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Identification of Protease-Producing Bacteria Isolated from Banyuwedang, Bali, and Characterization of its Protease

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Abstract

Proteases or peptidases is known as a largest group of hydrolytic enzymes and have been applied in various industries such as food, pharmacy, leather, detergent and waste treatment. Although they are also produced by plants and animals, microbes remain the main source of proteases in the world market which mostly derived from *Bacillus* sp. Aims of this research were to identify isolate BII-1 and study its protease. Analysis of 16Sr RNA sequencing showed the identity of BII-1 as *Bacillus subtilis* (99% similarity with the same species in GenBank). It was found that protease from BII-1 exhibited optimal temperature and pH of 50 ^{o}C and 8-9, respectively. It was activated by Li^{2+}, Na^{2+}, Mg^{2+} and K^{+}. The degenerated primer for protease gene was designed, and a partial protease gene was amplified from BII-1. The sequencing result showed that this amplified gene shared 100 and 99% similarity with those from *Geobacillus thermophiles* and *Bacillus subtilis* in the GenBank, respectively.

Keywords: protease, bacteria, *Bacillus subtilis, Geobacillus thermophylus*

1. Introduction

Proteases are enzymes that catalyze proteolysis, which are classified as class 3 in enzyme classification. Based on their site of action, proteases are grouped into two majors, exopeptidases and endopeptidases. Proteases can be further classified based on catalytic sites as serine proteases, aspartic proteases, cysteine proteases and metalloproteases. Based on the optimal pH range of proteolytic activity, they are classified into acid, alkaline, and neutral proteases (Rao, M.B., Tanksale, A.M., Ghatge, M. S., & Deshpande, V.V. 1998).

Proteases have been applied in various industrial processes such as degumming of silk, leather, pharmaceutical, food, detergent, processing of keratin residues, contact lens cleansing, biofilm removal, selective delignification of hemp, isolation of nucleic acid, photography and pest control (Kumar & Bhalla, 2005; Pawar, R., Zambare, V., Barve, S., & Paratkar, G., 2009; Mahmoodi, N.M,, Moghimi, F., Arami, M., & Mazaheri, F., 2010; Giri et al., 2011; Harde, S.M., Bajaj, I.B., & Singhal, R.S., 2011; Leslie, 2011; Joshi & Satyanarayana, 2013; Khan, 2013; Motyan, J.A., Toth, F., & Tozser, J., 2013; Kumar, D., & Bhalla, T.C., 2015; Singh & Bajaj, 2016; Suwannaphan, S., Fufeungsombut, E., Promboon, A., & Chim-Anage, P., 2017). More recently proteases have been applied to produce high quality food supplement from hydrolyzed protein (Moreno et al., 2017).

Proteases found in the market is mostly produced by microorganisms although there are some produced by plants and animals. The total value of protease covers 60% of the total worldwide enzyme sales (Rani, K., Rana, R., & Datt, S., 2012). This is because microbial properties exhibit advantageous properties that are useful for biotechnological processes such as their resilience under extreme temperature, pH and the presence of inhibitors (Singh & Bajaj, 2017).

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Hot spring become a promising source for discovering novel enzyme, especially thermostable enzyme as some proteases isolated from hot spring showed characteristics which meet industrial needs. *Bacillus* sp. isolated from hot springs in Limpopo Province, South Africa was reported producing 3 kinds of protease which can reduce phenol concentration. This is a substansial characteristic to be used for bioremediation (Jardine, J.L., Stoychev, S., Mavumengwana, V., & Ubomba-Jaswa, E. 2018). *Brevibacillus thermoruber* OA30 produced protease 32-F38 which showed a great thermostability after 240 min. It also showed a highly stable activity in the presence of different detergents and solvents (Gomri et al., 2018). An anaerobic thermophic bacteria had been isolated from a hydrothermal hot spring in Algeria, *Caldicoprobacter algeriensis* strain TH7C1, which showed capability of producing protease with high keratinolitic activity. The activity reach 21000U/ml in just 24 hours of incubation at 50 ^{o}C. The properties of keratinases produced by this strain is promising to be applied for bioremediation and for the dehairing in the leather processing industry (Bouacem et al., 2016).

Indonesia is located on the ring of fire with countless of high temperature environments such as hydrothermal vent and hot springs which are yet to be explored. Exploration of microbial diversity to obtain novel proteases suitable for industrial processes is urgently needed. Aims of this research were to examine the properties of a protease produced by the bacterial isolate of BII-1 isolated from hot spring in Banyuwedang, Bali, identify the isolate and its partial protease gene .

