Pertanika J. Trop. Agric. Sc. 41 (3): 1437 - 1448 (2018)

PERTANIKA JOURNALS

TROPICAL AGRICULTURAL SCIENCE

Journal homepage: http://www.pertanika.upm.edu.my/

Isolating and Characterising Chitinolytic Thermophilic Bacteria from Cangar Hot Spring, East Java

Ruth Chrisnasari*, Devi Verina, Aime Clorinda Tapatfeto, Stefan Pranata, Tjandra Patjajani, Mariana Wahjudi and Maria Goretti Marianti Purwanto

Department of Biology, Faculty of Biotechnology, University of Surabaya, Jalan Raya Kalirungkut, Surabaya 60293, Indonesia

ABSTRACT

In the present study, chitinolytic thermophilic bacteria were collected from Cangar hot spring, East Java, Indonesia and screened. The 16S rRNA gene sequencing was used to identify the isolated bacterium which showed highest chitinolytic activity. The identified isolate was then characterised based on morphological and physiological analyses. The results showed the isolated bacterium belonged to *Bacillus licheniformis*. This isolate produced large amounts of chitinase on 0.9% (w/v) colloidal chitin (pH 7.0) at 52°C in a very short time (24 hours). Two pairs of primer were designed to detect the presence of glycosyl hydrolase (GH) 18 chitin domain sequences in the isolated bacterium. Two amplicons sized ~250 bp and ~1000 bp were obtained from PCR process. Then the amplicons were sequenced and analysed. The sequencing results showed the isolated *Bacillus licheniformis* was proven to have genes encoding *ChiA* and *ChiC* domain.

Keywords: Bacillus licheniformis, ChiA, ChiC, thermophilic bacteria, thermostable chitinase

ARTICLE INFO

Article history:
Received: 18 September 2017
Accepted: 25 June 2018
Published: 29 August 2018

E-mail addresses:
ruth_c @staff.ubaya.ac.id (Ruth Chrisnasari)
devi.verina@yahoo.com (Devi Verina)
aimee_tjung@yahoo.com (Aime Clorinda Tapatfeto)
stefanp192@gmail.com (Stefan Pranata)
tjandra@staff.ubaya.ac.id (Tjandra Patjajani)
mariana_wahyudi@staff.ubaya.ac.id (Mariana Wahjudi)
maria_gmp@staff.ubaya.ac.id (Maria Goretti Marianti Purwanto)
* Corresponding author

INTRODUCTION

Chitinases (EC 3.2.1.14) are grouped into either Family 18 or Family 19 under glycosyl hydrolases superfamily which is capable of degrading chitin into its derivates by hydrolysing the β-1,4-glycosidic bonds between the N-acetylglucosamine residues (Shaikh & Deshpande, 1993). Nowadays, the demand for chitinase with new or desirable properties has increased due to a wide-range of industrial application of chitin derivates, such as chitooligosaccharides and

ISSN: 1511-3701
e-ISSN: 2231-8542

© Universiti Putra Malaysia Press

N-acetylD-glucosamine (Ramirez-Coutino, Marin-Cervantes, Huerta, Revah, & Shirai, 2006). Chitooligosaccharides produced by enzymatic hydrolysis of chitin has been especially used in pharmaceuticals fields as antioxidant, immunostimulant (Shahidi, Arachchi, & Jeon, 1999), antihypertensive, antibacterial, antifungal, and as a food quality enhancer (Bhattacharya, Nagpure, & Gupta, 2007).

Chitinases are produced by various microbes and recognised as extracellular inducible enzymes. Most bacteria secrete Family 18 chitinases to degrade chitin and utilise it as an energy source (Hart, Pfluger, Monzingo, Hoihi, & Robertus, 1995). The superiority of chitinase-producing bacteria is one of the key factors in the enzyme production. The high biodiversity in Indonesia presents a great opportunity to get potential bacteria with special characteristic to be used as enzymes producer. Therefore, the exploration of the chitinase-producing bacteria is vital Indonesia. Chitinolytic thermophilic bacteria can be isolated from both soil and aquatic thermophile habitats i.e. hot spring and crater. The advantage of using thermophilic bacteria is their ability to synthesise the heat stable molecule, including enzymes. Thermostable enzymes produced by thermophilic bacteria are very effective and beneficial for industrial processes that need high temperature — e.g. chitin degradation in pharmaceutical industries and waste processing in seafood industry. High temperature can improve reaction speed, increase the solubility of the reactants and non-volatile products as well as reducing mesophilic microbial contamination (Martin, Delatorre, & Camila, 2007).

