INNSPUB

International Journal of Biosciences | IJB |
ISSN: 2220-6655 (Print) 2222-5234 (Online)
http://www.innspub.net
Vol. 5, No. 6, p. 128-134, 2014

RESEARCH PAPER OPEN ACCESS

Protease activity of bacterial isolates TP5K1 and TP6K5 in tofu solid waste substrate and identification of isolates based on 16S rDNA

Baital Izzatul Badriyah, Tri Ardyati*, Sri Widyarti

Department of Biology, Brawijaya University, Malang, Indonesia

Key words: Bacterial isolates TP5K1 and TP6K5, protease, tofu solid waste, 16S rDNA.

http://dx.doi.org/10.12692/ijb/5.6.128-134 Article published on September 20, 2014

Abstract

In Indonesia the utilization of tofu solid waste is limited, only used as animal feed and traditional food although tofu solid waste contains 23.7 % protein. This study focused on using tofu solid waste as substrate for protease production of bacteria isolates TP5K1 and TP6K5. The aim of this research were to observe the highest protease spesific activity from isolates TP5K1 and TP6K5 and to identify the isolates using 16S rDNA. Identification of those isolates was carried out using universal primer 27F and 1495R and amplification of 16S rDNA. Ten percent (w/v) of tofu solid waste broth medium was used as growth medium for isolates TP5K1 and TP6K5, whereas tofu solid waste 0.5 % and 1 % (w/v) was used as substrate for crude protease activity measurement compared to control 1 % (w/v) casein. The result showed that the highest specific activity of isolate TP5K1 was 663.4 U/mg occured at 18 hours, whereas isolate TP6K5 was 324.5 U/mg occurred at 24 hours. The highest protease specific activity of isolates TP5K1 occurred in 0.5 % (w/v) tofu solid waste substrate reached 110.06 U/mg and TP6K5 occurred in 1 % (w/v) tofu solid waste substrate reached 10.10 U/mg. Based on 16S rDNA, isolate TP5K1 was identified as *Exiguobacterium indicum* PCWCW1 and isolate TP6K5 was identified as *Exiguobacterium* sp. TDWCW9.

* **Corresponding Author:** Tri Ardyati ✉ tri_ardyati@yahoo.com

Introduction

Protease is the second of total enzyme that is used in various industrial fields and reaches 60 % of the sales in the world (Kumar, 2002; Adinarayana *et al.*, 2003; Beg *et al.*, 2003). Protease is very important in the industry and are widely applied in industries of feed, food, detergent, and pharmaceutical industries, etc. (Mohen *et al.*, 2005; Bhaskar *et al.,* 2007; Jellouli *et al.*, 2009; Deng *et al.*, 2010).

Enzyme necessity in Indonesia almost 100 % are mostly imported from other countries (Rajasa, 2003; Noguiera *et al.*, 2006). The high necessity for enzyme and abundant of natural resources are an opportunity to develop industrial enzymes in Indonesia (Akhdiya, 2003). One alternative strategy to anticipate dependence on imported enzyme is to optimize the utilization of biological resources in Indonesia like utilizing cheap and available substrates (Suhartono, 2000). The cost of substrate used to produce enzymes at industrial level is 30-40 % (El Enshasy *et al.,* 2008). Low cost substrate to produce proteases in industrial scale can be done by utilize tofu solid waste. Tofu solid waste contains 23.7 % protein, however in Indonesia it only used as animal feed and traditional food (*tempeh gembus* and *oncom*) (Marwaha & Kennedy, 1988).

In the previous study, we obtained two proteolytic bacteria isolates TP5K1 and TP6K5 from tofu solid waste of Tofu Home Industry, Malang-Indonesia (Badriyah & Ardyati, 2013). Both isolates were characterized morphologically, however phylogenetic characteristic have not yet studied. In this study identification of isolates TP5K1 and TP6K5 based on phenotypic and phylogenetic characters was studied. Besides, the highest spesific activity of bacteria isolates TP5K1 and TP6K5 on tofu solid waste was observed.

