

SHORT COMMUNICATION

Optimization of Culture Conditions to Produce Thermostable Keratinolytic Protease of *Brevibacillus thermoruber* LII Isolated from the Padang Cermin Hot Spring, Lampung, Indonesia

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Hot springs represent one of the most promising sources for the isolation of thermostable enzyme producers. The microorganisms living in a hot spring not only have to withstand elevated temperatures but also extreme environmental pH and certain chemical compounds that are often toxic to other microbes. A bacterial strain denoted as *Brevibacillus thermoruber* LII has been isolated from Padang Cermin Hot Spring, Lampung, Indonesia. Optimization of the conditions for protease production by this strain revealed that the isolate produced a thermostable protease optimally at temperature and pH ranges 45-55 °C and 6-7, respectively, with keratin as substrate. The strain's keratinolytic activity was shown by the ability to degrade untreated chicken feathers after 24 h incubation in liquid medium.

Key words: *Brevibacillus thermoruber*; hot spring, thermostable protease

Sumber air panas merupakan salah satu sumber yang paling menjanjikan untuk isolasi penghasil enzim tahan panas. Mikroorganisme yang hidup di sumber air panas tidak hanya tahan terhadap suhu tinggi tapi juga terhadap pH lingkungan yang ekstrim dan senyawa kimia yang seringkali bersifat racun bagi mikroorganisme lain. Isolat *Brevibacillus thermoruber* LII telah diisolasi dari sumber air panas Padang Cermin, Lampung, Indonesia. Optimasi kondisi untuk menghasilkan protease oleh isolat tersebut menunjukkan bahwa isolat ini menghasilkan protease tahan panas secara optimal pada suhu dan pH masing-masing 45-55 °C dan 6-7 dengan keratin sebagai substrat. Aktivitas keratinolitik strain ini ditunjukkan dengan kemampuannya mendegradasi bulu ayam tanpa perlakuan setelah inkubasi 24 jam pada medium cair.

Kata kunci: *Brevibacillus thermoruber*, protease tahan panas, sumber air panas

Proteolytic enzymes do not only play important roles in cellular metabolic processes but have also gained considerable interest for industrial applications. Proteases account for nearly 60 % of the total annual turnover of the world enzyme market (Chen *et al.* 2004; Chu 2007). They are widely applied in detergent, protein modification, leather, meat, brewing, photographic, dairy, membrane cleansing, and waste treatment industries (Kumar *et al.* 2002; Chu 2007).

The advantages of using thermostable enzymes in industry include; the capability to use elevated temperatures, thus facilitating faster reactions, increasing the solubility of nongaseous reactants/products and reducing the incidence of microbial contamination by mesophilic organisms (Sokkheo *et al.* 2000; Chen *et al.* 2004). Thermostable proteases are active above 60-70 °C and withstand organic solvents, detergents, low and high

pH values and other denaturing agents (Covan *et al.* 1985; Covan 1997; Gupta and Khare 2006). These properties are of particular interest for industrial processes. As the substrate of the proteases is unfolded at elevated temperatures, thermostable enzymes have due to their characteristics-higher specific activities. They also appear to be rather useful in the synthesis of high molecular weight peptides due to their resistance against organic solvents, when processes are performed in media with low water content. (Sellek and Chaudhuri 1998; Bruins *et al.* 2001; Synowiecki 2008).

Hot springs are promising sources for direct isolation of thermostable enzymes producers. Microorganisms in a hot spring are not only adapted to the elevated temperature but also to the prevalent pH and the presence of certain chemical compounds.

Due to the growing market and the potential applications of proteases, there is an increasing interest in the isolation of new bacterial species as proteolytic

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enzyme producers with novel properties for industrial applications. Therefore, rarely exploited regions and niche habitats are likely to yield such strains.

There are many hot springs spread in Lampung province which is connected to Anak Krakatau Mountain, the active volcano located in Sunda Strait. One of those is hot spring which is an unnamed and unexplored spring located in Padang Cermin. There are many such springs in Lampung, hence no reports on exploration of thermophile in this area exist.

B. thermoruber was reported at first as a new species of *Bacillus* by Manachini *et al* (1988) and showed protease producing capacity at optimum temperature 45 °C and optimum pH 9. Wang *et al.* (2012) reported about a *Brevibacillus* species that produced thermostable protease with slightly different characters to the one produced by strain LII (optimum temperature 75 °C and optimum pH 9). Another experiment on this bacteria was reported by Lee *et al* (2004). Lee's group reported the presence of a gene encoding Lon Protease in *B. thermoruber* WR-249. Lon proteases are ATP-dependent serine peptidases belonging to the MEROPS peptidase family S16 (lon protease family, clan SF). *B. thermoruber* had also been reported for its ability to degrade fibroin which is one of the substrates of protease (Suzuki *et al.* 2009). All reports on protease produced by *B. thermoruber* showed that thermostable protease produced by LII had unique characters regarding optimum temperature and stability against thermal.

