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Production, optimization, purification, kinetic analysis, and applications of alkaline proteases produced from *Bacillus subtilis* through solid-state fermentation of agricultural byproducts

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Abstract

Proteases have gained commercial value due to multiple applications in different industrial sectors. The current research aimed to use cheaper agricultural waste for optimal protease production. The maximum level of protease production was achieved at 37 °C, an incubation period of 24 h, pH 9.0, inoculum size 3%, 1.5 g sucrose as a carbon source, and 30% moisture content by using solid-state fermentation. Ammonium nitrate and yeast extract tremendously increased protease production among the various inorganic and organic nitrogen sources. Ca^{2+} ions and Tween 40 showed optimal protease production among the tested metal ions and surfactants. Protease purification was carried out by ammonium sulphate precipitation followed by Sephadex G-100 gel filtration chromatography. The purification resulted in 1.3-fold of purified protease with 51.5U/mg specific activity and a total yield of 37.5 %. The molecular weight of purified protease was confirmed upon SDS-PAGE with a single band of ~36 kDa. The protease was stable over a temperature range of 35-45 °C and pH 7-9, with maximal activity at 40 °C and pH 9. The kinetic parameters Vmax (maximum rate) and Km (Michaelis-Menten constant) were calculated as 0.307 U/g and 11.2 mg/mL, respectively. The alkaline protease significantly dehair the goat skin and successfully removed the animal blood stain from cotton cloth.

Keywords: *Bacillus*; chromatography; protease; Sephadex G-100; soya bean meal.

1. Introduction

Proteases are the hydrolytic group of enzymes that cleave proteins by degrading peptide bonds (Niyonzima & More, 2015). Proteases are commercially important enzymes and have gained more attention among all the hydrolytic enzymes because of their wide range of industrial, environmental, and biotechnological applications. They play a vital role in the food, pharmaceutical, detergent, chemical, silk, and leather industries. Microbial sources are more widely exploited than any other source due to their simple production method (Das & Prasad, 2010). Proteases are produced by various major bacterial genera, including *Aeromonas, Arthrobacter, Alcaligenes, Bacillus, Halomonas, Serratia,* and *Pseudomonas*. *Bacillus* species are the chief source that secretes different extracellular soluble enzymes (Selvam *et al*., 2016). Solid state fermentation (SSF) has acquired economic interest due to its ability to utilize cheap raw materials. SSF is a process that involves a solid matrix and occurs without

free water. The substrate must contain adequate moisture to support the organism's metabolism and growth (Pandey, 2003). SSF has gained more interest over submerged fermentation because of reduced production costs by utilizing moderately processed or unprocessed raw materials (Adelere & Lateef, 2016; Çakmak & Aydoğdu, 2021). Additional advantages of SSF are superior productivity and improved recovery of products (Pandey *et al*., 2000). Production of the enzyme is generally influenced by media components, cultural factors such as time, pH, temperature, and nutritional factors, especially nitrogen and carbon sources, surfactants, and metal ions. Several studies used municipal solid wastes (MSW) as cheap raw materials to produce enzymes (Hasan *et al*., 2017; Iqbal *et al*., 2018; Hakim *et al*., 2018). The MSW comprises 65- 75-% of organic material, including carbohydrates, peptides, proteins, fatty acids, and their esters. These organic waste materials can be used as raw material in fermentation processes to produce pure and commercially essential products, reducing environmental pollution and other health hazards (Iqbal *et al*., 2015; Hasan *et al*., 2017; Iqbal *et al*., 2018;). However, very few studies have been done to produce enzymes with agricultural byproducts.

Thermo stability is one of the industrial enzymes major and unique properties, and it is beneficial because it reduces microbial contaminants. Purification overall involves the recovery of protein with a high degree of purity. Furthermore, the specific activity of enzymes is also increased by the purification process, making them more specific for different industrial applications.

The present work focused on the production, optimization of significant parameters, purification, kinetic analysis, and applications of alkaline proteases by *Bacillus subtilis* NCIMB-10144. This may satisfy the future need for alkaline proteases and open up the path for industrial applications.

2. Materials and methods

2.1 Microorganism

The strain of *Bacillus subtilis* NCIMB-10144 was taken from Industrial Biotechnology Laboratory, Food, and Biotechnology Research center, PCSIR.

2.2 Preparation of Inoculum

A loopful of *Bacillus subtilis* strain was aseptically transferred into the 50ml sterilized nutrient broth. The inoculated broth was placed in a shaker at 37ºC, 150 rpm for 24 hours. The obtained vegetative cells after 24 h of incubation were used as inoculum.