Materials and methods

Bacteria Isolates

Isolates bacteria used were TP5K1 and TP6K5. Both isolates were non-pathogenic, produce clear zone on skim milk medium and has protease activity in 2 % rice bran medium (Badriyah & Ardyati, 2013).

Protease Activity of Isolates TP5K1 and TP6K5 on 10 % (w/v) tofu solid waste broth medium

A loopfull of each isolates TP5K1 and TP6K5 was inoculated into 10 mL of media CCB (calcium casseinate broth), incubated at 30 ^{o}C, 120 rpm, 24 hours. Five milliliters of each culture then inoculated into 45 mL of 10 % (w/v) tofu solid waste broth medium (pH 7). This broth called inoculum stock and incubated at 30^{o} C, 120 rpm, 24 hours (Madigan *et al.*, 2003). Fifteen milliliters of inoculum stock (containing 10^{7} cell/mL of bacteria) was added into 135 mL of 10 % tofu solid waste broth medium (pH 7). This is called production medium. Bacteria suspension was incubated at 30 ^{o}C, 120 rpm. Samples were taken at 0, 1, 2, 4, 6, 10, 14, 18, 24, 30 and 48 hours. Each sample then centrifuged at 4 ^{o}C, 4.000 rpm, 15 minutes. The method of protease activity measurement was reffered by Enggel *et al.* (2004).

Detection of Protease Activity Using Tofu Solid Waste Substrate and Protein Content Measurement

Crude protease samples from 10 % tofu solid waste broth medium of each isolates TP5K1 and TP6K5 was assayed for protease activity. The substrate used in this study was 0.5 % and 1 % (w/v) tofu solid waste and 1 % casein as control. All measurement were triplicate. Protein content of the crude enzyme was estimated using method of Bradford *et al.* (1976).

DNA Extraction of Isolates TP5K1 and TP6K5

Isolates TP5K1 and TP6K5 were subcultured in Luria Berthani broth medium for 24 hours and incubated at 120 rpm, 30 ^{o}C. Whole genome extraction of TP5K1 and TP6K5 was carried out based on modified method of Ausubel *et al.* (1995).

DNA Amplification, Purification, and Sequencing

Sequence of 16S rDNA of isolates TP5K1 and TP6K5 were amplified using pair primer 27F (5'GAGAGTTTGATCCTGGCTCAG3') and 1495R (5'CATCGGCTACCTTGTTACGA3') (Taylor *et al.*, 2000), PCR solutions adjusted according of Intron Biotechnology, PCR program of Randazzo *et al.*

(2002) (Table 1.). Product of amplification was separated by 1.5 % electrophoresis agarose *gel* based on modified method of Suharjono *et al.* (2010) and using *GeneRuler*™ DNA *LadderMix* 100 as DNA marker. Purification and sequencing were performed in Macrogen Co., Korea.

Results and discussion

Protease Activity of Isolates TP5K1 and TP6K5 on Tofu Solid Waste Broth Medium

Tofu solid waste was used as medium for the growth of proteolytic bacteria because it contains protein of 21.16-23.7 % (Marwaha & Kennedy, 1988; Lahoni, 2003;). Tofu solid waste was used as a growth medium because of the price is cheap and rich of nitrogen source. In addition tofu solid waste also source of carbon, proteins, fats, vitamins, and minerals (Ca, Mg, and Fe). Therefore tofu solid waste was favoured for grown the proteolytic bacteria (Kasmidjo, 1991; Kuswardani & Wijajaseputra, 1998).

Table 1. PCR reaction condition to amplify DNA of isolates TP5K1 and TP6K5.

Reaction	Temperature (°C)	Time (minute)	Cycle
Pre-denaturation	94	3	1
Denaturation	94	0.5	30
Annealing	52	0.5	
Extension	68	1.5	
Post Extension	68	7	1

Table 2. Spesific activity from bacteria isolates TP5K1 and TP6K5.

