Vol.67: e24210355, 2024
https://doi.org/10.1590/1678-4324-2024210355
ISSN 1678-4324 Online Edition

BABT
Brazilian Archives of Biology and Technology

Article - Agriculture, Agribusiness and Biotechnology

Production, Partial Purification and Characterization of Protease through Response Surface Methodology by *Bacillus subtilis* K-5

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Editor-in-Chief: Alexandre Rasi Aoki
Associate Editor: Luiz Gustavo Lacerda

Received: 03-Jun-2021; Accepted: 09-Sep-2023

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HIGHLIGHTS

- *Bacillus subtilis* K-5 is thermophilic and alkaliphilic strain for the production of thermostable alkaline protease.
- Maximum protease production was achieved by using wheat bran as a substrate from agro-industrial by-product through solid state fermentation.
- Kinetics parameters of protease indicate that it has high affinity towards substrate and very stable enzyme to be used in poultry feed industry.

Abstract: The aim of present study was the production of protease from local *Bacillus subtilis* through solid state fermentation. Response Surface Methodology (RSM) was used for the optimization of all the culture conditions. Casein (1% w/v) was used as a substrate in nutrient agar medium for the screening of enzyme production potential and showed maximum zone of clearance (4.6 cm). It was identified as *Bacillus subtilis* K-5 by genetic identification based on 16S rRNA and blast technology of NCBI. Among culture conditions, incubation

temperature, incubation time, pH of the medium and moisture level of the substrate were optimized. Maximum protease production was observed at 37°C, pH 9.0 with incubation time of 36 h and moisture to substrate ratio of 1: 0.75. Maximum protease production of 70.21 U/mL was recorded when wheat bran was used as an agro-industrial substrate. The activity of crude protease was observed 99.63 % at 60°C and pH 10.0 with protein concentration 0.63 mg/mL and specific activity of 111.56 U/mg. Protein contents of 0.57 mg/mL (specific activity of 124.72 U/mg) and protein contents of 0.44 mg/mL (specific activity of 143.65 U/mg) were observed by 70% saturation with ammonium sulphate and gel chromatography, respectively. Line Weaver Burk plot was used to find its Vmax and Km, which were 344. 83 mg/mL/min and 100.04 mg/mL, respectively. The study concluded that *Bacillus subtilis* K-5 is thermophilic and alkaliphilic strain which produces active protease and can be used as potential microorganism for industries.

Keywords: Protease; *Bacillus subtilis*; casein; gel chromatography; ammonium sulfate precipitation.

INTRODUCTION

Proteases are one of the most important groups of industrial enzymes and account for nearly 60% of the total enzyme sale. These are biocatalysts which hydrolyses peptide bonds of proteins and single class of enzymes which occupy a pivotal position due to their wide application in detergent, pharmaceutical, leather, photography, food, poultry feeds, meat processing and agriculture industries [12]. Proteases occur naturally in all plants, animals and microorganisms. The quantity of the enzyme produced from plants and animal sources are very limited, both for economic and technical reasons. Microorganisms produce a large variety of enzymes so they are known to play a vital role in technology for the production of extracellular enzymes on industrial scale [9].

Proteases are mainly produced from microbial sources such as bacteria, yeast and fungi. Among the various proteases, bacterial proteases are the most significant, compared with fungal proteases [13]. *Bacillus* species produce a variety of proteases of which an alkaline protease (subtilisin) and a neutral metalloprotease are extracellular, whereas at least two serine proteases are produced. However, the proteases produced by *bacillus* are mostly alkaline in nature and highly stable against extreme temperature, pH and other conditions [36]. The industrial sectors frequently use *Bacillus subtilis* for the production of commercial protease. *Bacillus subtilis* is found mainly in the soil and is also known as hay bacillus and grass bacillus. It is a rod-shaped organism, which can make a tough protective endospore and can withstand extreme environmental conditions [20].

