biochemical properties, thermoalkalophilic xylanase.

Bakteri alkalofil dan enzim yang dihasilkannya bermanfaat dalam proses industri. Karena itu, penemuan bakteri baru dari sumber biodiversitas mikrob lokal Indonesia masih diperlukan. Pada penelitian ini, satu galur bakteri alkalotermofil berhasil diisolasi dari endapan sumber air panas, Cimanggu, Jawa Barat, Indonesia. Sumber air panas ini mempunyai pH 8 dan suhu 60 °C. Bakteri yang telah diisolasi dapat bertahan hidup pada pH 11 dan suhu 55 °C, dan menghasilkan xilanase yang mempunyai pH dan suhu optimum masing-masing 9 dan 70 °C. Hasil karakterisasi menggunakan analisis sekuens DNA dari gen 16S rRNA dan secara biokimia menunjukkan bakteri ini satu kelompok dengan *Bacillus halodurans* dan mempunyai homologi 99% dengan *Bacillus halodurans* C-125. Galur bakteri ini juga potensial menghasilkan enzim industri lainnya seperti protease, amylase, dan gelatinase.

**Kata kunci:** 16S rRNA, *Bacillus halodurans*, karakter biokimia, xilanase termoalkalofil.

Alkalophiles have made a great impact in industrial application, for their capability to produce alkalophilic or alkaline-stable enzymes. Alkalophilic microorganisms can be isolated from normal environments such as garden soil, although viable counts of alkalophiles are higher in samples from alkaline environments (Horikoshi 2004). A thermoalkalophilic *Bacillus halodurans* JB99 was reported as a source of alkaline thermostable keratinolytic protease for dehairing process in leather industry (Shrinivas and Naik 2010). *Bacillus halodurans* S7 was also reported as a producer of thermostable alkaline endo- $\beta$ -1-4-xylanase, which is potential for application in pulp and paper industry (Mamo *et al.* 2006).

In pulp and paper industry, enzymatic processes are attractive futuristic alternatives to the current harsh chemical procedures. The search for alternative procedures to reduce persistent use of toxic chemicals has been going on for many years. Pulp bleaching

process is conducted at high temperature and alkaline environment; therefore, the use of thermostable alkaline xylanase is very attractive from the economical and technical point of view (Mamo *et al.* 2006).

A number of bacteria were isolated from various environments and screened for potential producers of xylanolytic enzymes. *Bacillus licheniformis* I5 is an isolated endoxylanase producer, and from this strain an endoxylanase gene has been cloned (Helianti *et al.* 2008). *Bacillus subtilis* AQ1, which was isolated from sediment of an aquarium, was also reported as endoxylanase producer. The original AQ1 endoxylanase promoter and the signal peptide gave a very high constitutive extracellular expression in *E.coli* DH5 $\alpha$ , however this endoxylanase showed less activity in alkaline condition (Helianti *et al.* 2010).

Recently, we isolated a thermoalkalophilic bacterium from sediment of mineral rich, local hot spring, Cimanggu, West Java. The temperature of the spot was 60 °C and the pH was 8. About 0.5 mL of material from colloidal sediment was inoculated to alkaline medium for selection and incubated at 50 °C

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with shaking. After 18-20 hours incubation, the culture was spread on the same solid medium containing xylan. The selection medium was based on the previous work (Ohta *et al.* 1974) with the following component: polypeptone 10 g, meat extract 10 g, yeast extract 3 g, glucose 3 g,  $\text{Na}_2\text{CO}_3$  (anhydrous) 10 g in 1 L deionized water with range pH 10 to 11. Solid medium was prepared by the addition of 2% ( $\text{w v}^{-1}$ ) agar. A pure bacterial strain that grew at pH 10 and 50 °C and produced clear zone on solid medium containing 0.5% xylan was isolated (Fig 1a). It was designated as Cm1.

The supernatant (obtained by centrifugation 5600 g, 4 °C) of the culture grown in liquid medium containing 0.5% xylan was used to assay the xylanase activity. The effect of temperature on xylanase activity was measured in temperature range of 60-100 °C at pH 9 Tris-HCl buffer. The effect of pH on the activity was measured at 70 °C at pH range 7-11 using the following buffers: 50 mM phosphate buffer (pH 7), 50 mM Tris-HCl (pH 8-9), and 50 mM Tris-Glycine buffer (pH 10-11). The thermostability was measured by incubating the enzyme at 60 °C and pH 9 for 15, 30, 45, and 60 min.

