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RESEARCH PAPER

Immobilisation, characterisation and zymogram analysis of alkaline protease produced by *Bacillus cereus* FT11

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Abstract
The alkaline protease produced by *Bacillus cereus* FT 11 was partially purified by ammonium sulphate precipitation and immobilised in 3.5% alginate beads. The characterisation studies of immobilised enzyme showed that it has maximum activity at a temperature of 65 °C (450 U/ml), pH of 10.5 (295 U/ml) and protein substrate (casein) concentration of 3.5% (305 U/ml). The Vmax of immobilised enzyme was found to be 303 U/ml and Km value, 2.51 mg/ml, which indicates a high affinity of enzyme towards its substrate. The immobilized enzyme also showed stability at wide ranges of temperatures from 5 °C to 65 °C with 96% residual activity and wide ranges of pH from 8.0 to 10.5 with 95% residual activity. The enzyme was further purified by ion exchange chromatography method with DEAE cellulose as the matrix and the activity was analysed through zymogram with gelatine. The specific activity of purified enzyme was calculated to be 421 U/mg.

Keywords Alkaline protease · *Bacillus cereus* · Enzyme immobilisation · Ion exchange chromatography · DEAE cellulose · Zymography

Introduction

Microbial alkaline proteases are the hydrolytic enzymes studied extensively in enzymology. The rapid growth of microorganisms, easy genetic manipulation and less space requirement for cultivation make microbial enzymes preferable in production of industrially important enzymes. They possess the highest score in enzyme market. There are several products in the market which are based on microbial alkaline proteases (Gupta et al. 2002; Rao et al. 1998; Singhal et al. 2012). Industries with different applications of microbial protease include food, pharmaceutical, detergent, leather and textile industries. In detergents, alkaline protease enzymes with broad substrate specificity facilitate to remove stains produced due to blood, body secretions and food. The major prerequisite of a protease enzyme to be used in detergent is their activity and stability at higher temperature and pH ranges and compatibility with the added oxidising agents and chelating molecules in the detergent. In leather industry, alkaline proteases facilitate selective hydrolysis of non-collagenous skin constituents, removal of non-fibrillar proteins, dehairing and faster water absorption by the hide to reduce its soaking time in leather processing. Use of microbial enzymes in leather industry has successfully improved the quality of leather and reduced environmental pollution as it replaced conventional chemical treatments. In food industry, proteases have role in baking, cheese production and meat tenderization (Pawar et al. 2023; Rao et al. 1998; Zhou et al. 2018).

The commercially used microbial proteases are considered eco-friendly as their producers are non-pathogenic and non-toxin producers in nature (Gupta et al. 2002; Kirk et al. 2002; Van Beilen and Li 2002). The most important properties of industrial enzymes are their thermo activity, thermo stability, ability to withstand different ranges of pH and substrate concentrations. These properties of an enzyme can be improved through methods like, introduction of disulphide

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bonds, chemical modification or crosslinking (Perry and Wetzel 1984; Braxton and Wells 1992). But, in such methods the reusability of enzymes and recovery of products are limited. To overcome this situation, immobilisation technique is employed which has advantages like easy product recovery, reusability, controlled product formation and easy removal of enzyme from the reaction mixture (Hanefeld et al. 2009; Mohidem et al. 2023; Rodrigues et al. 2011; Sheldon and van Pelt 2013). Immobilisation also improves the thermal stability of enzymes (Aissaoui et al. 2013; Gracia-Galan et al. 2011).

Immobilisation by entrapment in calcium alginate is employed in this study as it is relatively cheap, rapid, non-toxic and the versatile method. (Betigeri and Neau 2002; Maghraby et al. 2023). The work was aimed at the production, partial purification, immobilisation and characterisation of alkaline protease enzyme produced by soil isolated *Bacillus cereus* FT11. The enzyme was also purified by ion exchange chromatography technique and the enzyme activity was visualised through zymography technique.

