Thermostable Chitinase Producing Bacterium from Ijen Hot Spring – Indonesia: Isolation, Identification, and Characterization

Ruth Chrisnasari[1,2*], *Liony* Priscilla Sutanto[1], *Dian* Paulina[1], *Alicia* Wahjudi[1], and *Tjandra* Pantjajani[1].

[1]Faculty of Biotechnology, University of Surabaya, Raya Kalirungkut, Surabaya 60293, East Java, Indonesia
[2]Laboratory of Food Chemistry, Wageningen University & Research, Bornse Weilanden 9, 6708 WG Wageningen, The Netherlands

Abstract. The high industrial demand for thermostable chitinase with new and desirable characteristics has led to the exploration of chitinolytic bacteria from extreme environments. Therefore, this study aimed to isolate, screen, and identify chitinase–producing bacteria from Ijen hot spring, Indonesia. The highest chitinolytic activity bacterium was identified by 16S rRNA gene sequencing and its characteristics were confirmed by morphological and physiological analyses. Chitinase production activity of selected bacterium under variation of agitation and aeration as well as its chitinase properties were characterized afterward. Twelve chitinolytic bacterial colonies were isolated and screened for their growth activity on Thermus colloidal chitin medium, in which chitin was used as the sole carbon source. Among these twelve isolates, isolate B2 showed the highest chitinolytic activity. The molecular, morphological, and physiological analyses confirmed that isolate B2 belonged to *Bacillus licheniformis*. This isolate produced a huge amount of chitinase on Thermus colloidal chitin medium at 50 °C within 30 h. The highest growth and chitinase production activity were recorded at 3 vvm aeration rate and 300 rpm agitation speed. Chitinase produced by this isolate was optimally active at pH of 7 and temperature of 55 °C and evidently proofed as thermostable due to its high residual activity after several thermal stability tests.

Keywords: *Bacillus licheniformis*, chitooligosaccharides (CHOS), chitinolytic enzyme, environmentally-friendly technology

1 Introduction

Chitin is a homopolymer of β–1,4 linked N–acetyl–D–glucosamine (GlcNAc) which becomes the second largest biopolymer present in the world after cellulose. This

* Corresponding author: ruth_c @staff.ubaya.ac.id

biopolymer is widely spread in the environment as a structural component of crustaceans, insects, mollusks, and fungi [1, 2]. Most of this insoluble polymer is produced on a huge scale from the seafood processing industry, *i.e* shrimp, lobster, and crab industries, and it is considered as waste that may cause an environmental problem due to their very slow decomposition [3].

On the other hand, bioactive properties of chitin derivates, chitooligosaccharides (CHOS), are suited for broad application in the food and pharmaceutical industries. CHOS have been reported to possess antibacterial, antifungal, antitumor, antioxidant, anti-inflammatory, antidiabetic and immunomodulators properties thus making them widely applied in drug development, gene therapy, wound dressings, functional food development, *etc*. [4–6].

CHOS can be produced from chitinous wastes through enzymatic hydrolysis by chitinases, which are claimed to be environmentally friendly, easy to control, low cost, and specified processes [7, 8]. Chitinases (EC 3.2.1.14) belongs to the group of glycosyl hydrolases superfamily which is capable of degrading chitin into its derivates by acting on β–1,4–glycosidic bonds between N–acetylglucosamine residues [9]. Enzymatic hydrolysis of chitin is usually preceded by heating to loosen the chitin structure and make it accessible by the enzyme [10]. Moreover, the high-temperature process provides many advantages including improvement of reaction speed, increasing the solubility of the reactants as well as reducing mesophilic microbial contamination [11]. Therefore, a chitinolytic enzyme with high thermal stability properties has gained more interest because its effectiveness to be applied in high temperature possesses industry.

As an extracellular inducible enzyme, chitinases are produced by various microbes, including bacteria, fungi, and archaea. The superiority of the microbes plays an important role in enzyme production. Indonesia with high biodiversity of microbes provides a great opportunity to find thermostable chitinase–producing bacteria. Thermostable enzymes are commonly synthesized by thermophiles as they have adapted to the condition in which they have to live and survive [12]. Chitinolytic thermophilic bacteria can be present in both soil and aquatic thermophile habitats *i.e*. hot springs and crater.

This work was aimed to isolate the most prominent thermostable chitinase–producing bacterium from Ijen Hot Spring, Indonesia. The obtained isolate then was identified through molecular, morphological, and physiological analyses followed by characterization of its growth and chitinase productivity under specific conditions.