Xylanase activity was measured duplicates using the Miller (1959) method using dinitrosalicylic acid to quantify reducing sugar. D-xylose was used as a standard. 50  $\mu\text{L}$  crude extract at appropriate dilutions in phosphate buffer was mix with 450  $\mu\text{L}$  of 1% oat spelt xylan in 50 mM of buffer at the indicated pH. The mixture was then incubated at the indicated temperature for 5 min. Subsequently, 750  $\mu\text{L}$  DNS reagent (1% dinitrosalicylic acid, 0.2% phenol, 0.05% sodium sulfite, and 1% sodium hydroxide, 20% ( $\text{w v}^{-1}$ ) potassium sodium tartrate) was added to stop the reaction. Then the mixture was boiled at 100 °C for 5 min, and kept at room temperature. Afterwards 250  $\mu\text{L}$  of water was added and the mixture was centrifuged to obtain a clear supernatant. The absorbance was measured at 540 nm. As blank we used the same mixture as in the above sample; however, enzymes were added following addition of DNS into the reaction mixture. One unit xylanase activity was defined as the amount of enzyme that releases 1  $\mu\text{mol}$  of xylose per min under the assay condition.

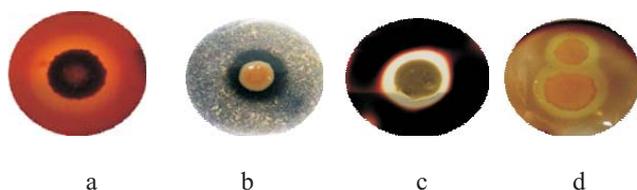


Fig 1 Isolate CM1 in Luria Bertani media containing specific substrate: a, xylan; b, skim milk; c, starch; d, gelatin. To make the clear zone looked clearer, KI<sub>1</sub> reagent was added to starch hydrolyzing medium.

The optimum pH and temperature for the enzyme was pH 9 and 70 °C, respectively, showing thermoalkalophilicity of the xylanase produced by this CM1 isolate. The activity at the pH 9 and temperature 70 °C was  $126.67 \pm 0.85 \text{ U mg}^{-1}$ . The pH profile showed that the crude enzyme was active in the range of pH 7-11. The crude enzyme still retained 65% of its activity after incubation at 60 °C and pH 9 for 30 min (Fig 2a, b, and c).

Other enzyme activities of this strain were investigated with CMC, skim milk, starch, and gelatin. The strain was streaked on the Luria Bertani Agar medium containing 0.5% of each substrate. The isolate showed protease, gelatinase, and amylase activity, but no cellulose activity (Fig 1 b, c, d).

