

Full Length Research Paper

Production of thermostable and organic solvent-tolerant alkaline protease from *Bacillus coagulans* PSB-07 under different submerged fermentation conditions

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An alkaliphilic bacterium producing organic solvent-tolerant and thermostable alkaline protease was isolated from poultry litter site and identified as *Bacillus coagulans* PSB-07. Protease production under different submerged fermentation conditions were investigated with the aim of optimizing yield of enzyme. *B. coagulans* PSB-07 produced protease optimally at the beginning of stationary phase over broad pH range of 6.0 to 11.0 and temperature range of 30 to 50°C. The optimum enzyme production was at 37°C and pH 8.0. The media composition that supported maximum yield of protease (760.4 U/ml) contained sucrose (0.5%), peptone (0.75%), NaCl (0.05%) and MgSO₄·7H₂O (0.01%). The protease had optimum activity at 60°C and pH 8.0 with casein as substrate. The enzyme was stable over a broad pH of 6.0 to 12.0 and showed good thermostability retaining 91.4 and 50.5% of its original activity after incubation at 50 and 60°C for 60 min at pH 8.0 in the presence of CaCl₂. The protease was strongly activated by metal ions, Ca²⁺ and Mg²⁺. The enzyme showed remarkable activity and stability in the presence of all organic solvents studied except benzene. The exhibited properties of the protease suggest the suitability of the enzyme for applications in peptide synthesis, detergent formulation and bio-transformation in non-aqueous medium.

Key words: Alkaline protease, *Bacillus coagulans* PSB-07, organic solvent-tolerant, thermostable, production.

INTRODUCTION

Proteases cover up to 60% of total enzyme market and are valuable commercial enzymes that have biotechnological as well as industrial applications; however, the present known proteases are not sufficient to meet most of the industrial demands. Alkaline proteases hold a great potential for application in the detergent and leather industries (Gupta et al., 2002; Kumar and Takagi, 1999). Proteases have been isolated from microbes, plants and animals. In bacteria, serine and metallo-proteases are the principal classes of proteases found in several species such as *Bacillus subtilis*, *Bacillus amyloliquefaciens*, *Bacillus coagulans*,

Pseudomonas sp., *Lysobacter enzymogenes* and *Escherichia coli* (Fujishige et al., 1992).

In recent years, studies have been carried out to characterize alkaline protease from various microorganisms. However, many of the alkaline proteases for industrial purposes have limitations such as low stability towards temperature, pH, metal ions and production cost of the enzymes arising from growth medium (Joo and Chang, 2005). Research efforts have been directed mainly towards evaluating the effect of various cost-effective substrates on the yield of enzymes, requirement of divalent metal ions in the fermentation medium and

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optimization of environmental and fermentation parameters but up to date, no defined medium has been established for the optimum production of proteases from different microbial sources (Gouda, 2006).

In this study, the production of extracellular alkaline protease from a newly isolated *B. coagulans* PSB-07 was investigated under different submerged fermentation conditions and some physicochemical properties of the enzyme were determined.

MATERIALS AND METHODS

Bacterial strain

The microorganism used was an alkaliphilic bacterium isolated from soil sample of a poultry litter site. This strain was identified as *B. coagulans* PSB-07 by the Biotechnology Unit of Federal Institute of Industrial Research, Lagos based on methods described in Bergey's Manual of Systematic Bacteriology (2009). This bacterial strain was maintained on nutrient agar slants, and stored at 4°C.

Inoculum preparation and protease production

Seed inoculum was prepared by growing a loopful of slant culture of *B. coagulans* PSB-07 in 20 ml of seed medium containing 0.75% (w/v) peptone, 0.5% (w/v) glucose, 0.05% (w/v) NaCl and 0.01% (w/v) MgSO₄·7H₂O in a 200 ml conical flask with pH adjusted to 8.0. The inoculated seed medium was incubated at 37°C for 24 h at 180 rpm in a shaking incubator (Stuart, UK). The 24 h old seed culture was used as inoculum for the production media. 2.5 ml of seed inoculum (constituting 5% v/v) was transferred into 50 ml of production media which had same composition as the seed medium. At the end of 48 h cultivation period, cultures were harvested by centrifugation at 10,000 rpm for 15 min at 4°C using refrigerated bench-top centrifuge (Eppendorf 5810R). The cell free supernatant was recovered as crude enzyme preparation and used for further studies.

Assay of protease activity

Protease activity was determined by a modified procedure of Fujiwara et al. (1993) using 1.0% casein in 50 mM Tris-HCl buffer (pH 8.0) as substrate. The assay mixture consisted of 2.0 ml of substrate and 0.5 ml of enzyme solution in 50 mM Tris-HCl buffer, pH 8.0. The reaction mixture was incubated at 40°C for 30 min and reaction was terminated by the addition of 2.5 ml of 10% (w/v) trichloroacetic acid (TCA). The mixture was allowed to stand for 15 min and then centrifuged at 10,000 rpm for 10 min at 4°C to remove the resulting precipitate. Protease activity was determined by measuring the absorbance of the supernatant at 280 nm. One unit of protease activity was defined as the amount of enzyme required to release 1 µg of tyrosine per ml per minute under the standard assay conditions.

