**Brevibacillus thermoruber**: thermophilic bacteria isolated from hot spring with the promising potential as a biomolecule producer

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**Abstract.** Activities and evolution of organisms are controlled by temperature, one of the most important environmental factors. Some microorganisms, have been known as thermophile, need thermal environment for growth and reproduction. They have been topics for much research during the last two decades. One of the interesting and potential thermophilic bacteria is *Br. thermoruber*. It is originated from hot spring that have been found and showed some abilities to produce important macromolecules. *Br. Thermoruber* is one of four *Brevibacillus* species recorded to produce a thermostable enzyme, such as keratinase, fibroinolytic enzyme and potential mitochondrial enzyme, which plays a major role in the degradation of intracellular proteins. It is also known as the exopolysaccharide cell factory. The genome sequencing and analysis of this species showed important information on the existence of some other potential enzymes. This project also opens up opportunities to conduct further research on production mechanisms of some enzyme previously found and to predict any potency that *Br. thermoruber* have.

1. Introduction

Thermophiles are microorganisms growing optimally between 55 - 80 °C. They are classified into thermophiles (50° C or higher), extreme thermophiles (65–79°C) and hyperthermophiles (above 80 °C) [1]. They are found either as Gram positive or negative, spore-forming or not, and may exhibit an aerobic or anaerobic metabolism. The intensive studies have been focused on their ability to produce thermostable enzymes with the properties that meet industrial needs. Some thermostable enzymes with novel properties produced by thermophiles were reported in last few years such as thermostable protease [2,3] Xylanases [4–6], cellulase [7,8] lipase [9,10] polymerases [11], beta-galactosidases [12] and esterases. The environment for microorganism to grow is not only characterized by temperature but also extreme pH and salinity [13].

The genus *Brevibacillus* contains organisms with significant biotechnological characteristics. One of them is *Brevibacillus brevis* which was reported having capability to degrade triphenyltin which contaminates the environment worldwide [14]. This species was also reported producing Edeines, a typical cationic peptides, with broad antimicrobial activity spectrum [15]. Another species was identified as a host for the expression system, *Brevibacillus choshinensis*. The system "*Brevibacillus* in vivo cloning" recently developed by the Higeta Shoyu Community facilitates efficient output screening. This expression mechanism has developed recombinant interferon μ, a form of cytokine that has been shown to promote adjuvant and growth in poultry and animals and is capable of being used as antibiotic alternatives [16]. Hybrid protein, Cholera toxin B subunit-insulin B chain peptide (a nasal vaccine against autoimmune diabetes), was also produced by *Brevibacillus choshinensis* [17]. It was also used to produce antigenic protein VP28, a recombinant protein which protect shrimp from
white spot syndrome disease [18] and single-chain antibody (scFv) which is produced by completely functional antigen-binding fragment technique [19].

Manachini et al. [20] reported for the first time that *Bacillus thermoruber* cells are gram positive, straight rods, with lateral flagellation with oval endospores. They are heat loving microorganisms and produce red pigment. Colonies are red colored, circular, entire, and convex and have glossy and mucilaginous surfaces [20]. Most of *Br. thermoruber* were isolated from hot spring [21–24]. This explains the optimum growth temperature (45 to 48°C) that differed from other *Brevibacillus* species (30°C) [25].

*Brevibacillus thermoruber* is one of four thermophilic *Brevibacillus* species i.e *Brevibacillus aydinogluensis* [26], *Brevibacillus borstelensis* [25] and *Brevibacillus limnophilus*. In this study, the recent update on the potential of *Br. thermoruber* as a producer of thermostable enzymes active in hard-to-degrade proteins (keratinase and fibroinolytic enzymes) and LON proteases, which play an important role in the intracellular degradation of proteins. *Br. thermoruber* is also a promising microbial cell factory for exopolysaccharide production.

2. Classification

Until 1996, the morphological identification of *B. brevis* were based on Laubach [27]. Shida et al. [25] listed it through genetic reclassification, along with nine other species as a new *Brevibacillus* genus i.e. *B. brevis*, *B. agric*, *B. centrosporus*, *B. choshinensis*, *B. parabrevis*, *B. reusseri*, *B. formosus*, *B. borstelensis*, *B. laterosporus*, and *Br. thermoruber*. Phylogenetic relationships of *Brevibacillus* and other *Bacillus* species based on 16S rRNA gene sequences was present in Figure 1. In the time of writing, the genus *Brevibacillus* includes in 21 species after Xian et al. [28] reported the novel species, *Brevibacillus sediminis* sp. (Figure 2.)

