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

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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

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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

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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 was 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 compare 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

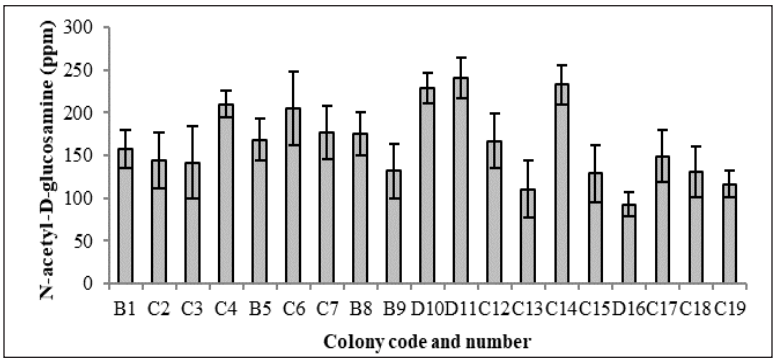

Figure 1. The screening based on chitinolytic activity of 19 isolates obtained from Cangar Hot Spring

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.*

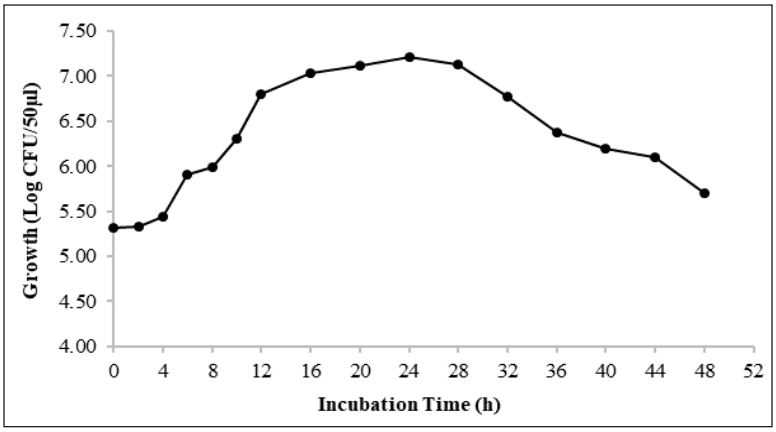

Figure 2. The growth of *Bacillus licheniformis* D11in thermus colloidal chitin broth medium pH 7.0 at 52ºC for 48 hours

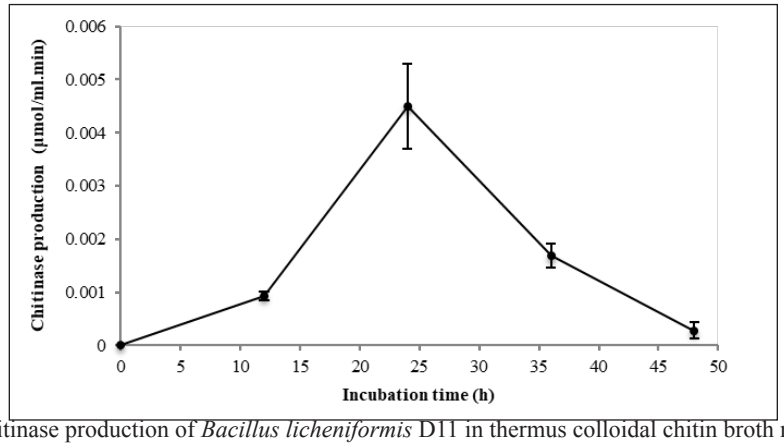

Figure 3. Chitinase production of *Bacillus licheniformis* D11 in thermus colloidal chitin broth medium (pH 7.0) at 52ºC