Materials and methods

Crude enzyme production

Alkaline protease enzyme produced by soil isolated *Bacillus cereus* FT11 strain previously isolated in our laboratory was selected for the present study. The crude enzyme was prepared by culturing the selected strain in protease production medium at 35 °C for 48 h in a rotary shaker at 200 rpm. [Protease production medium composition (g/L): Lactose: 10 g; Casein: 10 g; Yeast extract: 5 g; KH_2PO_4: 2 g; K_2HPO_4: 2 g; $MgSO_4.7H_2O$: 1 g; pH: 7.2 (Tambekar and Tambekar 2013)]. The medium was then centrifuged at 5000 rpm for 20 min at 4 °C and cell free supernatant was collected which can be used as crude enzyme extract. The total protein content of the crude extract was calculated according to the Lowry's method (Lowry et al. 1951) of protein estimation.

Partial purification by ammonium sulphate precipitation and dialysis

The crude enzyme was partially purified by ammonium sulphate precipitation and dialysis. A saturated solution of ammonium sulphate (Sigma-Aldrich) was prepared at a temperature of 25 °C. The whole process was completed at a temperature of 25 °C. An appropriate volume of saturated ammonium sulphate solution was added to 100 ml cell free supernatant to get the 20% ammonium sulphate concentration (The volume of saturated ammonium sulphate to be added was determined from table presented in the technical bulletin obtained with the chemical). The solution was equilibrated by continuously stirring for 1 h and kept overnight for the precipitation to complete. The solution was then centrifuged at 10,000 rpm for 15 min to pellet out the precipitated protein. The supernatant was taken to the next step. The next concentrations of 40%, 60%, 80% and 95% were made by the addition of appropriate volumes of saturated ammonium sulphate solution and by repeated stirring and centrifugation. The protein pellets obtained from each concentration were dissolved in Tris buffer, pH: 9.0, and further, each fraction was dialysed against the same buffer (Adetunji and Olaniran 2023; Ahmed et al. 2011; Ullah et al. 2022).

The dialysis membrane used in the study was HiMedia LA395 Dialysis membrane-110. The required numbers of dialysis membrane were cut out with equal length. The membranes were boiled in 200 ml of 2% Na_2CO_3 containing 1 mM EDTA, for 10 min. The solution was cooled and the membranes were rinsed thoroughly in distilled water. The dialysis membranes were again boiled in distilled water containing 1 mM EDTA for 10 min. The membranes were again washed with distilled water 2—3 times. The washed membranes were stored in 5% ethanol under refrigeration. The different protein fractions dissolved in buffer were placed in the dialysis membrane and both sides of the membrane were tied. The dialysis membranes were then immersed in the ice-cold buffer (Tris, pH: 9.0), in a beaker and kept at 4 °C. The buffer in the beaker was changed frequently (within 2 h, twice, and then after 6 h and 12 h) (Roe 2006).

Protease assay

Protease activity of protein fractions was calculated by conducting a modified universal protease assay (Cupp-Enyard 2008). The protein fractions obtained from ammonium sulphate precipitation (1 ml each) was mixed with 1 ml of 2% casein solution in alkaline buffer (pH 9) in test tubes and incubated at 37 °C for 10 min. Control tubes were kept with distilled water instead of enzyme solution. After 10 min, the reaction was terminated by adding 3 ml of 20% trichloro acetic acid (TCA) to the tubes. The contents in the test tubes were centrifuged at 5000 rpm for 15 min and supernatant was collected. In to 0.5 ml of the supernatant, 2.5 ml of 0.5 M Na_2CO_3 solution was added, mixed well and incubated for 20 min at room temperature. After incubation, 0.5 ml of Phenol-Folins reagent was added to this mixture and kept for 10 min and absorbance of the solutions was read at 660 nm using spectrophotometer. The absorbance values obtained in protease activity assay was interpreted using tyrosine standard graph and protease enzyme activity was calculated. The amount of enzyme required to liberate 1 µg tyrosine per ml per minute under the standard conditions defined one unit of protease activity (Hameed et al. 1999). Protein estimation of fractions obtained from ammonium

sulphate precipitation was done according to the Lowry's method (Lowry et al. 1951) and specific activity of the fractions was also calculated (Ahmed et al. 2016; Vishnupriya et al. 2016).