Isolate	Specific Activty (U/mg)		
	Casein 1 %	Tofu Solid Waste 0,5 %	Tofu Solid Waste 1 %
TP5K1	589.33±80,37 (d)	110.06±11.85 (c)	69.06±9.96 (b)
TP6K5	703.48±92.04 (e)	3.60±0.24 (a)	10.10±1.81 (a)

The highest protease activity (U/mg) of isolates TP5K1 was 663.4 after 18 hours incubation, whereas isolates TP6K5 was 324.5 after 24 hours incubation (Fig. 1). Incubation time of proteolytic bacterial isolates TP5K1 and TP6K5 with optimum protease activity in 10 % tofu solid waste broth medium (Fig 1.) was used as samples for protease specific activity measurement assay using 0.5 % and 1 % of tofu solid waste substrates and 1 % casein as control. The highest protease specific activity of bacteria isolates TP5K1 occurred in 0.5 % tofu solid waste substrate (110.06 U/mg), whereas TP6K5 occurred in 1 % (10.10 U/mg) (Table 1).

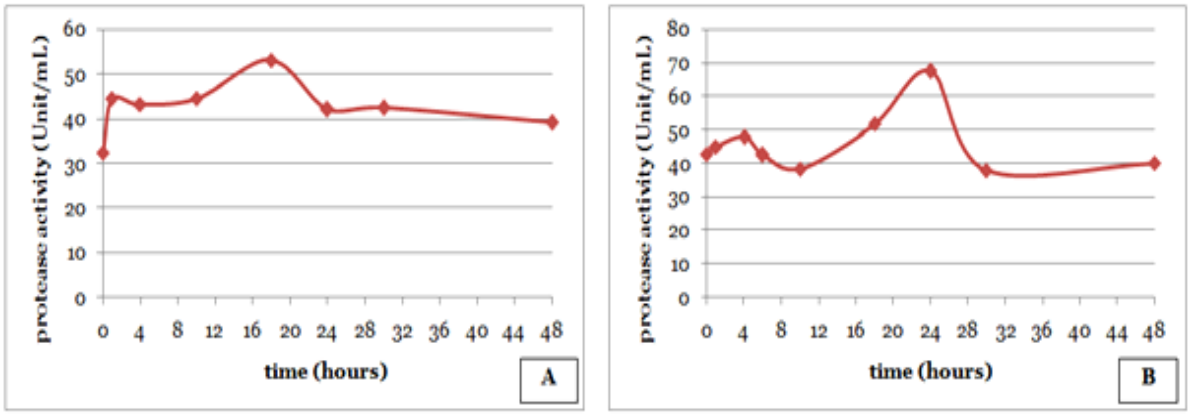

Fig 1. Protease activity of isolate TP5K1 (A) and TP6K5 (B) in 10 % tofu solid waste broth medium.

Recently, many members of *Exiguobacterium* genus have been reported able to produce alkali-tolerant protease. *Exiguobacterium* sp. YS1 was able to produce protease at pH 10 in basal medium containing 1 % skim milk (w / v). Optimum protease activity of *Exiguobacterium* sp. YS1 was at pH 9 occured after 48 hours incubation about 85 % equal to 170 U at pH 8.5-10 in anaerobic condition (Suga & Koyama, 2000; Yumoto *et al.,* 2004; Borsodi *et al.*, 2005; and Kasana & Yadav, 2007). *Exiguobacterium acetylicum* MTCC 9115 grown in PYE medium able to produce specific activity of 105.2 U/mg of 285 mg protein in crude protease sample.