2. Materials and Methods

2.1. Microorganisms and Cultured Condition

The protease-producing bacterial isolate BII-1 was isolated from Banyuwedang, Bali, and stored as a glycerol stock in -80 ^{o}C. An aliquot of the glycerol stock was inoculated into a 150-ml flask containing 50 ml LB medium. After 24 h of incubation at 37 ^{o}C, 10 % of starter culture was transferred into a 500-ml flask containing 150 ml production medium (0.1% of K_2HPO_4, 0.1% NaCl, 0.5% yeast extract, 0.01% $Mg_2SO_4.7H_2O$ and 2% of skim milk). The cultivated microbial cells were harvested after 36 hours by centrifugation at 10.000 xg for 10 minutes for being used in 16S rRNA analysis, and the resulting supernatant was used for enzyme characterization.

2.2. DNA Extraction and 16S rRNA Identification

Identification of protease-producing bacterium was conducted using 16S rRNA gene sequencing and analysis.Genomic DNA was initially extracted using TIANamp Bacteria DNA Kit protocol. The extracted DNA was amplified using the universal 16S rRNA primers 27F-5'-AGAGTTTGATCCTGGCT CAG-3' and 1492R 5'-GGTTACCTTGTTACGACTT-3' (Lane, 1991). The sequence was subjected to a homology search against NCBI DNA database using BLAST Nn (Basic Local Alignment Search Toll) (Altscul, S.F., Gish, W., Miller, W., Myers, E.W., & Lipman, D.J. 1990).) then aligned using Clustal W program (Higgins, D.G., Thompson, J.D., & Gibson, T.J. (1996). The neighbor-joining mid-point analysis (Kim, 1993) were performed using MEGA7 (Kumar, S., Stecher, G., & Tamura, K. 2016).

2.3. Partial Protease Gene Cloning

Degenerated primer pair for amplifying partial protease genes (KerF 5'-TAYAYHGTNGGNTTYAAR-3'; KerR 5' 5'NARNACYTTNACNCCRTA-3' /IDT-Integrated DNA Technologies) was designed and used to amplified a partial protease gene from the isolate BII – 1. The correct-sized PCR product (approximately 331 bp) was run on 1% agarose with 1 kb plus marker (Thermo Scientific). The appropriate size of band was purified using peqGOLD Gel Extraction Kit and sent for sequencing.

2.4. Protease Assay

Protease activity was determined by applying a modified method given by Takami, H., Akiba, T., & Horikoshi, K. (1989). The mixture contained of 250 µL of 1% casein (in Tris-Cl buffer pH of optimum enzyme) was incubated at optimum temperature with 250 µL of protease sample for 10 minutes. The reaction was stopped by adding 500 µL of 0.4 M TCA. The mixture was centrifuged at 10.000 x 9 for 10 minutes. Supernatant (500 µl) was mixed with 2500 µL of 0.4 M Na_2CO_3 and 250 µL of 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 GenesysTM. Tyrosine standard solution, in the range of 0-1000 mg/L was prepared in triplicates to obtain a standard curve. One unit (U) of protease was defined as the amount of tyrosine (µg/ml) in one minute under the defined assay conditions.

2.5. Protease Characterization

The protease activity at different pHs, temperatures and metal ions was investigated. To determine the optimum temperature of enzyme activity, 250 µl of enzyme was mixed with 250 µL of 1 % casein in TrisCl Buffer pH 9 and incubated at different temperatures (50–70^{o}C) for 10 minutes and the reaction was

terminated by adding 500 µL of 0.4M trichloro acetic acid (TCA). Then the protease activity was measured as described earlier. The effect of different pHs on protease activity was determined by incubating 250 µL enzyme with 250 µL 1% casein in 0.05 M buffer of acetate (pH 5-6), phosphate (pH 6-8) and TrisCl (pH 8-10) for 10 minutes at 50°C. The enzyme activity was terminated by adding 500µL of 0.4M TCA. Effect of various metal ions on enzyme activity was determined by incubating the enzyme with 1% of casein in appropriate buffer containing metal ions (Co^{2+}, Mg^{2+}, Li^{2+}, Fe^{2+}, Cu^{2+}, Zn^{2+}; Mn^{2+}, and Ca^{2+}) in the form of chloride (Cl) salt at the final concentration of 5 mM at 50°C for 10 minutes. The enzyme activity without metal ion was used as the negative control and was considered as 100% activity.