The aim of this study was to isolate the most prominent local chitinolytic thermophilic bacteria from Cangar Hot Spring, East Java for thermostable chitinase production. The obtained isolate then was identified based on molecular, morphological and physiological analyses. The identified isolate was used to produce chitinase under specific condition. The isolate was then further characterised by detection of glycosyl hydrolase (GH) 18 chitin domain sequences in the isolate genome using PCR based method.

MATERIALS AND METHODS

Enrichment and Cultural Medium

Nutrient Broth (NB) (Merck) and Luria Bertani (LB) broth (Scharlou) were used as enrichment medium. Thermus colloidal chitin (TCC) broth containing 0.7% (w/v) $(NH_4)_2SO_4$, 0.1% (w/v) K_2HPO_4, 0.1% NaCl, 0.01% (w/v) $MgSO_4 \cdot 7H_2O$, 0.05% (w/v) yeast extract, 0.1% (w/v) bactotryptone and 0.5% colloidal chitin (Yuli, Suhartono, Rukayadi, Hwang, & Pyun, 2004) was used as culture medium. The TCC agar medium for screening process was made by adding 15 g L^{-1}bacto agar in the TCC broth medium. The chitin was produced from shrimp shell and the colloidal chitin was made based on Hsu & Lockwood (1975).

Bacterial Isolation, Screening and Identification

A total of four different soil and water mixture samples were aseptically collected from different regions of Cangar Hot Spring, East Java, Indonesia. The four samples were enriched in NB and LB broth solution respectively with sample and medium ratio 1:3. The enriched samples were incubated for 24 hours at 52°C with 150 rpm of shaking speed. Bacterial strains were isolated and screened from enriched medium following standard procedures using spread plate technique on TCC agar plates. Morphologically distinct colonies were sub-cultured in TCC broth and purified to single species level using streak plating repeatedly on TCC agar plates. Pure isolates were maintained by sub-culturing on TCC slants and stored at 4°C.

The pure isolates were screened for chitinase activity in TCC broth. The isolates were previously grown in LB broth at 52°C until each isolate reach 0.5 of OD_{600}. As much as 1 mL of each isolate taken and added to 9 mL of TCC broth and incubated for 36 hours at 52°C. The samples were then centrifuged at 4000 rpm for 3 minutes. The supernatant was used for N-acetyl D-glucosamine detection using Nelson–Somogyi assay (Nelson, 1944).

The selected isolate was identified through partial 16S rRNA gene sequencing analysis. Chromosomal DNA of the isolate was extracted from the pure culture using Fungal/ Bacterial DNA MiniPrep Kit (Zymo Research) and amplified using a pair of 16S universal primer (Botha, Botes, Loos, Smith, & Dicks, 2012) ordered from Macrogen, Korea (Forward: 5'-CACGGATCCAGACTTTGATY MTGGCTCAG-3' and Reverse: 5'-GTGAAGCTTACGGYTAGCTTGTTA CGACTT-3'). The amplification reaction mixture contained 5 µl of 16S forward primer 10 µM/µl, 5 µl of 16S reverse primer 10 µM/µl, 25 µl of GoTaq Green Master Mix 2X (Intron), 2.5 µl of DMSO, and 12.5 µl of double-distilled water (ddH_2O). The amplification was performed with initial denaturation at 94 ºC for 5 min, 30 cycles of denaturation at 94 ºC for 45 sec, annealing at 55 ºC for 1 min, and elongation at 72 ºC for 1.5 min followed by final elongation at 72 ºC for 5 minutes. The preparation of samples for sequencing analysis was as follows: (1) the PCR products were purified using PCR Purification Kit (Roche), cloned into pGEMT-Easy (Promega) and transformed to *E. coli* DH5α competent, (2) the transformed cells were confirmed by colony PCR method, (3) DNA plasmid was extracted from the transformed cells using Plasmid Isolation Kit (Roche) and analysed for sequencing (Macrogen, Korea). The homology analysis of 16S rRNA gene sequence was conducted using BLAST algorithm in GenBank (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Bacterial confirmation and characterisation through morphological and physiological properties were conducted based on Bergey's Manual of Systematic Bacteriology (De Vos et al., 2009).

Chitinase Production

As much as 10% (v/v) of isolate was inoculated into TCC broth medium and agitated at 180 rpm (Yin Der shaker incubator). The fermentation conditions were 0.9% (w/v) of colloidal chitin concentration, pH 7.0 and a temperature of 52ºC. Sub-sample of the culture (50 mL) at initial and final fermentation was concentrated and analysed for chitinase activity assay (Rahayu, Fredy, Maggy, Hwang, & Pyun, 1999).