2.3 Fermentation process

20 g of sterilized solid substrate moistened with 15 ml of sterilized mineral salt medium (g/100ml): $CaCl_2$, 0.5, NaCl 0.05, K_2HPO_4 0.5, $MgSO_4$, 0.05, Sucrose 1.5, Yeast extract 2, Ammonium nitrate 0.5 was inoculated with vegetative inoculum (1.0 mL) of *Bacillus subtilis* and incubated for 48h at 37 °C (Imtiaz *et al.,* 2013). After a fixed time interval, 50 ml of phosphate buffer (0.25M, pH 9.0) was added to each flask and placed for 30 min in a shaker

at 150 rpm. The enzyme solution was centrifuged at 6,000 rpm for 15 min and used for the estimation of enzyme activity (Shivasharana & Naik, 2012).

2.4 Proximate analysis

The physiochemical value of soya bean meal (i.e., moisture, fat, ash, and protein contents were evaluated according to AOAC (2000).

2.5 Protease assay and total protein content determination

The enzyme assay was measured by following the Anson (1938) method. One unit of protease activity is represented as “the amount of enzyme required to release 1μmol of tyrosine per minute from casein under standard assay conditions" (Bajaj *et al.,* 2009). At the same time, total protein was estimated by following the Lowry *et al*. (1951) method.

2.6 Enzyme purification

Three steps were used to purify the protease enzyme. Initially, the crude extract was precipitated to its saturation level by adding ammonium sulfate 50 -80% (w/v). The precipitated fraction was then recovered by centrifugation at 7000 rpm for 15 min (4 °C). After centrifugation, the obtained pellet was re-suspended in 0.05 M Tris-HCl buffer (pH 9.0) and finally dialyzed in dialyzing tubing (12,000 Da) against the same buffer overnight with two or three changes.

The dialyzed sample was subjected to a Sephadex column (G 100, Pharmacia; 1.5 cm x 30 cm) by using the FPLC system (Bio-Rad, Biologic LP, USA), which was pre-equilibrated with (pH 9.0, 0.05 M) Tris-HCl buffer. At the flow rate of 60 mL/h, the active fractions of 2 mL were collected with the same buffer. All the active fractions that showed the maximum protease activity were collected carefully and stored at −20 °C for further analysis.

2.7 Molecular weight determination

Molecular weight was determined according to the suggested method by Laemmli (1970).

2.8 Protease characterization

2.8.1 Effect of different temperatures, pH, and metal ions

To determine protease activity, the purified enzyme was incubated within the temperature range of 30-50ºC for 10 min. Its stability was assessed between 30-50ºC for 2 hours. However, the effect of different initial pH ranges (4-11) on protease activity was also evaluated. The optimal enzyme activity was investigated by using the following buffers (0.05 M) Citrate buffer (pH 4-5), potassium Phosphate buffer (pH 6-7), and Tris-HCl buffer (pH 8.0, 9.0, and 10.0). The residual activity of the protease enzyme was studied within a pH range of (4-11) for 2 hours. The impact of several metal ions (5 mM) on the protease activity was also tested for 30 min in different ionic solutions.

2.9 Applications of alkaline Protease

2.9.1 De-staining property of protease

A white cotton cloth (4cm x 4cm) was stained with animal blood. Afterwards, 1.0 mL of enzyme solution for the de-staining process was prepared as defined by Adinarayana *et al.* (2003) and applied for 50 minutes.

2.9.2 Dehairing agent

To perform the leather Dehairing process, the goat skin was cut into $4.5 \times 4.5\ cm^2$ size, washed, and dried. First, the hairy side of the goat hide was completely soaked into the mixture containing protease enzyme along with 14 % Sodium sulphide and lime. Later the piece was incubated at 37º C for different periods (3-18 h). However, goat hide treated only with distilled water without the addition of protease enzyme was used as a control. Each treatment's softness and hair loss were observed (Mukhtar & Haq, 2008; Nadeem *et al.,* 2010).

2.10 Statistical analysis

All the data were expressed as the average of three replicates in each experiment. The SPSS 22.0 version was used for all the statistical analyses.