The growth and enzyme production of the organism are strongly influenced by the medium components like carbon and nitrogen sources. Besides the nutritional factors the culture parameters like substrate, moisture to substrate ratio, pH of the medium, incubation temperature and incubation time also plays major role in the enzyme production [30] so optimization of the media components and culture parameters is the primary task in a biological process. Conversion of agro-industrial wastes by microorganisms into useful biomass and their enzymes is a new trend, a new protease producing organisms and perfected fermentation technology are needed to meet the ever growing demand of this enzyme. Pakistan is an agricultural country so it has a wide variety of agro-industrial by-products which are cheaper and easily available in market. These by-products are good sources of proteins, carbohydrates and minerals needed for the growth of microorganism and synthesis of microbial enzymes [29].

The demand for microbial proteases is increasing for the industrial purpose because of their wide range of applications. Since the emergence of enzymology, microbial proteases have been investigated intensively as one of the most important hydrolytic enzymes. Nearly two-third of the commercial available proteases are produced from bacterial origin [44]. The use of protease in poultry industry of Pakistan has increased much for the last ten years. In poultry, substantial amount of protein passes undigested from the gastro-intestinal tract of the bird. This undigested protein fraction creates an opportunity to add exogenous protease in the poultry diets. The protease can be supplemented with the aim to reduce dietary protein levels without compromising bird's performance, hence environmental burden can be minimized by lowering nitrogen excretion and protein waste [46]. The high cost of imported protease limits the maximal use in poultry feed formulation. However, a cheaper protease, if available in the market will increase its utilization. This would definitely have a positive impact in reducing feed cost by using alternate feed ingredients in the poultry diets. Taking into account the importance of protease in utilizing alternative feedstuffs in poultry diets, it is of great importance to have indigenous production of this enzyme.

It was hypothesized that novel protease could save poultry feed cost as compared to commercial protease and could improve bird performance by improving protein digestion in low protein diets while using alternate cheaper feed ingredients. So, the objectives of present study was the production of cheaper alkaline protease from local strain of *Bacillus subtilis* K-5 through solid state fermentation and its application in broiler chickens.

MATERIALS AND METHODS

Collection and Isolation of *Bacillus subtilis*

In the present study, *Bacillus subtilis* was obtained from Industrial and Environmental Biotechnology Laboratory, University Institute of Biochemistry and Biotechnology, PMAS Arid Agriculture Univerisity, Rawalpindi, Pakistan. It was recultured on Yeast Extract Peptone (YEP) agar media and after 48 hours pure bactrerial colonies were obtained. Slants were prepared from pure colonies to preserve the culture for further use.

Screening of *Bacillus subtilis* for protease production

The loopful culture of *Bacillus subtilis* was streaked on nutrient agar plate containing casein (1% w/v) as a substrate. The plates were incubated for 24 h at 37^{o}C and enzyme activity was observed. The proteolytic activity was detected by the presence of clear zone of hydrolysis on casein agar by the procedure described by [27].

Identification of *Bacillus subtilis*

Bacteria was identified using 16S rRNA sequencing from Macrogen Inc. South Korea. Then multiple sequence alignments was tested using blast program (NCBI) to access similarity.

Preparation of Liquid Broth

Yeast extract broth was prepared in agar less media for further use in fermentation process. The pH of the broth was adjusted to 6.8 by using 1N solution of NaOH and HCl. A glass marble was placed inside the broth flask to prevent clumping of spores in liquid medium. The flask was cotton plugged, autoclaved and loop full of bacterial colonies was transferred from pure culture into flasks containing liquid broth under aseptic conditions. Flasks were then placed in shaking incubator at 120 rpm and 30^{o}C for 24 hours.

