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Isolation and Characterization of Thermophilic *Bacillus subtilis* subsp. inaquosorum CGR-1 from Cangar Hot Springs

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ABSTRACT

Bioindustries often involve biochemical processes that occur at higher temperatures. However, most proteins, including enzymes, lose their structural integrity and functionality at higher temperatures. Thus, thermostable enzymes from thermophilic microorganisms are best suited candidates for successful bioprocessing under such conditions. Indonesia is one of the best study sites for performing bioprospecting of thermostable enzyme-producing thermophilic microorganisms due to the numerous hot springs. To explore the biodiversity of thermophilic microorganisms with potential industrial applications, we isolated and characterized thermophilic bacteria from the Cangar hot spring, Batu, East Java, Indonesia. One isolate (CGR-1) showed growth at 60˚C and was identified as *Bacillus subtilis* subsp. *inaquosorum* based on 16s rRNA gene sequencing followed by bioinformatic analysis. This is the first report on the isolation of *Bacillus subtilis* subsp. *inaquosorum* CGR-1 from Indonesia, especially from a hot spring environment. This isolate showed cellulolytic and amylolytic activity at 50˚C, which would encourage further exploration on the industrial and environmental applications.

1. Introduction

Bioindustries strive to produce high value chemicals such as pharmaceutically active compounds, biopolymers, and biofuels using economical and sustainable raw materials and mainly in/using microorganisms. However, the mesophilic microorganisms or enzymes used in most bioindustries cannot endure high temperatures that are typically used in some biochemical processes [[1]]. For example, high temperatures between 60°C–105°C are needed for the liquefaction of starch and solubilization of lignocellulosic materials in biofuel industries, and also for the process of bio-bleaching in pulp & paper industries [1–3]. Thus, the development and application of thermostable enzymes would help to broaden the operating conditions and increase the efficiency of bioprocessing.

Genetic and protein engineering provide powerful means to construct thermostable enzymes, however, these approaches are expensive, labor-intensive, and time consuming. Alternatively, exploring naturally available thermostable enzymes is attractive. Thermostable enzymes are mainly produced by

thermophilic microorganisms which grow optimally at temperatures more than 50℃. Indonesia, with around 70 active volcanoes and a large number of geothermal areas and hot springs, is an ideal place for exploring the biodiversity of thermophilic microorganisms and for obtaining potential novel thermostable industrial enzymes [4].

In this study, we isolated and characterized thermophilic bacteria from Cangar hot spring Batu, East Java, Indonesia. Cangar hot springs are reportedly rich in biodiversity of thermophilic bacteria, especially belonging to the genus *Bacillus*, which are identified producers of thermostable industrial enzymes such as α-amylase, chitinase, and lipase [5–7]. Based on 16s rRNA gene sequencing and followed by bioinformatic analysis, the bacterial isolate obtained in this study was identified as *Bacillus subtilis* subsp. inaquosorum, which was not previously isolated in Indonesia. The isolate was capable of producing cellulolytic and amylolytic enzymes at high temperatures and thus has significant potential for industrial application.

2. Materials and methods

2.1. Sampling site and method

The Cangar hot spring is situated in the Raden Soerjo Forest Park, Batu, East Java, Indonesia at 112° 32’ 0’’ E longitude and 7° 44’ 31’’ S latitude. Samples (water and sediments) were collected from different sites of the hot spring in sterile insulated water bottles and immediately transported to a laboratory for further analysis.

2.2. Isolation of bacteria and determination of thermotolerance

Bacteria were isolated in nutrient agar (NA) media (Merck, Germany) using the serial dilution technique. Ten grams of soil sample was diluted in 90 ml of sterile NaCl solution (0.85%) in 250 ml conical flasks and shaken on an orbital shaker at 100 rpm for 1 h to obtain a homogenized soil suspension. The suspension is then let stand until the suspension separates into two parts. The top layer was then serially diluted and dilutions of 10−2 and 10−3 were spread onto NA plates and incubated at 50°C for 48 h. Single colonies of different morphological characteristics, including shape, color, elevation, and margin, were identified from the different plates. Colonies were transferred into freshly prepared NA slants and incubated at 50 °C, 60 °C, and 70 °C for 48 h. The isolated colonies cultured on freshly prepared NA slants at 50°C for 48 h were subsequently also kept at 4 °C for further studies.