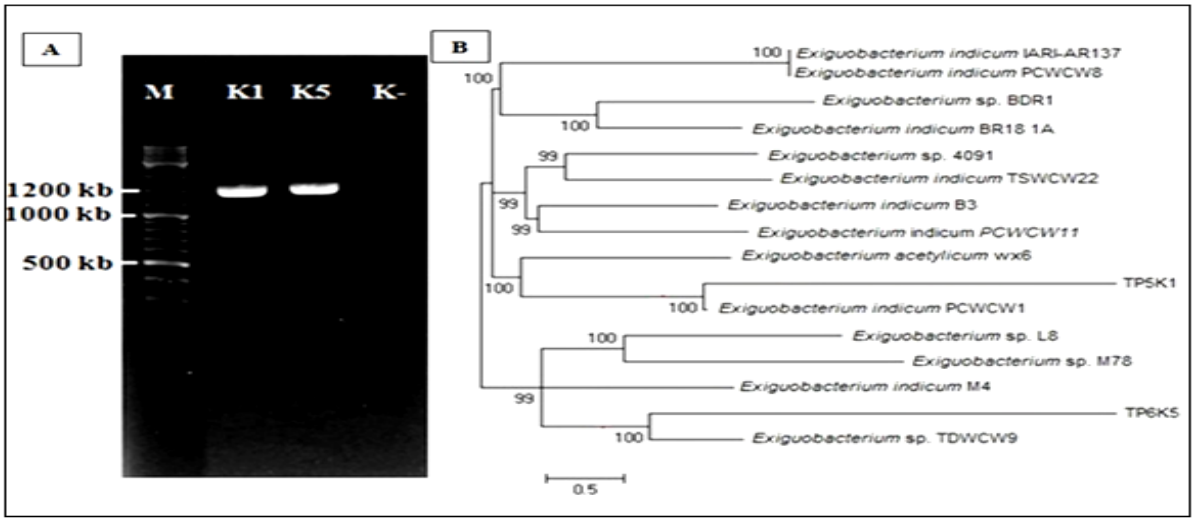

Fig. 2. DNA amplicons of isolates TP5K1 TP6K5 on 1.5 % agarose gel. M (marker *GeneRuler*™ DNA *LadderMix* 100), K1 (TP5K1), K5 (TP6K5), K (negative control) (A); Phylogeny tree which showed phylogenetics of proteolytic bacterial isolates TP5K1 and TP6K5 with reference isolates from Gene Bank based on 16S rDNA sequence which construsted with *Maximum Likelihood* algorithm and *Tamura-Nei* method (B).

Identification of Isolates TP5K1 and TP6K5 Based on 16S rDNA

This study used 16S rDNA as molecular marker to identify isolates TP5K1 and TP5K6. Amplicon DNA of isolates TP5K1 and TP6K5 was shown by the appearance of 1200 bp band on 1.5 % agarose gel electrophoresis (Figure 2. A). Klindworth *et al.* (2013) reported that the amplification of bacterial DNA using 16S rDNA markers able to produce ≥ 1200 bp amplicon. The study done by Huang *et al.* (2004) also shown the 1200 bp amplicon using 16S rDNA. The results of phylogenetic analysis based on Maximum Likelihood algorithm showed that isolates TP5K1 was identified as *Exiguobacterium indicum* PCWCW1, whereas isolates TP6K5 was identified as *Exiguobacterium* sp. TDWCW9 (Figure 2. B).

Conclusion

The highest protease specific activity from bacteria isolates TP5K1 occurred in 0.5 % tofu solid waste substrate reached 110.06 U/mg, whereas TP6K5 occurred in 1 % tofu solid waste substrate reached 10.10 U/mg. Identification based on 16S rRNA sequences showed that isolate TP5K1 was identified as *Exiguobacterium indicum* PCWCW1 and isolate TP6K5 was identified as *Exiguobacterium* sp. TDWCW9.

References

Adinarayana K, Ellaiah P, Prasad DS. 2003. Purification and partial characterization of thermostable serine alkaline protease from a newly isolated *Bacillus subtilis* PE-11. American Association of Pharmaceutical Scientists Pharmaceutical Science Technician **4**, 56-63.
http://dx.doi.org/10.1208/pt040456

Akhdiya A. 2003. Isolasi Bakteri Penghasil Enzim

Protease Alkalin Termostabil. Buletin Plasma Nutfah **9(2).**

Ausubel FM, Brent RE, Kingston DD, Moore JG, Seidman JA, Smith Struht. 1995. Short Protocol in Molecular Biology. Willey: New York.

Badriyah BI, Ardyati T. 2013. Deteksi aktivitas proteolitik isolat bakteri asal ampas tahu pada substrat bekatul. Jurnal Biotropika **1(3),** 109-113.