Preparation of substrate

For the production of protease from *Bacillus subtilis* through solid state fermentation different agro-industrial by-products (Wheat bran, corn cob, rice polish, waste bread and wheat straw) were used which are easily available at cheaper price. All substrates were dried to zero moisture level, grinded to powder, then sieved to separate into 40 and 80 mesh sizes and packed in air tight containers. The substrate with maximum enzyme production was selected for bulk protease production.

Optimization of protease production

Growth parameters including incubation time, incubation temperature, pH of the medium and substrate to moisture ratio were optimized for the maximum production of alkaline protease through solid state fermentation. The optimization was performed through Response Surface Methodology (RSM) and experiments were designed with JMP 13.0 software. There were 26 experiments with 2 central points. Incubation time was checked from 24 hrs to 48 hrs, incubation temperature from 25^{o}C to 45^{o}C, pH from 6 to 12 and moisture to substrate ratio of 1:0.5, 1:0.75 and 1:1 i.e., 50, 75 and 100 % to maximize protease production.

Enzyme Extraction

After specific time of incubation, the flasks were taken out from incubator and 50 mL of distilled water was added in it. The culture was mixed and then placed in shaking incubator at 30^{o}C for 60 minutes for homogenization. Later the mixture was filtered through Whatmann No. 1 filter paper and was centrifuged at 10,000 rpm for 15 minutes at 4^{o}C. The supernatant was then collected and stored as crude enzyme at 4^{o}C for further protease assay.

ENZYME ASSAY

Protease enzyme assay

Protease activity in crude enzyme extract was determined by method reported by [7], using casein as a substrate. Casein solution (0.65%) was prepared in 0.1M Tris-HCl buffer (pH 8.5). Five mL of 0.65 % casein solution was taken in glass vials, which were kept in oven at 37 ^{o}C for 5-10 minutes. One mL of crude enzyme was added in each vial. It was homogenized by gentle shaking and kept in oven at 37^{o}C for 30 minutes. Reaction was stop by adding 5 m chilled Trichloroacetic acid (TCA 10%). These vials were left for 30 minutes at room temperature and solution from each vial was then sieved through 0.45 µm syringe filters. Then 2 mL of filtrate was taken in another vial. Five mL of 0.5 M Na_2CO_3 was added in this filtrate in all vials. One mL of Follin's Ciocalteus reagent (2 folds diluted) was added instantly after Na_2CO_3. The resulting solution was placed in dark place for 30 minutes at room temperature until the appearance of brilliant blue color. Then absorbance of blue color was noted at the wavelength of 660 nm against the reagent blank by spectrophotometer which was kept ready after calibration.

Preparation of tyrosine standards

L-tyrosine standard stock solution (1.1 mM) was prepared by adding 0.2 mg/mL L-tyrosine in distilled water and heated gently until tyrosine dissolves. This L-tyrosine standard solution was allowed to cool at room temperature. The dilutions from tyrosine standards were made according to Table 3. Five mL of 0.5M Na_2CO_3 and 1 mL Follin's Ciocalteus reagent was added in each vial including blank. The solution was mixed immediately and kept for 20 min at room temperature. Finally, the absorbance was measured at wavelength of 660 nm using spectrophotometer and tyrosine standard curve was plotted by the procedure described by [21].

Calculation of enzyme activity

Tyrosine concentration was calculated using the regression equation from tyrosine standard curve. The enzyme activity (U/mL) was calculated using the formula given by [41].

$$\text{Protease activity (U/mL)} = \frac{\mu\text{g tyrosine released} \times \text{Total voulme of reaction mixture}}{\text{Time} \times \text{Enzyme volume} \times \text{Volume in the cuvette}}$$

One unit of protease activity was defined as the amount of enzyme required to hydrolyze casein to give 1 µg of tyrosine in one minute at standard assay conditions of pH and temperature [14].

CHARACTERIZATION OF PROTEASE ENZYME

The protease was further characterized for the determination of following parameters.