2.3. Assessment of cellulolytic activity

The cellulolytic activity of one isolate (CGR-1), which showed growth both at 50 °C and 60 °C, was evaluated using the CMC plate assay [8]. A single CGR-1 colony was streaked onto CMC agar plates and incubated at 40 °C, 50 °C, and 60 °C for 48 hours. After incubation, the agar medium was overflown with Congo red solution (1% w/v) for 30 min. The Congo red solution was then discarded and the plates were further treated by flooding with 1 M NaCl for 30 min. The formation of a clear zone of hydrolysis indicated cellulose degradation.

2.4. Assessment of amylolytic activity

The amylolytic activity of CGR-1 was evaluated using the starch agar plate assay [9]. A single CGR-1 colony was streaked onto starch agar plates and incubated at 40 °C, 50 °C, and 60 °C for 48 hours. After incubation, the agar medium was overflown with Lugol’s Iodine. A clearance zone around the colony indicated starch degradation.

2.5. Identification and characterization of the bacterial isolate.

The CGR-1 isolate was characterized microscopically by the Gram staining technique. Biochemical identification of the isolate was also conducted using Microbact™ 24E system kit (Thermo Fisher Scientific, USA).

2.6. 16S rRNA gene amplification and sequencing.

Genomic DNA of CGR-1 was extracted and purified using Quick-DNA™ Fungal/Bacterial Miniprep Kit (Zymo Research, USA). Amplification of the target region of the 16s rRNA 27F gene was done using 27F (5′- AGA GTT TGA TCM TGG CTC AG-3′) and 1492R (5′- TAC GGY TAC CTT GTT ACG ACT T-3′) primers [10]. Purification of the PCR product was performed using DNA Clean & Concentrator™-5 (Zymo Research, USA). The purified PCR products were sequenced by First Base (Malaysia).

The deduced sequences were compared for homology against the NCBI database of 16s ribosomal RNA sequences using Nucleotide BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi). A phylogenetic tree (Neighbor-Joining (Unrooted Tree)) was constructed using the NCBI Blast Tree Method. The results of BLAST analysis were also confirmed using 16s-based ID from EZBioCloud [11].

3. Results and discussion

3.1. Characteristics of sampling site

Sampling was conducted in February (rainy season in Indonesia), and the temperature of the sampling site was 50.1 °C and the pH was 6.3. The sampling site was fed with plant litter, and the presence of thermophilic microorganisms at this site might indicate these are capable of utilizing the cellulosic materials from the fed dead plant materials.

3.2. Morphological and biochemical characteristics of the isolate

Only one bacterial colony was isolated from the sampled water and sediment. This might be due to the high incubation temperature (50 °C) used for the incubation of inoculated water and sediment, whose purpose is to isolate thermophilic and thermotolerance bacteria only. The bacterial isolates were screened for thermotolerance between 50 °C–70 °C. The isolate (CGR-1) could survive at temperatures of 60 °C **(Figure 1a)**, and no colony growth was observed at 70°C. The isolate formed white round colonies with raised elevation and a lobate margin. Microscopic observation revealed the isolate to be rod-shaped Gram-positive bacteria **(Figure 1b).** Biochemical tests using Microbact™ 24E system were also performed **(Table 1).** Based on the morphological characteristics, microscopic observation, and biochemical tests including catalase, nitrate reduction, and citrate utilization, the CGR-1 isolate was hypothesized to belong to the genus *Bacillus* [8].