3. Results and discussion

3.1 Screening of agro-residues as substrates for the production of protease

The current study conducted the protease production through SSF by *Bacillus subtilis* NCIMB-10144. SSF is an environment friendly technique and is greatly influenced by the nature of solid substrates. In addition, this solid material plays a dual role in fermentation by supplying nutrients to the microbial culture and anchorages the growth cells. In the present study various solid substrates (soya bean meal, wheat bran, and chickpea) were tested for the protease production. Figure 1a revealed that among all the substrates, the use of soybean meal significantly increased the enzyme activity in contrast to other substrates. This could be due to the reason that soya bean is a good source of proteinaceous nitrogen, carbohydrates, and other nutritious substances essential for the growth of bacteria. In continuation, different concentrations ranging from 5-30 g of soybean meal (SBM) were also tested for protease production. Figure 1b showed that the soybean meal at 5g gave maximal protease productivity a greater or lesser concentration of SBM than the optimal value resulting in less production. Present results agreed with Su *et al.* (2018), who reported a positive effect of soya bean meal on the protease production by *Clostridium butyricum*. Moreover, proximate analysis of the soya bean meal was also carried out. In Figure 2 high percentage of protein contents were observed in the soya bean meal compared to the crude fiber (CF), fat, moisture, and ash content.

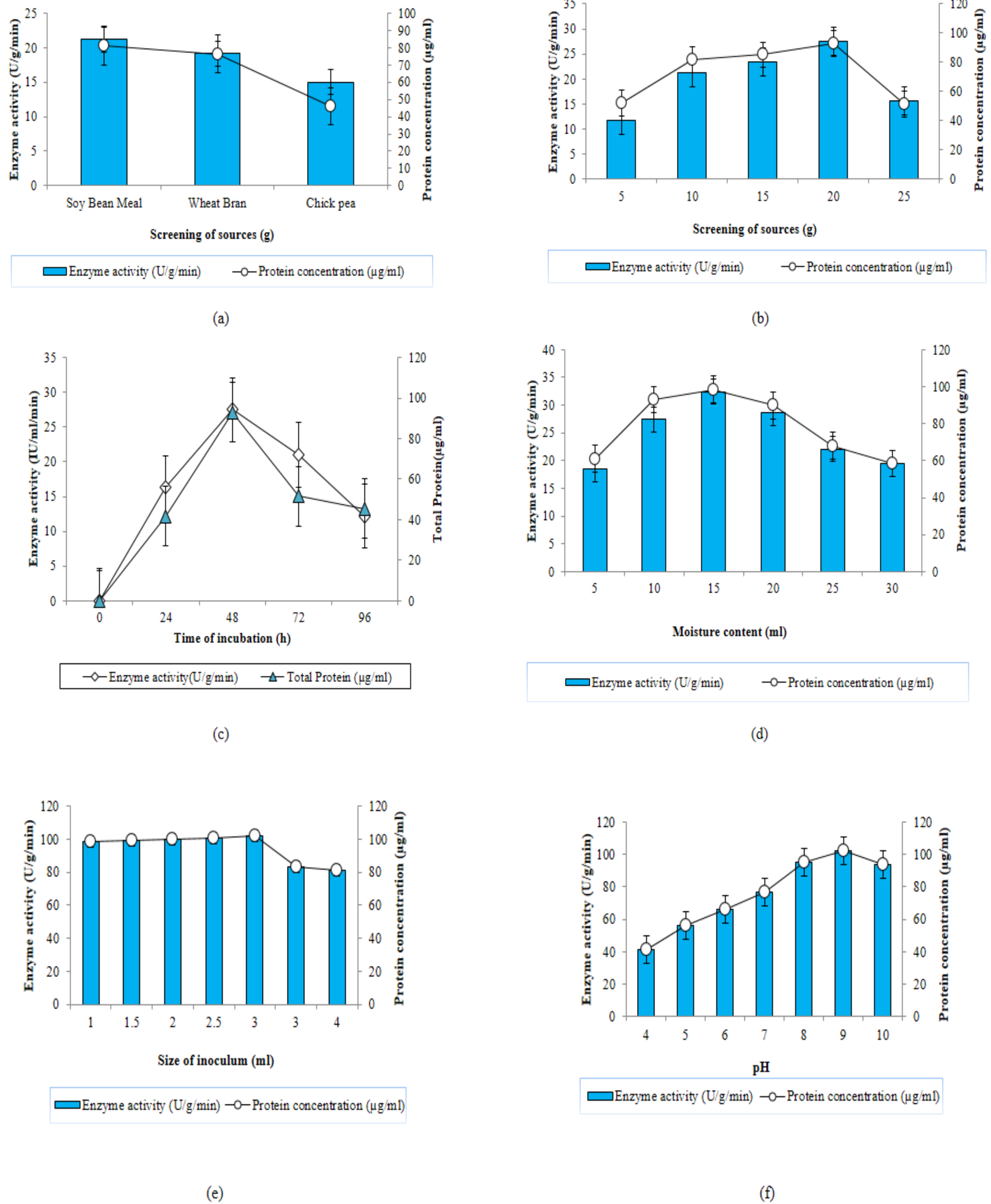

Fig. 1. Impact of various physical factors on the protease production by *Bacillus subtilis* NCIMB-10144 (a) Different substrates (b) Soya bean meal conc. (c) Time of incubation (d) Moisture contents (e) Size of inoculum (f) pH