B. thermoruber LII was isolated from the hot spring at Padang Cermin, Lampung after extensive screening on Minimal Synthetic Medium (MSM plate agar containing 0.1% NaCl, 0.1% K₂HPO₄, 0.1 % NaCl, 0.01% MgSO₄.7H₂O, and 0.05 % yeast extract supplemented with 1% skim milk). The purified cultures were preserved in 40% glycerol and stored at -70 °C.

Preliminary investigations revealed that out of three proteolytic isolates (clear zone around colony), strain LII produced a thermostable protease with highest activity when fermented at 50 °C in liquid medium after 22 h incubation. The protease was stable up to 100 min at 75 °C.

For proper identification of LII by 16S rRNA analysis, the DNA of the bacterium was extracted using the TIANamp DNA Kit. The 16S rDNA (1.47 kbp) fragment was amplified by PCR using a pair of universal primers 16S rDNA-27F and 16S rDNA-1492R (Gee *et al.* 2003) and 50 µL of the reaction mixture containing 1 µL chromosomal DNA, 2µL

primer mix, 22 µL ultrapure water, 25 µL 2x Tag PCR Master Mix (TIangen Biotech, China). After denaturation at 95 °C for 5 min, 30 cycles of annealing at 55 °C for 30 sec, extension at 72 °C for 90 sec and denaturation at 95 °C for 60 sec were performed, followed by a final extension at 72 °C for 10 min. The nucleotide sequence of the amplified fragment was identified on a 3730 DNA sequencer (Applied Biosystems, CA, USA) and subjected to a homology search against a DNA database using the FASTA program (<http://fasta.ddbj.nig.ac.jp/>). The nucleotide sequence was deposited at GenBank with accession number AB757752.1. The phylogenetic tree constructed on the basis of the sequence and presented in Fig 1 identifies the strain as a representative of the *B. thermoruber* species.

The protease activity was determined using a modified Takami method (1989). The mixture containing 0.25 mL 1% casein in 0.025 M Tris-Cl buffer, pH 7 was incubated with 0.25 mL of the enzyme for 10 minutes. The reaction was stopped by adding 0.5 mL 0.4 M TCA. The mixture was centrifuged at 10.000 rpm (Beckman Coulter™ Micfuge® Centrifuge F 241.5P) for 10 min. Supernatant (0.5 mL) was mixed with 2.5 mL 0.4 M Na₂CO₃ and 0.25 mL Folin-Ciocalteu's Phenol Solution and incubated for 30 minutes at room temperature. The absorbance of the solutions were read against the sample blanks at 660 nm using Spectronic® 20 Genesys™. (Spectronic Instrument Inc, Rochester, NY). Tyrosin standard solution, in the range of 0-1000 mg L⁻¹ was prepared in triplicate to obtain a standard curve. One unit (U) of protease was defined as the amount of enzyme that could produce 1 µmol of tyrosine in one min under the defined assay conditions.

Zymogram analysis was carried out to check the finger print of the thermostable protease produced by strain LII based on the effect of incubation condition and substrate supplement. The samples, without prior heating, were loaded on a 10 % acrylamid gel supplemented with 1% gelatin for 1.5-2 h. Before staining, the gel was immersed in 0.025 M Tris-Cl buffer pH 9 at the optimum temperature 75 °C for 30 min. The protease band appeared as clear zone surrounded by dark blue color of the gel.

The optimum temperature for enzyme production was investigated in 15 mL culture medium by supplementing 1.5 % skim milk in MSM. The culture was incubated in a shaking incubator (150 rpm) (Gymorax™ Amerex instrument Inc.USA) at temperatures 45, 50, 55, and 60 °C. One loop-full of

