

## SCREENING OF THERMOSTABLE PROTEASE PRODUCING MICROORGANISMS ISOLATED FROM INDONESIAN HOTSPRING

### *Penapisan Mikroorganisme Penghasil Protease Tahan Panas yang Diisolasi dari Sumber Air Panas Indonesia*

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#### ABSTRACT

Although many proteases had been studied and characterized, only a few of them are commercially available. Protease thermostability is one of the crucial properties for industrial application. This research aimed to isolate and to screen the potential isolate which produce thermostable protease. There were 6 isolates (BII-1, BII-2, BII-3, BII-4, BII-6 and LII), isolated using solid Minimal Synthetic Medium (MSM) supplemented with 1.5% skim milk, that have, protease activity. Based on the 16S-rRNA gene sequencing analysis, isolates BII-1, BII-2 and BII-6 were identified as *Bacillus licheniformis*, isolates BII-3 and BII-4 were identified as *Bacillus subtilis*, while isolate LII was identified as *Brevibacillus thermoruber*. Three isolates (BII-6, BII-4 and LII) were then further investigated for the second screening step using liquid MSM supplemented with 1% skim milk. The isolates (BII-6, BII-4 and LII) optimally produced protease when they were cultivated at 35, 30 and 50 °C respectively after 22 h of incubation. Protease produced by BII-6, BII-4 and LII had optimum temperature of 65, 60 and 85 °C, optimum pH at 7-8, 8 and 9 and stable up to 100 min at 55, 60 and 75 °C respectively.

**Keywords:** thermostable protease, *Bacillus subtilis*, *Bacillus licheniformis*, *Brevibacillus thermoruber*

#### ABSTRAK

Meskipun banyak protease yang sudah dipelajari dan dikarakterisasi, hanya beberapa yang tersedia secara komersial. Ketahanan protease terhadap panas merupakan salah satu sifat penting untuk diaplikasikan di industri. Penelitian ini bertujuan untuk mengisolasi dan menapis isolat potensial yang menghasilkan protease yang tahan terhadap panas. Ada 6 isolat bakteri (BII-1, BII-2, BII-3, BII-4, BII-6 dan LII) yang diisolasi dengan menggunakan media padat *Minimal Synthetic Medium* (MSM) dengan penambahan 1,5% *skim milk* mampu menghasilkan protease. Analisis susunan gen 16S rRNA terhadap keenam isolat menunjukkan bahwa hanya ada 3 isolat berbeda yang teridentifikasi sebagai *Bacillus licheniformis* (BII-1, BII-2 dan BII-6), *Bacillus subtilis* (BII-3 dan BII-4) dan *Brevibacillus thermoruber*(LII). Tiga isolat (BII-6, BII-4, dan LII) selanjutnya diteliti lebih lanjut untuk penapisan kedua menggunakan medium cair MSM dengan penambahan 1% *skim milk*. Isolat-isolat ini menghasilkan protease secara optimal ketika dikultivasi pada suhu 35, 30, dan 50 °C untuk masing-masing isolat setelah inkubasi selama 22 jam. Protease yang dihasilkan oleh BII-6, BII-4 dan LII berturut-turut mempunyai suhu optimum 65, 60 dan 85 °C, pH optimum 7-8, 8-9 dan stabil sampai 100 menit pada suhu 55, 60, dan 75 °C untuk masing-masing isolat.

**Kata kunci:** protease tahan panas, *Bacillus subtilis*, *Bacillus licheniformis* *Brevibacillus thermoruber*

#### INTRODUCTION

Proteases (EC 3.4.21-24 and 99; peptidyl-peptide hydrolases) are enzyme that hydrolyze proteins via the addition of water across peptide bonds and

catalyze peptide synthesis in organic solvents and in solvents with low water content (Beg et al., 2003). Proteases, which constitute 60% of the total enzyme market (Rao et al., 1998) is the most vital enzyme used industrially and academically. The use of

protease was estimated reaching to 250 billion US\$ by 2010 (Turk, 2006; Mario et al., 2009; Akanbi et al., 2010). They are widely applied in detergent, protein modification, leather, meat, brewing, photographic, dairy, membrane cleansing and waste treatment industries (Kumar et al., 2002; Chu, 2007). The demand of the enzyme will keep increasing due to the need of the enzyme which can withstand on harsh industrial process.