Effect of pH on the protease activity

The crude protease was incubated at different pH (6, 7, 8, 9, 10, 11 and 12) with the phosphate buffer. The effect on the enzyme activity was studied by incubating the mixture of enzyme and casein with optimized substrate to moisture ratio and at the optimized temperature for 20 minutes.

Effect of temperature on the protease activity

The crude protease was incubated at different temperatures (25, 35, 40, 45, 50, 55, 60, 65, 70 and 75^{o}C). The effect of temperature on activity of the protease was studied by incubating mixture enzyme and casein with optimized substrate to moisture ratio and at optimized pH for 30 minutes.

Protein Estimation of Crude Protease Enzyme

Total protein contents of the crude protease were determined according to the method described by [21] using Bovine Serum Albumin (BSA) as standard protein.

Specific Activity of Crude Protease Enzyme

The specific activity of crude protease was determined by the formula given below;

$$\text{Specific Activity (U/mg)} = \frac{\text{Protease enzyme activity (U/mL)}}{\text{Protein contents (mg/mL)}}$$

PURIFICATION OF CRUDE PROTEASE ENZYME

Alkaline protease produced under the optimized conditions was partially purified by ammonium sulphate precipitation method and Sephadex G-100 column chromatography.

Ammonium sulphate precipitation method

Proteins in the crude enzyme extract were purified by adding ammonium sulphate according to different saturation levels of salt including 10, 20, 30, 40, 50, 60, 70 and 80%. The samples were left for 4 hours at 4°C, later the samples were centrifuged for 15 minutes at 10,000 rpm at 4°C. The pellets were dissolved in 1.5 mL of Tris-HCl buffer (pH 8.5). The supernatant and pellets, both were subjected to protease assay. The fraction showing the highest protease activity was considered as the best purified.

Gel filtration chromatography

Gel filtration chromatography was also adopted for enzyme purification. For this purpose, 5% Sephadex G-100 solution was prepared by mixing 5 g Sephadex G-100 in distilled water. The final volume was maintained at 100 mL. The mixture was stirred gently and left overnight allowing the Sephadex beads to swell. The next day, the gel was mixed gently and the slurry was poured gently into the vertical column. The column was filled with the gel to its two third with the intervals so that the gel bed kept at uniform. The gel was equilibrated with 0.05M Tris-HCl buffer (pH 8.5) and eluted with the same buffer at a flow rate of 30 mL/hours. Total 30 elusions were collected. First 5 elusions were not considered. The next 25 elusions were subjected to protease assay.

Estimation of kinetic parameters

The reaction of the enzyme was checked for multiple casein concentrations made in 0.1M Tris-HCl buffer at the optimum values of pH and temperature. Using the linear equation of Lineweaver-Burk Plot, K_m and V_{max} for alkaline protease were determined.

RESULTS AND DISCUSSION

Screening of *Bacillus subtilis* for protease production

Bacillus subtilis was identified as protease producing strain by zone of hydrolysis around the colonies by using casein as a substrate which indicates proteolytic activity. The diameter of zone of hydrolysis was noted 4.6 cm. The Zone of hydrolysis for *Bacillus subtilis* on casein agar is shown is Figure 1.

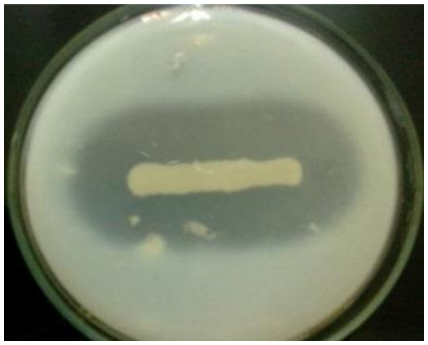

Figure 1. Screening of *Bacillus subtilis* for protease production

Identification of bacterial strain

Recultured *Bacillus subtilis* was subjected to 16S rRNA gene sequencing. The identified strain has 99.77 % similarity with *Bacillus subtilis* K-3.