2 Methods

2.1 Enrichment and cultural medium

Two enrichment media were used to enrich soil and water samples, *i.e* Nutrient Broth (NB) (Merck) and Luria Bertani (LB) broth (Scharlou). Thermus colloidal chitin (TCC) was used as cultural medium and prepared with composition of 2 % colloidal chitin, 0.7 % (w v^{-1}) $(NH_4)_2SO_4$, 0.1 % (w v^{-1}) bacto tryptone, 0.1 % (w v^{-1}) K_2HPO_4, 0.1 % NaCl, 0.01 % (w v^{-1}) $MgSO_4 \cdot 7H_2O$, and 0.05 % (w v^{-1}) yeast extract. For the streaking, screening, and selection process, TCC agar was used and prepared by adding 15 g L^{-1} bacto agar to the TCC broth.

2.2 Isolation, screening, and selection of the chitinolytic bacterium

Both soil and water samples were aseptically collected from three different regions of Ijen Hot Spring, Indonesia. The three samples then were enriched in NB and LB broth medium

respectively with a sample and medium ratio of 1:3. The enriched samples were incubated for 24 h at 50 °C at 150 rpm agitation speed (1 rpm = 1/60 Hz). Bacterial strains were isolated and screened from enriched medium following standard procedures using the spread plate technique on TCC agar plates. Morphologically distinct colonies with halo zones around were subcultured and purified to single-species level by streak plating repeatedly on TCC agar plates.

The pure isolates were subcultured on TCC agar plates and screened for chitinolytic activity by measuring the diameter of the halo zone after incubation at 50 °C for 24 h. Colony with the biggest halo zone which indicates the highest chitinolytic activity then was selected.

2.3 Identification and characterization of the isolate

The selected isolate was identified through 16S rRNA gene sequencing analysis. Genomic DNA of the selected isolate was extracted from the pure culture using Wizard Genomic DNA Purification Kit (Promega) and amplified using a pair of 16S universal primer, 27 F: 5′–AGAGTTTGATCCTGGCTCAG–3' and 1492 R: 5′–GRTACCTTGTTACGACTT–3′ (Macrogen). Amplifications reaction mixture were containing 50 µl GoTaq Green Master Mix 2X, 10 µl 27 F primer 10 µM/µl, 10 µl 1492 R primer 10 µM μl^{-1}, 27.4 µl ddH2O dan 2.6 µl genomic DNA.The amplification condition was arranged with an initial denaturation at 94 ºC for 5 min, 35 cycles of denaturation at 94 ºC for 45 sec, annealing at 49.7 ºC for 45 sec, and elongation at 72 ºC for 2 min followed by final elongation at 72 ºC for 5 min.

The PCR product then was purified using Wizard SV Gel and PCR Clean-up System (Promega), cloned into pGEMT–Easy vector (Promega), and transformed to *E. coli* DH5α. DNA plasmid was then extracted from the transformed cells using the High Pure Plasmid Isolation Kit (Roche) and analyzed for sequencing (Macrogen, Korea). BLAST algorithm (http://blast.ncbi.nlm.nih.gov/blast.cgi) was used to do homology analysis of 16S rRNA gene sequence. The selected isolate was also characterized through morphological and physiological analyses according to Bergey's Manual of Systematic Bacteriology [13] to confirm the molecular analysis identification result.

2.4 Chitinase production and characterization

The growth and chitinase production profile of the isolate which is grown in the culture medium at 50 °C and 150 rpm agitation speed for 48 h, was recorded in order to determine the best harvest time of the enzyme. The best harvest time then is used for further steps on optimization of enzyme production. The optimization of enzyme production was conducted in a 1 L fermenter (New Brunswick™ BioFlo®/CelliGen® 115) in the culture medium at 50 °C and pH of 7 under variation of agitation (200, 300, and 400) rpm and aeration (2, 3, and 4) vvm for 30 h. The cell was counted following the standard procedure of Total Plate Count (TPC) in TCC agar plates. The harvested enzyme then was characterized for its optimum pH and temperature and tested for its thermal stability. During the thermal stability test, the enzyme was preincubated without substrate at various temperatures of (50, 60, 70, 80, 90) °C for different time intervals of (0, 30, 60, 90, 120) min respectively before being tested for its activity as described in section 2.5.

2.5 Chitinase activity assay

Chitinase activity was determined by mixing 0.5 mL of the enzyme with 0.5 mL of colloidal chitin (0.1 g mL^{-1} dissolved in 0.02 M phosphate buffer at pH 7.0). The mixture then was incubated at 55 ºC, 180 rpm for 90 min. The reaction was stopped by adding the

mixture with 1 mL of Nelson solution (Nelson A:Nelson B is 25:1) and heated in the boiling water for 20 min. The mixture was then cooled down and added with 1 mL arsenomolybdate solution and 7 mL of aqua dest before vertexing. The mixture was then centrifuged at 10×10^3 rpm for 1 min and the supernatant was taken. The presence of N–acetyl–D–glucosamine (GlcNAc) in the supernatant as a result of chitin degradation was detected spectrophotometrically at 540 nm. N–acetyl glucosamine (Sigma) was used as a standard. The amount of enzyme that liberates 1 μmol of reducing sugar as N–acetyl–D–glucosamine equivalent per minute is defined as one unit of the chitinase activity (U).