Thermostability is the crucial properties of enzyme for industrial application. Thermostable proteases are stable and active above 60-70 °C and withstand to organic solvent, detergent, low and high pH and other denaturing agents (Covan et al., 1985; Covan, 1997; Gupta & Khare, 2006) so that it is particular interest in industrial process. Thermostable proteases has high specific activity due to the protein characteristic as substrate for proteases which unfolded at elevated temperature. They also useful in synthesis of high molecular weight peptide due to their resistance against organic solvents carried out in process with low water content (Sellek & Chaudhuri, 1998; Bruins et al., 2001; Synowiecki, 2008).

Commercially, protease are produced by plants, animals and microorganisms. Microorganisms are attractive sources for protease and other enzymes due to their ability to be cultured in large scale in short fermentation time with abundant desire product. To improve the enzyme performance, their genes can be manipulated easier than plants and animals (Tambekar et al., 2009; Ningthoujam & Kshetri, 2010).

Enzymologists have special interest to thermophilic microorganisms both at the fundamental and industrial level as natural source of enzymes that are active and stable at elevated temperatures. Hot spring as one of thermophile habitats is considered as promising source for the direct isolation of thermostable enzymes. The microorganisms living in hot spring are not only withstand to elevated temperature but also to the pH of environment and the presence of certain chemical compounds. Some of thermostable protease producing microorganisms were isolated from hot spring as reported by Pakpahan (2009) who isolated three unidentified isolates from Sipolohon hot spring, North Tapanuli, North Sumatera. Wilson & Remigio (2012) also reported novel moderate thermophilic bacterium (EP1001) as thermostable protease producer isolated from an alkaline hot spring, Zimbabwe.

The objective of the research was to isolate and to screen the potential isolate which produce thermostable protease from Indonesian hot spring, *i.e* Padang Cermin (Lampung) and Banyu Wedang (Bali). In this study, screening were conducted in two steps. The first step was carried out using solid media and

the second step was done using liquid media, followed by proteases characterization. The potential isolates were chosen based on its ability to produce protease which active and stable at highest temperature.

## MATERIAL AND METHOD

### Screening of Protease Producing Microorganisms Using Solid Medium

Thermostable producing bacteria was isolated from samples collected from hot springs at Padang Cermin, Lampung and Banyu Wedang, Bali using solid medium of Minimal Synthetic Medium (MSM) containing of 0.1% NaCl, 0.1% K<sub>2</sub>HPO<sub>4</sub>, 0.01% MgSO<sub>4</sub>·7H<sub>2</sub>O, 0.05% yeast extract and supplemented with 1% skim milk. The incubation was carried out at 37, 55 and 70 °C. The clear zone formed around the colony indicated the ability of the isolate to produce protease and designated as the Proteolytics Index (PI). The Proteolytics Index was determined by measuring diameter of clear zone around the colony compare to diameter of the colony. The purified cultures were preserved in 40% glycerol and stored at -70 °C.

### Phylogenetic Analysis by 16S rRNA Gene Sequencing

Bacteria's DNA was extracted using TIANamp Bacteria DNA Kit. A 16S-rRNA (1.47 kbp) fragment was amplified by PCR using a pair of universal primers (16S rDNA"27F and 16S rDNA"1492R). Fifty µl of the reaction mixture contained 1 µl chromosomal DNA, 2 µl primer, 22 µl ultrapure water and 25 µl 2xTag PCR Master Mix (Tiangen Biotech, China). The PCR reaction was set as: denaturation at 95 °C for 5 min and 30 cycles of annealing at 55 °C for 30 s, extension at 72 °C for 90 s and denaturation at 95 °C for 60 s. Final extension was carried out after 30 cycle at 72 °C for 10 min. The nucleotide sequences of the fragment were identified using 3730 DNA sequencer (Applied Biosystems, CA, USA) and subjected to a homology search against NCBI DNA database using BLAST (Basic Local Alignment Search Tool) (Altschul et al., 1990) then aligned using Clustal W program (Higgins et al., 1992) available at European Bioinformatics Institute website (<http://www.ebi.ac.uk/clustalw/>). The sequence retrieve from Gene Bank data base (Benson et al., 2003) available at the NCBI website (<http://www.ncbi.nlm.nih.gov/>). Clustal analysis (Sokal & Sneath, 1963) and the neighbor-joining mid point analysis (Saitou & Nei, 1987; Rohlf, 1993) were performed using TREECON for Windows (Version 1.3b) (Van der Peer & De Wachter, 1994). The consistency of each node was estimated by

bootstrapping over markers (Felsenstein, 1985) using 100 pseudoreplications.