Optimization of protease production

Protease production was optimized by the study of various growth conditions through RSM. Experimental combinations for all four parameters under study obtained with JMP software were performed in triplicate. The most suitable conditions for maximum protease production were 35ºC, pH 9, Incubation time 36 hours and moisture level 75 % (Figure 2).

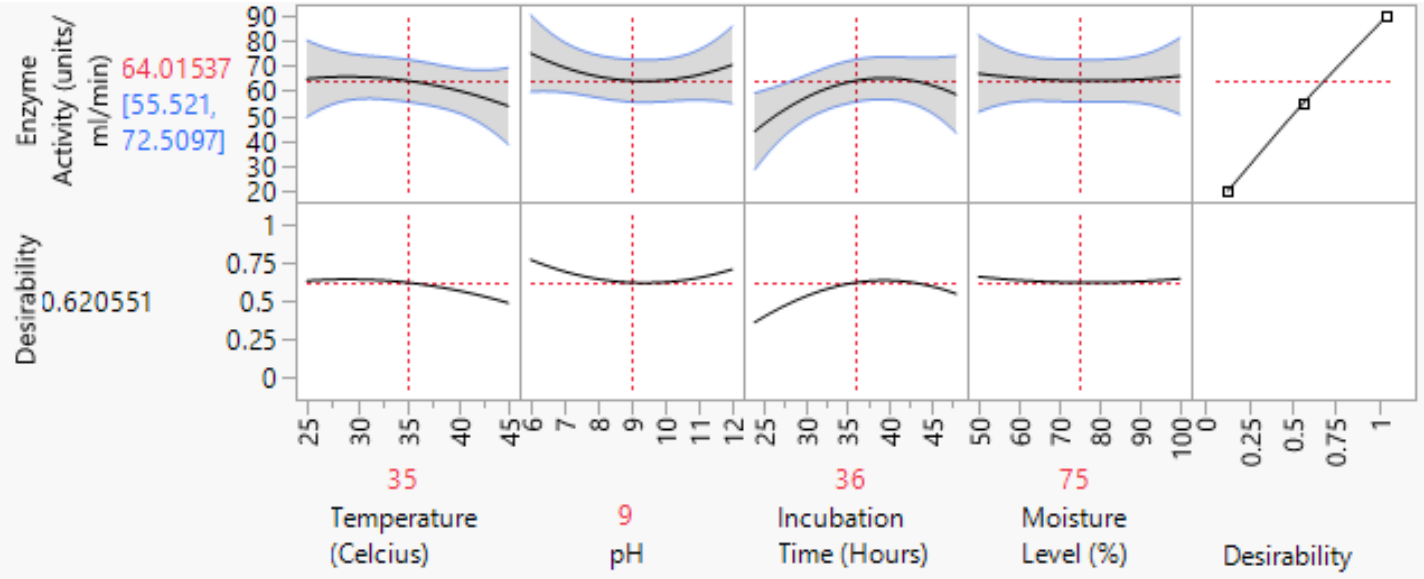

Figure 2. Most suitable culture conditions for maximum protease production

Effect of temperature on protease production

Effect of different temperatures ranging from 25-45ºC on protease production was carried out and maximum alkaline protease production (71.38 U/mL) was observed at 35ºC (Figure 3). It was observed that production of protease was increased from 25ºC to 35ºC and then started decreasing as temperature goes up from 35ºC to 45ºC. Higher temperature is found to have adverse effect on metabolic activities of microorganisms. The enzyme is denatured by losing its catalytic properties due to stretching and breaking of week hydrogen bonds within enzyme structure by increasing temperature [2]. Maximum growth of the bacteria is noted in temperature range of 30 to 40ºC [16]. Current study was in agreement of [15] who reported optimum temperature of 37ºC for alkaline protease production. [42] also reported that maximum protease production was observed at 37-40ºC from *Bacillus cereus* SU12 isolated from oyster *Asccostrea cucullata*. Similar results were reported with other *Bacillus* species. The optimum temperature for maximum protease production from *Bacillus amovivorus* [37] *Bacillus fastidiosus* [38] and *Pseudomonas fluorescens* CM1 [3] was 37ºC.