3 Results and discussions

Soil and water samples were taken from three different places in Ijen Hot Spring, namely locations A, B, and C. From these three locations, only from location B chitinolytic bacteria was found. A total of 12 chitinolytic isolates, which were shown by the presence of a halo zone around the colony, were obtained (Figure 1).

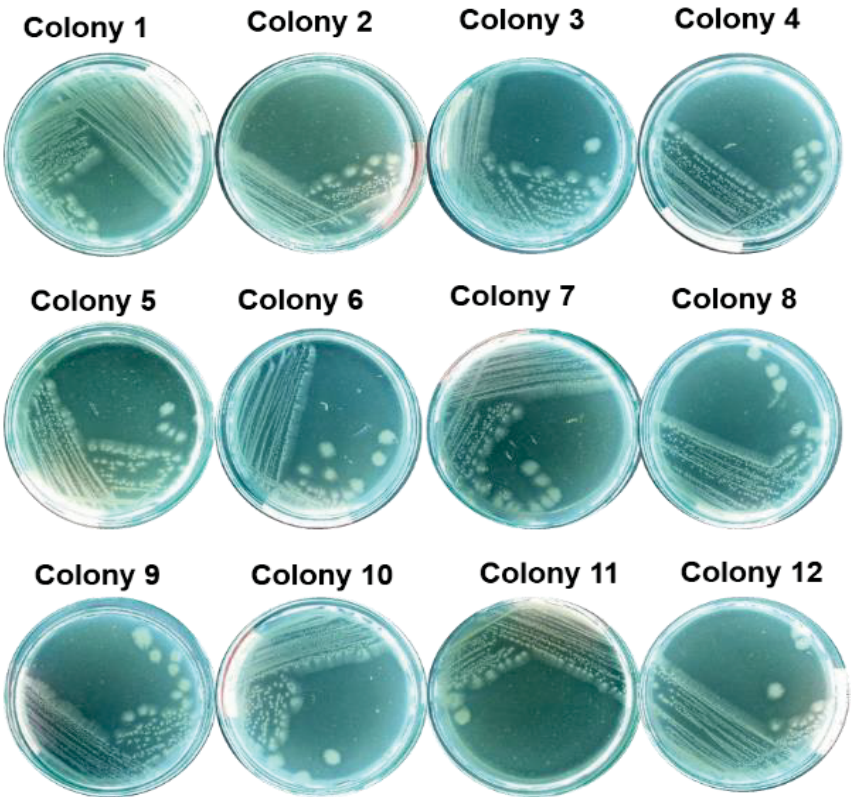

Fig.1. Twelve chitinolytic isolates were obtained from Ijen Hot Spring

Every single colony was then subcultured and tested for gram–staining and its chitinolytic activity on TCC agar plates. The halo zone around each colony was then calculated and the results were presented in Table 1. The three isolates with the biggest halo zone were selected and named isolates B2, B4, and B11.

Table 1. Screening of 12 chitinolytic isolates obtained from Location B Ijen Hot Spring.

Number of Isolate	Halo Zone Diameter (cm)	Gram Staining
1	0.079	-
2	0.119	+
3	0.086	+
4	0.112	+
5	0.084	+
6	0.108	+
7	0.109	+
8	0.088	-
9	0.092	+
10	0.099	+
11	0.110	-
12	0.080	+

The three selected isolates were then re-tested for their chitinolytic activity by sub-culturing the three isolates in the same TCC agar plates and it conducted triplicates. The results are presented in Table 2, in which isolate B2 consistently shows the biggest halo zone among the others. The isolate B2 then was selected for further processes.

Isolate B2 then was identified through partial 16S rRNA gene sequencing analysis. Chromosomal DNA of the isolate was extracted from the pure culture and the result is shown in Figure 2 (a). The chromosomal DNA was then amplified using a pair of 16S universal primer and the amplicon is visualized in Figure 2 (b).

Table 2. Screening of the three highest chitinolytic activity isolates.