### Screening in Liquid Medium

Three different species (based on 16S-rRNA analysis) of protease producing bacteria isolated from previous screening step were cultured in liquid MSM medium with 1% skim milk and incubated for 35 h at 25-60 °C. The free cell supernatant was measured for protease activity at optimum condition for each isolate.

### Protease Assay

Protease activity was determined by applying a modified method by Takami et al. (1989). The mixture containing of 0.25 ml of 1% casein in 0.025 Tris-Cl buffer pH 7 was incubated with 0.25 ml of enzyme for 10 min. The reaction was stopped by adding 0.5 mL of 0.4 M TCA. The mixture was centrifuged at 10,000 rpm for 10 min. Supernatant (0.5 ml) was mixed with 2.5 ml of 0.4 M Na<sub>2</sub>CO<sub>3</sub> and 0.25 ml of Folin-Ciocalteu's Phenol Solution and incubated for 30 min at room temperature. The absorbance of the solutions were read against the blank sample at 660 nm using Spectronic®20 Genesys™. Tyrosin standard solution, in the range of 0-1000 mg/L was prepared in triplicate to obtain a standard curve. One unit (U) of protease was defined as the amount of enzyme that could produce 1 µg of tyrosine in one minute under the defined assay conditions.

### Partial Characterization

**Optimum Temperature and pH.** Optimum temperature was determined by measuring enzyme

activity at 40 – 90 °C. Optimum pH for enzyme assay was determined by measuring the enzyme activity using substrate with pH of 4 -10 at optimum temperature.

**Thermal Stability.** The stability of enzyme against thermal was determined by incubating the enzyme in 20 mM buffer Tris-HCl at optimum pH for each isolate at three temperature levels (65, 60 and 55 °C for BII-6, 60, 55 and 50 °C for BII-4, 85, 80 and 75 °C for LII) up to 100 min. The enzyme activity was measured every 10 min and expressed as relative activity against the enzyme without thermal treatment as 100% enzyme activity.

## RESULT AND DISCUSSION

### Screening on Solid Medium

The sampling site were located at Padang Cermin, Lampung (5° 37' 59" LS; 105° 04' 20" BT) and Banyu Wedang, Bali (8° 10' 38" LS; 114° 35' 26"). The properties of samples were presented at Table 1.

The screening and isolation of protease producing bacteria were carried out at 55 °C to obtain the thermostable protease producing isolates. There were 6 isolates grown on MSM plate agar containing of 1.5% skim milk and formed clear zone around of the colony after 30 hours incubation at 55 °C (Fig 4.). The code and Proteolitics Index of the isolates were presented at Table 2. Isolates from Banyu Wedang had bigger PI value of 12.5-15.5 mm compared to that from Padang Cermin, which was 4 mm. However, the value of PI was not the only indicator for obtaining a

Table 1. Temperature and pH of *in situ* Sample

Code of Isolates	Salinity (‰)	pH	Temperature (°C)	Source of Isolates
LII	4-5	6,9	97	Padang Cermin, Lampung
BII	4	8,1	44,8	Banyu wedang, Bali

Table 2. Proteolitics Index of Protease Producing Microorganisms

Code of Isolates	Proteolitics Index (mm)
LII	4
BII	12.5-15.5



Figure 1. Protease producing microorganisms on agar plate with 1% skim milk.

potential protease. The indicator of thermostable protease producing bacteria is the ability of the isolate to produce protease which has optimum temperature and stable at the highest temperature.

Therefore, a second step of screening was conducted by growing the potential isolates in liquid medium and measuring the activity at a series of temperature and pH, and investigated their stability against thermal.