![Figure 1. Phylogenetic relationships of *Brevibacillus* and other *Bacillus* species based on 16S rRNA gene sequences](image-url)
3. Enzym producer for hard-to-degrade protein

Hard-to-degrade protein commonly used to mention the dense and strong structure protein which cannot be hydrolyzed using common protease such as trypsin or pepsin. These proteins, such as collagen, keratin, and prion protein, tend to gain plasticity, generating more susceptibility substrate for enzymatic degradation at elevated temperature [29]. This enables the use of thermophiles and their enzymes on such proteins to be successful. There were two strains of Br. thermoduric that have been reported producing thermostable protease which are active on keratin and fibroin degradation.

3.1. Keratinase

Keratin is an insoluble structural protein which has high stability i.e. feathers and wool [30]. Degradation of keratin is difficult as the polypeptide densely packed and strongly stabilized by several hydrogen bonds and hydrophobic interactions along with several disulfide bonds. The structural rigidity is warranted by composition and molecular configurations of its constituent amino acid. There are at least 30 different keratin polypeptides falling into two evolutionary families designated type I and type II. The helical rod domain of about 310 amino acids within each polypeptide chain is flanked by a shorter non helical head and tail domains which are thought to have a flexible conformation [31]. Keratin has mechanical stability and resistance to common proteolytic enzymes such as pepsin, trypsin, and papain due to its supercoiled polypeptide chain which is composed of α-helix (α-keratin) or β-sheet (β-keratin). Its tightly packed and cross linking of protein chain by cysteine bridges confers high mechanical stability and resistance to those enzymes [32].

Although a lot of important information on keratin hydrolysis available, the mechanism of keratin biodegradation by microorganisms is not completely elucidated. Nevertheless, the reduction of cysteine bridges may have a significant influence on keratin degradation [33]. Some investigations
revealed that most keratinases have the ability to hydrolyze diverse substrates [33,34], so that they are able to replace the used of conventional protease in leather industry and detergent additive. Recent application of keratinase as reported by Jang et al. [35] is to improve biosynthesis of silver nanoparticles that can be applied as anticoagulants, thrombolytic, anticancer, and larvicidal agents. Keratinase is also used for cosmetic formulation [36] and bioremediation [37].

A keratinolytic strain, Bevibacillus thermoruber T1E was isolated by Bihari et al [38]. The isolate was investigated as a gram-positive non-pathogenic rod with optimum growth at 50°C on native feathers. T1E was able to released 0.72 mg/ml protease after 7 days incubation. Keratinase T1E was reported as 2.23 kD protein which was active optimally at pH of 6. Although keratinase produced by T1E was claimed as the first one which was characterized from Brevibacillus, but the investigation on its characters was not complete.

Keratinase produced by Bevibacillus thermoruber LII isolated from Padang Cermin hotspring Lampung had been reported by Zilda et al. (2014) [21]. LII produced keratinase optimally at temperature of 50°C using minimal synthetic medium with 1% chicken feather after 36 hours. It was active optimally at temperature of 85°C and pH of 8-9. Degradation of feather keratin by Br. thermoruber is presented in Figure 3.

![Figure 3. High resolution scanning electron microscope image of chicken feather (500× magnification). (A) before incubation (B) after 24 hours incubation at 50°C with Br. thermoruber LII in liquid MSM medium [21].](image)

### 3.2. Fibroinase

Fibroin is the main component of silk produced by silkworm, Bombyx mori. The primary organization of fibroin, called crystalline structure, is divided into a heavy-chain (H-chain, 350 kDa) and the Light-chain (L-chain, 25 kDa), attached to a disulfide bond [39]. An accessory, glycoprotein (25 kDa) named P25, is also non-covalently linked to these chains [40]. The repeated amino acid sequences which are assembled into nano-crystals (β-sheet) form hydrophobic domains of polymeric chains. Secondary structure is constructed from these hydrophobic domains (consist of bulky and polar side chains) with hydrophilic links forming the amorphous part [41]. There are 18 different natural amino acids composing fibroin with the amino acid composition consists mainly of Gly (43%), Ala (30%), and Ser (12%) [42] with a molecular weight of 3.5 - 3.6 × 10^5 Da [43].