Chitin Domain Sequence Detection

Chitin Domain Sequence (CDS) was detected based on PCR method using 2 pairs of primer. The first primer was designed to detect *ChiA* (FChiA: 5'-GGYGTCGATVTSGACTGGGAGTAYCC-3' and RChiA: 5'-TCRTAGGTCATRATATTGATCCARTC-3'). The second primer was designed to detect *ChiB* (FChiB: 5'-CTACGCCGGAATACGAAGGGATCGGATA-3' and 5'-AACTCCGCTTCCTCACCAGGTT-3'). Amplification reaction was made in 100 µl containing 100 ng chromosomal DNA, 10 µM/µl forward and reverse primers respectively, 50 µl GoTaq Green Master Mix 2X, and ddH_2O. Amplification process was performed with initial denaturation at 95°C for 5 min, 35 cycles consist of denaturation 95°C for 45 sec, gradient annealing with varied temperature of 53-66°C for 45 sec, and elongation 72°C for 1 min, followed by final elongation 72°C for 10 minutes. PCR product was visualised using agarose gel electrophoresis. The remaining PCR product was purified and prepared for sequencing analysis.

RESULTS AND DISCUSSION

Soil and water mixture samples were taken from four different location of Cangar Hot Spring. Of the four locations (named as location "A", "B", "C" and "D"), 19 single colonies with chitinolytic activity were obtained, where 4 colonies obtained from location B, 12 colonies at locations C and 3 colonies at locations D. None of the colony obtained from location A. The 19 colonies then were screened for chitinolytic activity in TCC broth medium based on amount of N-acetyl D-glucosamine produced as presented at Figure 1. From the data, colony D11 showed highest chitinolytic activity compared to the other colonies, although it is not significantly different with colony C14 and D10 (p-value > 0.05). The D11 colony was then identified, characterised and used for further experiments.

Colony D11 was identified based on the homology of the partial 16S rRNA gene analysis. The homology analysis of gene sequence showed that colony D11 was 99% identical with *Bacillus licheniformis* strain ATCC 14580. *Bacillus licheniformis* have been reported to have multiple and thermostable chitinase (Takayanagi, Ajisaka, Takiguchi, & Shimahara, 1991; Tantimavanich, Pantuwatana, Bhumiratana, & Panbangred, 1998; Trachuk, Revina, Shemyakina, & Stepanov, 1996), making this species commonly used as antifungal biocontrol agents and suitable for industrial

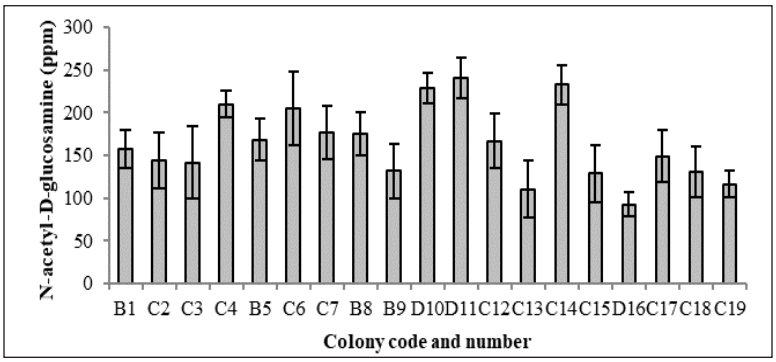

Figure 1. The screening based on chitinolytic activity of 19 isolates obtained from Cangar Hot Spring

chitin waste degradation (Kamil, Rizk, Saleh, & Moustafa, 2007; Veith et al., 2004).

The characterisation assay on morphological and physiological analysis based on Bergey's *Manual of Systematic Bacteriology* is presented in Table 1. *Bacillus licheniformis D11* showed a positive result in the following tests: catalase, amylase, oxidase, and gelatinase production; acid production from glucose, mannitol, arabinose, sucrose and glycerol; growth in 2-7% (w/v) NaCl; Voges-Proskauer test; nitrogen fixation; nitrate reduction, motility and anaerobic growth. *Bacillus licheniformis D11* showed a negative result in the following tests: acid production from lactose and xylose, hydrolysis of urea, utilization of acetate and citrate; indole formation; methyl red test and indole formation. The growth of *Bacillus licheniformis* D11 on TCC broth medium showed the lag (0-4 h), log (4-16 h), stationary (16-28 h) and the death phase (28-48 h) during incubation time (Figure 2).