#### Phylogenetic Analysis by 16S rRNA Gene Sequencing

The analysis of 16S-rRNA sequencing showed that BII-1, BII-2 and BII-6 gave 99% similarity to *Bacillus licheniformis*, while BII-3 and BII-4 had 99% similarity to *Bacillus subtilis* and LII had 99% similarity to *Brevibacillus thermoruber*.

Most of thermostable proteases were reported to be produced by *Bacillus* sp. such as *Bacillus stearothermophilus* (Salleh et al., 1977; Razak et al., 1993) *Bacillus caldolyticus* (Burg et al, 1991), *Bacillus subtilis* (Ningthoujam & Kshetri, 2010), *Bacillus cereus* (Jabeen & Qazi, 2011) and *Bacillus mojavensis* (Haddar, 2009). On the other hand, only few other types of bacteria that were reported to produce thermostable protease such as *Aquifex pyrophilus* (Choi et al., 1999) and *Pseudomonas* sp. (Asoodeh & Musaabadi, 2012).

#### Screening on Liquid Medium

The first step of screening was carried out at 55°C on solid medium supplemented with 1.5% skim milk resulted 6 isolates forming clear zone around the colony indicating that the isolates had protease activity (Fig 1.). The second screening step was conducted by testing the most potential isolate for

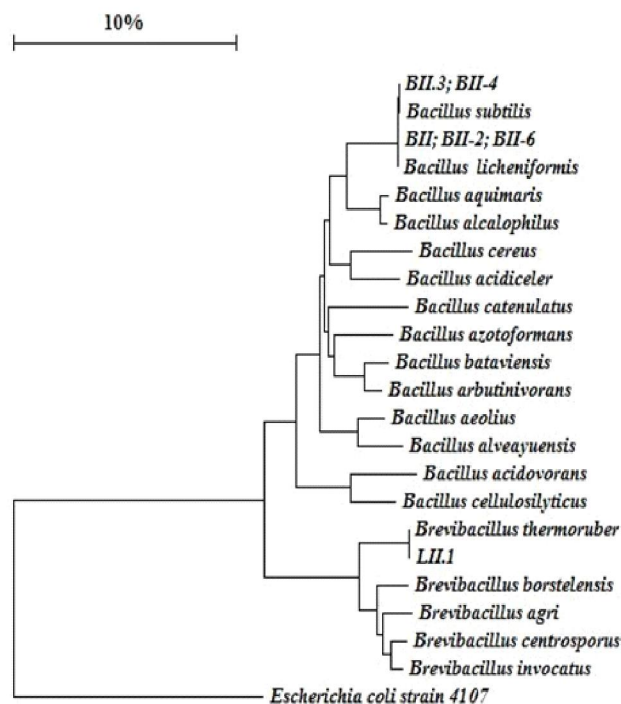


Figure 2. Phylogenetic tree of protease producing microorganisms.

their ability to produce protease in liquid medium which active and stable at highest temperature. Isolate BII-6 and BII-4 were not able to grow at 55°C and the 16S-rRNA identification showed that the isolates had 99% similarity to *Bacillus licheniformis* and *Bacillus subtilis* respectively. Hadaar (2009) and Ningthoujam & Kshetri (2010) reported two protease producing isolates that were classified as mesophile and grow optimally at 30–35 °C. Therefore, the three different species isolates (BII-6, BII-4 and LII ) were grown in liquid MSM supplemented with 1% skim milk and incubated up to 34 h at 25-40 °C for BII-6 and BII-4 isolates, and 45-55 °C for LII isolate.

The result showed that the highest protease activity was produced at temperature 35, 30 and 50 °C for BII-6, BII-4 and LII respectively (Fig 3.). The protease was produced by isolates after 22 h incubation. The optimum temperature of the proteases were 65, 60 and 85 °C (Fig 4.), and optimum pH were 7-8, 8, and 9 for BII-6, BII-4 and LII respectively (Fig 5.). The protease produced by LII showed the stability up to 100 min against thermal at highest temperature (75 °C) while protease from BII-2 and BII-6 at 55 and 50 °C respectively (Fig. 6). All isolates produced protease with optimum temperature above the optimum temperature for their growth and enzyme production.