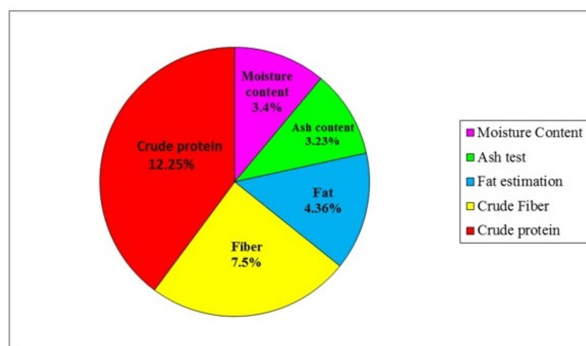

Fig. 2. Proximate analysis of soybean meal

3.2 Influence of incubation time and moisture content

Figure 1c depicted the impact of incubation time (0-96h) on enzyme production. Results revealed that the protease production gradually increased after 24 hours of incubation and reached a maximum at 48 hours. The decline in protease activity was noticed before and after the optimal incubation time. It could be due to the decomposition or denaturation of protease due to interaction with other compounds in the fermentation medium (Uyar & Baysal, 2004). *Bacillus* species generally produce optimum proteases in stationary or post-expression phases (Sharipova *et al*., 2000). However, depending upon the medium composition and other factors, it may produce maximum production in an exponential phase. Current results disagreed with George-Okafor and Mike-Anosike (2012), who reported maximum protease production after 72 h incubation.

In a solid state fermentation (SSF) water content of substrate is greatly influenced by the substrate's absorbing capacity and capillary forces. Moreover, moisture level significantly affects the substrate's physical properties, which makes it different from submerged fermentation. Figure 1d shows the impact of moisture content (5-30%) on protease production. Among all the levels, higher titers of protease enzyme were achieved at 15%. However, less production was observed with a further rise or drop in moisture content than 15%. It was believed that the increased amount of moisture content reduces the porosity of the substrate; thereby limiting the oxygen supply; on the other hand, lower moisture content causes sub-optimal growth and lowers the swelling of the substrate, which ultimately decreases protease production. The current study contradicts Akcan and Uyer (2011), who reported 30% moisture content for protease production.

3.3 Influence of different inoculum sizes and pH

Inoculum size is one of the main biological factors in enzyme production. Figure 1e depicted the impact of various inoculum sizes (1-4 %) on protease production. Among all the tested inoculum sizes, the maximum production was witnessed with 3.0 % of inoculum. Above or below the optimal level inoculum size influence the enzyme yield. A similar observation reported optimal protease production with 3 ml of inoculum (Muthulakshmi *et al*. 2011).

The pH of the fermentation medium helps in the enzymatic processes and the transportation of different components through the cell membrane. Figure 1f shows the effect of initial pH (4-11) on protease production. Results indicated a gradual increase in enzyme activity from pH 4 to 7; optimum production was noticed at pH 9. Current work agreed with Akcan and Uyer (2011), who reported maximum protease production at pH 9.

3.4 Effect of different carbon and inorganic-organic nitrogen sources

Nutrient sources were also found to be an important factor in protease production. Carbon is one of the primary nutrients for bacteria. The effect of carbon sources (sucrose, starch, maltose, fructose, and lactose) on the protease activity was also investigated. Figures 3a & b show that the maximal protease activity was achieved when sucrose served as the carbon source at 1%. Current work contradicts with Asha and Palaniswamy (2018), who reported lactose as the best carbon source for protease production. Nitrogen also serves as an important nutrient source next to carbon for protease production. The effect of inorganic nitrogen sources (ammonium nitrate, ammonium chloride, and ammonium acetate) and organic nitrogen sources (yeast extract, urea, tryptone, nutrient broth, and peptone) on protease production were studied. Among all the inorganic (Figures 3c &d) and organic nitrogen sources (Figures 3e & f), the maximum protease production was obtained with ammonium nitrate and Yeast extract at 0.5% and 2 %, respectively. Present results are in accordance with Badhe *et al.* (2016), who mentioned maximum protease production with yeast extract.