3. Results and Discussion

3.1. 16S rRNA Identification

Banyuwedang Bali is hot spring located in Desa Pejarakan, 140 km from Denpasar, Indonesia. The hot spring is in the center of mangrove forest with temperature of 44.8 °C and pH of 8.1. Some bacteria had been isolated from this site and kept as collection of Culture Collection laboratorium at Research and Development Center for Marine and Fisheries Product Processing and Biotechnology. One of those, BII-1, showed the capability to degrade skim milk by forming a large clear zone surrounding its colony (Figure 1). Its partial 16S rRNA gene of 1465 bp showed 99 % similar to *Bacillus subtilis.* (Figure 2).

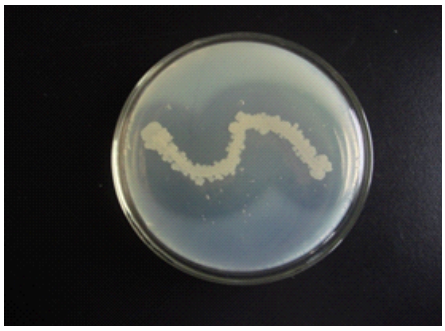

Figure 1. A clear zone formed by BII-1 on solid MSM with 2% of skim milk

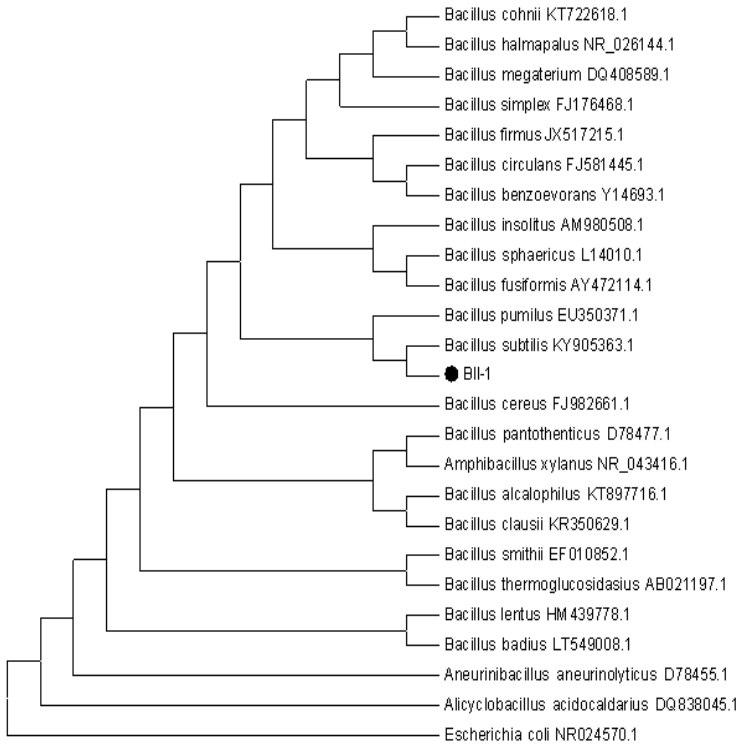

Figure 2. Neighbor-joining phylogenetic tree (bootsrap = 1000) constructed by MEGA7 showing the relationship of BII-1 with members of *Bacillus* present in GenBank based on 16S rRNA gene analysis

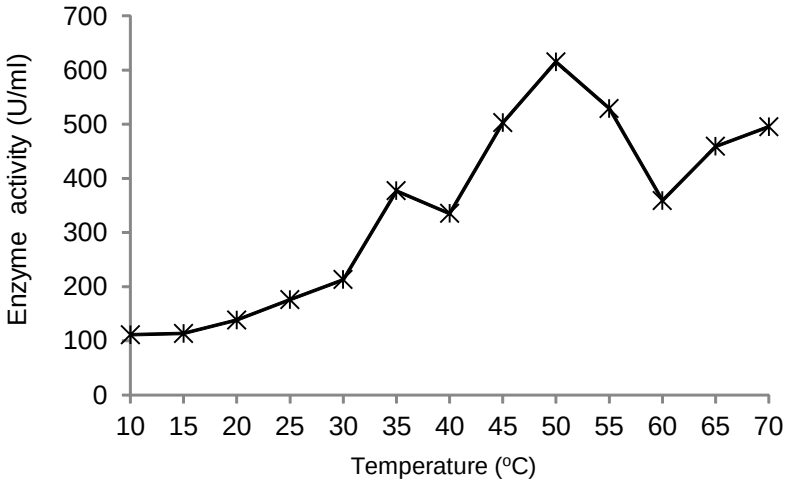

Figure 3. Protease BII activity at various of temperatures. The assay was performed using 1 % casein in 0.01 mM TrisCI buffer pH 9