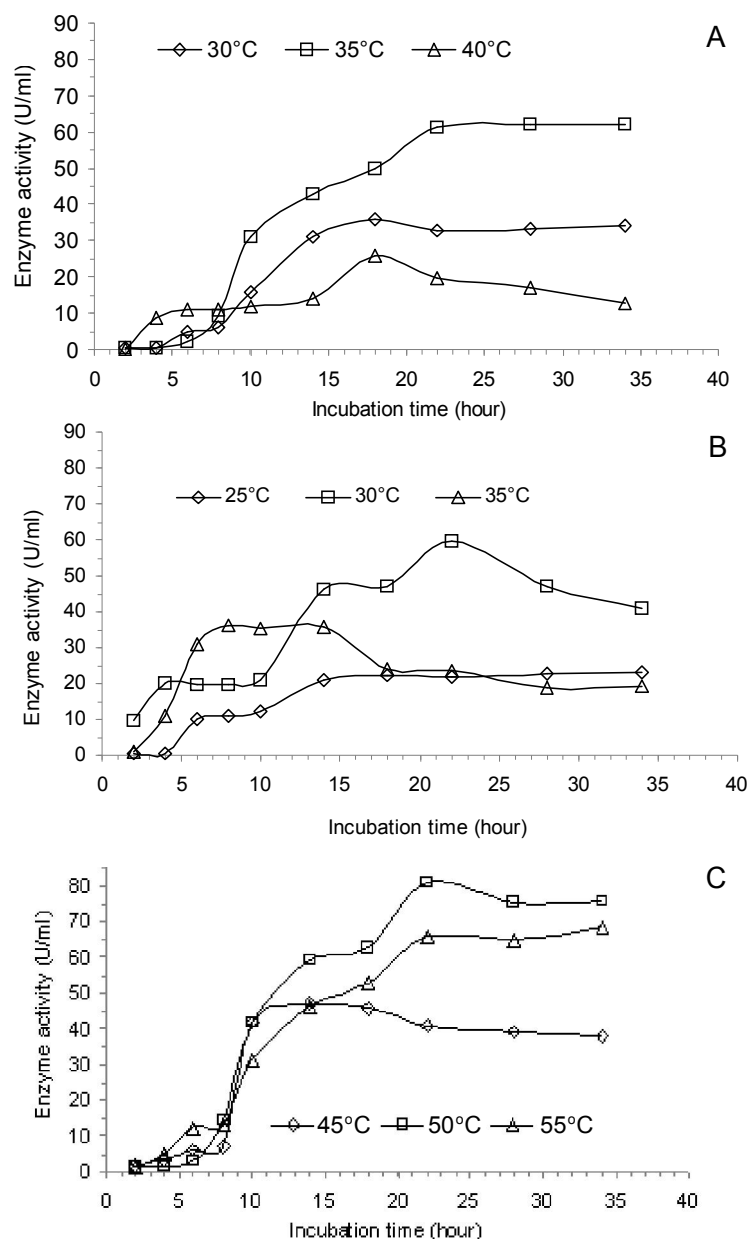


Figure 3. Effect of incubation temperatures on protease production (A): BII-6; (B): BII-4; (C): LII.