Effects of different fermentation conditions on protease production

Growth profile and production of protease

Growth profile of *B. coagulans* PSB-07 over the cultivation period and production of protease were studied by cultivating the microorganism for 12, 24, 36, 48, 60, 72 and 84 h at pH 8.0 and

37°C with shaking at 200 rpm. 50 mM Tris-HCl, pH 8.0 was used for media preparation. The growth of the microorganism was determined by measuring the absorbance of culture at 600 nm. The fermentation broth was centrifuged at the end of each cultivation period and the supernatant was used for determination of protease activity.

Effects of temperature on growth and protease production

Optimal temperature for growth and protease production by *B. coagulans* PSB-07 was determined by monitoring growth profile and protease production at fixed media concentration and pH with varied temperature. Cultures were grown at 20, 30, 37, 40, 45, 50, and 60°C at 200 rpm, pH 8 for 48 h which was the determined cultivation period for optimal growth of *B. coagulans* PSB-07 and protease production.

Effect of pH on growth and protease production

The effect of pH on growth of *B. coagulans* PSB-07 and protease production was investigated by growing the cultures of *B. coagulans* PSB-07 at fixed media concentration and temperature 37°C with varied pH of 6.0 to 11.0 at 200 rpm for 48 h using appropriate buffers. 50 mM of buffer solutions (sodium citrate, pH 6.0, Tris-HCl, pH 7.0 and 8.0, and glycine-NaOH, pH 9.0 to 11.0) were used for media preparation. Optimal pH for growth and protease production by *B. coagulans* PSB-07 was determined at the end of the cultivation period by measuring the absorbance of culture at 600 nm and determining protease activity in the cell free supernatant obtained after centrifugation at 10,000 rpm and 4°C for 15 min.

Effect of carbon sources on protease production

Some carbon sources were investigated for their effects on protease production by *B. coagulans* PSB-07. Fructose, maltose, lactose, yam (*Dioscorea alata*) flour, sucrose and soluble starch were tested at 0.5% w/v at the determined optimal pH and temperature for protease production by *B. coagulans* PSB-07 with glucose as control. Cultures were grown for 48 h at 200 rpm. Growth and protease production were measured at the end of the cultivation period to determine the carbon sources that supported high yield of enzyme.

Effect of nitrogen sources on protease production

Effect of some nitrogen sources (0.75%, w/v) on growth and protease production by *B. coagulans* was studied at the optimal temperature and pH for protease production. The nitrogen sources used were groundnut meal, beef extract, locust bean meal, gelatin, yeast extract and soybean meal. These were used to replace peptone (control) in different production media and cultures were grown for 48 h at 200 rpm. Growth and protease production were measured at the end of the cultivation period to determine the nitrogen sources that supported high yield of protease.

Determination of some properties of protease from *B. coagulans* PSB-07

Effect of temperature on enzyme activity and stability

Effect of temperature on activity of crude enzyme was determined by incubating the reaction mixture at temperatures ranging from 30

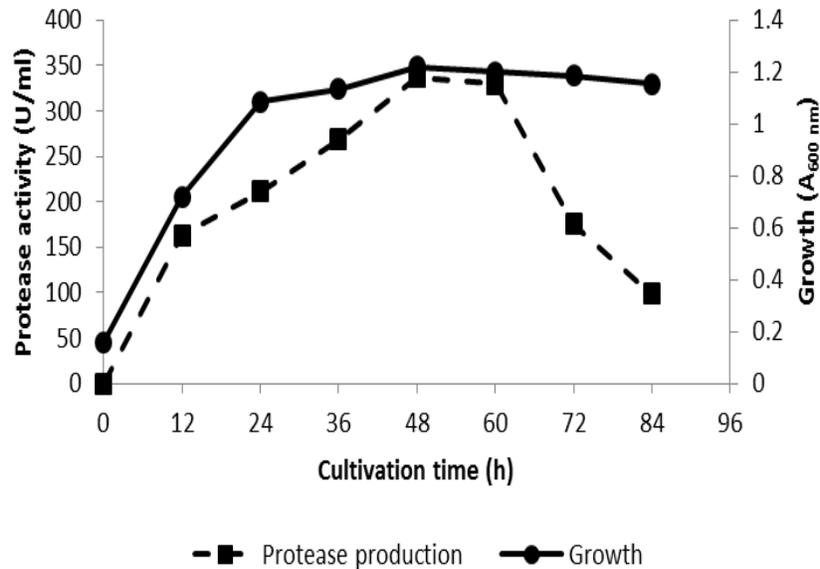


Figure 1. Growth profile and protease production of *Bacillus coagulans* PSB-07 over 84 h cultivation period. Protease activity (U/ml) was used to measure protease production.

to 80°C at pH 8.0 for 30 min in the presence and absence of 5 mM CaCl₂ and activity of protease was determined according to the standard assay procedure. The thermal stability of the protease was determined by incubating crude enzyme at temperatures ranging from 40 to 80°C in the presence and absence of 5 mM CaCl₂ for 60, 90 and 120 min, respectively. The residual protease activity was determined according to the standard assay procedure.