Beg QK, Sahai V, Gupta R. 2003. Statistical media optimization and alkaline protease production from *Bacillus mojavensis* in a bioreactor. Process Biochemistry **39,** 203-209. http://dx.doi.org/10.1016/S0141-0229(02)00293-4.

Bhaskar N, Sudeepa ES, Rashmi HN, Selvi AT. 2007. Partial purification and characterization of protease of *Bacillus proteolyticus*-CFR3001 isolated from fish processing waste and its antibacterial activities. Bioresource Technology **98,** 2758-2764. http://dx.doi.org/10.1016/j.biortech.2006.09.033

Borsodi AK, Micsinai A, Rusznyak A, Vladar P, Kovacs G, Toth EM, Marialigeti K. 2005. Diversity of alkaliphilic and alkalitolerant bacteria cultivated from decomposing reed rhizomes in a Hungarian soda lake. Microbiol Ecology **50(1),** 9-18. http://dx.doi.org/10.1007/s00248-004-0063-1

Bradford MM. 1976. A rapid and sensitive method for quantitation of microgram quantities of protein utilizing the principle of protein dye-binding. Analytical Biochemistry **72,** 234-254. http://dx.doi.org/10.1016/0003-2697(76)90527-3

Deng A, Wu J, Zhang Y, Zhang G, Wen T. 2010. Purification and characterization of a surfactant-stable high-alkaline protease from *Bacillus* sp. B001. Bioresource Technology **101,** 7100-7116. http://dx.doi.org/10.1016/j.biortech.2010.03.130

Enggel J, Meriandini A, Natalia L. 2004. Karakterisasi protease ekstraselular *Clostridium bifermentans* R14-1-b. Jurnal Mikrobiologi Indonesia **9(1),** 9-12.

El Enshasy H, Abuoul-Enein A, Helmy S, El Azaly Y. 2008. Optimization of the industrial production of alkaline protease by *Bacillus licheniformis* in different production scales. Australian Journal of Basic and Applied Sciences **2(3),** 583-593.

Huang Y, Fan X-G, Wang ZM, Zhou JH, Tian X-F, Li N. 2004. Identification of helicobacter species in human liver samples from patients with primary hepatocellular carcinoma. Journal Clinical Pathology **57,** 1273-1277. http://dx.doi.org/10.1136/jcp.2004.018556

Jellouli K, Bougatef A, Manni L, Agrebi R, Siala R, Younes I, Nasri M. 2009. Molecular and biochemical characterization of an extracellular serine-protease from *Vibrio etschnikovii* J1. Journal of Industrial Microbiology & Biotechnology **36,** 939-948. http://dx.doi.org/10.1007/s10295-009-0572-5

Kasana RC, Yadav SK. 2007. Isolation of a psychrotrophic *Exiguobacterium* sp. SKPB5 (MTCC 7803) and characterization of its alkaline protease. Current Microbiology **54(3),** 224-229. http://dx.doi.org/10.1007/s00284-006-0402-1

Kasmidjo. 1991. Penanganan Limbah Perkebunan dan Limbah Pangan. Yogyakarta: Universitas Gajahmada.

Klindworth A, Pruesse E, Schweer T, Peplies J, Quast C, Horn M, Glockner FO. 2013. Evaluation of general 16S ribosomal RNA gene PCR primers for classical and next-generation sequencing-based diversity studies. Nucleic Acids Research **41(1),** 1-11. http://dx.doi.org/10.1093/nar/gks808

Kumar CG. 2002. Purification and characterization of a termostable alkaline protease from alkalophilic

Bacillus pumilus. Letters in Applied Microbiology **34,** 13-7.
http://dx.doi.org/10.1046/j.1472-765x.2002.01044.x

Kuswardani I, Wijajaseputra AI. 1998. Produksi protein sel tunggal *Phanerochaete chrysosporium* pada media limbah cair tahu yang diperkaya: kajian optimasi waktu panen. *Prosiding* Seminar Nasional Teknologi Pangan dan Gizi. 604-613.