Isolate Number	Diameter (cm)			
	Colony	Halo zone + Colony	Halo Zone	Average Halo zone
B–11	0.322	0.55	0.228	0.203 ± 0.0676
	0.314	0.568	0.254	
	0.281	0.407	0.126	
B–2	0.398	0.74	0.342	0.284 ± 0.0548
	0.393	0.626	0.233	
	0.466	0.743	0.277	
B–4	0.278	0.411	0.133	0.132 ± 0.0111
	0.212	0.332	0.12	
	0.354	0.496	0.142	

Homology analysis of the gene sequence showed that colony B2 was 99 % identical with *Bacillus licheniformis,* as presented in the phylogenetic tree (Figure 3). Morphological and physiological analyses of B2 isolate are presented in Table 3. All the characteristics listed are corresponding to the characteristic of *B. licheniformis* [13, 14], except on citrate utilization. The Isolate B2 showed negative, while the references were positive. Each strain of *B. licheniformis* may respond differently to citrate utilization, ranging from 20 % to 80 % positive reactions [15]. It has been reported that *B. licheniformis* isolated from rotten vegetables also showed negative activity on citrate utilization [16].

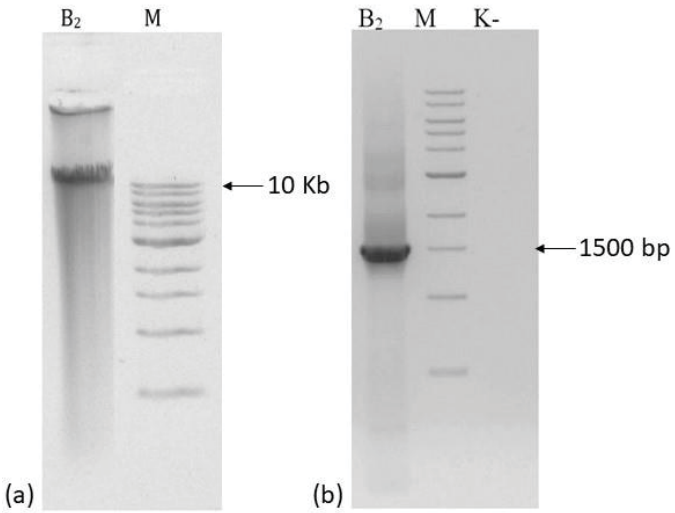

Fig 2.(a) Genomic DNA of B2 isolate; (b) Amplicon of 16S rRNA gene of B2 Isolate Marker 1 Kb Plus DNA Ladder; K- = negative control

The ability of *B. licheniformis* B2 to produce chitinase in TCC broth was tested, in which chitin was used as the solely carbon source. The fermentation process was maintained at 50 °C and a pH of 7.0 for 48 h. The growth and chitinase production by the isolate are presented in Figure 4. Corresponds to the growth curve, chitinase is produced from the log phase (12 h) and it reaches the peak at the middle of the stationary phase (30 h to 36 h). Chitinases produced by bacteria are mainly for chitin degradation and its utilization as an energy source [17]. The enzyme production decreased at 36 h to 48 h, this happened due to the lack of nutrients or secretion of toxic substances which may inactivate the enzymes [18].

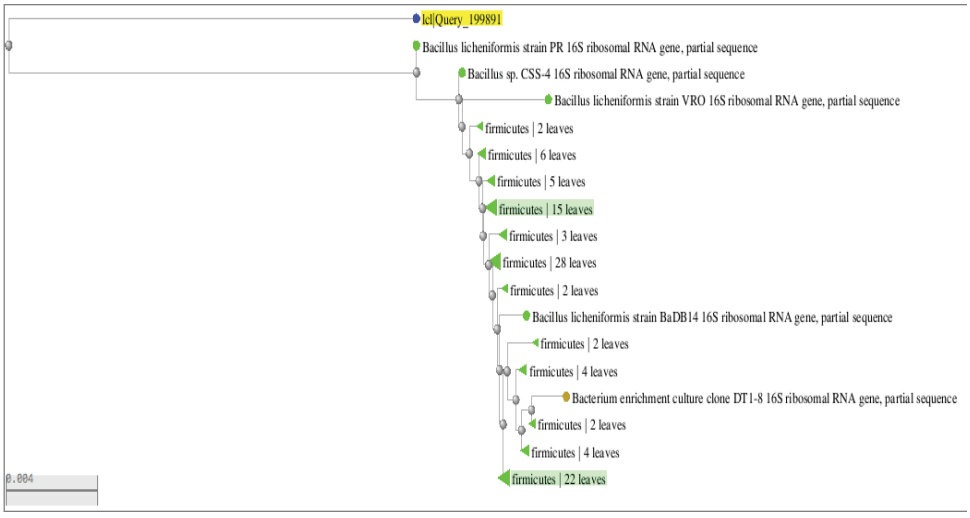

Fig 3. Phylogenetic tree based on 16S rDNA sequence analysis of B2 isolate showing homology with *B, licheniformis*

Table 3. Morphological and physiological characteristics of isolate B2.