Table 1. Biochemical characteristics of thermophilic *Bacillus mycoides*

No	Characteristics	Observation result	No	Characteristics	Observation result
1	Oxidase	+	**15**	TDA	-
2	Motility	-	**16**	Gelatin	+
3	Nitrate	+	**17**	Malonate	-
4	Lysine	-	**18**	Inositol	-
5	Ornithine	-	**19**	Sorbitol	-
6	H_2s		**20**	Rhamnose	-
7	Glucose	-	**21**	Sucrose	-
8	Mannitol	-	**22**	Lactose	-
9	Xylose	-	**23**	Arabinose	-
10	ONPG	+	**24**	Adonitol	-
11	Indole	-	**25**	Raffinose	-
12	Urease	+	**26**	Salicin	-
13	VP	-	**27**	Arginine	-
14	Citrate	-	**28**	Catalase	+

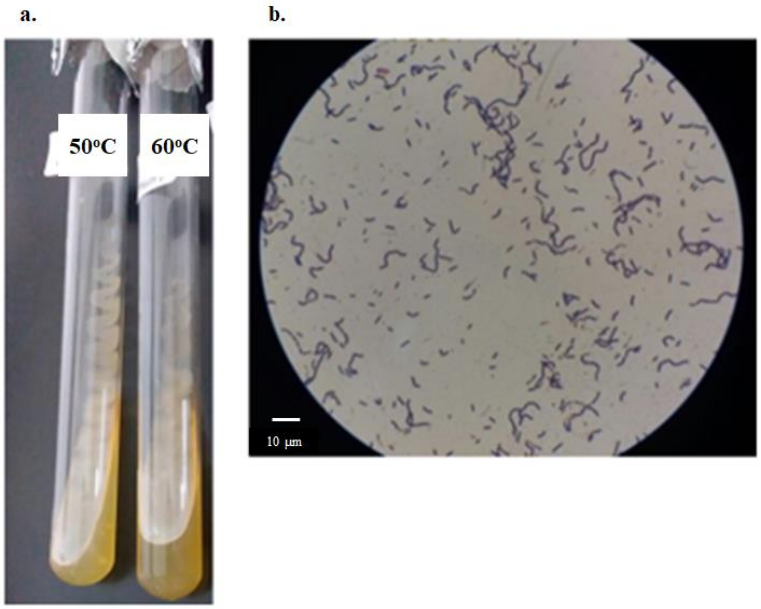

Figure 1. (a) Isolate growth on Nutrient Agar media after 48 hours incubation at 50ºC and 60ºC. **(b)** Gram staining result of the isolate

3.3. Molecular identification of the CGR-1 isolate

The CGR-1 isolate was identified based on partial 16s rRNA gene sequencing. Alignment with reference sequences from NCBI database of 16s ribosomal RNA sequences using BLAST and 16s-based ID from EZBioCloud revealed that CGR-1 was 99.93% and 99.86% identical with *Bacillus subtilis* subsp. inaquosorum, respectively. A neighbor-joining tree indicating the phylogenetic position of CGR-1 isolate was also constructed **(Figure 2).**

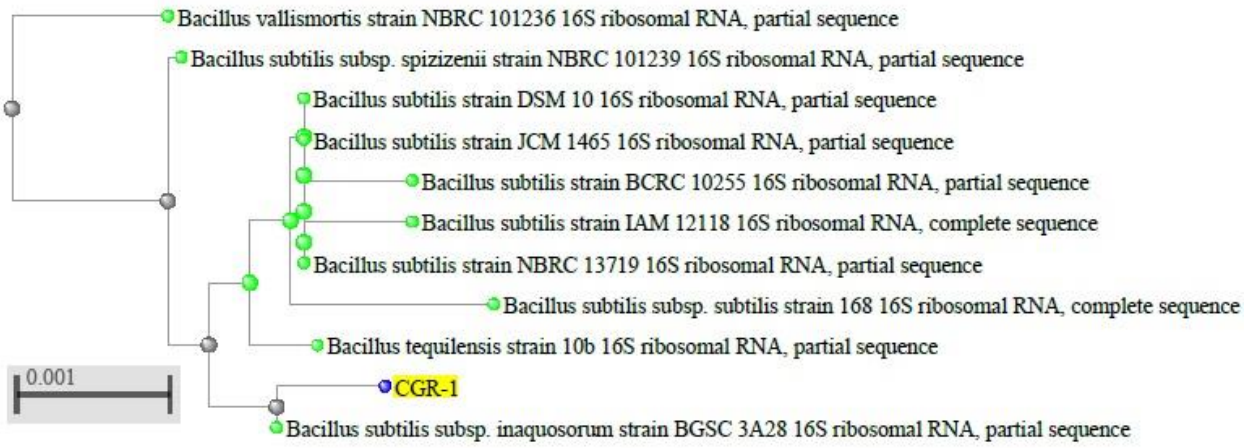

Figure 2. Neighbor-joining tree showing the phylogenetic position of CGR-1 isolate.