Effect of pH on enzyme activity and stability

The effect of pH on activity of protease was determined by assaying for enzyme activity at different pH values ranging from 4.0 to 12.0. The pH was adjusted using 50 mM of the following buffer solutions: sodium acetate, pH 4.0 and 5.0, sodium citrate, pH 6.0, Tris-HCl, pH 7.0 and 8.0 and glycine-NaOH, pH 9.0 to 12.0. Reaction mixtures were incubated at 40°C for 30 min and the activity of the protease was measured according to standard assay procedure. Effect of pH on stability of the protease was determined by incubating the enzyme in relevant buffers of varied pH (4.0 to 12.0) without substrate for 60 min at 40°C and the residual protease activity was determined as described earlier.

Effect of metal ions on protease activity

The effects of metal ions (Ca²⁺, Mg²⁺, Hg²⁺) and combinations of the divalent cations (Ca²⁺ + Mg²⁺ and Ca²⁺ + Hg²⁺) on protease activity were investigated by adding 5mM of each metallic chloride or combination to the reaction mixture. Protease activity was determined following the standard assay procedure.

Hydrolysis of protein substrates

Hydrolysis of protein substrates such as casein, bovine serum albumin (BSA), ovalbumin and gelatin by the crude protease was determined. Protease activity was assayed by mixing 0.5 ml of crude enzyme with 2.0 ml assay buffer containing 1% (w/v) of

protein substrate. After incubation at 40°C for 30 min, the reaction was stopped by adding 2.5 ml of 10% w/v TCA. The mixture was allowed to stand for 15 min and then centrifuged at 10,000 rpm for 10 min at 4°C to remove the resulting precipitate. Protease activity was determined by estimating the amount of tyrosine in the supernatant. Activity towards casein was used as control.

Effect of organic solvents on protease activity and stability

Effects of glycerol, methanol, isopropanol, dimethyl sulfoxide (DMSO), benzene, and acetone on crude protease activity were studied by introducing the selected organic solvent into the reaction mixture at concentration of 0.5% (v/v) and 1% (v/v) and protease activity was determined according to the standard assay procedure. Organic solvent stability of protease was investigated by pre-incubating 0.75 ml of crude protease with 0.25 ml of organic solvent at 40°C for 30 min with shaking. The residual protease activity was determined and compared with the control which had distilled water in place of the tested organic solvents.

RESULTS AND DISCUSSION

Effects of different fermentation conditions on protease production

Growth profile and protease production

Growth profile of *B. coagulans* PSB-07 and protease production were studied to determine the cultivation period for optimal yield of enzyme and evaluate the effect of microbial growth on protease production. *B. coagulans* PSB-07 exhibited exponential growth up to 48 h, followed by stationary phase (Figure 1). Protease production by *B. coagulans* PSB-07 is also shown in Figure 1. Enzyme activity started appearing in the medium at about 12 h of

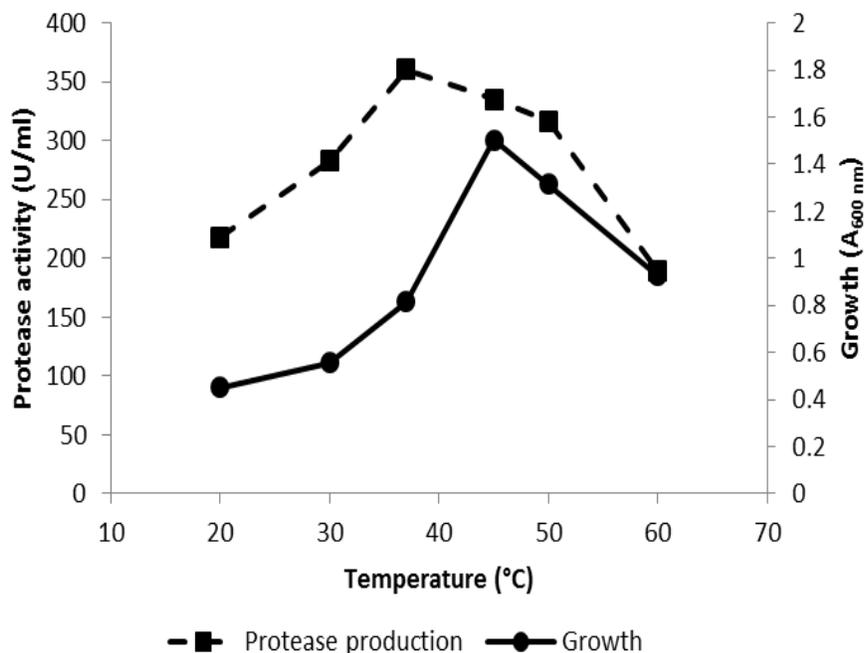


Figure 2. Effects of temperature on growth and protease production over 48 h cultivation period.

cultivation. Protease production continued to increase till the 48 h with maximum enzyme activity of 337.35 U/ml. This suggests that production of protease by this *Bacillus* sp. is dependent on cell growth.