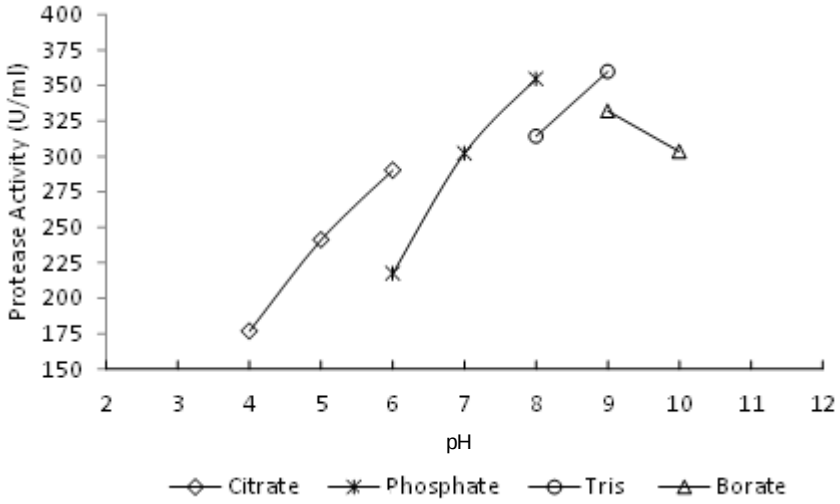

Figure 4. Activity of BII-1 protease at various of pHs. The assay was performed using 1 % casein at 50 °C

Members of the genus *Bacillus* are important producers of proteases that account for 35% of the global market of industrially useful microbial enzymes (Jayakumar, R., Jayashree, S., Annapurna, B., & Seshadri, S. 2012). Some preeminence characteristic of protease-producing *Bacillus* is their ability to produce enzymes that are suitable for industrial processes (Priya et al., 2014; Rehman et al., 2017), which are stable at extreme temperature, pH conditions, and the presence of solvents, detergents and other potential enzyme inhibitors (Joshi & Satyanarayana 2013). Particularly, *Bacillus subtilis*, *B. amyloliquefaciens*, and *B. licheniformis* showed excellent fermentation properties with high product yields and lack of toxic byproducts (van Dijl, J., & Hecker, M. 2013; Singh & Bajaj, 2016; Guleria, S., Walia, A., Chauhan, A., & Shirkot, C.K. 2016).

3.2. The Properties of Protease

Activity of the protease from BII-1 was investigated at different temperatures ranging from 10 to 70 °C using 1 % casein as substrate. BII-1 protease showed an optimum temperature at 50 °C (Figure 2). The activity significantly enhanced at 35 °C and reached the highest at 50 °C. Most of thermostable proteases reported so far were produced by members of *Bacillus* (Anandharaj, M., Sivasankari, B., Siddharthan, N., Rani, R. P., & Sivakumar, S. 2016); Singh & Bajaj, 2017). Effect of temperature on the activity of BII-1 protease showed similar pattern with that produced by *Bacillus tequilensis* P15 with the optimum temperature of 50 °C (Bose, A., Pathan, S., Pathak, K., & Keharia, H. 2014). *Bacillus pumilus* MP