3.4. Cellulolytic and amylolytic activity of the CGR-1 isolate

Bacillus subtilis strains reportedly possess cellulases and amylases, and therefore the CGR-1 isolate was also assessed for its cellulolytic and amylolytic activities [12–14]. After 48 h incubation at 50 °C and treatment with Congo red, a clear zone was observed in CMC agar around CGR-1 colonies, which indicated the hydrolysis of CMC via cellulases **(Figure 3a)**. Amylolytic activity was also observed in CGR-1 colonies as shown by the presence of a clear zone around the CGR-1 colonies after 48h incubation at 50 °C and treatment with Lugol's iodine, indicating starch hydrolysis by amylase **(Figure 3b)**.

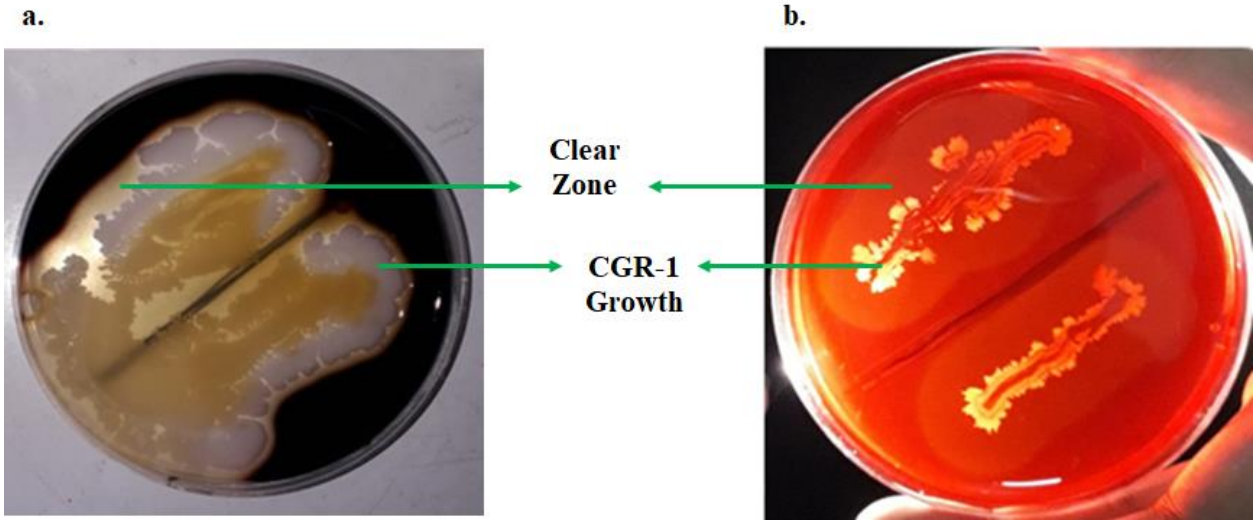

Figure 3. (a) Amylolytic and **(b)** cellulolytic activities of CGR-1 isolate indicated by clear zone surrounding the bacterial growth after treatment with Lugol's iodine and Congo red, respectively.

3.5. Discussion

Hot springs are one of the most explored ecosystems for biocuration of thermostable enzyme-producing thermophilic microorganisms. Due to its location in the Pacific ring of fire, Indonesia provides a rich ecosystem at hot spring sites where numerous thermophilic bacteria species can thrive

successfully. A few of these bacterial isolates were reported to produce thermostable industrial enzymes such as amylase, lipase, protease, and xylanase [15–17]. Interestingly most of the isolates that have been isolated from the Cangar hot spring, the current study site, are identified as members of genus *Bacillus*, which predominantly includes *Bacillus licheniformis* strains [5–7].