In correlation to the cell growth curve of Figure 2, chitinase had been produced since the log phase and achieved the optimum at the middle of stationary phase (24 h). The enzyme production was then decreased at 36-48 hours due to lack of nutrients or secretion of toxic substances which inactivated the enzymes (Saima, Roohi, & Ahmad, 2013). *Bacillus licheniformis* D11 achieved optimum amounts of chitinase in a very short time (Figure 3), 24 hours, compared with the other chitinase producer bacteria. *Microbispora* sp. (Nawani, Kapadnis, Das, Rao, & Mahajan, 2002), *B. cereus*, *B. sphaericus* and *B. alvei* (Wang & Hwang, 2001), as well as *Aeromonas punctata* and *Aeromonas hydrophila* (Saima et al., 2013) produced the highest chitinase after 48 h. *Bacillus* sp. HSA,3-1a had been reported to produce the highest chitinase at the end of the stationary phase after 72 h incubation time (Natsir, Patong, Suhartono, & Ahmad, 2010). The short production time revealed *Bacillus licheniformis* D11 to be one of the prominent chitinase producers.

Detecting the presence of glycosyl hydrolase (GH) 18 Chitin Domain Sequence (CDS) in *Bacillus licheniformis D11* genome was done by PCR method using 2 pairs of primer. The first primer was designed to

Table 1
Morphological and physiological characteristic of d11 isolate

Characteristic	Colony Properties	Reference*
Colony shape	Irregular	Irregular
Elevation	Flat	Flat
Margin	Undulate	Undulate
Colony colour	White	White
Cellular morphology	Rod-shaped	Rod-shaped
Gram staining	Gram positive	Gram positive
Spore	Oval endospore	Oval endospore
Catalase	+	+
Amylase	+	+
Urease	-	-
Oxidase	+	+
Gelatinase	+	+
Acid from:		
- Glucose	+	+
- Lactose	-	-
- Mannitol	+	+
- Xylose	-	-
- Arabinose	+	+
- Sucrose	+	+
- Glycerol	+	+
Utilisation of:		
- Acetate	-	-
- Citrate	-	-
Growth in salinity		
- 2 % NaCl	+	+
- 5% NaCl	+	+
- 7% NaCl	+	+
Indole formation	-	-
Methyl red test	-	-
Voges-Proskauer test	+	+
Nitrogen fixation	+	+
Nitrate reduction	+	+
Motility	+	+
Anaerobic growth	+	+

*Data compiled from De Vos et al. (2009); Oziengbe & Onilude (2012); Sankaralingam, Shankar, Ramasubburayan, Prakash and Kumar (2012); Waldeck, Daum, Bisping and Meinhardt (2006).

detect *ChiA*. Amplification using this primer by gradient thermocycler in variation of annealing temperature (T_a47-60°C) produced one amplicon sized ~250 bp (Figure 4) which was later sequenced and analysed. Based on sequence alignment (BLASTn) result, this primer was able to detect *ChiA* domain sequence in *B. licheniformis* (Table 2). *ChiA* domain sequence can be found in some strains of *Bacillus* sp. i.e *B.*

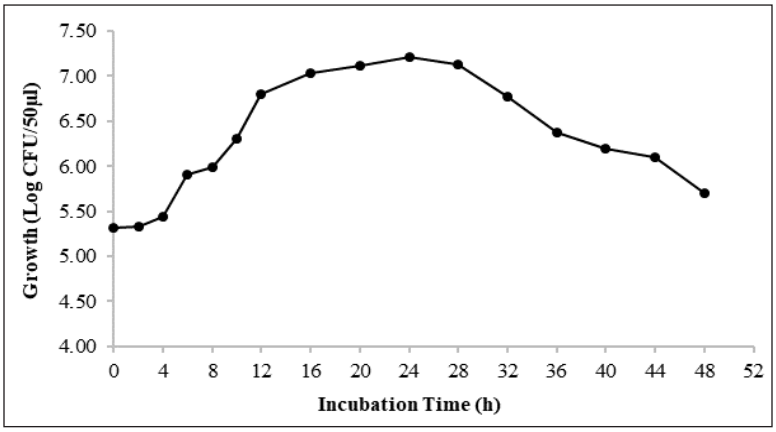

Figure 2. The growth of *Bacillus licheniformis* D11in thermus colloidal chitin broth medium pH 7.0 at 52ºC for 48 hours