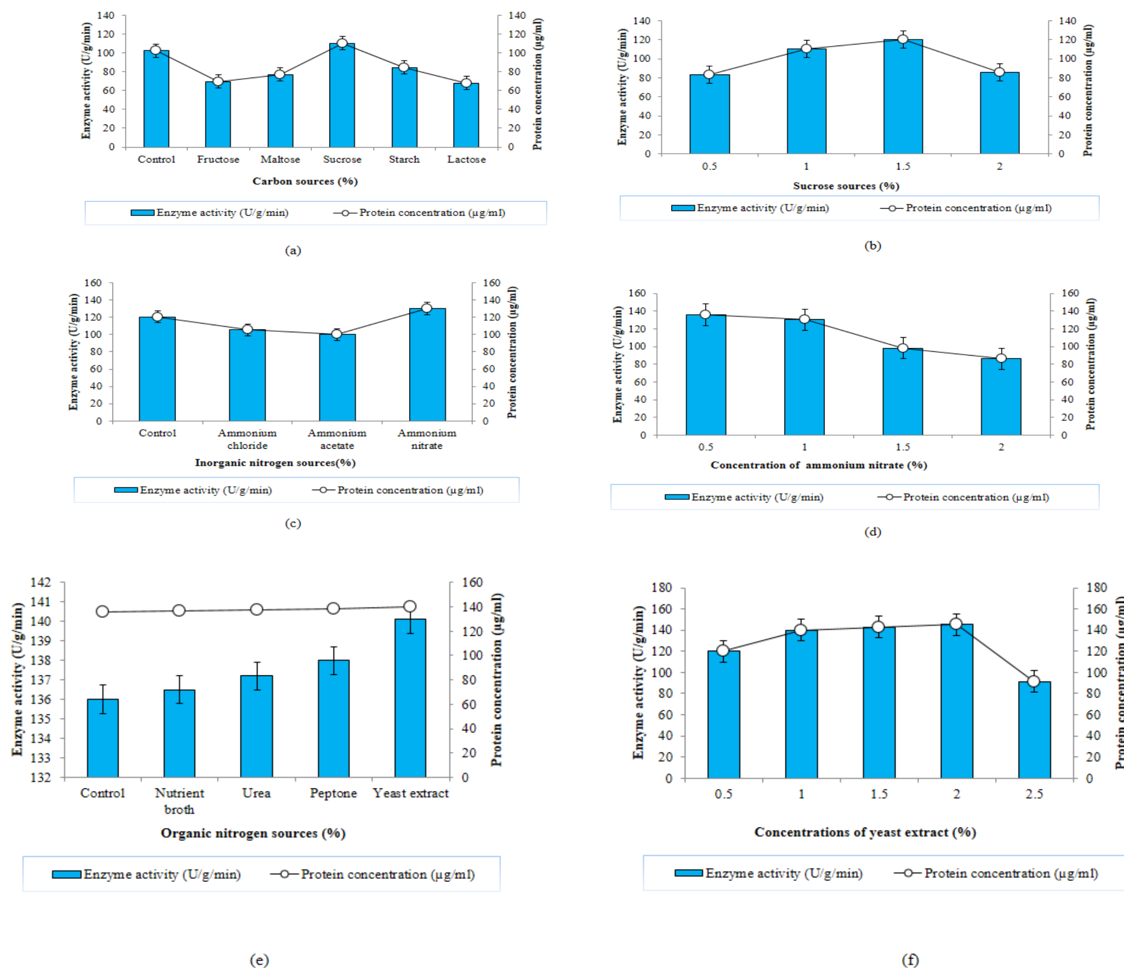

Fig. 3. Impact of various nutritional parameters on the protease production by *Bacillus subtilis* NCIMB-10144 (a) Carbon sources (b) Sucrose conc. (c) Inorganic nitrogen sources (d) Ammonium nitrate conc. (e) Organic nitrogen sources (f) Yeast extract conc.

3.5 Impact of various metal ions and surfactants

Metal ions play an important role in enzyme action and their structural modification. The influence of supplementation of metal ions ($CaCl_2$, KCl, $ZnCl_2$, and $MgSO_4$) and surfactants (Tween 80, Tween 20, Tween 40, and SDS) for protease production were also studied (Figures 4a & b). The results showed that adding of Ca^{2+} ions to the medium resulted in high protease activity. The use of surfactants significantly affected the extracellular enzyme production by bacteria, including *Bacillus* species (Nascimento & Martins, 2006). Figure 4c depicted that enzyme production was higher in the presence of Tween 40. Current research differs from Ananthan (2014), who reported the positive effect of Tween 80 by *Vibrio* sp.