Characteristic	**B2 Isolate**	***B. licheniformis****
Colony shape	Irregular	Irregular
Elevation	Flat	Flat
Margin	Undulate	Undulate
Colony color	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 – Mannitol – Xylose – Arabinose – Sucrose – Glycerol	 + + + + + +	 + + + + + +
Utilization of 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	+	+
Growth on: 10 ℃ 20 ℃ 30 ℃ 40 ℃ 50 ℃ 65 ℃	 - + + + + -	 - + + + + -

*Data compiled from [13] and [14]

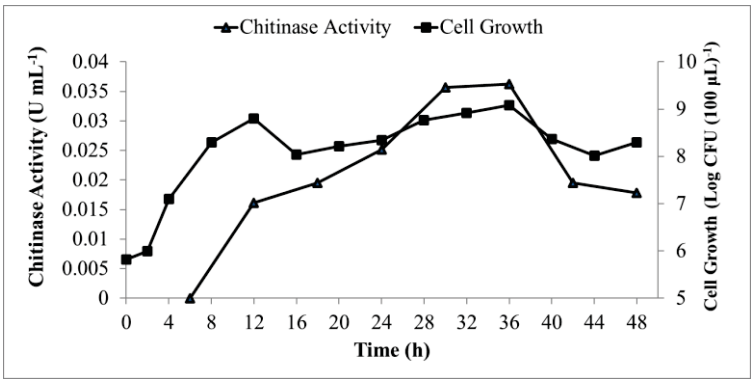

Fig. 4. The growth *of Bacillus licheniformis* B2 in Thermus colloidal chitin broth medium pH 7.0 at 50 ºC for 48 h.

Based on the chitinases production curve in Figure 4, the 30th h was defined as the enzyme harvest time and used for further optimization of fermentation processes. The chitinase production and cell growth of *Bacillus licheniformis* B2 under variation of aeration rate using a batch fermenter are shown in Figure 5. Both of the cell growth and chitinases activity values varied with a change in aeration rates and the maximum appeared at a value of 3 vvm. The increase in aeration rates would yield higher cell biomass and increase enzyme production. However, a decrease both in cell growth and enzyme production was recorded at 4 vvm aeration rate which may be derived from improper oxygen transfer in the growth medium. A higher flow rate with low agitation speed increases the airflow into the vessel along the agitator/impeller shaft, this condition is called impeller 'flooding' [19]. This phenomenon should be avoided, as the impeller surrounded by an air column, will no longer contact the liquid properly, resulting in poor mixing, reduced air dispersion, and oxygen transfer rates [19]. The poor nutrient mixing and air dispersion in the fermentation medium at a higher aeration speed can reduce the growth of microorganisms as well as enzyme production.

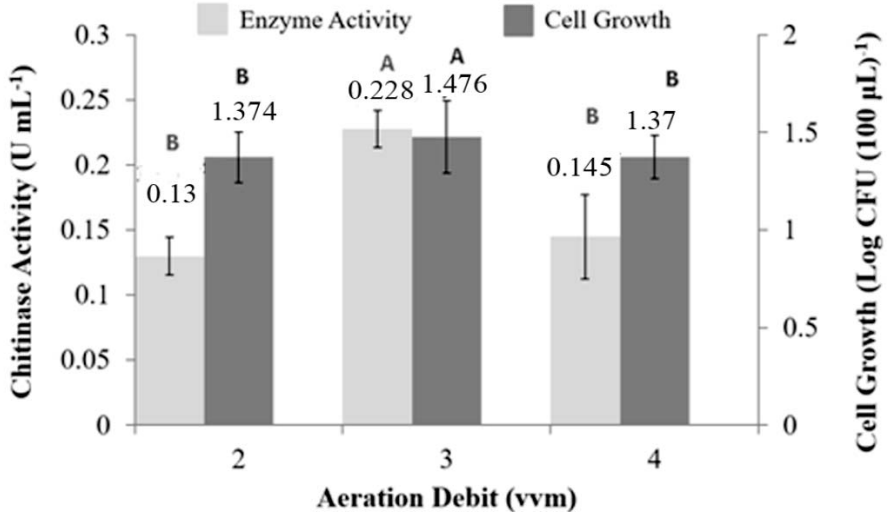

Fig 5. Enzyme production and cell growth of B2 Isolate under variation of aeration rate using batch fermenter. The different letters in each parameter indicate a significant difference ($P < 0.05$)

The agitation rate is one of the required parameters for the proper nutrient homogenization and oxygen transfer in the fermentation system. Therefore, the effect of three agitation speeds (200, 300, and 400) rpm on chitinase production and the cell growth at constant aeration speed 3 vvm were studied. Figure 6 represents the increase of cell growth and enzyme production with the increase of agitation speed from 200 rpm to 300 rpm. The enzyme activity and the cell growth reached the optimum at 300 rpm agitation speed. However, the higher agitations at 400 rpm reduced both cell growth and chitinase production due to sheer stress and heterogeneous mixing effects [19]. The results of optimization on aeration and agitation rate suggested that proper mixing is critical for better oxygen transfer rate and maximizing the chitinases production.