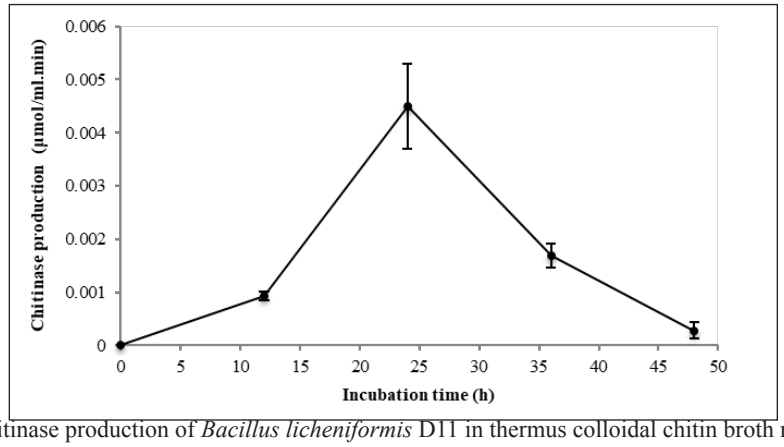

Figure 3. Chitinase production of *Bacillus licheniformis* D11 in thermus colloidal chitin broth medium (pH 7.0) at 52ºC

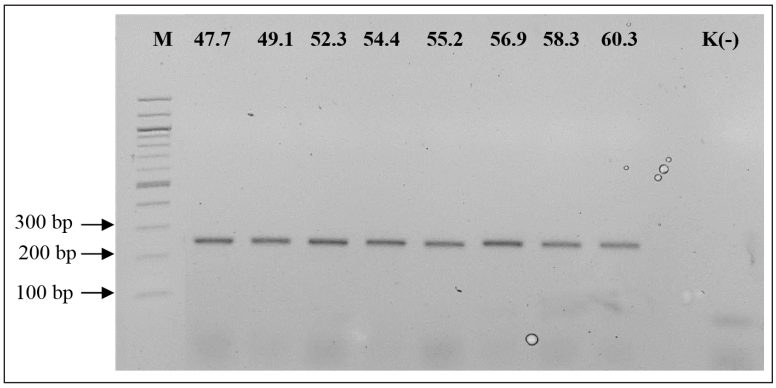

Figure 4. Visualisation of PCR product using *ChiA* primer in variation of 47.7-60.3°C annealing temperature on 2% agarose gel electrophoresis. M= marker 100 bp, 47.7-60.3= annealing temperature in °C, K(-)= negative control (without DNA template).

licheniformis, *B. cereus*, *B. thuringiensis*, and *B. pumilus*. In bacteria, the function of this gene is to degrade insoluble chitin into its derivates and plays an important role in the defence mechanism against pathogens (Funkhouser & Aronson, 2007). *ChiA* domain sequence consists of catalytic domain (GH18), fibronectin domain III (Fn3), and chitin binding domain (CBD) (Herdyastuti, Tri, Mudasir, & Sabirin, 2009; Islam et al., 2010). Amplification using *ChiB* primer by gradient thermocycler in variation of annealing temperature (T_a 53-66°C) produced one amplicon sized ~1000 bp (Figure 5) which was sequenced and analysed. Based on sequence alignment (BLASTn) result, this sequence had high levels of similarities with *ChiA* and *ChiC* domain sequence in *B. licheniformis* (*B. licheniformis* strain HRBL-15TDI7, *B. licheniformis* WX-02, dan *B. licheniformis* chiB gene strain F11) (Table 3). This result confirmed *ChiB* primer can detect the presence of *ChiA* and *ChiC* domain sequence in *B. licheniformis* D11 due to high level of similarity between the domains.

ChiA, *ChiB*, and *ChiC* belong to the group GH18. From the amino acid sequence, *ChiC* has different amino acid sequence compared with *ChiA* and *ChiB*. *ChiB* has a lower specific activity than *ChiA* because of the absence of fibronectin domain III. In addition, *ChiB* cuts GlcNAc oligomers shorter than *ChiA* (Brurberg, Nesl, & Eijsink, 1996). *ChiB* can be found in *Aspergillus fumigatus*, *Photorhabdus themperata*, and some strains of *B. licheniformis*. ChiC has three functional domains, namely N-terminal domain, fibronectin domain III, and catalytic domain. N-terminal domain in