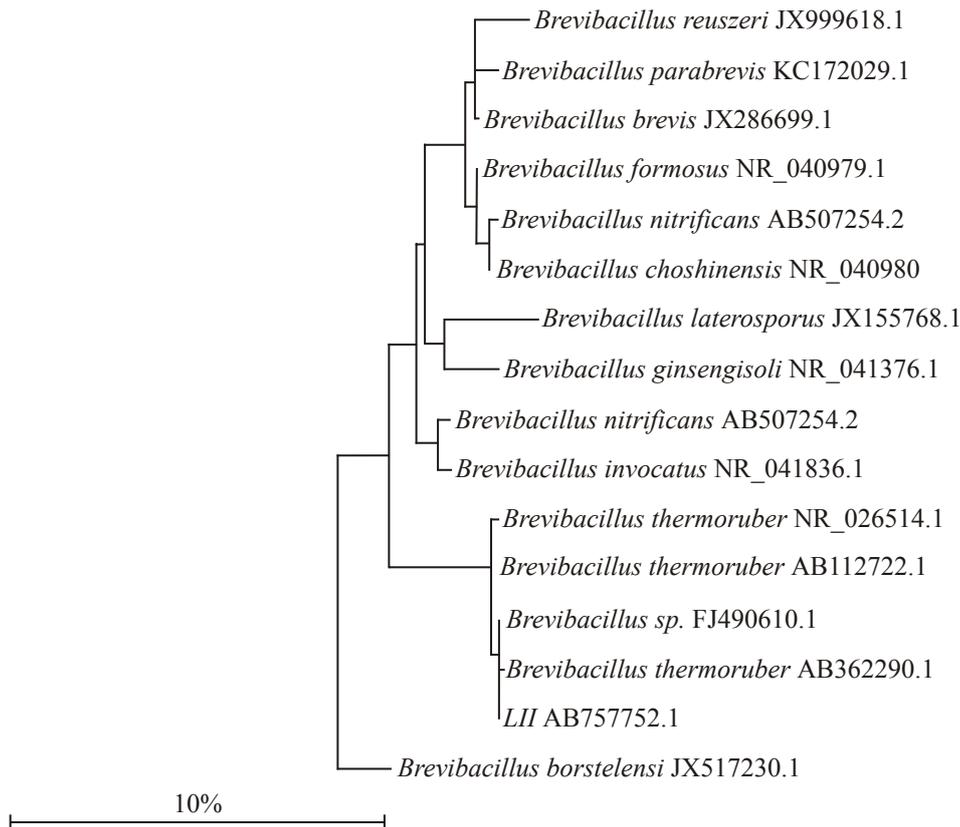


Fig 1 Phylogenetic tree of strain LII based on 16s rRNA gene sequences. GenBank accession numbers are given after the species name. Bar, 10 substitution per 100 nucleotides.

24 h culture from plate agar was inoculated into starter medium which had the same composition with working medium and incubated at temperature previously mentioned. After 24 h incubation, 5% of the starter culture was inoculated into 15 mL working medium. The cell free supernatant was measured for the activity after 22 h incubation.

The pH optimization of the *B. thermoruber* LII culture medium to produce the thermostable protease was carried out at pH 4, 5, 6, 7, 8, 9, and 10.

For determination of the optimum substrate, four kinds of proteins were used; 1 % casein, 1 % collagen, 1 % skim milk, and 0.5 % keratin. Keratin was prepared by washing chicken feathers with tap water followed by sun drying. Subsequently, the dried feathers were boiled in 0.0125 M NaOH for 20 min, neutralized with HCl and then again sun dried. The above procedure was repeated twice before the samples were ground and used as the substrate.

Strain LII is a Gram positive, short rod with terminal or subterminal oval endospore. and grows optimally at temperatures range 45-55 °C.

16S rRNA gene sequencing showed that LII belongs to *B. thermoruber* with 98% homolog. *B. thermoruber* LII produced the highest activity of

thermostable protease when cultivated at 50 °C. Nevertheless, high activity was found at 45 and 55 °C and although not too high, the activity of thermostable protease was still detected when cultivated at 60 °C (Fig 2A) Some reports showed the different temperatures for enzyme production, for examples *Bacillus subtilis* strain 38's optimum production temperature was 47 °C (Chantawannakula *et al.* 2002), *Bacillus sp.* MTG 30 °C (Gauda, 2006) and *Bacillus sp* SMTA-2 60 °C (Nascimento & Martins 2004). Frankea *et al.* (1986) revealed on their study that the link between enzyme synthesis and energy metabolisms in bacteria was controlled by temperature as well secretion of extracellular enzyme. The temperature possibly changed the physical properties of the cell membrane for enzyme secretion (Rahman *et al.* 2005). The activity of thermostable protease was detected over a broad pH range with the optimum pH at 6 (Fig 2B).