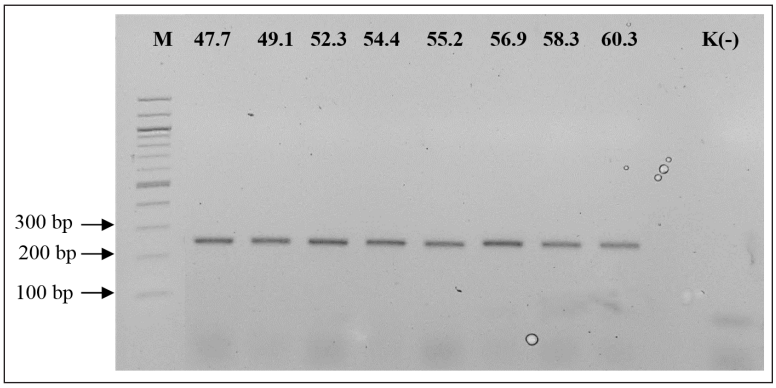

Figure 4. Visualisation of PCR product using *ChiA* primer in variation of 47.7-60.3°C annealing temperature on 2% agarose gel electrophoresis. M= marker 100 bp, 47.7-60.3= annealing temperature in °C, K(-)= negative control (without DNA template).

licheniformis, *B. cereus*, *B. thuringiensis*, and *B. pumilus*. In bacteria, the function of this gene is to degrade in soluble chitin 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*

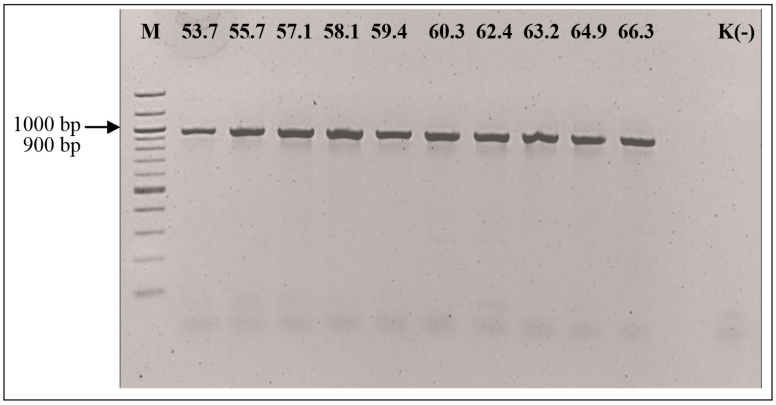

Figure 5. Visualisation of PCR product using *ChiB* primer in variation of 53.7-66.3°C annealing temperature on 1.5% agarose gel electrophoresis. M= marker 100 bp, 53.7-66.3= annealing temperature in °C, K(-)= negative control (without DNA template).

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.

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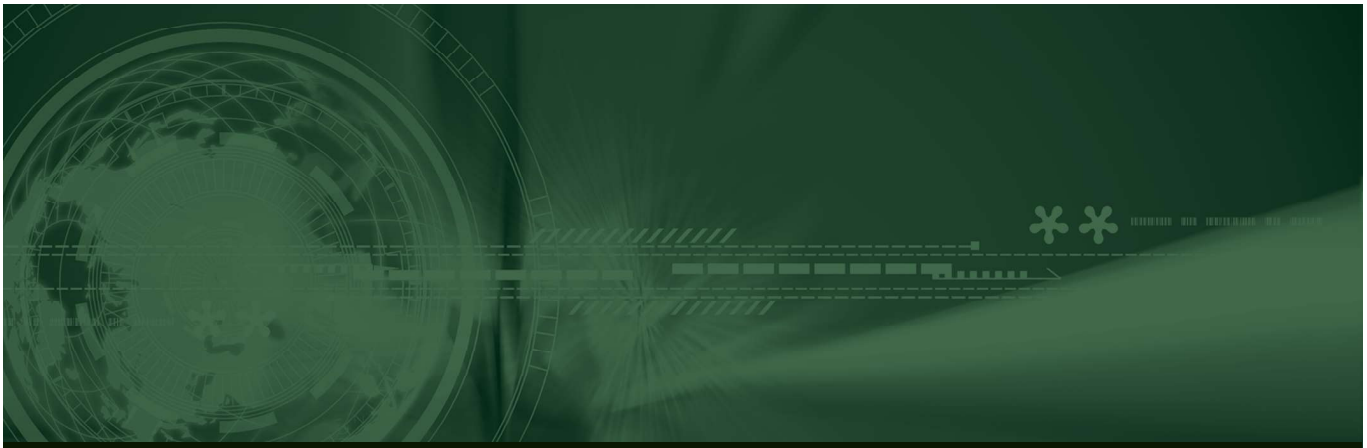

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JTAS

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Journal of Tropical Agricultural Science
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About the Journal

Overview

Pertanika Journal of Tropical Agricultural Science (JTAS) is the official journal of Universiti Putra Malaysia published by UPM Press. It is an open-access online scientific journal which is free of charge. It publishes the scientific outputs. It neither accepts nor commissions third party content.