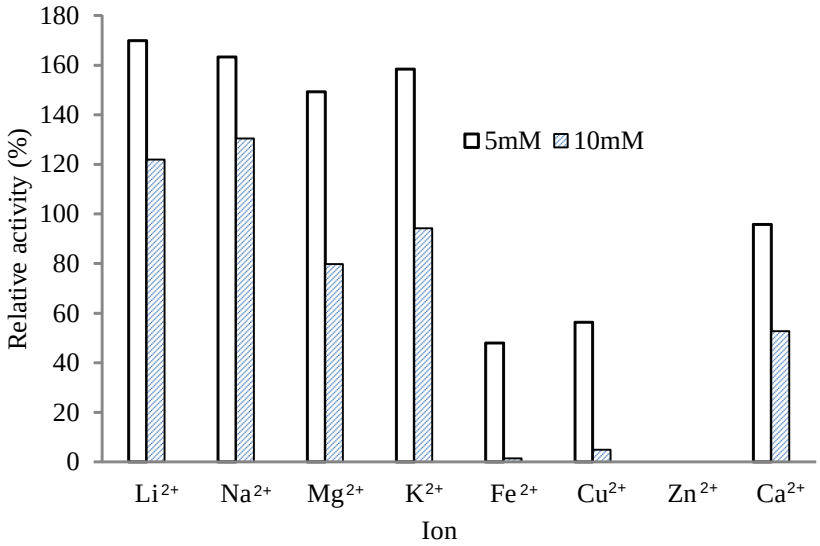

Figure 5. Activity of protease produced by BII-1 in the present of 5 and 1 mM Metal ion. The activity of protease was measured at 50 °C in Tris –Cl buffer pH of 9

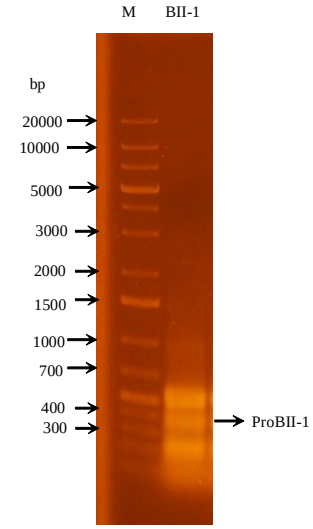

Figure 6 . ProBII-1 on 1% agarose. The size of protease band amplified with primer pair of KerF 5'-TAYAYHG TNGGNTTYAAR-3'; KerR 5' 5'NARNACYTTNACNCCRTA-3' is .350 bp (M=Marker)

27 isolated from sea water samples of Laut Selatan produced protease with the same optimum temperature (Baweja, M., Tiwari, R., Singh, P. K., Nain, L., & Shukla, P. 2016). Marine *Bacillus subtilis* AP-MSU 6 produced protease with the highest activity at 40 °C (Maruthiah, T., Esakkiraj, P., Prabakaran, G., Palavesam, A., & Immanuel, G. 2013) and *B. subtilis* GA CAS8 at 50 °C (Sathishkumar, R., Ananthan, G., & Arun, J. 2015). It proved that the same species of microorganism could produce enzyme with different properties.

The effect of pH on the activity of BII protease was investigated under various pH ranging from 4 -10. The protease produced by BII-1 showed high activity at the pH range of 5 -10 with the highest activity at pH 9 in Tris buffer (Figure 3). The result showed that such buffer used for determining the optimum pH influencing the enzyme activity. As indicated in Figure 3, the enzyme activity was significantly different between phosphate and Tris buffer at the pH of 8. Similar activity pattern was also observed for citrate and phosphate buffers at pH of 6.

The activity of Protease BII-1 was activated by Li^{2+}, Na^{2+}, Mg^{2+} and K^{+} and enhanced the activity by 70, 60, 45 and 55% repectively while Fe^{2+}, Cu 2^{2+} and Zn^{2+} inhibited the enzyme activity (Figure 5). Most of

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BII-1pro    TTTCCAACAATATGGATGTTATTAACATGAGCCTTGGCGGACCTTCTGGTTCTACAGCGC  60
GQJK2pro    TTTCCAACAATATGGATGTTATTAACATGAGCCTTGGCGGACCTTCTGGTTCTACAGCGC  1079821
GeoPro      TTTCCAACAATATGGATGTTATTAACATGAGCCTTGGCGGACCTTCTGGTTCTACAGCGC  60
BacPro      TTTCCAACAATATGGATGTTATTAACATGAGCCTTGGCGGACCTTCTGGTTCTACAGCGC  721

BII-1pro    TGAAAACAGTCGTTGATAAAGCCGTTTCCAGCGGTATCGTCGTTGCTGCCGCTGCCGGAA  120
GQJK2pro    TGAAAACAGTCGTTGATAAAGCCGTTTCCAGCGGTATCGTCGTTGCTGCCGCTGCCGGAA  1079761
GeoPro      TGAAAACAGTCGTTGATAAAGCCGTTTCCAGCGGTATCGTCGTTGCTGCCGCTGCCGGAA  1389
BacPro      TGAAAACAGTCGTTGATAAAGCCGTTTCCAGCGGTATCGTCGTTGCTGCCGCTGCCGGAA  781