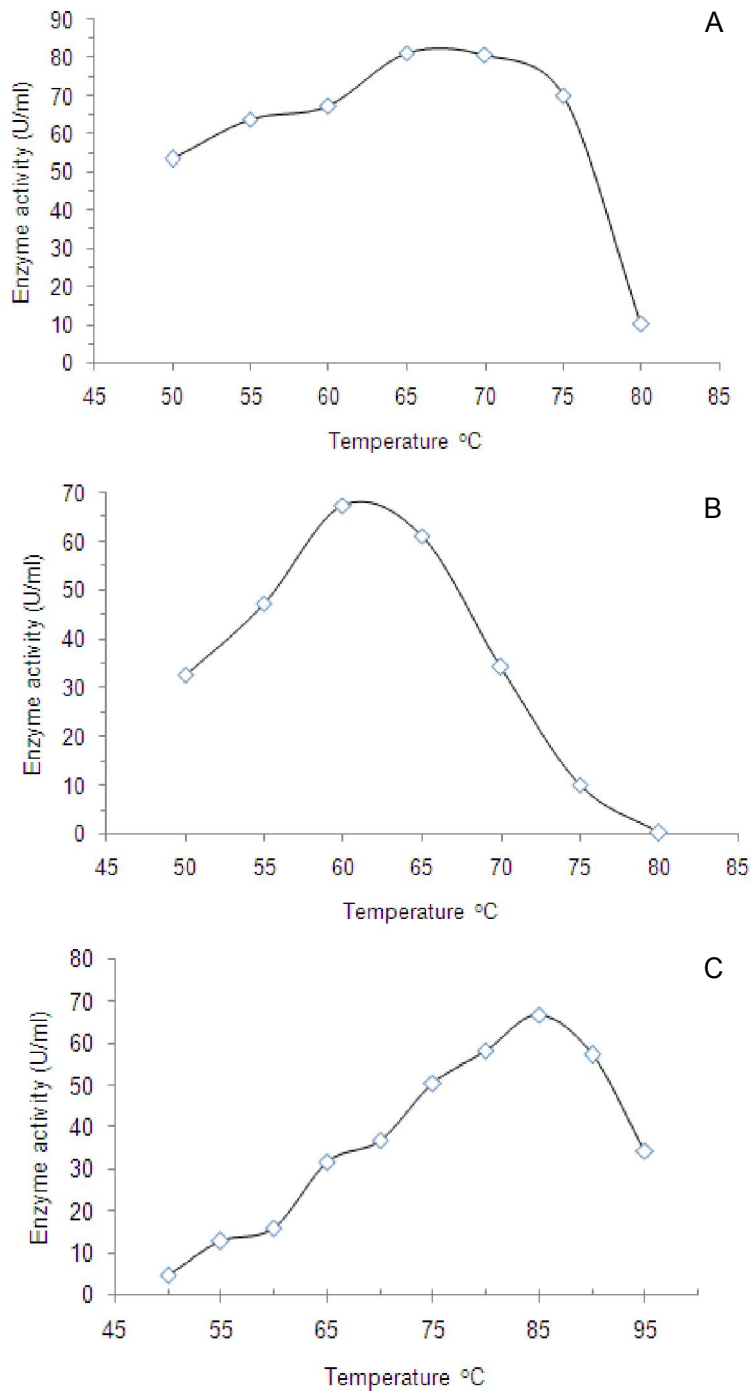


Figure 4. Effect of temperature on enzyme activity (A): BII-6; (B): BII-4; (C): LII.

These were in accordance with the previous result as shown by the thermophilic bacterium EP1001 which produced protease at 45 °C with optimum temperature for the enzyme activity of 75 °C (Wilson & Remigio, 2012), and *Bacillus subtilis* SH1 produced protease at 30 °C with optimum temperature for the enzyme activity of 50 °C (Ningthoujam & Kshetri, 2010). Other researches also reported the similar result (Salleh et

al., 1977; Burg et al, 1991; Razak et al., 1993; Choi et al., 1999; Haddar, 2009; Jabeen & Qazi, 2011; Asoodeh & Musaabadi, 2012).

This study also showed that both *Bacillus licheniformis* BII-6 and *Bacillus subtilis* BII-4 isolated from Banyu Wedang hot spring at temperature of 44.8 °C, were mesophile bacteria. The two isolates produced thermostable protease when cultivated at