Recognized internationally as the leading peer-reviewed interdisciplinary journal devoted to the publication of original papers, it serves as a forum for practical approaches to improving quality in issues pertaining to tropical agriculture and its related fields.

JTAS is a **quarterly** (*February, May, August and November*) periodical that considers for publication original articles as per its scope. The journal publishes in **English** and it is open to authors around the world regardless of the nationality.

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The *Introduction* explains the scope and objective of the study in the light of current knowledge on the subject; the *Materials and Methods* describes how the study was conducted; the *Results* section reports what was found in the study; and the *Discussion* section explains meaning and significance of the results and provides suggestions for future directions of research. The manuscript must be prepared according to the Journal's **Instructions to Authors**.

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Notification of the editorial decision is usually provided within ten to fourteen weeks from the receipt of manuscript. Publication of solicited manuscripts is not guaranteed. In most cases, manuscripts are accepted conditionally, pending an author's revision of the material.

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3. The chief executive editor, in consultation with the editor-in-chief, examines the reviews and decides whether to reject the manuscript, invite the author(s) to revise and resubmit the manuscript, or seek additional reviews. Final acceptance or rejection rests with the Edito-in-Chief, who reserves the right to refuse any material for publication. In rare instances, the manuscript is accepted with almost no revision. Almost without exception, reviewers' comments (to the author) are forwarded to the author. If a revision is indicated, the editor provides guidelines for attending to the reviewers' suggestions and perhaps additional advice about revising the manuscript.

4. The authors decide whether and how to address the reviewers' comments and criticisms and the editor's concerns. The authors return a revised version of the paper to the chief executive editor along with specific information describing how they have answered' the concerns of the reviewers and the editor, usually in a tabular form. The author(s) may also submit a rebuttal if there is a need especially when the author disagrees with certain comments provided by reviewer(s).

5. The chief executive editor sends the revised paper out for re-review. Typically, at least one of the original reviewers will be asked to examine the article.

6. When the reviewers have completed their work, the chief executive editor in consultation with the editorial board and the editor-in-chief examine their comments and decide whether the paper is ready to be published, needs another round of revisions, or should be rejected.

7. If the decision is to accept, an acceptance letter is sent to all the author(s), the paper is sent to the Press. The article should appear in print in approximately three months.

   The Publisher ensures that the paper adheres to the correct style (in-text citations, the reference list, and tables are typical areas of concern, clarity, and grammar). The authors are asked to respond to any minor queries by the Publisher. Following these corrections, page proofs are mailed to the corresponding authors for their final approval. At this point, **only essential changes are accepted**. Finally, the article appears in the pages of the Journal and is posted on-line.

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Pertanika Journal of Tropical Agricultural Science
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Contents

Foreword

Welcome to the **Third Issue 2018** of the Journal of Tropical Agricultural Science (JTAS)!

JTAS is an open-access journal for studies in Tropical Agricultural Science published by Universiti Putra Malaysia Press. It is independently owned and managed by the university and run on a non-profit basis for the benefit of the world-wide science community.

This issue contains 45 articles, out of which one is a review paper, two are short communications and the rest (42) are regular articles. The authors of these articles come from different countries, namely Malaysia, Indonesia, Thailand, Iran, Nigeria, India, Korea and Japan. Indonesia alone contributed 19 articles, the highest number of articles.

Articles submitted in this issue cover wide range of agricultural science fields including agricultural economics and management, agronomy, animal products, aquaculture, biotechnology, botany, ecology, fisheries sciences, food and nutrition development, forestry science, genetics and molecular biology, marine science, microbiology, nature products, organic chemistry, plant physiology, soil and water science, and zoology. An article is outlined from each of three favoured field in this issue: biotechnology; food and nutrition development; and plant physiology.