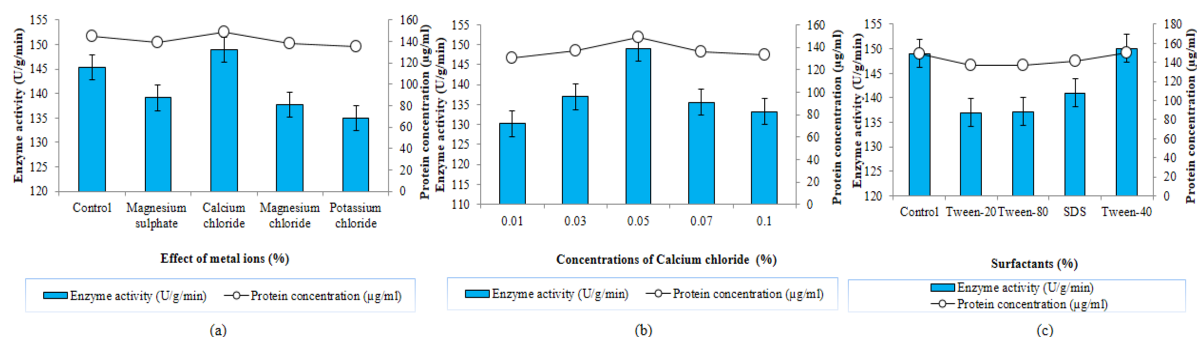

Fig. 4. Impact of metal ions and surfactant on the protease production by *Bacillus subtilis* NCIMB-10144 (a) Different metal ions (b) Calcium chloride conc. (c) Surfactants

3.6 Purification of protease

The purification scheme of protease by *Bacillus subtilis* NCIMB-10144 was presented in Table 1. Initially, the crude extract was precipitated with 80% ammonium sulfate. A 1.9-fold purification with 66.6 % yield purification along with 73.68 U/mg specific activity was obtained. Then, the concentrated active fractions were further purified using Sephadex-G 100 (Pharmacia) column (1.5 cm x 30 cm). Figure 5a shows the elution profile of the protease enzyme. The active fractions of the single peak indicated 1.3-fold purification with a purification yield of 37.5% and 51.5 U/mg of a specific activity. Current results are in accordance with Ravindran *et al*. (2011), who reported protease purification by Sephadex column (G-100). The purity of the protease enzyme was confirmed by a single band of~36 kDa molecular weight (Figure 5b). Similar results were presented by Vijayaraghavan *et al*. (2016).

Table 1. Stepwise purification of protease enzyme

Sr #	Steps of Partial purification	Volume of enzyme (ml)	The specific activity of the protein (U/mg)	Total protein contents (mg)	Total activity of protease (IU)	Fold Purification	Yield percentage (%)
1	Crude enzyme	50 ml	38.3	99.5	103.4	1	100
2	$(NH_4)_2SO_4$ Precipitation	25.8	40.5	65.14	74.03	1.0	71.5
3	Dialysis	5	73.68	34.6	68.9	1.9	66.6
4	Sephadex-G 100	2	51.5	27.57	38.72	1.3	37.5

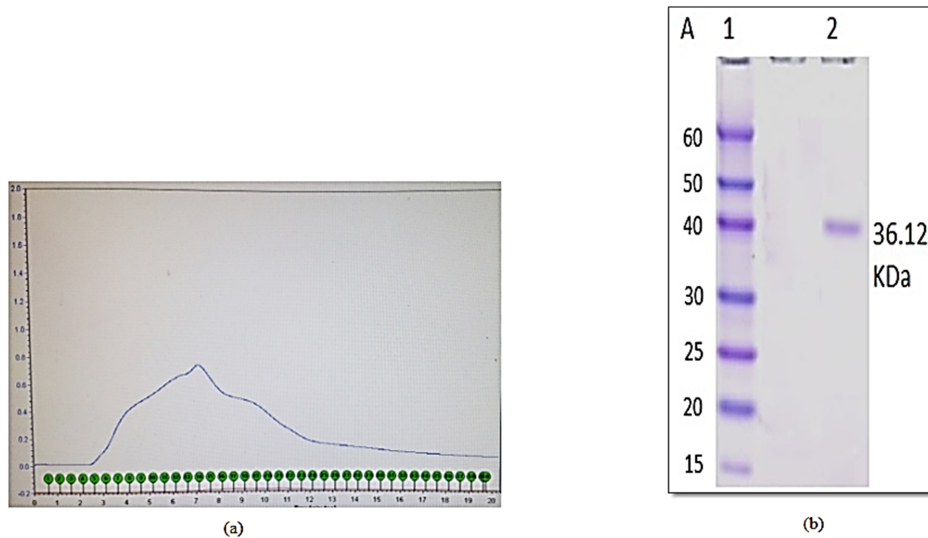

Fig. 5. Purified alkaline protease (a) Elution profile on Sephadex-G 100 (b) Molecular weight on SDS PAGE

3.7 Characterization of protease

The maximum activity of purified protease was recorded at 40°C; it gradually decreased above 40 °C and gave minimum activity at 50°C. The protease retained 100% residual activity for 2 h of incubation at 40 °C (Figure 6a).