Effect of temperature on growth and protease production

Optimum growth of *B. coagulans* PSB-07 was observed at 45°C after which the growth decreased (Figure 2), revealing that *B. coagulans* PSB-07 is mesophilic. Previous studies on the characteristics of alkalophilic *Bacillus* strains producing alkaline proteases reveal that most strains have temperature optima between 30 to 45°C (Kanekar et al., 2002; Joo et al., 2003; Secades et al., 2001). Maximum protease production was obtained at 37°C (Figure 2). This agrees with recently reported optimum temperature for protease production by *B. amyloliquefaciens* PFB-01 (Olajuyigbe and Ogunyewo, 2013).

Effect of pH on growth and protease production

The growth of *B.coagulans* PSB-07 increased from pH 6.0 to 8.0 but began to decrease from pH 9.0 to 11.0 (Figure 3). Also, maximum protease production was obtained at pH 8.0 after which there was a decline (Figure 3). Protease from *B. subtilis* studied by Das et al. (2010) showed similar trend. However, *Bacillus clausii* I-

52 produced protease maximally at pH 11.0 (Joo et al., 2003).

Effect of carbon sources on growth and protease production

All the carbon sources studied supported growth and production of protease by *B. coagulans* PSB-07 (Figure 4). The growth of *B. coagulans* PSB-07 was maximum using glucose as carbon source. Protease production was supported by sucrose, glucose, fructose and maltose showing that these carbon sources were most effectively utilized by the organism for enzyme production. Sucrose supported maximum enzyme production of 760.37 U/ml (Figure 4). These results are similar with those reported by Madzak et al. (2000) that sucrose was the best substrate for production of extracellular proteases in their study. Some earlier reports also indicated that production of protease was enhanced in the presence of maltose and glucose as carbon sources (Gupta et al., 2005; Mehrotra et al., 1999). However, repression of protease production in the presence of glucose, maltose and sucrose had been previously reported (Sen and Satyanarayana, 1993).

Effect of nitrogen sources on growth and protease production

Effects of different nitrogen sources on growth and

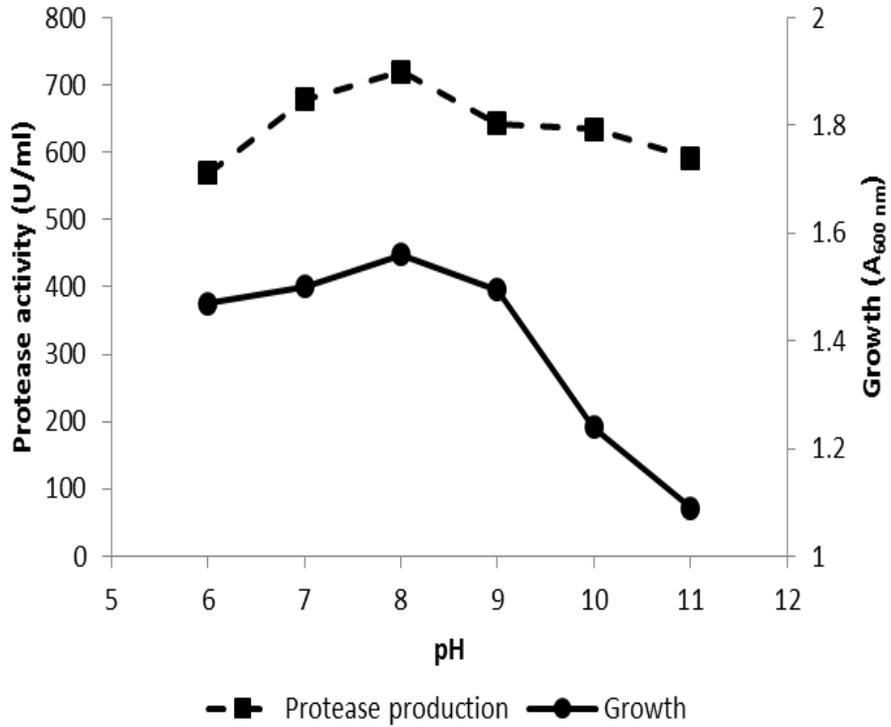


Figure 3. Effects of pH on growth and protease production over 48 hours cultivation period.