Selected from biotechnology field is a favourable article on bioactive potential of *Cosmos Caudatus* Kunth's leaves (locally known as '*ulam raja*') in scavenging free radicals and inhibiting α-glucosidase enzyme. The study was conducted by fellow researchers from Universiti Putra Malaysia (*Wan Nadilah Wan Ahmad, Khozirah Shaari, Alfi Khatib, Azizah Abdul Hamid* and *Muhajir Hamid*), Malaysia. The study shed some lights for future studies on plant phytochemicals and further development of the medicinal plant for health benefits. Details of the study is available on page 1367.

Selected from the field of food and nutrition development is an interesting article on contamination of pesticide and heavy metals in some vegetables and fruits, by Thailand scholars (*Sirikul Thummajitsakul, Rawitsara Subsinsungnern, Ngamrat Treerassapanich, Nutthida Kunsanprasit, Leeyaporn Puttirat, Patarapong Kroeksakul* and *Kun Silprasit*). The study samples were obtained from a local market and family farm in Ongkharak District of Nakhon Nayok Province, Thailand. They found high percentage of pesticides contamination and high level of heavy metals in the samples. This raises concern on health risk of the consumption of vegetables and fruits contaminated with pesticides and heavy metals. Details of the study is available on page 987.

Selected from the field of plant physiology is a pleasing article on effects of harmonic frequency and sound intensity levels on the opening of stomata, the growth and yield of soybeans, by fellow researchers from Indonesia (*Istirochah Pujiwati, Nurul Aini, Setyawan P. Sakti* and *Bambang Guritno*). They suggested the best combination of treatment to improve the productivity of soybean plants in Indonesia was exposure at a frequency of 4 kHz and sound intensity of 50 dB, followed by application of recommended dosage of leaf fertiliser. Details of the article is available on page 963.

We anticipate that you will find the evidence presented in this issue to be intriguing, thought-provoking and useful in reaching new milestones in your own research. Please recommend the journal to your colleagues and students to make this endeavour meaningful.

All the papers published in this edition underwent Pertanika's stringent peer-review process involving a minimum of two reviewers comprising internal as well as external referees. This is to ensure that the quality of the papers justified the high ranking of the journal, which is renowned as a heavily-cited journal not only by authors and researchers in Malaysia but by those in other countries around the world as well.

We would also like to express our gratitude to all the contributors, namely the authors, reviewers and editors, who have made this issue possible.

JTAS is currently accepting manuscripts for upcoming issues based on original qualitative or quantitative research that opens new areas of inquiry and investigation.

Chief Executive Editor
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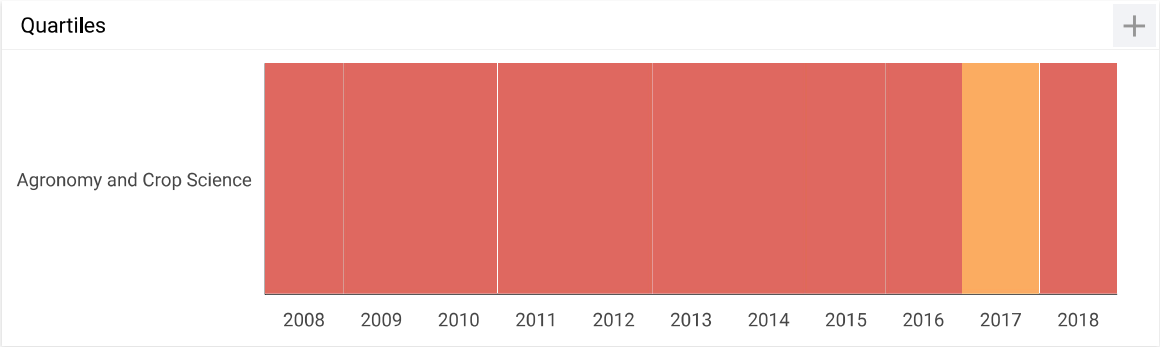