BII-1pro    ACGAAGGTTCGTCCGGAAGCTCAAGCACAGTCGGCTACCCTGCAAAATATCCTTCTACTA  180
GQJK2pro    ACGAAGGTTCGTCCGGAAGCTCAAGCACAGTCGGCTACCCTGCAAAATATCCTTCTACTA  1079701
GeoPro      ACGAAGGTTCGTCCGGAAGCTCAAGCACAGTCGGCTACCCTGCAAAATATCCTTCTACTA  1449
BacPro      ACGAAGGTTCGTCCGGAAGCTCAAGCACAGTCGGCTACCCTGCAAAATATCCTTCCACTA  841

BII-1pro    TTGCGGTAGGTGCGGTAAACAGCAGCAACCAAAGAGCTTCATTCTCAAGCGCAGGTTCTG  240
GQJK2pro    TTGCGGTAGGTGCGGTAAACAGCAGCAACCAAAGAGCTTCATTCTCAAGCGCAGGTTCTG  1079641
GeoPro      TTGCGGTAGGTGCGGTAAACAGCAGCAACCAAAGAGCTTCATTCTCAAGCGCAGGTTCTG  1509
BacPro      TTGCGGTAGGTGCGGTAAACAGCAGCAACCAAAGAGCTTCATTCTCAAGCGCAGGTTCTG  901

BII-1pro    AGCTTGATGTGATGGCTCCTGGCGTATCCATCCAAAGCACACTTCCTGGAGGCACTTACG  300
GQJK2pro    AGCTTGATGTGATGGCTCCTGGCGTATCCATCCAAAGCACACTTCCTGGAGGCACTTACG  1079581
GeoPro      AGCTTGATGTGATGGCTCCTGGCGTATCCATCCAAAGCACACTTCCTGGAGGCACTTACG  1569
BacPro      AGCTTGATGTGATGGCTCCTGGCGTATCCATCCAAAGCACACTTCCTGGAGGCACTTACG  961

BII-1pro    GTGCTTACAACGGCACGTCCATGGCATCTCC  331
GQJK2pro    GTGCTTACAACGGCACGTCCATGGCGACTCC  1079550
GeoPro      GTGCTTACAACGGCACGTCCATGGC  1594
BacPro      GTGCTTACAACGGCACGTCCATGGC  986
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Figure 7. The sequence of ProBII-1 compared to protease gene of *Bacillus subtilis* GQJK2 (CP021498.1), *Geobacillus thermohilus* aprJ (M64743.1), and *Bacillus subtilis* aprE (AB734701.1)

alkaline protease were activated by Ca^{2+}. But protease BII-1 was not influenced by Ca^{2+}. Protease produced by *Bacillus subtilis* AP-MSU 6 showed different reaction to Cu^{2+} which enhance its activity (Maruthiah, T., Esakkiraj, P., Prabakaran, G., Palavesam, A., & Immanuel, G. 2013) as well those produced by *V. fluvialis* TKU005 (Wang, S.L., Chio, Y.H., Yen, Y.H., & Wang, C.L. 2007) and *Thermoplasma volcanium* (Kocabiyik & Özel, 2007).

3.3. Identification of BII Protease Gene

A partial region of BII protease (*ProBII-1*) was amplified with a pair of degenerated primers with the PCR product size of 331 bp designated in this work (Figure 6). The result showed that *ProBII-1* was 99 % similar to that of *Bacillus subtilis* strain GQJK2 (CP021498.1), 100 % of *Geobacillus thermophilus* aprJ (M64743.1) and 99 % of *Bacillus subtilis* aprE (AB734701.1) *(*Figure 7.). It was previously reported by Jang et al (1992) that recombinant protease from *B. subtilis* GQJK2 showed 50 % activity after being heated for 30 minutes at 60 °C. The optimum temperature of heterologously expressed protease from *B. subtilis* aprE was approximally 50 °C (Han et al., 2013).

4. Conclusion

Protease produced by bacterial isolate BII-1 was expected to be thermostable protease as it showed clear zone on solid medium that incubated at 55 °C. It refer to the fact that protease partial gene of BII-1 also showed similarity to two thermostable protease genes belong to *Bacillus subtilis* aprE and *Geobacillus thermophylus* aprJ which were reported as thermostable protease.

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