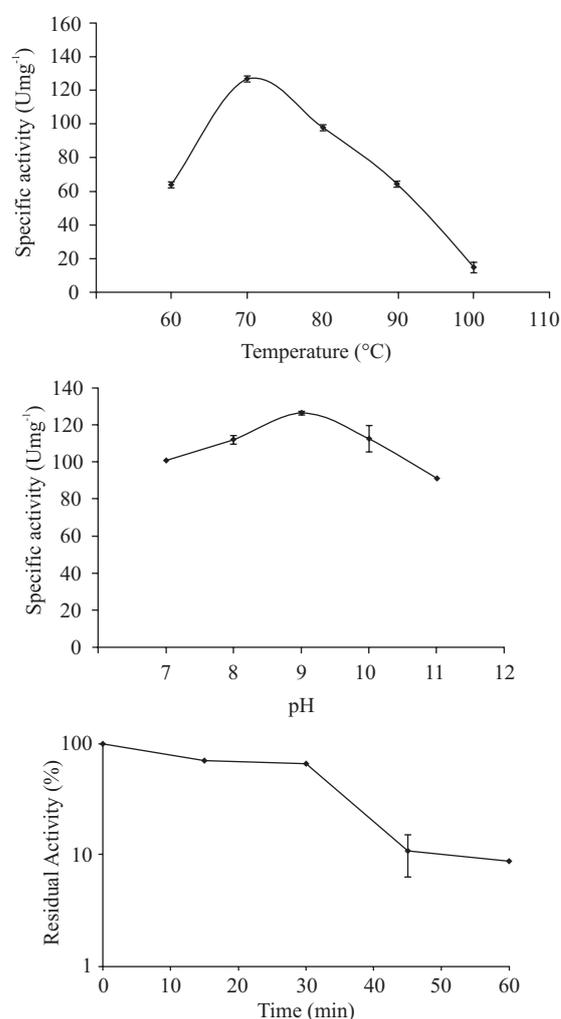


Fig 2 Temperature and pH profile, and thermostability of crude xylanase from isolate CM1: a, The effect of temperature on Cm1 crude xylanase activity. For this temperature profile, enzymatic activity was measured in 50 mM sodium phosphate pH 9. b, The effect of pH on CM1 crude xylanase activity. For this pH profile, enzymatic activity was measured in temperature 70 °C. The reaction pHs were adjusted to 7-11 with the following buffers: 50 mM phosphate buffer (pH 7), 50 mM Tris-HCl (pH 8-9), and 50 mM Tris-Glycine buffer (pH 10-11). c, Thermostability of CM1 crude xylanase. The enzyme used for the determination of pH stability was diluted in 50 mM Tris-HCl pH 9 in incubated at 60 °C for the designed time periods before the activity was assayed at 60 °C for 5 min. The assay results shown are the means of duplicated independent experiments, and the error bars indicate the standard deviation of these results.

For further characterization of the isolates, the analysis of 16S rRNA gene was conducted. Genomic DNA was extracted by the standard phenol-chloroform-isoamyl alcohol method as described previously (Helianti *et al.* 2008). The 16S rRNA gene fragment was amplified by PCR with universal primers 16S-27f (5'-GAGTTTGATCCTGGCTCAG- 3') and 16S-1525r (5'-AGAAAGGAGGTGATCCAGCC- 3'). The PCR was conducted using DNA Taq polymerase (KAPPA, USA) under the following conditions: denaturation at 94 °C for 1 min, annealing 61 °C 35 sec, extension 72 °C 2 min for 30 cycles followed by elongation at 72 °C for 5 min using a thermal cycler (Eppendorf, Germany). It was ligated to pGEM-T Easy vector at 4 °C over night. The white/blue screening with X-Gal/IPTG on the LB agar medium containing ampicillin 100 µg mL<sup>-1</sup> was done. The positive clones were then further verified using restriction enzyme and the confirmed clone was sequenced and analyzed. The sequencing was performed by ABI 3100 DNA sequencer. The DNA sequence was compared to other bacterial 16S rRNA sequences in NCBI GenBank using BLAST program. Then, the DNA sequence and the analysis result were submitted to the Genbank. The accession number of the sequence was JN903769. We characterized the strain based on this partial 16S rRNA sequences and its position in phylogenetic tree. The 1544 bp length of sequence of 16S rDNA showed 99% identity to *Bacillus halodurans* C-125, and phylogenetic tree analyses showed that this isolate belongs to the group of this species (Fig 3).

To complete the characterization of the isolate, morphology, biochemical characteristics, and carbohydrate metabolic profile was also investigated. Standard microbiological techniques were applied to determine microbiological characteristics such as

colony, spore, cell shape, gram staining, and Voges-Proskauer reaction. Carbohydrate source utilization experiments were carried out applying the API 50 CHB system (bio Merieux, Nurtingen, Germany). A single colony from 16-18 h culture incubated in 55 °C was inoculated to alkaline medium. One milliliter culture was then centrifuged at 5800 g, 4 °C for 15 min. The pellet was resuspended in 2 mL NaCl 0.85% then used to inoculate, dropwise, 5 mL NaCl 0.85% until the turbidity reached 2 McFarland. Then, twice the inoculum volume was used to inoculate 10 mL of API 50 CHB medium. The cell suspension was used to fill the wells of the API 50 CHB strips. The strips were then incubated at 37 °C for 24 - 48 h. A positive test corresponds to acidification revealed by the phenol red indicator contained in the medium changing to yellow. So far, there was no report on the complete biochemical properties of *Bacillus halodurans* except *Bacillus halodurans* C-125. There is a report about the purification and characterization of endoxylanase from *Bacillus halodurans* strain which had 100% identity of 16 rRNA to *Bacillus halodurans* C-125 (Mamo *et al.* 2006). However, they did not describe the detailed biochemical properties. Isolate C-125 was, at first, identified as *Bacillus lentus* (Aono 1995). However it was reidentified as *Bacillus halodurans* C-125 (Takami and Horikoshi 1999) mainly due to the 16S rRNA sequence and DNA-DNA hybridization data. Among *Bacillus halodurans* strains, only this one has been investigated biochemically. Therefore, these biochemical properties of the newly isolated bacterial strain were compared to those of *Bacillus halodurans* C-125. The newly isolated CM1 was less tolerant against NaCl (could not resist NaCl concentration above 7%) and metabolized sorbitol, whereas the *Bacillus halodurans*