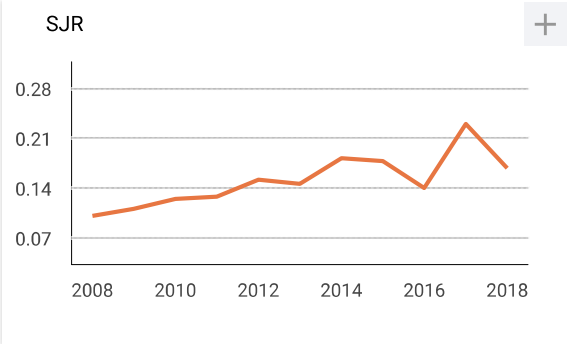

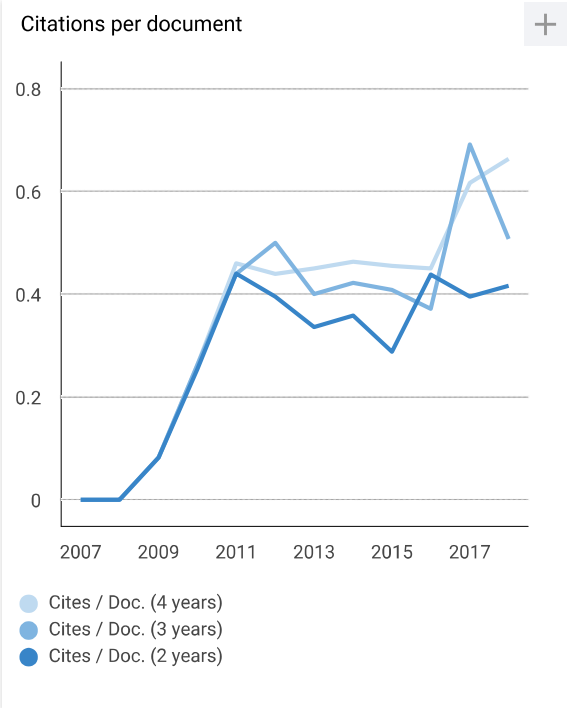

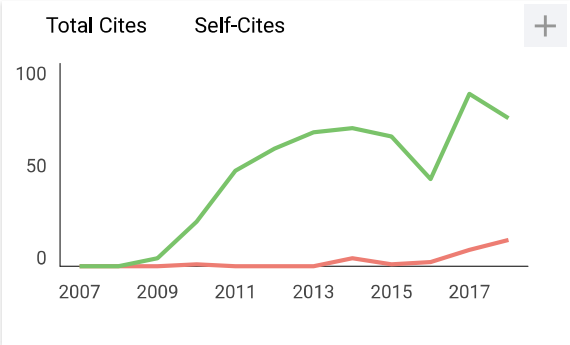

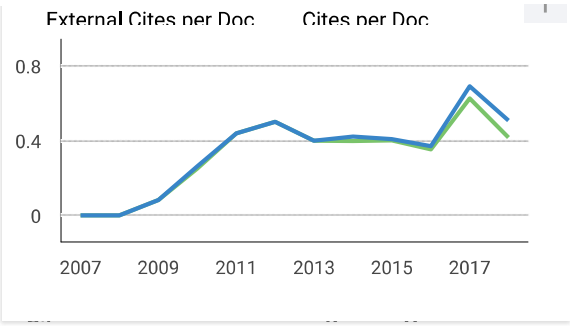

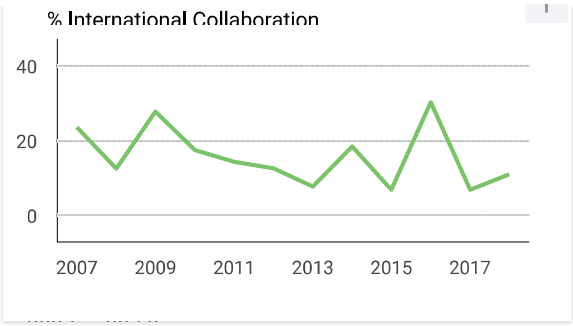

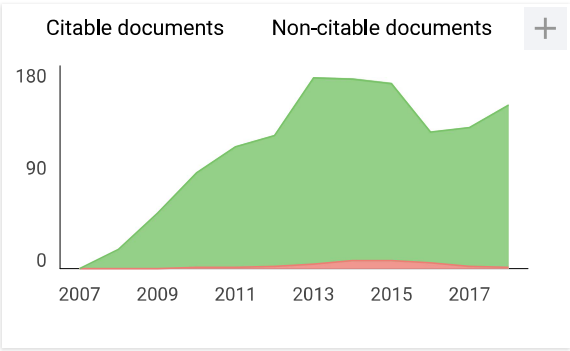

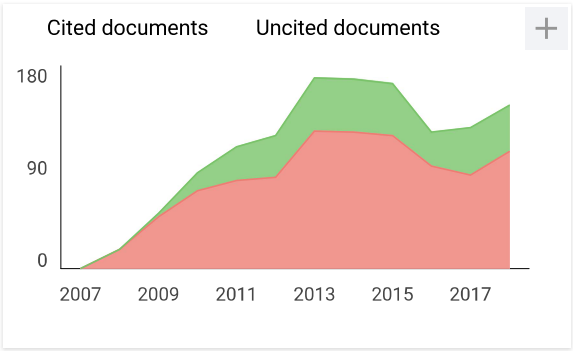

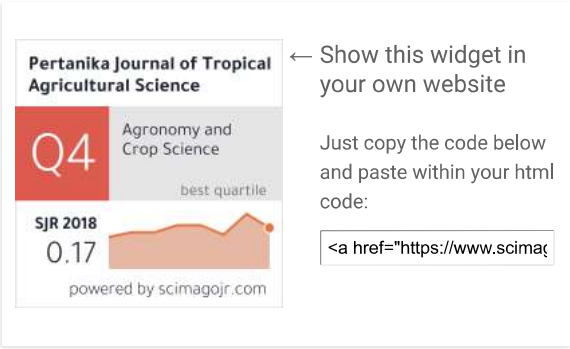

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Isolating and Characterising Chitinolytic Thermophilic Bacteria from Cangar Hot Spring, East Java

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

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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

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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

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, Hoilis, & 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, 2006).