For years, this protein polymer attracts many interests for developing innovative applications due to its unique chemical and mechanical properties. As a protein, fibroin is susceptible to biological degradation by proteolytic enzymes. The rate and extent of degradation may be highly variable, depending on a series of factors related to structural and morphological features of the polymer (fiber, film, sponge), processing conditions, as well as characteristics of the biological environment, and the presence of different mechanical and chemical stresses. The fragmentation of fibroin through mild
hydrolysis by fibroinolytic enzymes leads to its conversion to a soluble form without causing significant damage to its natural conformation which in contrast with fragmentation by powerful chemical reagents. Enzymatically generated peptides from fibroin showed various biomedical functions. The application of peptide have been expanding in the manufacturing of foods, cosmetics, pharmaceuticals, and other products [44,45]. Fibroin hydrolysates had been reported having anti-diabetic and hypertensive effects [46]. Nanoparticle prepared from Silk fibroin have received considerable attention for drug delivery due to its high binding capacity for various drugs, control of drug release properties, and mild preparation conditions [47].

The strain of Br. thermoruber, YAS-1, was isolated by Suzuki et al. [48] from the Manza hot springs Gunma, Japan which was known to produce strongest fibroin-degrading activity. The strain was identified as B. thermoruber based on the phylogenetic analysis on 16S rRNA sequence which was shared 99.5% identity with B. thermoruber DSM 7064T. Biochemical characteristics (i.e. gram positive, aerobic, short rod 0.8–1.0 ~ 2.5–4.8 μm) have been reported as belonging to B. thermoruber species by the development, optimal cultivation or subterminal of oval endospores in swollen sporangia at temperatures from 45 to 55 °C.

Suzuki et al. [48] incubated fibrous fibroin (100 mg) from the culture supernatant (25 ml, obtained at 24 h) at 55 °C for 24 h with reciprocal shaking (150 rpm). The weight of insoluble fibrous fibroin was gradually decreased to nearly 30% of the initial weight after 14 days, indicating that 70% of the fibrous fibroin had been degraded to become soluble. The result showed that YAS-1 strain produced fibroinolytic enzyme that seemed to degrade the crystalline regions to some extent in addition to the amorphous regions of fibroin during incubation since the crystalline region is about 60% of the B. mori’s fibroin, based on the digestibility by chymotrypsin [49]. This investigation also reported that the fibroinolytic enzyme produced by strain YAS-1 was more specific to the H-chains. The significant damaged of fibrous fibroin by the fibroinolytic enzyme produced by strain YAS-1 is presented in Fig 4.

![Figure 4. Scanning electron micrographs of the fibrous fibroins. (A) Starting fibroin; (B) after 14 days incubation with enzyme produced by YAS-1 (modified from Suzuki et al., [48]).](image)

4. Lon protease producer

Lon protease is a family of proteases which is found in archaea, bacteria, and eukaryotes. Lon protease (La) is a member of AAA+ superfamily [50] this comprises an N-terminal domain component that includes, i.e., a central ATPase domain with ATP binding and hydrolysis activity and a C-terminal protease dominance on one single polypeptide [51]. Although La is active as a homooligomer, nevertheless the oligomeric state of La is still indistinct. In E. coli, La is found as a tetramer or an octamer [52], as a tetramer to a hexamer in Mycobacterium [53] and as a hexamer or a heptamer in yeast mitochondria [54].

Lon protease plays an important role in intracellular protein degradation. Critical roles played by bacterial LonA are in general protein quality control and in cell regulation by degrading abnormal
proteins and cleavage of specific regulatory proteins, respectively [55]. These roles are important for radiation tolerance, cell division, capsular oligosaccharide synthesis and biofilm formation [56]. Under stress conditions, Lon play a role to prevent proteotoxicity and maintain organelle quality control and mtDNA biogenesis by targeting aberrant proteins and conditional digest of regulatory proteins [57]. Quiro’s et al. [58] ATP-Dependent Lon Protease was found to regulate the bioenergy of tumors through the reprogramming of mitochondrial activity. The experiment on generation of mice deficient in Lon protease (LONP1) has showed the essential roles of Lon protease in cell and organismal viability.

Br.thermorumber WR-249 lonprotease gene was cloned and expressed in E.coli and its protein product was purified and characterized by Lee et al. [59]. They reported that Br. thermorumber WR-249 produced an 88 kDa protein encoded by Bt-Lon which consisted of an N-terminal domain, a central ATPase domain which includes an SSD (sensor- and substrate-discrimination) domain, and a C-terminal protease domain. Like those produced by B. Thermoruber, Bt-Lon is a thermostable enzyme. The structure was homoheaxamer and heat inducible gene with optimal temperature of 70°C for ATPase activity and 50°C for peptidase and DNA-binding activities. This implies that to regulate their physiological needs, the functions of Lon protease in thermophilic bacteria may be switched, depending on temperature with melting temperature was estimated at 71.5 °C. Compared to Lon produced by mesophilic E. coli which have homotetrameric structure, Bt-Lon is a homohexamer of 88 kDa subunits. This result corresponded with statement of Vieille, C. and Zeikus, G.J. [60] that thermophilic protein composed of more oligomer than mesophilic one. The sequence of amino acid makes the properties of Bt-LON differences from those produced by mesophilic in the rigidity, electrostatic interactions, and hydrogen bonding which is relevant to thermostability.