Immobilisation

The protein fraction with higher protease activity and greater specific activity obtained from ammonium sulphate precipitation was taken as the partially purified enzyme. It was selected for immobilisation and characterisation studies. Equal volumes of enzyme and 3.5% sodium alginate solution (10 mL each) were mixed thoroughly and dripped with continuous stirring in to 0.2 M $CaCl_2$ solution. The concentration of sodium alginate was selected based on the various experiments conducted using different concentrations of it. At concentrations below 3.5%, the beads were unstable at higher temperatures. At concentration above 3.5%, the enzyme activity decreased, which may be due to high density of alginate that trapped the enzyme inside and prevented it from interacting with the substrate. The beads formed were stabilized at 4 °C for 24 h and taken for characterisation studies (Adetunji and Olaniran 2023; Betigeri and Neau 2002; Jesionowski 2014). As 10 mL of enzyme was used for preparing the beads and the weight of beads obtained was approximately 5 g, it was assumed that 1 ml enzyme solution was entrapped in 0.5 g of beads (Sharma and Tripathi 2013).

Characterisation of immobilised enzyme

The protease assay of immobilised enzyme was conducted based on the following parameters, temperature 37 °C, pH 9, substrate concentration 2% and incubation time 10 min (Rezakhani et al. 2014). The activity and stability of immobilised enzyme at different temperature, pH and substrate concentration (casein) were checked through protease enzyme assay (Cupp-Enyard, 2008). The activity of immobilised enzyme was checked by conducting the assay at different ranges of temperature (20 °C–70 °C), pH (6.0–11.0) and substrate concentration (0.5% to 4.5%). Control tubes were kept by replacing the enzyme with distilled water. Pre incubation for 30 min and 1 h was done at different ranges of temperature (5 °C–70 °C) and pH (2.5–11.0) to check the stability of immobilised enzyme. After pre incubation, protease assay was conducted and the residual activity of enzyme was also calculated to evaluate the change in enzyme activity occurred after pre incubation. Protease assay conducted without any pre incubation was considered as control. The enzyme activity of control was taken as 100% while calculating the residual activity of test samples. The percentage of remaining enzyme activity of the test samples compared to the control after preincubation was considered as the residual enzyme activity. (Anwar et al. 2009; Sharma and Tripathi 2013; Rezakhani et al. 2014; Ullah et al. 2022).

Statistical analysis

The data analysis was performed using Microsoft excel 2019 (Microsoft corporation) and the results designate mean standard deviation (Mean ± SD) of three experiment replicates performed independently.

The Michaelis–Menten constant (Km) and Vmax value for the immobilised enzyme was determined from Lineweaver–Burk plot drawn between reciprocal of substrate concentrations (1/S) and enzyme activity (1/V). The values were calculated based on the Lineweaver–Burk plot equation given below;

$$1/V = \left[\mathrm{Km}/\mathrm{V\,max}\right] 1/S + 1/\mathrm{V\,max}$$

Km/Vmax is known as gradient. Vmax is the rate of reaction when the enzyme is fully saturated with the substrate and Km value is the concentration of substrate which permits the enzyme to achieve half Vmax (Adetunji and Olaniran 2023; Anwar et al. 2009; Sharma and Tripathi 2013; Raval et al. 2014).

Purification of enzyme by ion exchange chromatography

Purification of partially purified alkaline protease produced by *Bacillus cereus* FT11 was done by ion exchange chromatography method. DEAE cellulose was used as the matrix (Mothe and Reddy 2016). The matrix was pre-treated using 0.1 M NaOH and 0.1 M HCl, filled in the column and equilibrated with Tris HCl (pH 7.4). After running sample, the elution was done using same buffer with different NaCl gradients (range 0.05–0.5 M in increments of 0.05 M). Different fractions were collected separately and protease assay for each fraction was conducted for detecting the protease activity (Banik et al. 2018; Colwell and Grigorova 1987; Yendri et al. 2018). NaCl residues from the active fraction was removed by dialysis and concentrated using poly ethylene glycol (50%). Total protein content and specific activity of the purified enzyme were also detected (Ahmed et al. 2016; Dubey et al. 2010; Howe et al. 1964; Vishnupriya et al. 2016).