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'-CACGGATCCAGACTTTGATYMTGGCTCAG-3' and Reverse: 5'-GTGAAGCTTACGGYTAGCTTGTTACGACTT-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 2x Taq 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 was 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 compare 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

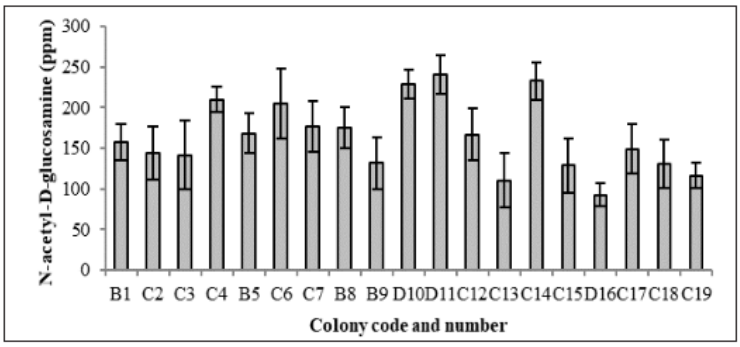

Figure 1. The screening based on chitinolytic activity of 19 isolates obtained from Cangar Hot Spring

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	+	+
...ple 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.*

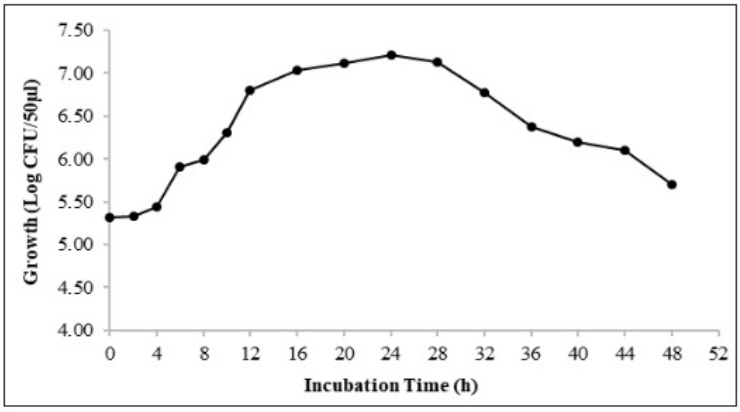

Figure 2. The growth of *Bacillus licheniformis* D11in thermus colloidal chitin broth medium pH 7.0 at 52ºC for 48 hours