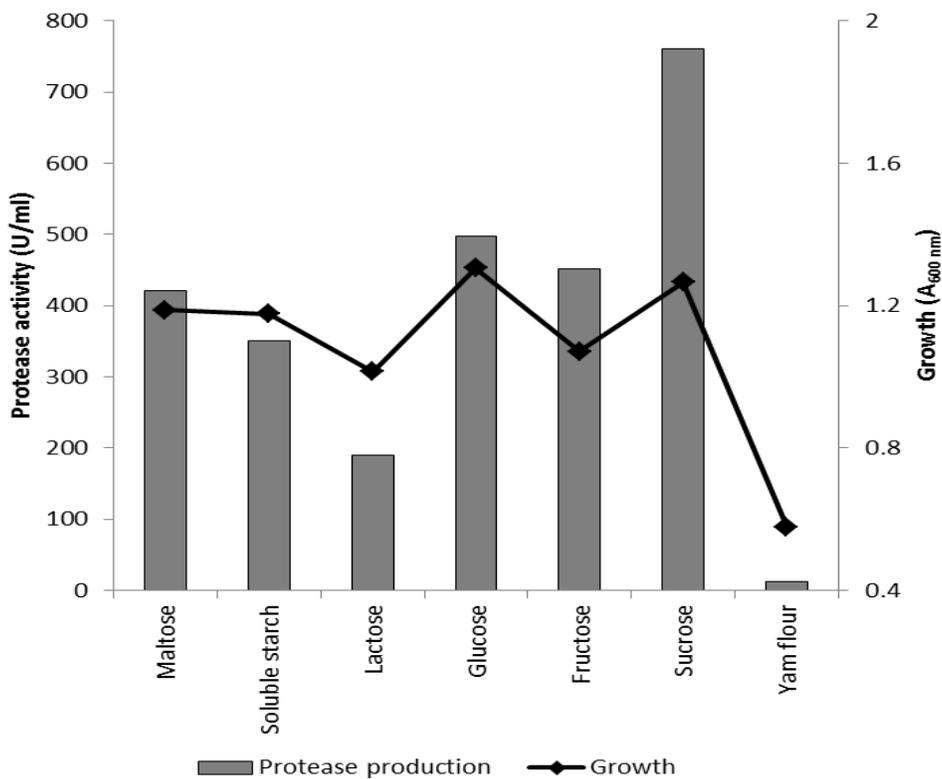


Figure 4. Effects of some carbon sources on growth and protease production of *Bacillus coagulans* PSB-07.

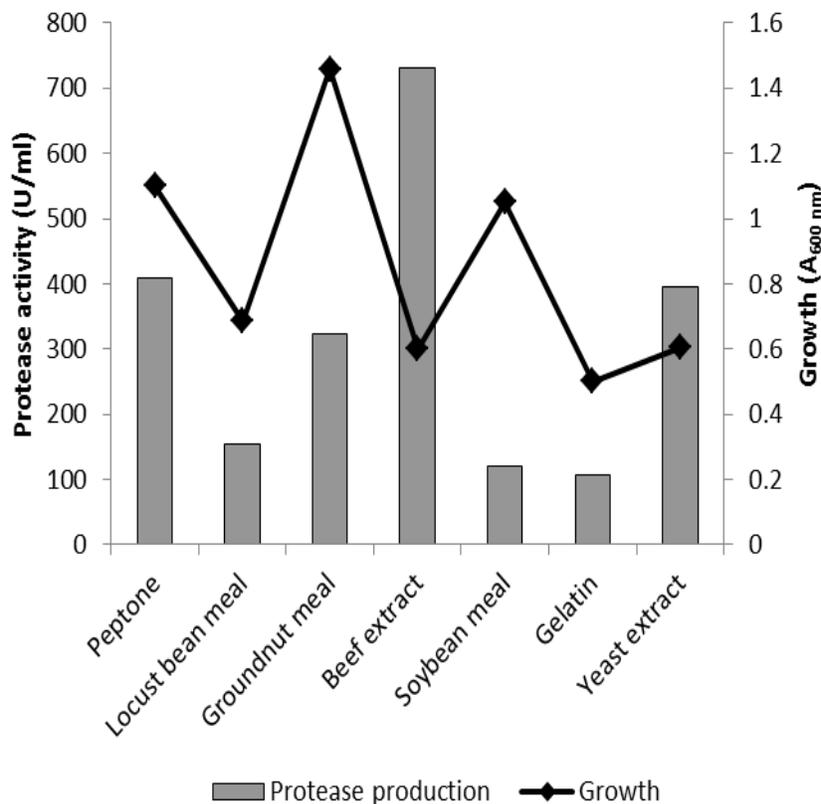


Figure 5. Effects of somenitrogen sources on growth and protease production of *Bacillus coagulans* PSB-07.

protease production at pH 8.0 and temperature 37°C are shown in Figure 5. Groundnut meal supported optimum growth of *B. coagulans* PSB-07 while optimum protease production of 731.64 U/ml was achieved with beef extract. Similar finding was reported by Udandi et al. (2009) on beef extract as the best substrate for protease production by *Bacillus* sp. It was observed in this study that locust bean meal, soybean meal and gelatin supported growth *B. coagulans* PSB-07 but with low yield of enzyme.

Characterization of protease from *Bacillus coagulans* PSB-07

Effect of temperature on protease activity and stability

The optimum temperature for protease from *B. coagulans* PSB-07 was 60°C both in the presence and absence of CaCl₂ (Figure 6). The enzyme activity declined sharply at temperatures above 60°C. The protease from *B. coagulans* PSB-07 showed high thermostability retaining 91% of its original activity at 50°C and 50.5% residual activity at 60°C after 60 min of incubation at pH 8.0 in the presence of 5 mM CaCl₂ (Figure 7). Surprisingly, this

protease still retained 29% residual activity after 120 min incubation at 70°C in the presence of 5 mM CaCl₂. The thermostability of the protease was enhanced in the presence of Ca²⁺.