Usually, the commercially produced microbial proteases by *Bacillus* sp. have an alkaline pH range between 8 to 12 (Rao *et al.*, 1998). In the current study, purified protease from *Bacillus subtilis*, NCIMB-10144, exhibited maximum activity at pH 9. It retained 97% activity at pH 9 and 50% at pH 11 (Figure 6b). In response to various metal ions, the Ca^{2+} ion positively regulates the optimal protease activity and stability (Figure 6c). These results follow the observations of Shivakumar *et al.* (2012). They reported maximum protease activity by Ca^{2+} ion.

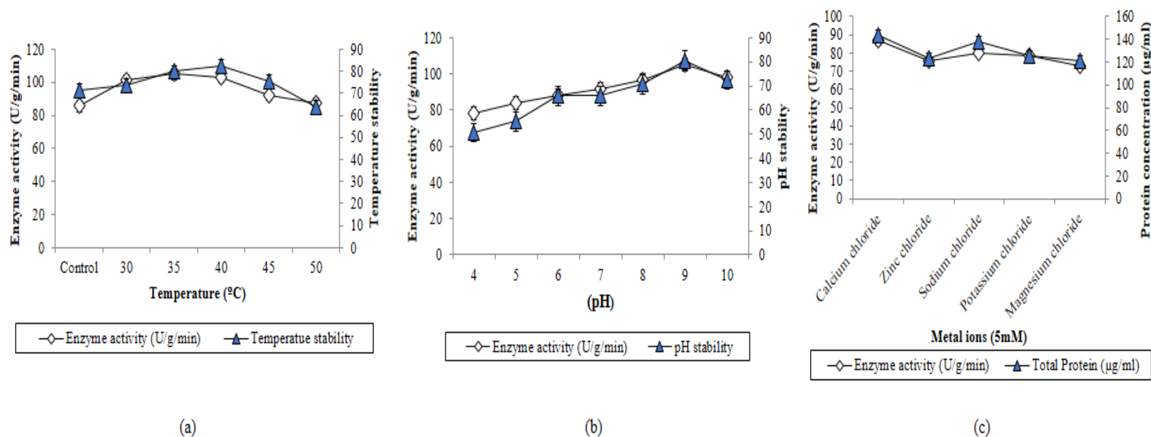

Fig. 6. Impact of protease activity and stability of purified protease by *Bacillus subtilis* NCIMB-10144 (a) Temperature (b) pH (c) Metal ions

3.8 Kinetic analysis

The Lineweaver-Burk (LB) plot for the proteolytic reaction of casein revealed that the *V*max of the reaction was 0.307 U/g. The Michaelis-Menten constant (*K*m) was 11.2 mg/mL (Figure 7).