The enzyme activity was visualised by zymogram analysis using poly acrylamide gel (12%) containing 1% gelatine. The purified protein fraction with highest enzyme activity was selected for the study. The sample was loaded and appropriate voltage and current was applied. After completing the run, the gel was placed in the solution of Coomassie blue and checked for clear zone of gelatine hydrolysis by the

purified enzyme (Wilkesman and Kurz 2009; Dubey et al. 2010; Lagziana and Asoodeha 2012).

Results and discussion

Crude enzyme production

The cell free supernatant collected was taken for the partial purification and immobilisation studies. On estimating the total protein content, it was found that the cell free supernatant contains 2.2 mg/mL protein.

Partial purification

Among the different fractions of proteins obtained from ammonium sulphate precipitation and dialysis, the highest protease activity and specific activity was shown by the fraction obtained from 80% concentration of ammonium sulphate. The protein fraction was showing an enzyme activity of 336 U/ml and specific activity of 280 U/mg and was selected for immobilisation and characterisation studies. The total protein content of the partially purified enzyme was 1.2 mg/mL (Vishnupriya et al. 2016). Many researchers have reported the salting out of proteins using ammonium sulphate resulting in the separation of proteases at varying concentrations. Partial purification of protease enzyme with 80% ammonium sulphate was reported for *Geobacillus toebii* LBT 77 and *Bacillus brevis* MWB-01 (Olajuyigbe and Falade 2014; Thebti et al. 2016). The strain *Bacillus subtilis* PE 11 produced a protease enzyme which got separated at 60% concentration of ammonium sulphate (Adinarayana et al. 2003) and the strain *Bacillus subtilis* KIBGE-HAS produced a protease which got precipitated at 40% concentration of ammonium sulphate (Anwar et al. 2009).

Immobilisation and characterisation of enzyme

The immobilisation of partially purified enzyme with 3.5% sodium alginate solution resulted in the formation of well-formed beads. On characterisation studies, the immobilised enzyme showed the highest enzyme activity (450 U/ ml) at a temperature of 65 °C (Table 1**;** Fig. 1) and on checking the stability at different temperature ranges (5 °C–70 °C), the immobilised enzyme showed residual activity above 96% on incubation for up to 1 h at all the temperatures tested except 70 °C (Table 2**;** Fig. 2**).** This result suggests that, the immobilised enzyme can be stored at different temperatures without considerable loss of its activity. The activity of protease enzyme at high temperature and maintenance of enzyme activity at different temperature ranges make them industrially important where the activity and storage of enzyme is carried out at different temperature ranges (Anwar et al. 2009; Sharma and Tripathi 2013; Wani et al. 2024). Scientists suggests the reasons for increased thermal stability of immobilised enzymes as the inability of external factors like temperature to modify the chemical structure of entrapped enzymes or the high rate of reaction of immobilised enzymes due to which the reaction is completed within a short time period and hence low risk of enzyme denaturation (Sharma and Tripathi 2013).

A maximum enzyme activity (295 U/ ml) by the immobilised enzyme was shown at a pH of 10.5 (Table 3**;** Fig. 3). It also maintained the residual activity above 95% at pH range from 8.0 to 10.5 when pre incubated for up to 1 h with 100% residual activity at pH 9.5. Also, the residual activity was above 85% at pH ranges from 6.5 to 7.5 (Table 4**;** Fig. 4). The maintenance of high residual activity may be due to the ionic nature of alginate used and modification of

Table 1 Protease activity of immobilised enzyme at different temperatures

Temperature (˚C)	Protease activity (U/mL)
20	215 ± 2
25	222 ± 2
30	255 ± 1
35	270 ± 1
40	275 ± 3
45	295 ± 3
50	355 ± 2
55	415 ± 3
60	435 ± 2
65	450 ± 1
70	310 ± 1
75	296 ± 2

The values given are mean ± SD of experiments carried out in triplicates

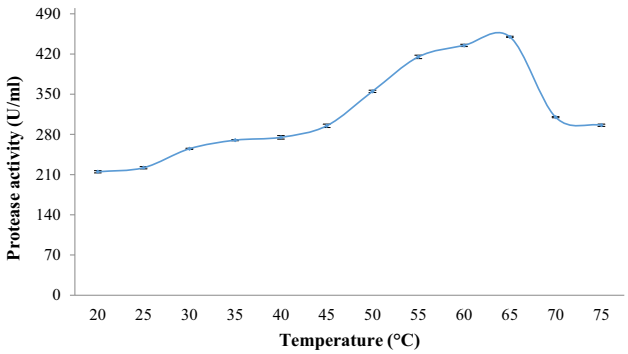

Fig. 1 The effect of temperature on the activity of immobilised enzyme. The activity increases gradually till 65 °C and declines thereafter. The results are mean of three independent determinants. Bars correspond to standard deviation