Effect of pH on protease activity and stability

The optimum pH for activity of protease from *B. coagulans* PSB-07 was 8.0 (Figure 8). The protease activity began to decline above pH 8.0 with the enzyme having relative activity of 23.94 and 15.76% at pH 9.0 and 10.0. However, the protease had optimum stability at pH 9.0. The protease was stable under alkaline conditions indicating its potential use in detergent formulation and leather (Saeki et al., 2007; Dias et al., 2008).

Effect of metal ions on protease activity

Protease activity was enhanced in the presence of Ca²⁺ and Mg²⁺ with relative activity of 141.7 and 118.8%, respectively (Table 1). The combination of the two divalent cations improved protease activity when compared with enzyme activity in the presence of only

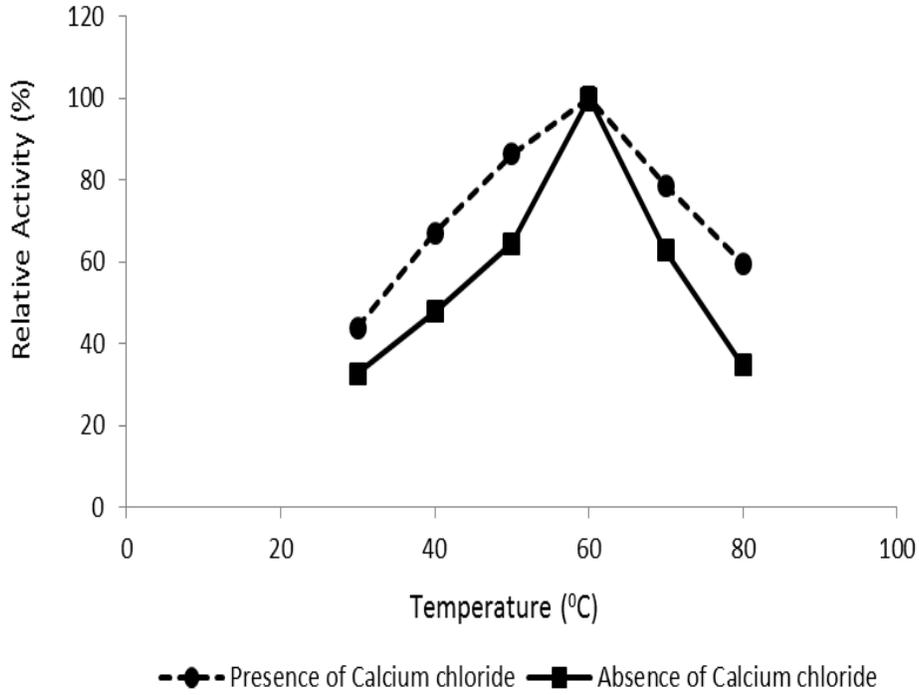


Figure 6. Effects of temperature on activity of protease from *Bacillus coagulans* PSB-07 in the presence and absence of CaCl₂.