Effect of pH for protease production

The maximum protease production (72.09 U/mL) was noted at pH of 9.0 (Figure 4). Any change in pH from this value leads to decrease in enzymatic activity possibly due to un-stability of enzyme. Similar results were obtained by [15] who reported that optimum pH was 9.5 for maximum production of protease from *Bacillus subtilis* IH_{72}. The production of protease is greatly influenced by pH of the fermentation medium. Changes in pH of medium causes denaturation of enzyme resulting in the loss of catalytic activity [15]. [5] also reported that maximum protease production from *Bacillus* sp. was obtained at pH 9.5. [39] reported that *Bacillus* species are known to grow in fermentation medium having alkaline pH. [2] also reported that initial pH of culture media is very important for the protease production so pH of 9.0 was optimum for maximum enzyme production from

Bacillus subtilis RSSK96. This suggest that bacterial strain was alkaliphilic in nature [28]. Most substrates used in solid state fermentations are known to possess excellent buffering capacity and this explains the fairly constant pH for the greater part of the fermentation [32,8]. Results of current study were in contrast with [42] who observed pH 7.0 for maximum protease production from *Bacillus cereus* SU12.

Effect of incubation time on protease production

Experiments were carried out at different incubation times ranging from 24-48 hours and maximum protease activity (72.42 U/mL) was observed by incubating fermentation media for 36h (Figure 5). Increase in enzyme production was observed when incubation time was increased from 25 to 36 hours but further increase in incubation time resulted in decreased enzyme production. The incubation period is directly related with the production of enzyme and other metabolites up to certain extent. After that, enzyme production starts to decrease which can be attributed to decreased supply of nutrients to microorganisms [31]. This study was in agreement with the studies of [5] and [35] who reported that *Bacillus* species are known to produce maximum amount of protease in stationary phase or post exponential phase of their growth which starts at 28 hours and lasts 48 hours of incubation in the fermenter. However, some other *Bacilli* synthesize protease during the exponential growth phase but it depends on medium composition. [23] also observed that *Bacillus subtilis* IH-72 produced maximum protease at incubation time of 40 hours. They further added that there was very little enzyme production during lag and log phase of bacterial growth. Same was reported by [42] that highest biomass yield was recorded at 36 hours of incubation time and maximum protease production was recorded at 30 hours. Results of present study were in contrast with the findings of [15] who reported optimum incubation time of 48 hours for the production of alkaline protease. It was also reported that *Bacillus* sp. produce maximum alkaline protease after 48 hours of incubation in alkaline medium [11].

Effect of moisture level on protease production

The initial moisture content significantly influence hydrolytic enzyme production in solid state fermentation. The moisture/water activity is one of the most critical factors for microbial growth and enzyme production using a particular substrate. Because solid state fermentation process is different from submerged fermentation culturing, since microbial growth and product formation occurs at or near the surface of the solid substrate particle having low moisture content [26]. The correlation between enzyme production and water contents of solid state fermentation is important factor so different substrate to moisture ratio ranging from 1:0.5 - 1:1 (75-100 %) were tested in different experiments and maximum protease production (71.43 U/mL) was noted at 1:7.5 (75 % moisture) level (Figure 6). It might be due to the fact that less quantity of diluent was insufficient to fulfil the moisture requirements of the organism. So culture conditions become suitable for bacterial growth by increasing quantity of the diluent for maximum protease production. [15] reported that 1:1 (100 %) moisture to substrate ratio was optimum for maximum protease production from *Bacillus subtilis*. Further increase in the moisture level resulted in the formation of a paste and condition became anaerobic which were not favorable for the growth of the organism and hence protease production was decreased. Our findings were in contrast with [2] who reported highest protease production from *Bacillus subtilis* RSSK96 at 30 % initial moisture contents.