Table 2
Sequence alignment result of ChiA amplicon using BLAST-n NCBI

Subject description	Query cover	Ident	Protein name	Domain
B. licheniformis strain LHH 100 chitinase (*ChiA*-65) gene, complete cds	76%	70%	ChiA-65	*ChiA*
B. licheniformis strain HRBL-15TDI7, complete genome	79%	69%	Chitinase A	*ChiA*
B. licheniformis WX-02 genome	79%	69%	GH18	*ChiA*
B. licheniformis strain UTM104 chitinase gene, partial cds	76%	69%	Chitinase A	*ChiA*
B. licheniformis strain KNUC 213 chitinase, partial cds	76%	69%	Chitinase A	*ChiA*
B. licheniformis strain DSM13 chitinase gene, partial cds	76%	69%	Chitinase A	*ChiA*
B. licheniformis strain N1 chitinase gene, complete cds	76%	69%	Chitinase A	*ChiA*
B. licheniformis strain CBFOS-03 chitinase (chi 18B), complete cds	76%	69%	Glycosyl Hydrolase	*ChiA*
B. licheniformis strain DSM 8785 chitinase (chiA) gene, partial cds	76%	69%	Chitinase A	*ChiA*
B. licheniformis strain A1 chitinase B gene, complete cds	76%	69%	Chitinase B	*ChiA*
B. licheniformis ATCC 14580, complete genome	79%	69%	GH18/Chitinase A	*ChiA*

Table 3
Sequence alignment result of ChiB amplicon using BLAST-n NCBI

Subject description	Query cover	Ident	Protein name	Domain
B. licheniformis strain HRBL-15TDI7, complete genome cds	100%	99%	Chi C, GH18, Chi A	*ChiC, ChiA*
B. licheniformis WX-02 genome	100%	99%	Chi C, GH18, Chi A	*ChiC, ChiA*
B. licheniformis chiB gene, chiA gene, mpr gene and ycdF gene, strain F11	100%	99%	Chi C (*binding domain*), Precursor ChiB, Putative Dehidrogenase	*ChiA, ChiC*
B. licheniformis ATCC 14580, complete genome	100%	99%	Chi C, GH18, Chi A	*ChiC, ChiA*
B. licheniformis strain SK-1 chitinase precursor (chiB) and putative chitinase precursor	100%	99%	Putative Chitinase	*ChiA*
B. licheniformis DSM13 = ATCC 14580, complete genome	100%	99%	Chi C, GH18, Chi A	*ChiC, ChiA*
B. licheniformis chiB gene, chiA gene, mpr gene and ycdF, strain F5	100%	99%	Putative Chitinase Precursor ChiB	*ChiB*
B. paralicheniformis strain BL-09, complete genome	100%	99%	Glycosyl Hydrolase	*ChiA*
B. paralicheniformis strain ATCC 9945a, complete genome	100%	94%	Putative Chitinase Precursor	*ChiA*
B. licheniformis strain MS-3 chitinase A-BL3 (chiA) gene, complete cds	100%	94%	Chitinase A-BL3	*ChiA*
B. licheniformis gh18D gene for glycoside hydrolase, complete cds	100%	94%	Glycosyl Hydrolase	*ChiA*
Bacillus sp. AV2-9 chitinase large (chiL) gene, complete cds	99%	82%	Chitinase L	*ChiA*

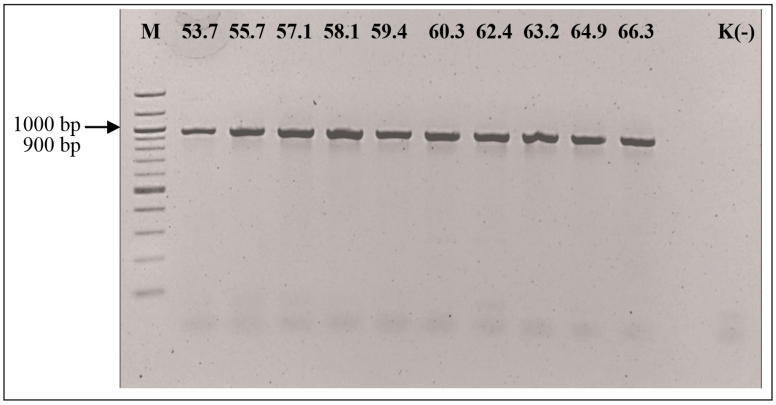

Figure 5. Visualisation of PCR product using *ChiB* primer in variation of 53.7-66.3°C annealing temperature on 1.5% agarose gel electrophoresis. M= marker 100 bp, 53.7-66.3= annealing temperature in °C, K(-)= negative control (without DNA template).

ChiC is similar to the C-terminal extension of *ChiA* (Tsujibo et al., 1998). Chitinase gene with *ChiC* domain can be found in *Streptomyces lividans*, *Paenibacillus* spp., *Pseudomonas* sp., *Serratia marcescens* and *Bacillus weihenstephanensis*.