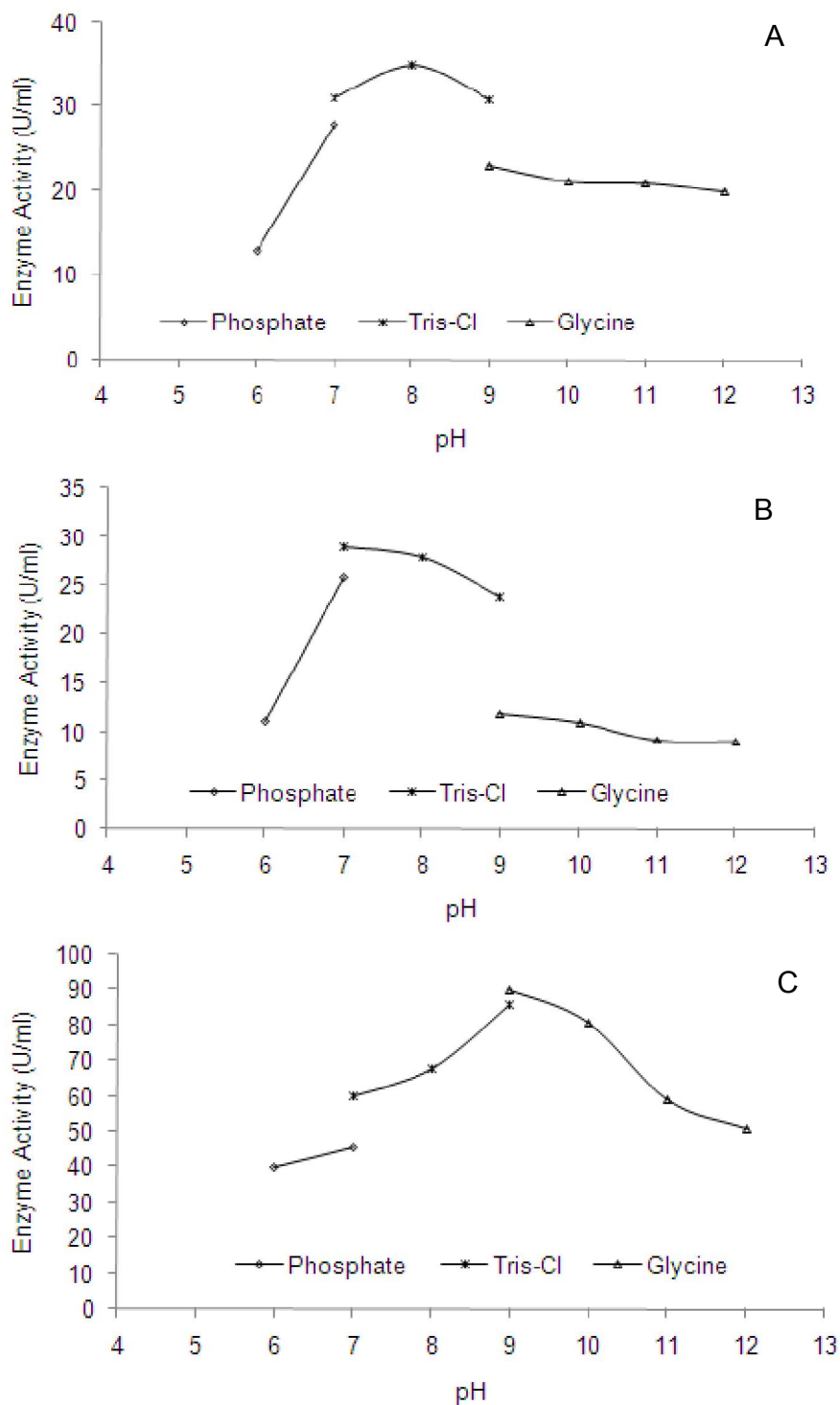


Figure 5. Effect of pH on enzyme activity (A): BII-6; (B): BII-4; (C): LII.

35 and 30 °C, even though both isolates could survive at temperature up to 55 °C and produce clear zones around the colony on agar plate containing skim milk.

The investigation showed that the clear zone around the colony or Proteolitics Indeks (PI) on agar plate can only be used to screen protease producing

bacteria but not to compare the ability of isolates to produce the potential protease as reported by Srinivasan et al. (2009); Ningthoujam & Kshetri (2010); and Bayoumi & Bahobil (2011). The characteristics of potential thermostable protease, signed by its ability to withstand against thermal, was showed by further