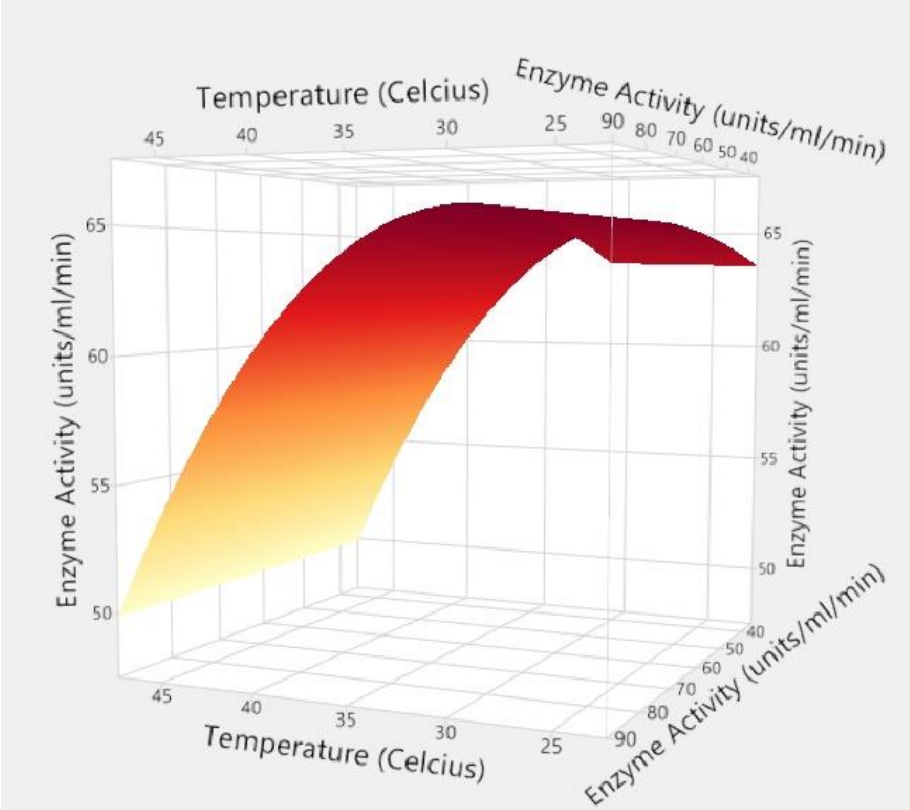

Figure 3. Effect of temperature on protease production

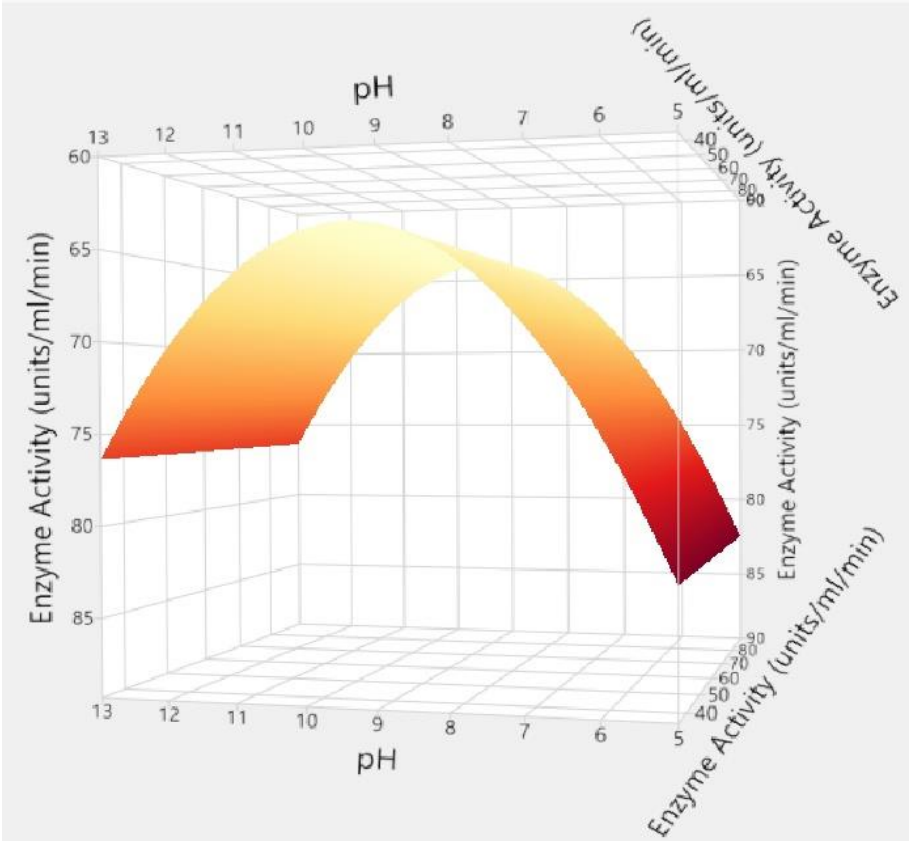