CONCLUSION

A total of 19 chitinolytic thermophilic bacteria were collected from Cangar hot spring, East Java, Indonesia. From the screening process, D11 isolate had the highest chitinolytic activity. The D11 isolate was identified as *Bacillus licheniformis* through molecular, morphological and physiological analyses. This isolate produced large amounts of chitinase ($4.49x10^{-3}$µmol/ml. minutes) on 0.9% (w/v) colloidal chitin (pH 7.0) at 52 °C in a very short time, 24 hours compared with other *Bacillus sp.* The sequence analysis showed that the isolated *Bacillus licheniformis* was proven to have genes encoding *ChiA* and *ChiC* domain. This isolate can be used for further application on chitinous waste degradation or chitin derivates production in pharmaceutical industries.

ACKNOWLEDGMENT

The authors thank Lembaga Penelitian dan Pengabdian Masyarakat (LPPM), University of Surabaya for its research funding through Hibah Penelitian Lanjut, ST. 087/Lit/LPPM-01/FTB/VIII/2013 and Hibah Kompetitif, ST. 007/Lit/LPPM-01/FTB/III/2016.

REFERENCES

Bhattacharya, D., Nagpure, A., & Gupta, R. K. (2007). Bacterial chitinases: Properties and potential. *Critical Reviews in Biotechnology, 27*, 21–28.

Botha, M., Botes, M., Loos, B., Smith, C., & Dicks, L. M. T. (2012). *Lactobacillus equigenerosi* strain Le1 invades equine epithelial cells. *Applied and Environmental Microbiology, 78*(12), 4248-4255.

Brurberg, M. B., Nesl, I. F., & Eijsink, V. G. H. (1996). Comparative studies of chitinases A and B from *Serratia marcescens*. *Microbiology*, *142*, 1581–1589.

De Vos, P., Garrity, G. M., Jones, D., Krieg, N. R., Ludwig, W., Rainey, F.A., & Whitman, W. B. (2009). *Bergey's manual of systematic bacteriology second edition: Volume 3: The firmicutes*. New York, NY: Springer.

Funkhouser, J. D., & Aronson J. (2007). Chitinase family GH18: Evolutionary insight from genomic history of a diverse protein family. *BMC Evolutionary Biology*, *7*(96), 1–16.

Hart, P. J., Pfluger, H. D., Monzingo, A. F., Hoihi, T., & Robertus, J. D. (1995). The refined crystal structure of an endochitinase from *Hordeum vulgare* L. seeds at 1.8 Å resolution. *Journal of Molecular Biology, 248*, 402–413.

Herdyastuti, N., Tri, J. R., Mudasir, Sabirin, M. (2009). Kitinase dan mikroorganisme kitinolitik: isolasi, karakterisasi dan manfaatnya [Chitinase and kitinolytic microorganisms: Isolation, characterization and its benefits]. *Indonesian Journal of Chemistry, 9*(1), 37–47.

Hsu, S. C., & Lockwood, J. L. (1975). Powdered chitin agar as a selective medium for enumeration of actinomycetes in water and soil. *Applied Microbiology*, *29*(3), 422–426.

Islam, S. M. A., Cho, K. M., Hong, S. J., Math, R. K., Kim, J. M., Yun, M. G., & Yun, H. D. (2010). Chitinase of *Bacillus licheniformis* from oyster

shell as a probe to detect chitin in marine shells. *Applied Microbiology and Biotechnology*, *86*(1), 119-129.

Kamil, Z., Rizk, M., Saleh, M., & Moustafa, S. (2007). Isolation and identification of rhizosphere soil chitinolytic bacteria and their potential in antifungal biocontrol. *Global Journal of Molecular Sciences, 2*(2), 57–66.

Martin, M. L. L., Delatorre, A. B. S., & Camila, R. (2007). Effect of culture conditions on the production of extracellular protease by thermophilic *Bacillus* sp. and some properties of the enzymatic activity. *Brazilian Journal of Microbiology, 38*, 253–258.

Natsir, H., Patong, A. R., Suhartono, M. T., & Ahmad, A. (2010). Production and characterization of chitinase enzymes from sulili hot spring in south Sulawesi, *Bacillus* sp. HSA, 3-1a. *Indonesian Journal of Chemistry, 10*(2), 263–267.

Nawani, N. N., Kapadnis, B. P., Das, A. D., Rao, A. S., & Mahajan, S. K. (2002). Purification and characterization of a thermophilic and acidophilic chitinase from Microbispora sp. V2. *Journal of Applied Microbiology, 93*, 965–975.