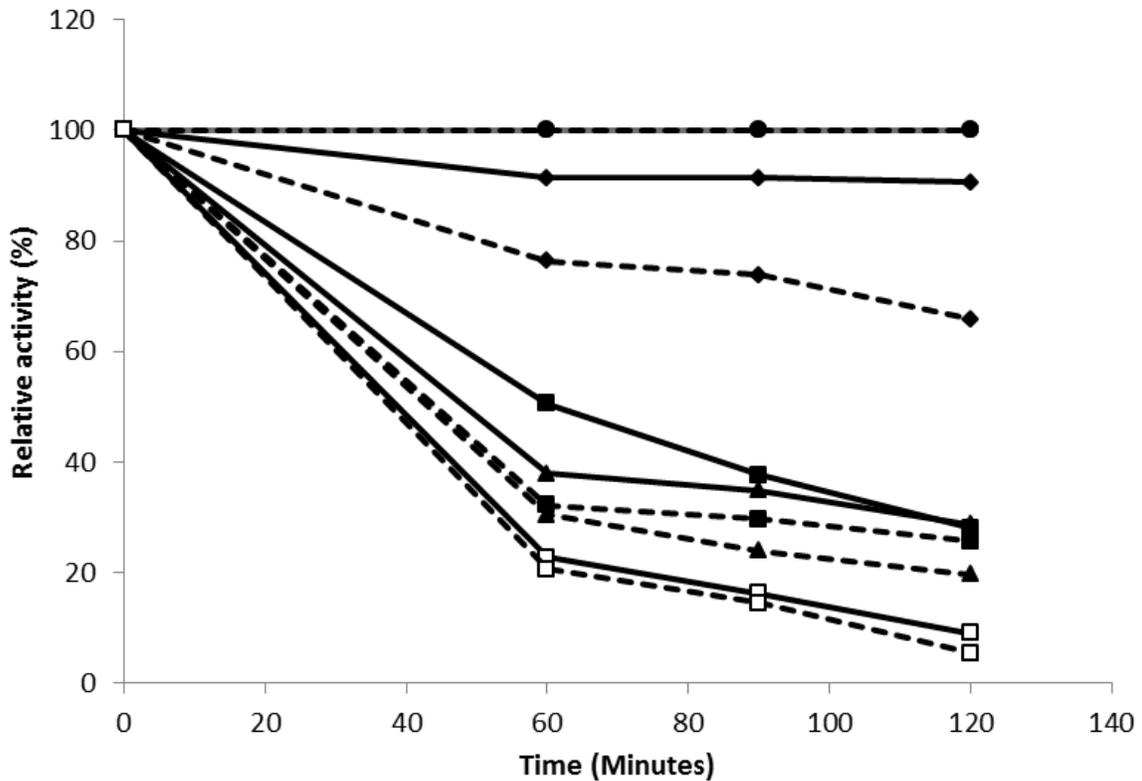


Figure 7. Effect of temperature on stability of protease from *Bacillus coagulans* PSB-07 in the presence (—) and absence (-----) of CaCl₂. Stability of enzyme at 40°C (●), 50°C (◆), 60°C (■), 70°C (▲) and 80°C (□) were determined after 60, 90 and 120 min of incubation at specified temperatures.

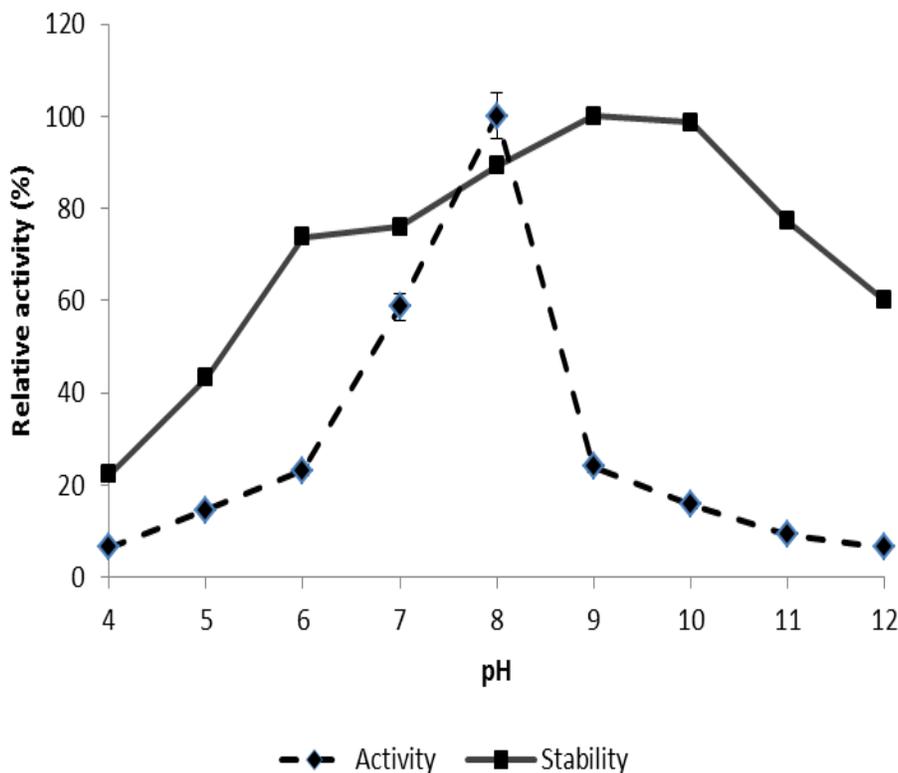


Figure 8. Effects of pH on activity and stability of protease from *Bacillus coagulans* PSB-07.

Table 1. Effect of some metal ions on alkaline protease activity.

Metal ions (5 mM)	Relative activity (%)
Control	100.0
Ca ²⁺ (CaCl ₂)	141.7
Mg ²⁺ (MgCl ₂)	118.8
Hg ²⁺ (HgCl ₂)	54.9
Ca ²⁺ +Mg ²⁺	128.3
Hg ²⁺ +Ca ²⁺	101.0

Mg²⁺. Nascimento and Martins (2004) reported that activity of protease from *Bacillus* sp. under their study was increased in the presence of Ca²⁺ and Mg²⁺. Our results reveal that protease activity was inhibited by almost 45% by Hg²⁺. Interestingly, the protease activity was increased by the combination of Hg²⁺ and Ca²⁺ (Table 1) with relative activity of 101%. Metal ions have been reported to play vital role in maintaining the active site conformation of the enzyme (Shivan and Jayaraman, 2011). Combination of divalent metal ions may be required for enhanced protease activity (Wan et al., 2009).

Hydrolysis of protein substrates

The protease under study showed highest hydrolytic

Table 2. Hydrolysis of protein substrates by protease from *Bacillus coagulans* PSB-07.