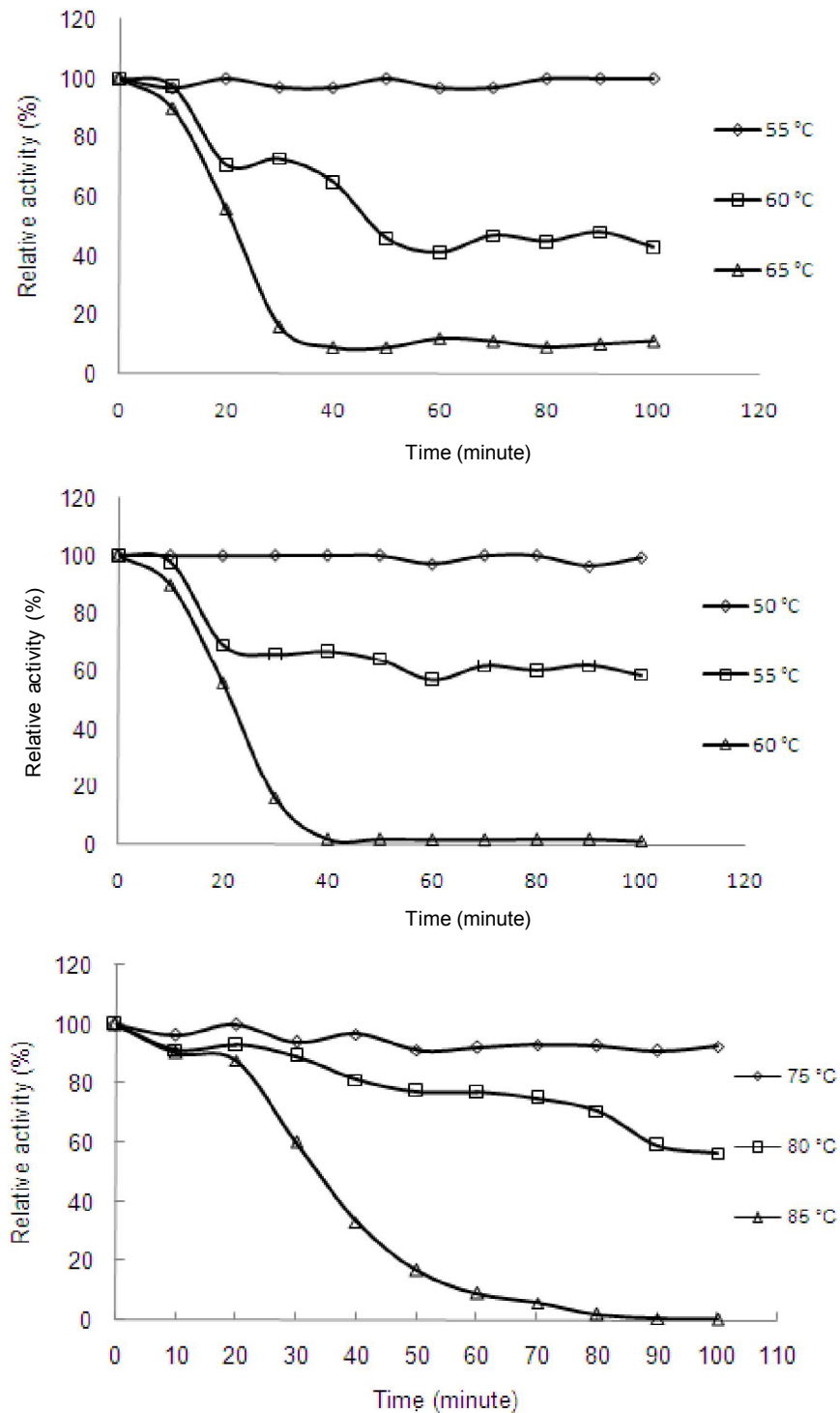


Figure 6. Effect of temperatures on enzyme stability (A): BII-6; (B): BII-4; (C): LII.

investigation, *i.e* by growing the isolates in liquid medium. Isolate LII, which only showed barely clear zone on agar plate (Fig. 1 and Table 2), produced thermostable protease with higher activity compare to others (Fig. 3). Protease produced by LII also showed the highest optimum temperature as well as the stability against thermal (Fig. 5 and Fig. 6).

## CONCLUSION

There were 6 protease producer isolates (BII-1, BII-2, BII-3, BII-4, BII-6 and LII) obtained from screening using solid MSM supplemented with 1.5% skim milk. Based on 16S-rRNA analysis those isolates were identified as *Bacillus licheniformis* (BII-1, BII-2 and



BII-6), *Bacillus subtilis* (BII-3 and BII-4) and *Brevibacillus thermoruber* (LII).

Further screening on three different isolates (BII-6, BII-4 and LII) in liquid medium revealed that the LII isolate as the most potential protease producer. The enzyme had characteristics of optimum temperature of 85°C, optimum pH of 9 and stable for up to 100 min at 75°C.

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