The result showed that LII preferred acidic to basic medium to produce the protease. Nevertheless, up to pH 10 the activity was still detected. The optimum pH of medium for protease production was different from those of *B. subtilis*, of which the optimum pH was 6.5 (Chantawannakula *et al.* 2002), and *Bacillus sp* strain

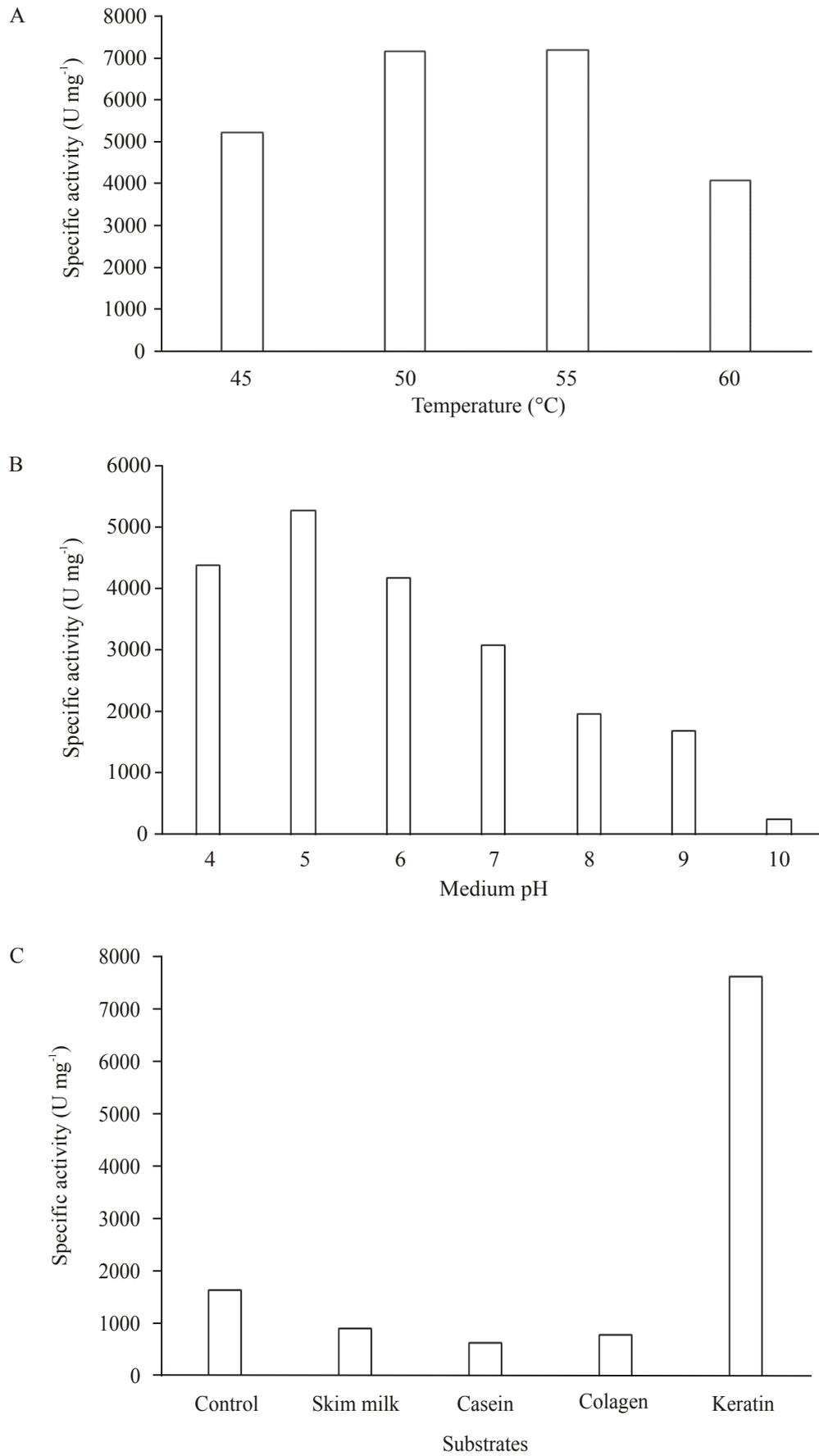


Fig 2 Effect of temperature, pH, and substrate supplementation of culture medium on protease production by *Bacillus thermoruber* LII. (A) Effect of incubation temperature on protease production; (B) Effect of medium production pH on protease production; (C) Effect of substrate supplementation in culture medium on protease production.

SMI-2 (Nascimento and Martins 2004), which produced protease optimally at pH 8. pH of culture medium affected many enzymatic processes and transport of various component across the cell membrane, as reported by Moon and Parulekar (1991).

The result showed that *B. thermoruber* LII produced protease optimally in medium supplemented with 0.1% feather keratin (Fig 2C). The activity in medium with collagen and casein supplementation showed lower activity than control.