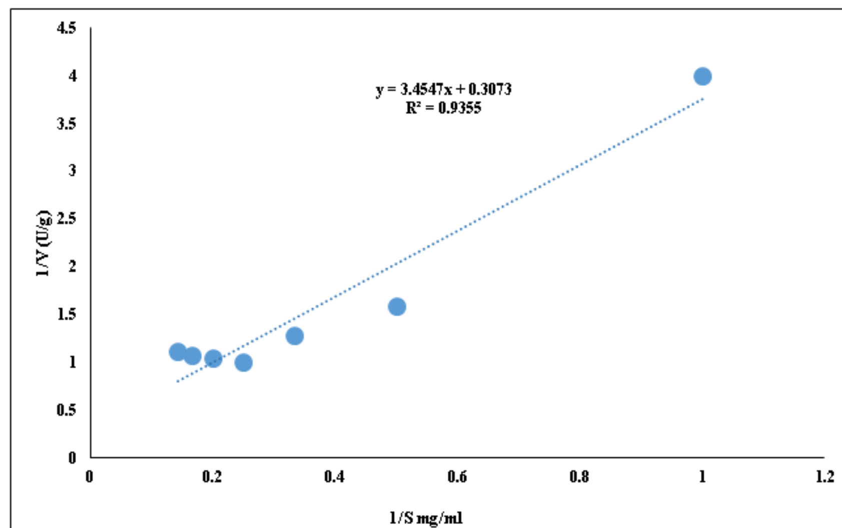

Fig. 7. Michaelis–Menten for protease activity

3.9 De-staining of blood

In the blood stain removal experiment, the compatibility of the protease enzyme was determined with the detergent. In the experiment, slight disappearance of blood stain was observed on the cotton cloth, which was washed only with the detergent, while no blood stain was removed from the cloth, which was soaked in the enzyme solution only. Contrary to this, blood stain was fully removed from the cotton cloth when dipped in 1 mL of the enzyme along with 1 ml (8 mg/mL) of detergent for 50 min (Figure 8). The experiment shows the cleaning effect of alkaline protease on white cotton cloth. It might be the reason that the protease enzyme enhances the stain removal property when used as an additive (Chimbekujwo & Moses, 2020). The present work was in accordance to Roshni *et al*. (2016), who used protease to remove animal blood stains from white cotton cloth.

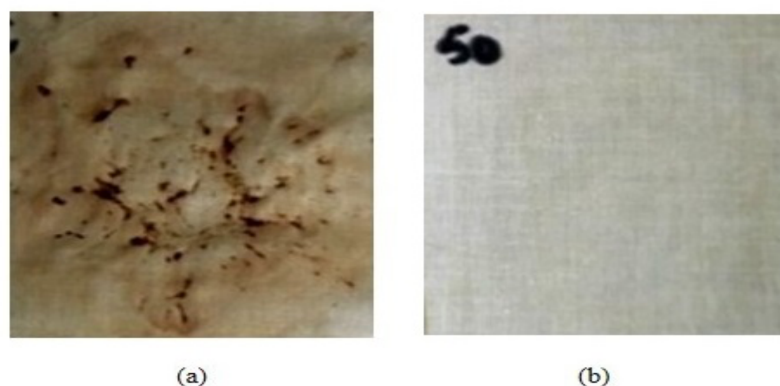

Fig. 8. Removal of blood stain (a) Control (b) Enzyme and detergent

3.10 The Dehairing of goat skin

Proteases produced from *Bacillus* sp. are proven efficient in leather processing (Zambare *et al*., 2007). In the current study, the Dehairing experiment successfully used protease on goat hide by removing the maximum amount of fine hairs. The protease enzyme can digest the collagen, so the Dehairing must be controlled to maintain the quality and avoid damage to the leather. Thus, the present results showed that the protease could be used in the Dehairing application. Figure 9b indicates that the complete dehairing of goat's hide was achieved after 15 h of incubation when treated with alkaline protease and sodium sulfate along with the lime; contrary to this, the skin that was treated only with distilled water (control) remained unaffected (Figure 9a). It was observed that the addition of the sodium sulphide de-haired the goat hide by removing all the hair above the epidermis due to which the leather did not become smooth and silky (Figure 9b). However, the protease enzyme attacked the hair below the epidermis and maintained the quality of the leather (Figure 9c) (Hakim *et al.,* 2018). A similar experiment was conducted by Hamza (2017), who used alkaline proteases on goat hide.

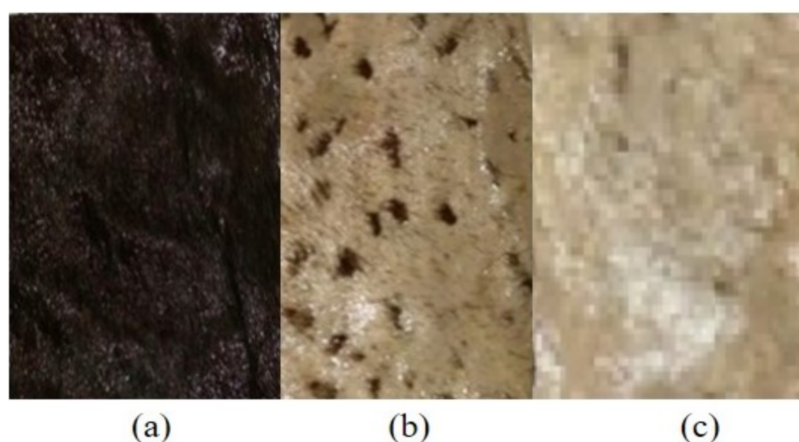

Fig. 9. Effect of protease on dehairing of goat hide(a) Control (b) partial hair removal from the goat hide (C) Full hair removal from the goat hide

4. Conclusion

In the current study, various physicochemical parameters were optimized for protease production. Considering as a cheap source soya bean meal was used as an ideal substrate for enzyme bioprocess. Furthermore, purified protease showed its stability and activity at high pH and temperature ranges. The practical application of blood stain removal from white cotton cloth and the Dehairing of goat hide indicates the potential use of alkaline protease in detergent and the leather industry.

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