Table 2 Residual activity of immobilised enzyme at different temperatures with preincubation for 30 min and 1 h

Temperature (˚C)	Residual activity 30 min (%)	Residual activity 1 h (%)
5	100 ± 0.5	98 ± 1
10	98 ± 1	98 ± 1.5
15	98 ± 1	97 ± 2
20	97 ± 1	97 ± 2
25	100 ± 0.5	100 ± 1.5
30	100 ± 0.5	100 ± 1
35	100 ± 1	98 ± 0.5
40	97 ± 1.5	97 ± 0.5
45	98 ± 1	97 ± 1
50	97 ± 1	97 ± 1
55	98 ± 1.5	97 ± 2
60	97 ± 1	97 ± 1.5
65	98 ± 1	97 ± 1
70	94 ± 2	92 ± 2

The values given are mean ± SD of experiments carried out in triplicates

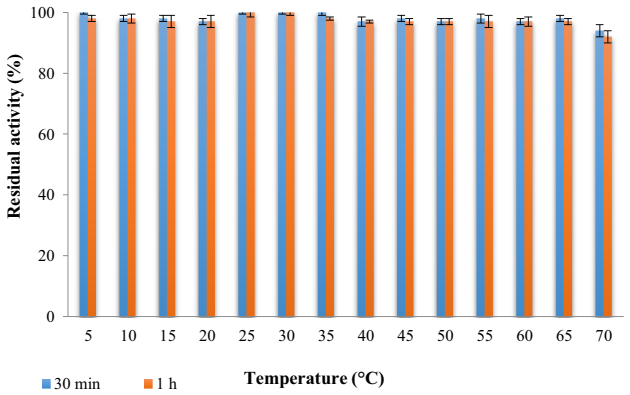

Fig. 2 Effect of temperature on stability of immobilised enzymes. The immobilised enzyme was showing residual activity above 96% on preincubation for 30 min and 1 h at all the temperatures tested except 70 °C. The results are mean of three independent determinants. Bars correspond to standard deviation

Table 3 Protease activity of immobilised enzyme at different pH

pH	Protease activity (U/mL)
6	245 ± 2
6.5	250 ± 2
7	250 ± 1
7.5	255 ± 3
8	260 ± 3
8.5	270 ± 3
9	280 ± 2
9.5	285 ± 2
10	290 ± 1
10.5	295 ± 1
11	260 ± 2

The values given are mean ± SD of experiments carried out in triplicates

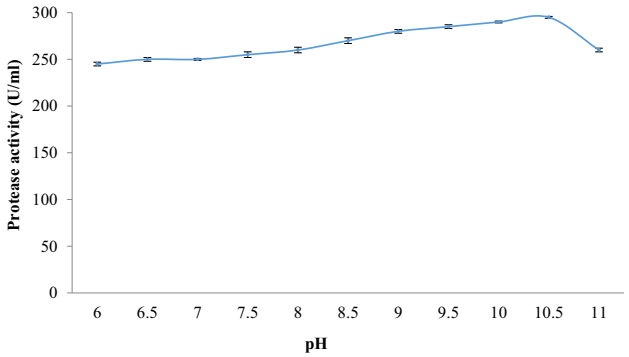

Fig. 3 The effect of pH on the activity of immobilised enzyme. The activity increases gradually till 10.5 pH and declines thereafter. The results are mean of three independent determinants. Bars correspond to standard deviation

microenvironment of the immobilised enzyme. Increased activity and stability of protease enzyme at alkaline pH ranges are also reported for *Aspergillus oryzae* AWT 20 (Sharma et al. 2006), *Bacillus subtilis* M-11 (Sahin et al. 2015) and *Conidiobolus macrospores* (Tanksale et al. 2001).