Lahoni E. 2003. Pengetahuan Bahan Makanan Ternak. Fakultas Peternakan. Institut Pertanian Bogor. Bogor.

Madigan MT, Martinko JH, Parker J. 2003. Brock Biology of Microorganism. Tenth Edition. Pearson Education, Inc.: USA.

Marwaha SS, Kennedy JF. 1988. Whey-pollution problem and potential utilization. International Journal of Food Science & Technology **23,** 323-336.
http://dx.doi.org/10.1111/j.13652621.1988.tb00586.x

Mohen FN, Dileep D, Deepthi D. 2005. Potential applicati on of protease isolated from *Pseudomonas aerogenosa* PD100. Electronic Journal of Biotechnology **8,** 197-203.
http://dx.doi.org/10.2225/vol8-issue2-fulltext-5

Nogueira FB, Krieger MA, Nirdé P, Goldenberg S, Romanha AJ, Murta SM. 2006. Increased expression of iron-containing superoxide dismutase-A (TcFeSOD-A) enzyme in *Trypanosoma cruzi* population with in vitro-induced resistance to benznidazole. Acta Tropica 100, 119-132.
http://dx.doi.org/10.1016/j.actatropica.2006.10.004

Pelczar MJ, Chan ECS. 2005. Dasar-dasar Mikrobiologi. Jakarta: UI-Press.

Rajasa H. 2003. Pidato pembukaan 3nd conference on industrial enzyme and biotechnology. Technology and Business Opportunity for Industrial Enzyme in Harmony with Environment. BPPT. Jakarta.

Randazzo CL, Torriani S, Akkerman ADL, de vos WM, Vaughan EE. 2002. Diversity, dynamics and activity of bacterial communities during production of an artificial silicia cheese as evaluated by 16S rRNA analysis. Applied and Environmental Microbiology **68,** 1882-1892.
http://dx.doi.org/10.1128/AEM.68.4.18821892.2002

Siddharth GP, Ambareesh KM, Basu S. 2008. Modification of Media using Low Cost Nitrogen Source for Protease Production by *Bacillus* spp. SRM University. A Bachelor Project Report.

Suga S, Koyama N. 2000. Purification and properties of a novel azide-sensitive ATPase of *Exiguobacterium aurantiacum*. Arch Microbiology **173 (33)**, 200-205.

Suharjono Mahendra APW, Triwiratno A, Wuryantini S, Rahayu LO. 2010. Sistematik filogenetik isolat-isolat kapang indigenous indonesia sebagai entomopatogen kutu sisik (*Lepidoshapesbeckii* Newman) hama tanaman jeruk. Biota **15(2),** 231-236.

Suhartono MT. 2000. Eksplorasi Protease Bakteri Asal Indonesia untuk Aplikasi Industri dan Riset Bioteknologi. Prosiding Seminar Nasional Industri Enzim dan Bioteknologi **II,** 125-133.

Swofford DL, Olsen GJ, Waddell PJ, Hillis DM. 1996. Phylogenetic Inference. Sinauer Associates: Sunderland, Massachusetts. 407-514.

Taylor MJ, Cross HF, Bilo K. 2000. Inflammatory responses induced by the filarial nematode Brugia malayi are mediated by lipopolysaccharide-like activity from endosymbiotic Wolbachia bacteria. Journal of Experimental Medicine
191, 1429-1436.
http://dx.doi.org/10.1084/jem.191.8.1429

Ward OP. 1985. *Proteolytic Enzymes. In:Comprehensive Biotechnology* Vol. **3.** M. Moo-Young (Ed). Pergamon Press: Oxford, UK.

Yumoto I, Hishinuma-Narisawa M, Hirota K, Shingyo T, Takebe F, Nodasaka Y, Matsuyama H, Hara I. 2004. *Exiguobacterium oxidotolerans* sp. nov., a novel alkaliphile exhibiting high catalase activity. International Journal of Systematic and Evolutionary Microbiology **54(6),** 2013-2017. http://dx.doi.org/10.1099/ijs.0.63129-0