Nelson, N. A. (1944). A photometric adaptation of the somogyi method for the determination of glucose. *The Journal of Biological Chemistry*, *153*, 375–380.

Oziengbe, E. O., & Onilude, A. A. (2012). Production of a thermostable α-amylase and its assay using *Bacillus licheniformis* isolated from excavated land sites in Ibadan, Nigeria. *Bajopas, 5*(1), 132–138.

Rahayu, S., Fredy, T., Maggy, T. S., Hwang, J. K., & Pyun, Y. R. (1999). Eksplorasi bakteri termofilik penghasil enzim kitinase asal Indonesia [Exploration of thermophilic bacteria producing enzyme kitinase origin Indonesia]. *Prosiding Seminar Hasil-Hasil Penelitian Bidang Ilmu Hayat* (pp. 349-356). Bogor, Indonesia: Pusat Antar Universitas Ilmu Hayat IPB.

Ramirez-Coutino, L., Marin-Cervantes, M. D. C., Huerta, S., Revah, S., & Shirai, K. (2006). Enzymatic hydrolysis of chitin in the production of oligosaccha-rides using *Lecanicillium fungicola* chitinases. *Process Biochemistry*, *41*, 1106–1110.

Saima, M. K., Roohi, I. Z., & Ahmad (2013). Isolation of novel chitinolytic bacteria and production optimization of extracellular chitinase. *Journal of Genetic Engineering and Biotechnology*, *11*, 39–46.

Sankaralingam S., Shankar, T., Ramasubburayan, R., Prakash, S., & Kumar, C. (2012). Optimization of culture conditions for the production of amylase from *Bacillus licheniformis* on submerged fermentation. *American-Eurasian Journal of Agricultural & Environmental Science, 12*(11), 1507–1513.

Shahidi, F., Arachchi, J. K. V., & Jeon, Y. J. (1999). Food applications of chitin and chitosan. *Trends in Food Science & Technology*, *10*, 37–51.

Shaikh, S. A. & Deshpande M. V. (1993). Chitinolytic enzymes: Their contribution to basic and applied research. *World Journal of Microbiology and Biotechnology*, *9*, 468–475.

Takayanagi, T., Ajisaka, K., Takiguchi, Y., & Shimahara, K. (1991). Isolation and characterization of thermostable chitinases from *Bacillus licheniformis* X-7u. *Biochimica et Biophysica Acta (BBA) - Protein Structure and Molecular, 1078*(3), 404–410.

Tantimavanich, S., Pantuwatana, S., Bhumiratana, A., & Panbangred, W. (1998). Multiple chitinase enzymes from a single gene of *Bacillus licheniformis* TP-1. *Journal of Fermentation and Bioengineering, 85*(3), 259–265.

Trachuk, L. A., Revina, L. P., Shemyakina, T. M., Chestukhina, G. G., & Stepanov, V. M. (1996). Chitinases of *Bacillus licheniformis* B-6839: Isolation and properties. *Canadian Journal of Microbiology, 42*(4), 307–315.

Tsujibo, H., Orikoshi, H., Shiotani, K., Hayashi, M., Umeda, J., Miyamoto, K., & Inamori, Y. (1998). Characterization of chitinase C from a marine bacterium, *Alteromonas* sp. strain O-7, and its corresponding gene and domain structure. *Applied and Environmental Microbiology*, *64*(2), 472-478.

Veith, B., Herzberg, C., Steckel, S., Feesche, J., Maurer, K. H., Ehrenreich, P., Gottschalk, G. (2004). The complete genome sequence of *Bacillus licheniformis* DSM13, an organism with great industrial potential. *Journal of Molecular Microbiology and Biotechnology, 7,* 204–211.

Waldeck J., Daum, G., Bisping, B., & Meinhardt, F. (2006). Isolation and molecular characterization of chitinase-deficient *Bacillus licheniformis* strains capable of deproteinization of shrimp shell waste to obtain highly viscous chitin. *Applied and Environmental Microbiology*, *72*(12), 7879–7885.

Wang, S., & Hwang, J. (2001). Microbial reclamation of shellfish wastes for the production of chitinases. *Enzyme and Microbial Technology*, *28*(4-5), 376–382.

Yuli, P. E., Suhartono, M. T. Y., Rukayadi, Y., Hwang, J. K., & Pyun, Y. R. (2004). Characteristic of thermostable chitinase enzymes from the Indonesian *Bacillus* sp.13.26, *Enzyme and Microbial Technology*, *35*, 147–153.