The immobilised enzyme showed the highest enzyme activity (305 U/ml) at a substrate concentration of 3.5%. Above this, the enzyme activity decreased (Table 5**;** Fig. 5**).** Vmax and Km value of the immobilised enzyme was calculated by plotting reciprocal of substrate concentrations tested on the x axis and reciprocal of enzyme activity obtained for each substrate concentration on y axis in a Lineweaver–Burk plot (Fig. 6). The equation obtained from the graph was; $y = 0.0083x + 0.0033$.

The value of 1/Vmax is 0.0033, from which the Vmax can be calculated. Km/Vmax is 0.0083, from which Km can be calculated. The value of Vmax for the immobilised enzyme was found to be 303 U/ml and Km value, 2.51 mg/ml. A smaller Km value indicates high affinity of enzyme towards the substrate (Lotfi et al. 2022; Tanksale 2001; Sharma and Tripathi 2013).

Purification of enzyme by ion exchange chromatography

The fraction eluted with 0.1 M NaCl showed maximum enzyme activity (320 U/ml), which is then considered as the purified enzyme. The total protein content of the

Table 4 Residual activity of immobilised enzyme at different pH with preincubation for 30 min and 1 h

pH	Residual activity 30 min (%)	Residual activity 1 h (%)
2.5	36 ± 0.5	33 ± 0.5
3	41 ± 1	36 ± 0.5
3.5	48 ± 1	38 ± 1
4	55 ± 2	38 ± 2
4.5	58 ± 2	39 ± 2
5	70 ± 1.5	64 ± 2
5.5	76 ± 1	73 ± 1
6	79 ± 0.5	77 ± 2
6.5	88 ± 1.5	85 ± 1.5
7	91 ± 1.5	89 ± 1.5
7.5	94 ± 2	94 ± 2
8	97 ± 2	95 ± 2
8.5	97 ± 2	95 ± 1
9	98 ± 1.5	98 ± 1
9.5	100 ± 1.5	100 ± 1
10	100 ± 1	97 ± 1.5
10.5	100 ± 1	97 ± 1.5
11	91 ± 1	89 ± 1.5

The values given are mean ± SD of experiments carried out in triplicates

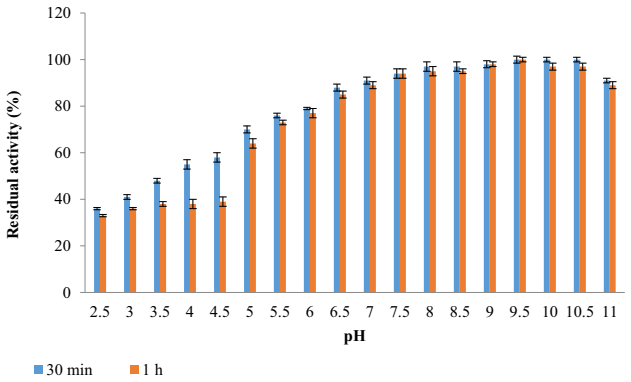

Fig. 4 Effect of pH on stability of immobilised enzymes. The enzyme maintained the residual activity above 95% at pH range from 8.0 to 10.5 when pre incubated for 30 min and 1 h with 100% residual activity at pH 9.5. The residual activity was above 85% at pH ranges from 6.5 to 7.5. The results are mean of three independent determinants. Bars correspond to standard deviation

fraction was 0.76 mg/mL and the specific activity of purified enzyme was calculated to be 421 U/mg. From the total protein contents of crude enzyme and purified enzyme, the total yield of the protease enzyme was calculated to be 34.5%. The zymogram of purified protein fraction showed clear zone of substrate degradation indicating the enzyme activity of the fraction thus suggesting the presence of

Table 5 Protease activity of immobilised enzyme at different substrate concentrations

Substrate concentration (%)	Protease activity (U/mL)
0.5	185 ± 1
1	225 ± 2
1.5	265 ± 3
2	280 ± 3
2.5	293 ± 3
3	300 ± 2
3.5	305 ± 2
4	300 ± 1
4.5	295 ± 2