The temperature and pH gave no effect on the type of thermostable protease produced by *B. thermoruber* LII as showed on acrylamid gel supplemented by 2 % gelatin (figure were not presented). However, the zymogram analysis showed that *B. thermoruber* LII produced more than one protease when the medium was supplemented with 1 % sikim milk as well as 1 % casein. The ability of *B. thermoruber* LII to produce

thermostable protease with high activity in liquid medium supplemented with 0.1% keratin showed that it had keratinolytic activity. The untreated chicken feather showed the damage after incubating with *B. thermoruber* in liquid medium as shown on Fig 4.

Keratinases are a class of proteolytic enzymes which have capability degrading keratin to soluble form. Several feather-degrading bacteria have been reported as keratinase producers that mostly belong to the genera *Streptomyces* and *Bacillus* (Marcedo *et al.* 2005; Kim *et al.* 2001; Bressolier *et al.* 1999; Chitte *et al.* 1999). Among Gram-positive bacteria, novel feather-degrading isolates have been identified as *Arthrobacter* sp. (Lucas *et al.* 2003), *Microbacterium* sp. (Thys *et al.* 2004), and *Kocuria rosea* (Bernal *et al.* 2006). Unfortunately, the properties of thermostable keratinases have been reported only in a few cases. Among them is keratinase isolated from

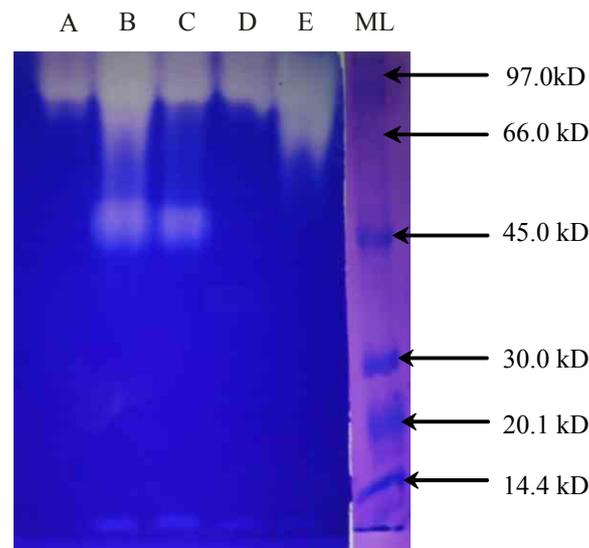


Fig 3 Effect of substrate supplementation in culture medium on protease finger print of *Brevibacillus thermoruber* LII. (A) control, (B) skim Milk, (C) casein, (D) Collagen, (E) Keratin, (M) Marker.

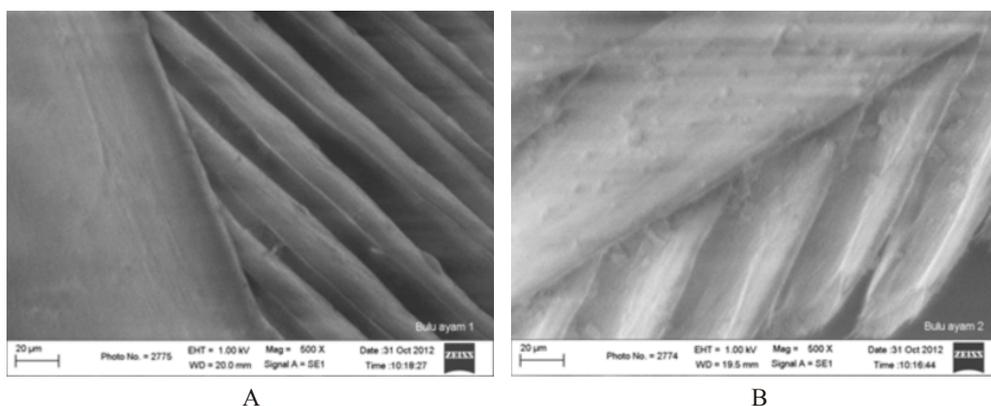


Fig 4 Scanning electron micrographs of feather. (A) Before incubation with *Brevibacillus thermoruber* LII culture; (B) After 24 h incubation with *B. thermoruber* LII culture.

Fervidobacterium islandicum AW-1, which had been characterized (Nam *et al.* 2002). This experiment showed that protease produced by *B. thermoruber* LII had different character to those produced by other strain of *B. thermoruber*. There are no known reports about thermostable protease produced by *B. thermoruber*, which has keratinolytic activity since this paper submitted. Further experiment to purify and characterize the enzyme is currently on work.

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