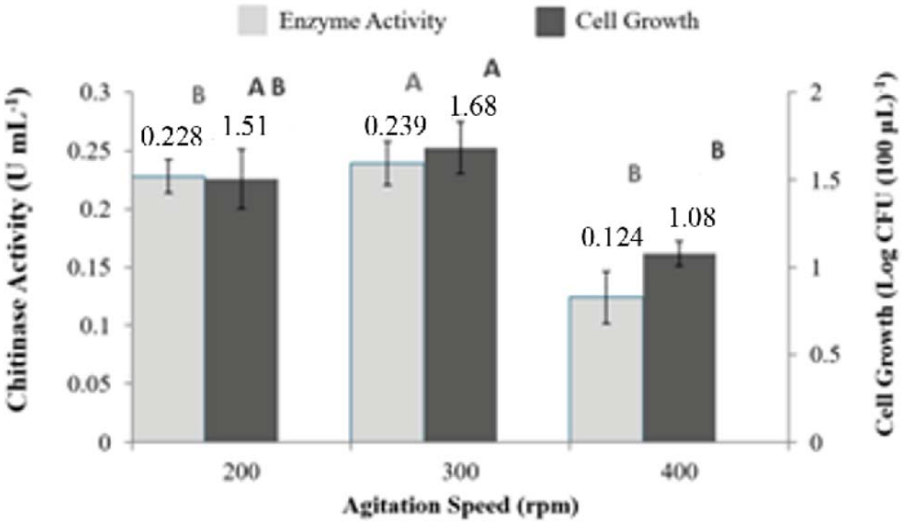

Fig 6. Enzyme production and cell growth of B2 Isolate under variation of agitation speed using batch fermenter. The different letters in each parameter indicate a significant difference ($P < 0.05$)

Enzyme activity can be affected by a variety of factors, i.e. pH and temperature. Enzymes work best in certain pH and temperature ranges, and sub-optimal conditions can cause them to lose their ability to bind to the substrate. Therefore, in this study characterization of chitinase produced by *B. licheniformis* B2 towards the variation of pH and temperature was conducted. The activity of chitinase was observed in variations of pH 5 to pH 8. The activity increased with the increase of pH until it reached the peak at pH of 7 and then decreased at the higher pH (Figure7). Each enzyme has its own optimum pH range. Conducting a reaction at pH outside of the optimum range makes the reaction runs slower. Moreover, extreme pH values can drive the enzymes to denature. The reported optimum pH of chitinases produced by *B. licheniformis* varied from pH 6 to pH 8 [20–22].

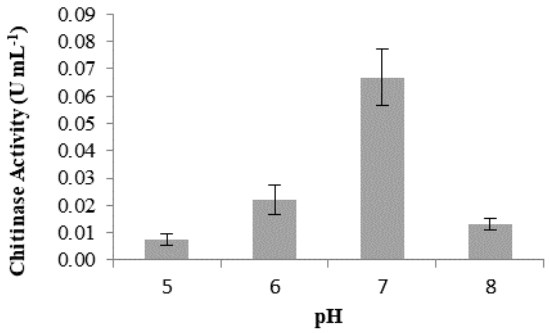

Fig 7. The activity of chitinase produced by B2 Isolate under variation of pH

The activity of the chitinase under variations of temperature from 40 to 65 °C is presented in Figure 8. The increasing temperature increased the enzyme activity until it reached the optimum at 55 °C and decreased at a higher temperature. The raising of temperature generally speeds up an enzymatic reaction, however lowering temperature slows down. Extreme high temperatures can cause an enzyme to denatured and be inactivated. Chitinases produced by *B. licheniformis* was reported to have an optimum temperature varied from 40 °C to 70 °C [20–22].

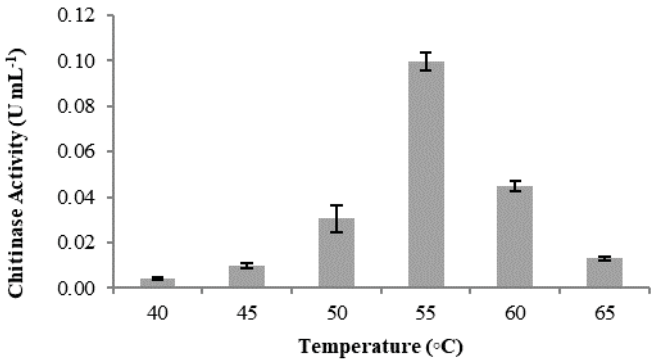

Fig 8. The activity of chitinase produced by B2 Isolate under variation of incubation temperature