The values given are mean ± SD of experiments carried out in triplicates

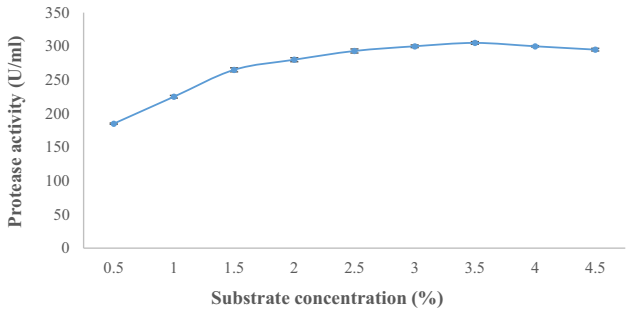

Fig. 5 The effect of substrate concentration on the activity of immobilised enzyme. The activity increases gradually till 3.5% concentration and declines thereafter. The results are mean of three independent determinants. Bars correspond to standard deviation

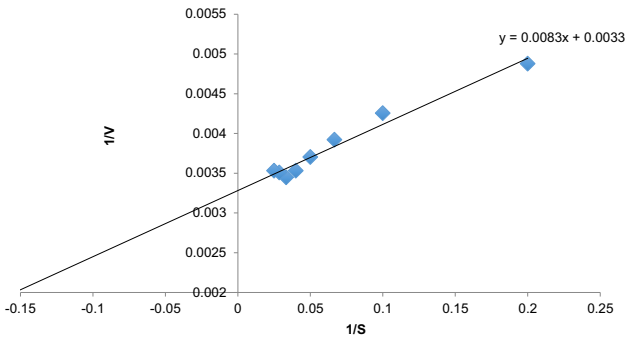

Fig. 6 Lineweaver–Burk plot of immobilised enzyme. The value of Vmax for the immobilised enzyme was found to be 303 U/ml and Km value, 2.51 mg/ml. A smaller Km value indicates high affinity of enzyme towards the substrate

enzyme (Fig. 7), a result similar to those reported by many researchers (Jobin and Grenier 2003; Seifzadeh et al. 2008; Ito et al. 2010; Budiarto et al. 2016).

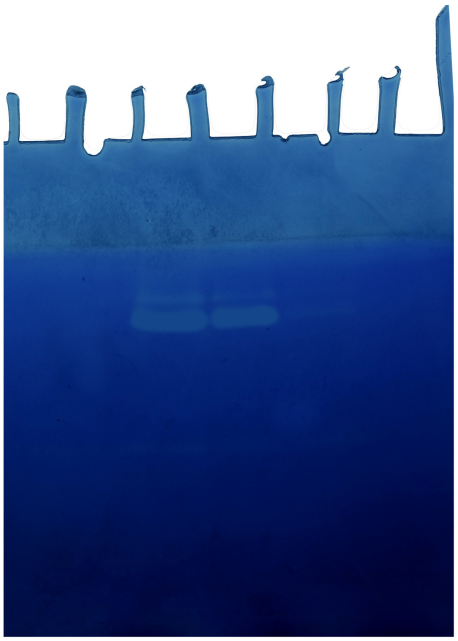

Fig. 7 Zymogram showing clear bands of gelatin hydrolysis

Conclusion

The immobilised alkaline protease enzyme produced by *Bacillus cereus* FT11 strain was found to be thermo active and thermo stable with highest enzyme activity at a temperature of 65 °C and stability at ranges of temperature from 5 °C to 65 °C. The immobilised enzyme was also active and stable at alkaline pH ranges. The consistent activity and stability of immobilised enzymes in harsh reaction conditions are major concerns faced during their industrial applications. The economic use of microbial enzymes depends on their reusability without losing much of its activity. By far, not much immobilised protease enzymes have been reported with increased activity and stability at higher temperature and pH ranges. The enzyme in this study shows consistent activity and stability at higher temperature and pH ranges which make it a unique candidate for industrial applications. Further studies can be done on specific applications of enzyme in different industries.

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Author contributions AB and PM were responsible for the conception and design of the study. AB and SNK performed the experiment. AB analysed the data and drafted the article. PM supervised the study and critically analysed the data. All authors approved the final manuscript.

Data availability The data generated in this study has been presented in the main text, tables and figures.

Declarations

Conflict of interest The authors declare no conflict of interest.

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