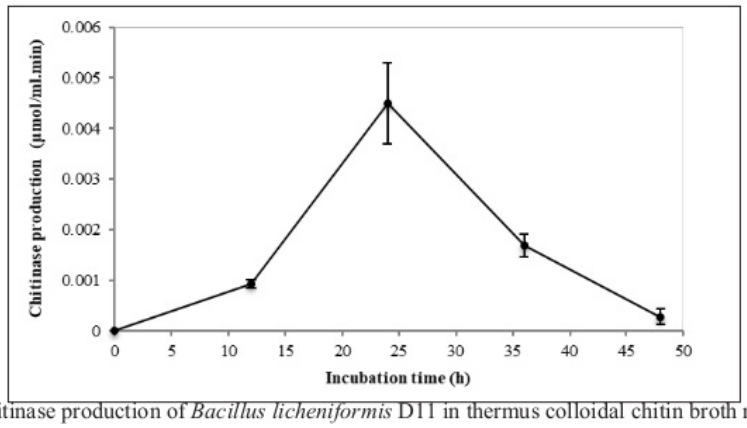

Figure 3. Chitinase production of *Bacillus licheniformis* D11 in thermus colloidal chitin broth medium (pH 7.0) at 52ºC

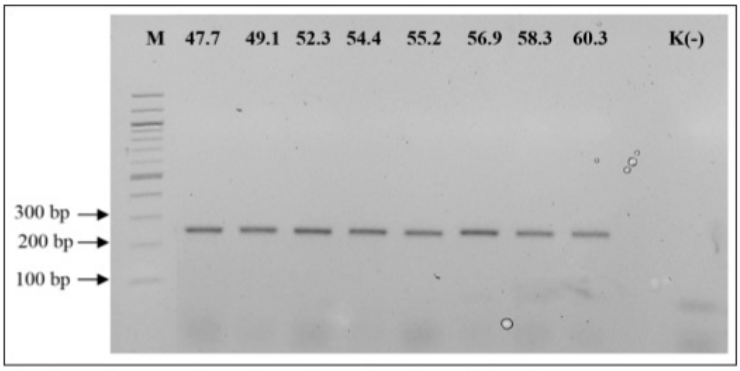

Figure 4. Visualisation of PCR product using *ChiA* primer in variation of 47.7-60.3°C annealing temperature on 2% agarose gel electrophoresis. M= marker 100 bp, 47.7-60.3= annealing temperature in °C, K(-)= negative control (without DNA template).

licheniformis, *B. cereus*, *B. thuringiensis*, and *B. pumilus*. In bacteria, the function of this gene is to degrade in soluble chitin 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	Do-main
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*

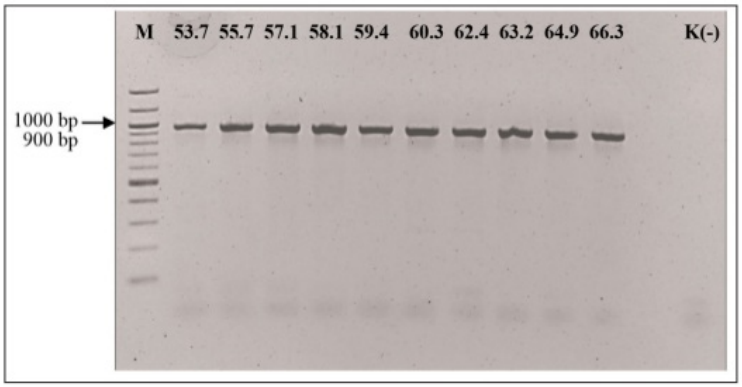

Figure 5. Visualisation of PCR product using *ChiB* primer in variation of 53.7-66.3°C annealing temperature on 1.5% agarose gel electrophoresis. M= marker 100 bp, 53.7-66.3= annealing temperature in °C, K(-)= negative control (without DNA template).

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 0.2% (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.

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