5. Cell factory for exopolysaccharide
Exopolysaccharides are polysaccharides which are synthesized and secreted extracellularly by microorganism. There are two group of exopolysaccharides based on monomeric composition, homopolysaccharides and heteropolysaccharides [61]. Based on the nomenclature, homopolysaccharides contain only one type of monosaccharide while heteropolysaccharides are composed by varying size from disaccharides to heptasaccharides Exopolysaccharides are water soluble polymers that can be readily prepared by a short process of fermentation. Because of their new chemical structures they compete with polysaccharides derived from plants and algae, and offer many new characteristics and applications. Their novelties are highlighted by recent publications encompassing the structure and function, chemical properties and their role in microbial ecology, medicine, dairy industry, biofilms, and corrosion and other biotechnology applications. Some of which produced in large scale are xanthan, gellan, and alginate. More upcoming industrial application of EPS which are in intensive search are as emulsifier, geller and as carbon source for polysaccharide conversion.

Many mesophilic EPS producing microorganisms with industrial value are pathogenic. Some thermophiles (obligate and hyperthermophile) which are known as EPS producer showed novel metabolic pathways [62] and in such a way they are also expected to produce exopolysaccharides with novel properties. EPS manufacturing with thermophile shows low accumulation of biomass and corresponding low EPS production, however it proposes short fermentation cycles, reduced fermentation broth viscosity, high molecular weight polymers and stable emulsions as well as nonpathogenic ones, suitable for use in food, pharmacy and cosmetic industries.

Radchenkova et al. [24] screened EPS producing bacteria isolated from nine hot springs located in three geographically separated areas in Bulgaria. A total of 38 aerobics showed the ability to produce EPS. From 12 strains which produced more than 20 μg/ml EPS, nine of them were identified as Br. thermorumber. Compared to mesophilic processes for EPS production, thermophile produced EPS in shorter period as showed by Br. thermorumber strain 423 with only less than one day [63]. Another strain, Br. thermorumber 438, even showed a very short period for EPS production that is in eight hours [24]. It is significantly different to those produced by mesophilic which needs at least few days to several weeks. Br. thermorumber strain 423 is highly recommended as potential microbial cell factory
for EPS both due to its high yield and productivity compared to other EPS-producing thermophilic bacteria.

6. The future potency based on genomic analysis

Draft genome sequence of *Br. thermoruber* was first submitted by Yildiz et al. [63]. Illumina HiSeq 2000 technology is used to sequence whole genome generating sequence data of 603 Mb which provides approximately 150-fold coverage. The size of nucleotide is 4,433,037 bp with total of 4446 coding sequences and 112 RNA genes which was generated from gene prediction and genome annotation. G+C content is 58.46% with coding region length of 3,830,130 bp. Among 3,020 protein-coding genes, 1,426 hypothetical proteins had no match to any known proteins which have high possibility encode novel protein with potential application.

Although genome analysis already been addressed by Yildiz et al. [64], it still calls for further laboratory works to obtain evidences on the exist of some genes. These results open up opportunities to conduct further study on the potency and mechanisms of any genes that previous found in *Br. thermoruber* such as: keratinolitic, fibroinolitic, Lon Protease, and EPS as describe above. These studies are important to design the strategies on optimizing of production and engineering for improving yield and properties of product so that it meets industrial needs. From genome analysis, Yildiz et al. [64] predicted some genes that code potential enzyme for industry as presented in Table below.

| Predicted Enzyme | Potency Industry |
|------------------|------------------|
| Lipase           | Dairy, baking, pulp and paper, polymer, detergent, leather, cosmetic, waste management |
| Glucoamylase     | Baking, Beverages, pulp and paper, detergent, leather, Beverages, Animal feed, |
| Endo-1,4-β-glucanase | Sweetener, prodrug in chemotherapy |
| Dipeptidase      | Detergent, textile, Meat, feed, leather, waste management, chemically industry, feed, medical uses, silver recovery |
| Serine protease  | Therapeutic, fermented food, sanack and biscuit, anaitic, fine chemical, cost drug |
| Amidase          | Carboxilic production, bioremediation, surface modification of polymer, |
| Nitrilase        | |

Yildiz et al. [64]

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