In this study, the only isolate obtained, *Bacillus subtilis* subsp. inaquosorum, showed growth at 60 °C. This subspecies was first isolated in 2009 from arid soils of the Death Valley in California, United States. Resultantly, it derived its name inaquosorum (from soils poor of water) [18]. Our finding is interesting since we isolated the exact subspecies from an aquatic environment in this study. Furthermore, to our knowledge, this is the first report on the isolation of *Bacillus subtilis* subsp. inaquosorum from Indonesia. This subspecies has been previously isolated from United States, South Korea, and India [19–21].

The *Bacillus subtilis* subsp. inaquosorum CGR-1 isolate in this study exhibited amylolytic and cellulolytic activities at 50 °C, similar to previous reports on thermostable enzyme-producing Bacillus subtilis strains [12–14]. Further research will be conducted on the characterization and production of thermostable amylolytic and cellulolytic enzymes. In addition to these enzymes, *Bacillus subtilis* subsp. inaquosorum reportedly also produces alkaline cellulase and alkaline mannanase, which are valuable enzymes in pulp and paper, food, and pharmaceutical industries. In addition, it also produces a lipopeptide biosurfactant, which is utilized in microbial enhanced oil recovery, bioremediation, and as an antifungal agent [19], [21–23]. Thus, the potential of our isolate to produce alkaline mannanase, alkaline cellulase, and biosurfactants will also be investigated in the future.

4. Conclusions

Biodiversity exploration of thermophilic microorganisms in the Cangar hot spring, Batu, East Java, Indonesia, resulted in the isolation of one bacterial strain (CGR-1) capable of growth at 60 °C and possessing cellulolytic and amylolytic activity at 50 °C. CGR-1 was identified as *Bacillus subtilis* subsp. inaquosorum CGR-1. In the future, the potential of *Bacillus subtilis* subsp. inaquosorum CGR-1 in the production of valuable thermostable hydrolytic enzymes and biosurfactants will be explored.

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References

[1] S. Elleuche, C. Schäfers, S. Blank, C. Schröder, G. Antranikian, Exploration of extremophiles for high temperature biotechnological processes. Biotechnol. Adv. 25, 2015, 116.
[2] S. Elleuche, C. Schröder, K. Sahm, G. Antranikian, Extremozymes--biocatalysts with unique properties from extremophilic microorganisms. Curr. Opin. Biotechnol. 29, 2014, 116.
[3] F. Rigoldi, S. Donini, A. Redaelli, E. Parisini, A. Gautieri, Review: Engineering of thermostable enzymes for industrial applications. APL Bioeng. 2, 2018, 011501.
[4] H. Yohandini, Julinar, M. Muharni, Isolation and phylogenetic analysis of thermophile community Within Tanjung Sakti Hot Spring, south Sumatera, Indonesia. HAYATI J. Biosci. 22, 2015, 143.