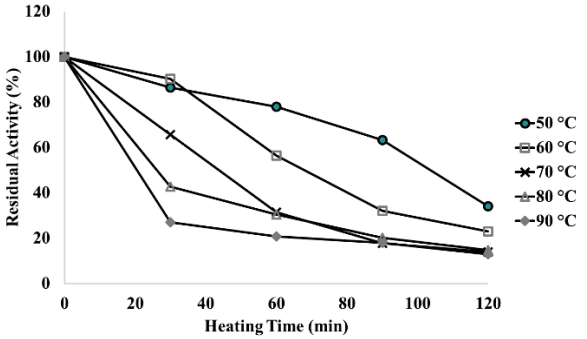

Fig 9. Residual activity of chitinase produced by B2 Isolate after heating periods

Chitinase from *B. licheniformis* B2 was also tested for its thermal stability under several heating periods. The results are shown in Figure 9. The enzyme exhibited complete heat

stability at 50 ºC for 60 min and 60 ºC for 30 min. The activity retained above 50 % after heating at 60 ºC for 60 min and 70 ºC for 30 min respectively. These findings indicate that chitinase produced by *B. licheniformis* B2 represents a high residual activity after several thermal stability tests and shows a better thermostability compared to the previously reported chitinases [23, 24].

4 Conclusions

There are 12 chitinolytic thermophilic bacteria that were successfully collected from Ijen hot spring, Indonesia. Isolate B2 which was identified as *Bacillus licheniformis*, performed the highest chitinolytic activity. This isolate produced large amounts of chitinase on Thermus colloidal chitin medium at 50 °C, pH of 7.0 within 30 h. The optimum cell growth and chitinase production were recorded at aeration and agitation rate 3 vvm and 300 rpm respectively. Chitinase produced by this isolate was optimally active at pH of 7 and a temperature of 55 °C. Moreover, it was evidently proofed as thermostable due to its high residual activity after several thermal stability tests giving a promising application in industrial processes.

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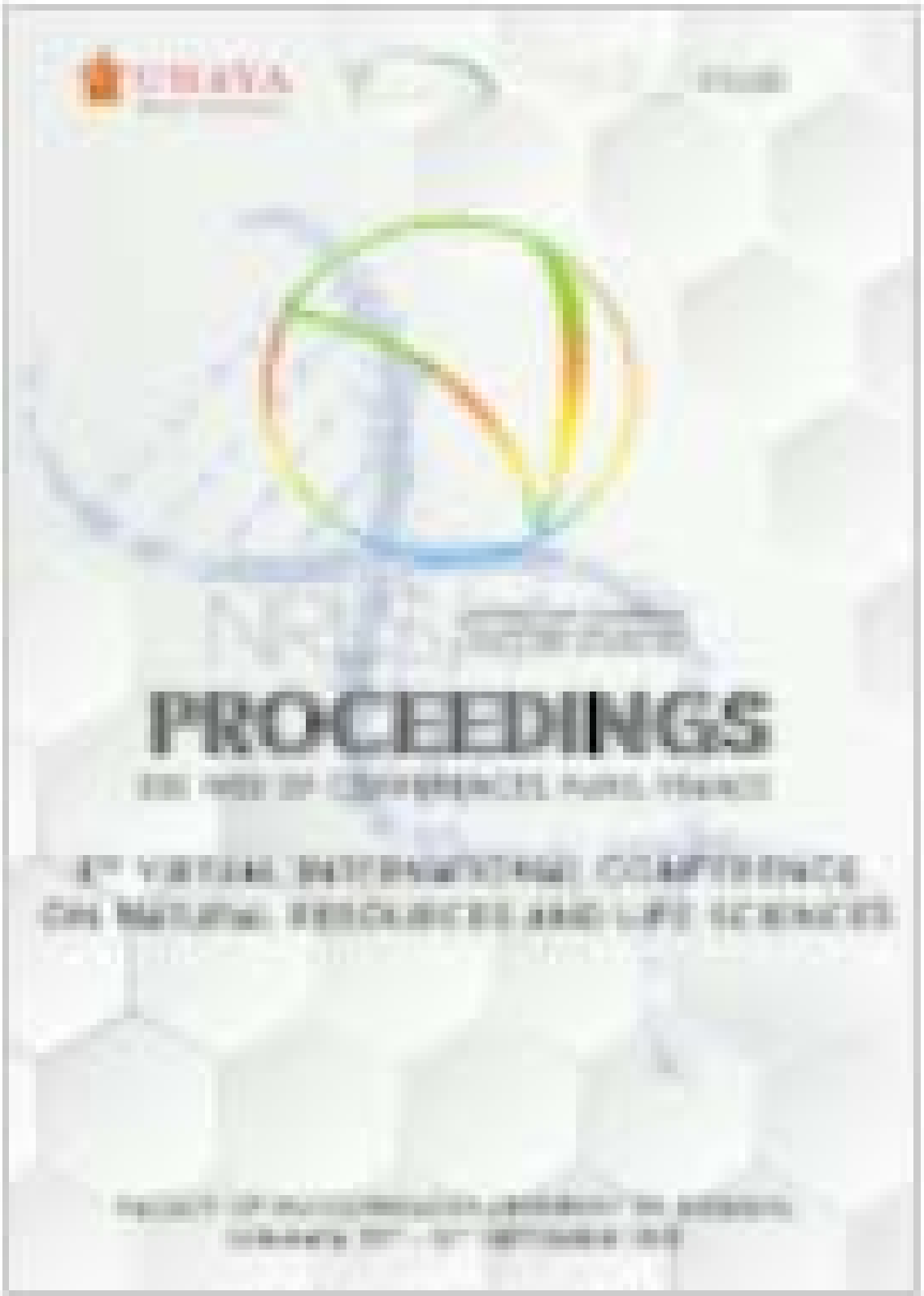
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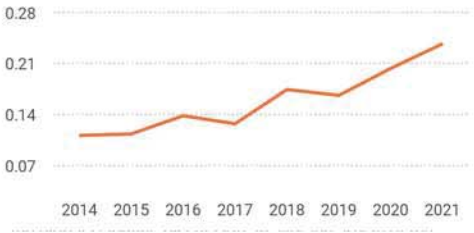