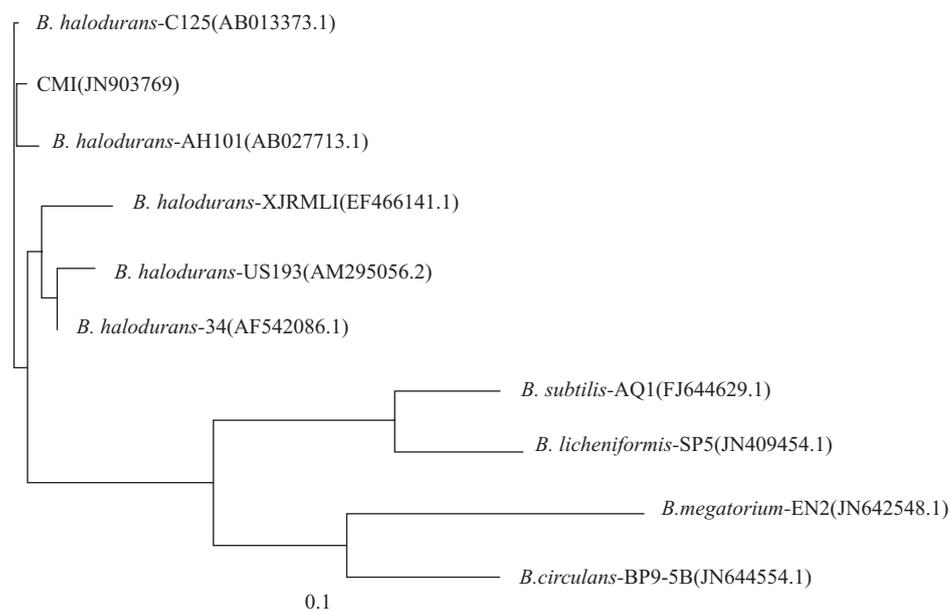


Fig 3 The position of CM1 strain in the phylogenetic tree of genus *Bacillus*. The tree was constructed using neighbor joining method. The bar below the tree represents a distance scale. The code in the bracket is a Genbank accession number.

Table 1 Characteristic of *Bacillus halodurans* CM1 compare to *Bacillus halodurans* C-125

| Characteristics          | Strain CM1        | Strain C-125        |
|--------------------------|-------------------|---------------------|
| Cell shape               | Rod               | Rod*                |
| Cell size                | 2.7 -5.5          | 0.6-0.7 x 2.5-4 µm* |
| Colony color             | white to brownish | Cream-white**       |
| Endospores               | +                 | + **                |
| Gram staining            | +                 | + *                 |
| Growth in anaerobic agar | +                 | + *                 |
| Voges-Proskauer          | -                 | - *                 |
| Growth at 40 °C          | +                 | + *                 |
| 50 °C                    | +                 | + *                 |
| 55 °C                    | +                 | + *                 |
| Growth at pH 5           | -                 | - *                 |
| pH 7                     | +                 | + *                 |
| pH 11                    | +                 | + **                |
| Growth in NaCl 5%        | +                 | + *                 |
| NaCl 7%                  | +                 | + *                 |
| NaCl 8%                  | -                 | + *                 |
| NaCl 10%                 | -                 | + *                 |
| Hydrolysis of Xylan      | +                 | + **                |
| Skim milk                | +                 | N.D                 |
| Casein                   | +                 | + **                |
| Starch                   | +                 | + **                |
| Gelatin                  | +                 | + **                |
| CMC                      | -                 | N.D                 |
| L-Arabinose              | +                 | + *                 |
| Ribose                   | +                 | + **                |
| D-Xylose                 | +                 | + *                 |
| Fructose                 | +                 | + *                 |
| Galactose                | +                 | + **                |
| Mannose                  | +                 | + **                |
| Melibiose                | +                 | + * *               |
| Lactose                  | +                 | + **                |
| Sucrose                  | +                 | + **                |
| Trehalose                | +                 | + **                |
| Rafinos                  | +                 | + **                |
| Sorbitol                 | +                 | - **                |

ND: Not described; \*Takami and Horikoshi 1999; \*\*Aono 1995.

C-125 was more tolerant to NaCl and could not degrade sorbitol (Table 1).

In conclusion, we have isolated an alkalophilic bacterium from mineral rich, local hot spring, Cimanggu, West Java. 16S rRNA sequence, morphology, and biochemical properties indicated that this new strain was *Bacillus halodurans* CM1.

The new bacterial strain produced alkalothermophilic xylanase which demonstrated optimum activity at pH 9 and 70 °C. This enzyme still retained 65% residual activity after 30 min incubation at 60 °C, thus promising its potential application in pulp and paper industry. Furthermore, this strain is also a potential producer of other enzymes such as protease, amylase, and gelatinase, so that the strain would be a good candidate of enzyme workhorse in industries.

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