[5] D. Ibrahim, H.L. Zhu, N. Yusof, Isnaeni, L.S. Hong, Bacillus licheniformis BT5.9 isolated from changar hot spring, Malang, Indonesia, as a potential producer of thermostable α-amylase. Trop. Life Sci. Res. 24, 2013, 71.
[6] F.A.A. Rashid, R.A. Rahim, D. Ibrahim, A. Balan, N.M.A. Bakar, Purification and properties of thermostable lipase from a thermophilic bacterium, bacillus licheniformis IBRL-CHS2. J. Pure Appl. Microbiol. 7, 2013, 1635.
[7] R. Chrisnasari, D. Verina, A.C. Tapatfeto, S. Pranata, T. Patjajani, M. Wahjudi, M.G. Purwanto, Isolating and characterising chitinolytic thermophilic bacteria from Cangar Hot Spring, east Java. Pertanika J. Trop. Agric. Sci. 41, 2018, 1437.
[8] B.T. Mohammad, H.I. Al Daghistani, A. Jaouani, S. Abdel-Latif, C. Kennes, Isolation and characterization of thermophilic bacteria from Jordanian hot springs: Bacillus licheniformis and Thermomonas hydrothermalis isolates as potential producers of thermostable enzymes. Int J Microbiol, 2017, 6943952.
[9] J.L. Jardine, S. Stoychev, V. Mavumengwana, E. Ubomba-Jaswa, Screening of potential bioremediation enzymes from hot spring bacteria using conventional plate assays and liquid chromatography-tandem mass spectrometry (Lc-Ms/Ms). J. Environ. Manage. 223, 2018, 787.
[10] G.K. Satyapal, S.K. Mishra, A. Srivastava, R.K. Ranjan, K. Prakash, R. Haque, N. Kumar, Possible bioremediation of arsenic toxicity by isolating indigenous bacteria from the middle Gangetic plain of Bihar, India. Biotechnol. Rep. 17, 2018, 117.
[11] S.H. Yoon, S.M. Ha, S. Kwon, J. Lim, Y. Kim, H. Seo, J. Chun, Introducing EzBioCloud: a taxonomically united database of 16S rRNA gene sequences and whole-genome assemblies. Int. J. Syst. Evol. Microbiol. 67, 2017, 1613.
[12] S. Maity, S. Mallik, R. Basuthakur, S. Gupta, Optimization of solid state fermentation conditions and characterization of thermostable alpha amylase from Bacillus subtilis (ATCC 6633). J. Bioprocess. Biotech. 5, 2015, 1.
[13] M. Sharma, B. Kumar Bajaj, Optimization of bioprocess variables for production of a thermostable and wide range pH stable carboxymethyl cellulase from Bacillus subtilis MS 54 under solid state fermentation. Environ Prog Sustain Energy. 36, 2017, 1123.
[14] A. Dhyani, R. Jain, A. Pandey, A. Sharma, K. Dhakar, V. Pande, Diauxic growth pattern in thermophilic Bacillus spp with respect to production of thermostable amylase. J. Curr. Microbiol. 1, 2018, 15.
[15] I. Febriani, R. Hertadi, P. Kahar, M.F. Akhmaloka, Isolation and purification of novel thermostable alkaline lipase from local thermophilic microorganism. Biosci. Biotechnol. Res. Asia. 7, 2010, 617.
[16] A. Arzita, S. Syamsuardi, A. Agustien, Y. Rilda, The diversity of the alkaline protease producers, thermophilic obligate Bacillus spp., from sungai Tutung Hot Spring, Kerinci, Jambi, Indonesia. J. Pure. Appl. Microbiol. 11, 2017, 1789.
[17] R.A. Arfah, A. Ahmad, M.N. Djide, M. Anis, M. Zakir, Production optimization and characterization of amylase enzyme isolated from Termofil bacteria Bacillus sp RSAII-1b from Lejja Hot Spring south Sulawesi. Am. J. Biomed. Life Sci. 3, 2015, 115.
[18] A.P. Rooney, N.P.J. Price, C. Ehrhardt, J.L. Swezey, J.D. Bannan, Phylogeny and molecular taxonomy of the Bacillus subtilis species complex and description of Bacillus subtilis subsp. inaquosorum subsp. nov. Int. J. Syst. Evol. Microbiol. 59, 2009, 2429.
[19] C.A. Knight, M.J. Bowman, L. Frederick, A. Day, C. Lee, C.A. Dunlap, The first report of antifungal lipopeptide production by a Bacillus subtilis subsp. inaquosorum strain. Microbiol. Res. 216, 2018, 40.

[20] J. Carrazco-Palafox, B.E. Rivera-Chavira, N. Ramírez-Baca, L.I. Manzanares-Papayanopoulos, G.V. Nevárez-Moorillón, Improved method for qualitative screening of lipolytic bacterial strains. MethodsX. 5, 2018, 68.
[21] S. Regmi, H.Y. Yoo, Y.H. Choi, Y.S. Choi, J.C. Yoo, S.W. Kim, Prospects for bio-industrial application of an extremely alkaline mannanase From Bacillus subtilis subsp. inaquosorum CSB31. Biotechnol. J. 12, 2017, 1700113.
[22] R. Gautam, J. Sharma, Optimization, purification of cellulase produced from Bacillus subtilis subsp. inaquosorum under solid state fermentation and its potential applications in denim industry. Int. J. Sci. Res. 3, 2012, 1759.
[23] T. Varadavenkatesan, V.R. Murty, Production and properties of a lipopeptide biosurfactant by B. subtilis subsp. inaquosorum. J. Microbiol. Biotechnol. Res. 3, 2013, 63.