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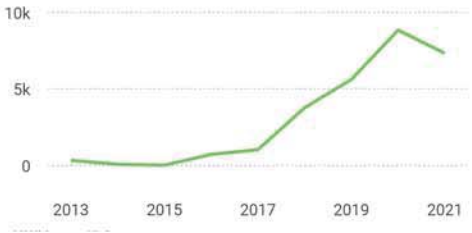

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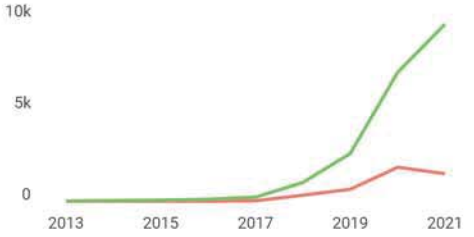

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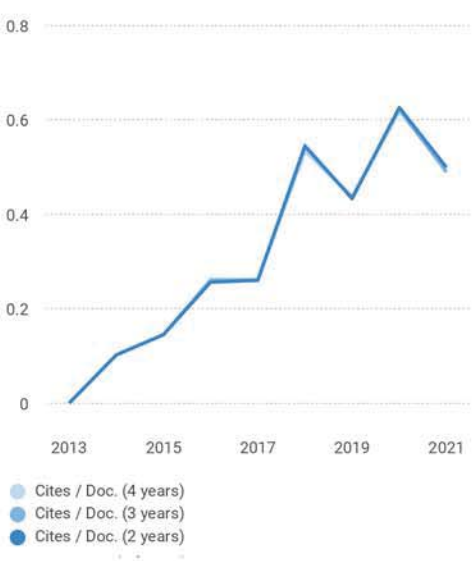

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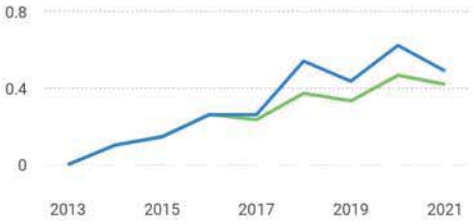

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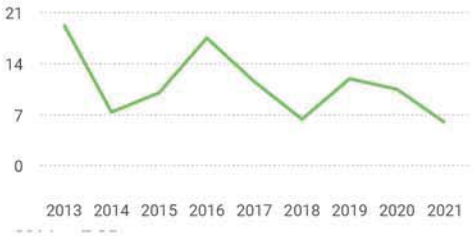

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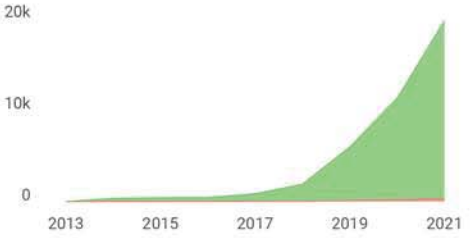

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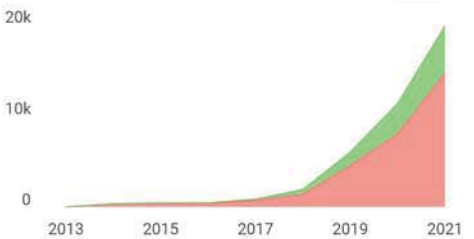

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