Substrate	Relative activity (%)
Casein	100
Gelatin	18
BSA	72
Egg albumin	45

BSA, Bovine serum albumin

activity towards casein (100%) which was the control. The hydrolysis of BSA was indicated with 72% relative activity. The protease exhibited poor hydrolytic activity on gelatine with 18% relative activity (Table 2). Yossana et al. (2006) had similar finding on hydrolytic activity of the alkaline protease under their study. The ability of protease from *B. coagulans* to hydrolyse different substrates tested may be advantageous for use of the enzyme in detergent formulation against a variety of stains (Gouda, 2006).

Effect of organic solvent on protease activity and stability

Effects of six organic solvents were evaluated on protease activity. The enzyme was active in all organic

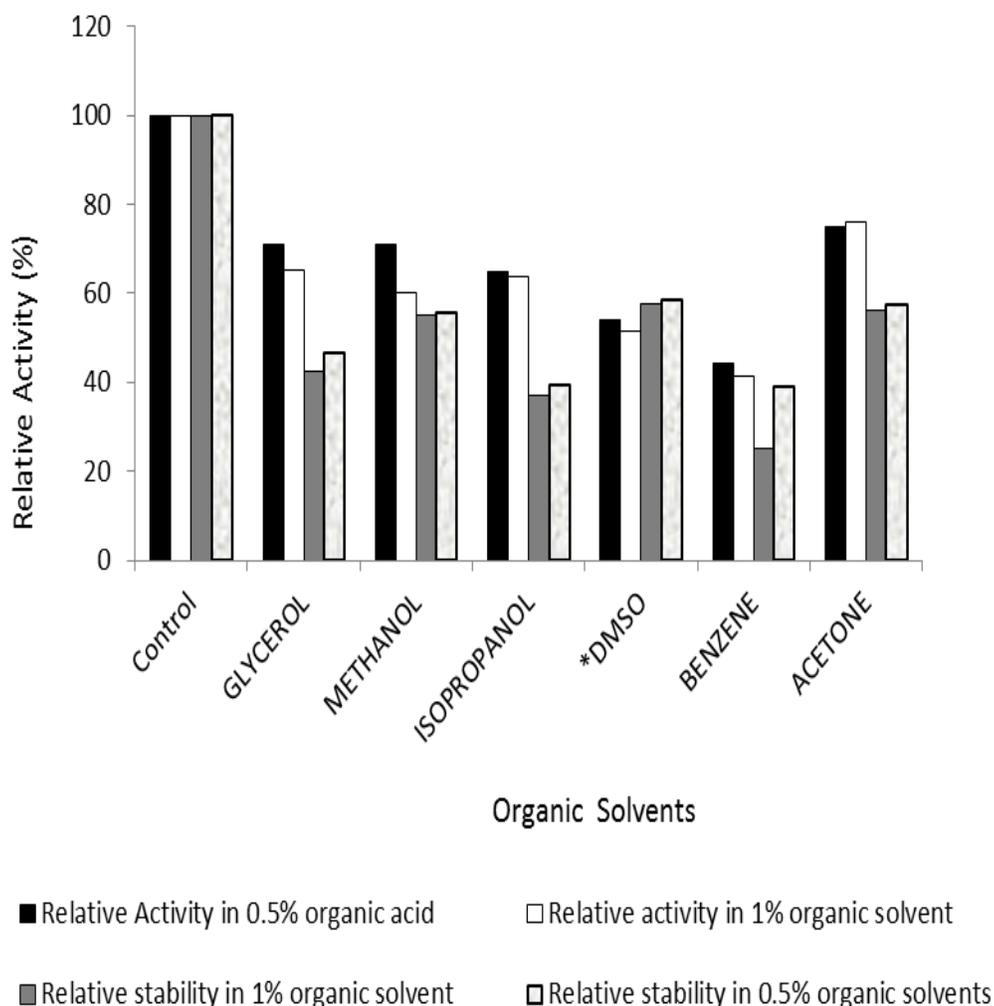


Figure 9. Effects of organic solvents (0.5% v/v and 1% v/v) on activity and stability of protease from *Bacillus coagulans* PSB-07. *DMSO: dimethyl sulfoxide.

solvents tested exhibiting above 70% relative activity in the presence of 0.5% (v/v) acetone, methanol and glycerol when compared with control (Figure 9). However, the enzyme lost 58.56% activity in the presence of 0.5% (v/v) benzene. The relative activity of the protease reduced drastically when 1% (v/v) organic solvents was used except for acetone. The protease was stable in 0.5% and 1% acetone, DMSO and methanol with over 50% residual activity. The increase of protease activity in the presence of DMSO could be explained by the enhancement of hydrophobic interactions inside protein molecules, which may cause a desirable conformational change for the interaction between the active site and substrate (Hadj-Ali et al., 2007). Results obtained in this study are particularly unique because enzymes have been reported to be inactivated and unstable in the presence of organic solvents (Ghorbel et al., 2003). This property makes protease from *B. coagulans* PSB-07 an excellent candidate as biocatalyst in aqueous-organic and organic solvent reactions.

Conclusion

The properties exhibited by the protease from *B. coagulans* PSB-07 under study show that *B. coagulans* PSB-07 is a good producer of thermostable and organic solvent tolerant alkaline protease with potential applications in peptide synthesis, ester synthesis and detergent industry.

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