Figure 4. Effect of pH on protease production

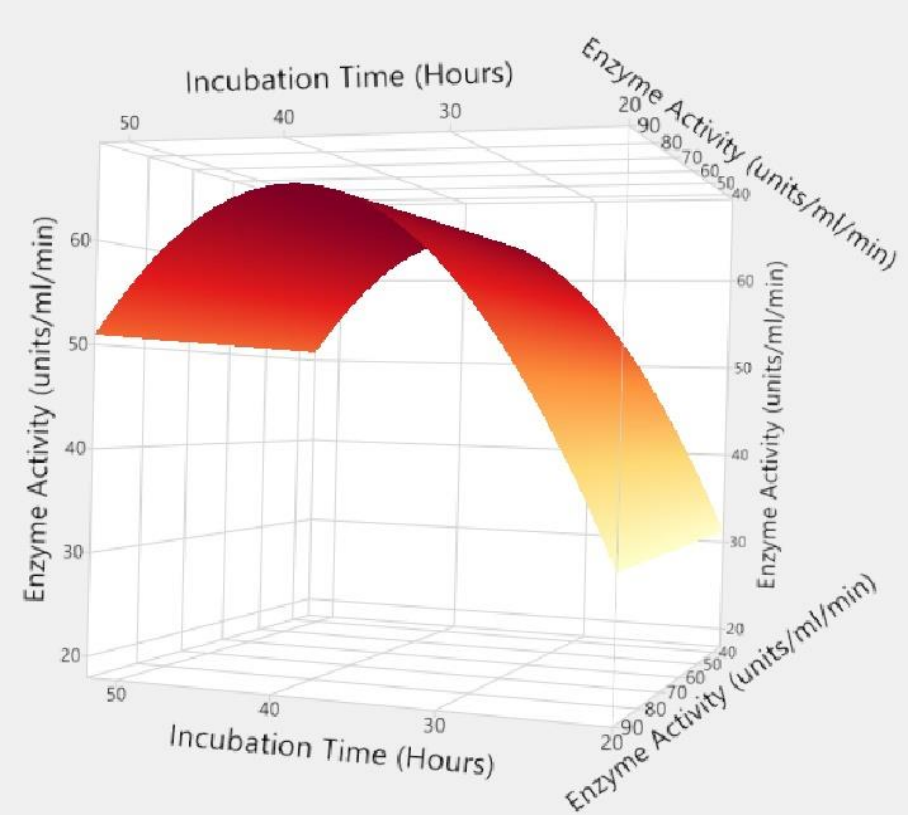

Figure 5. Effect of incubation time on protease Production.

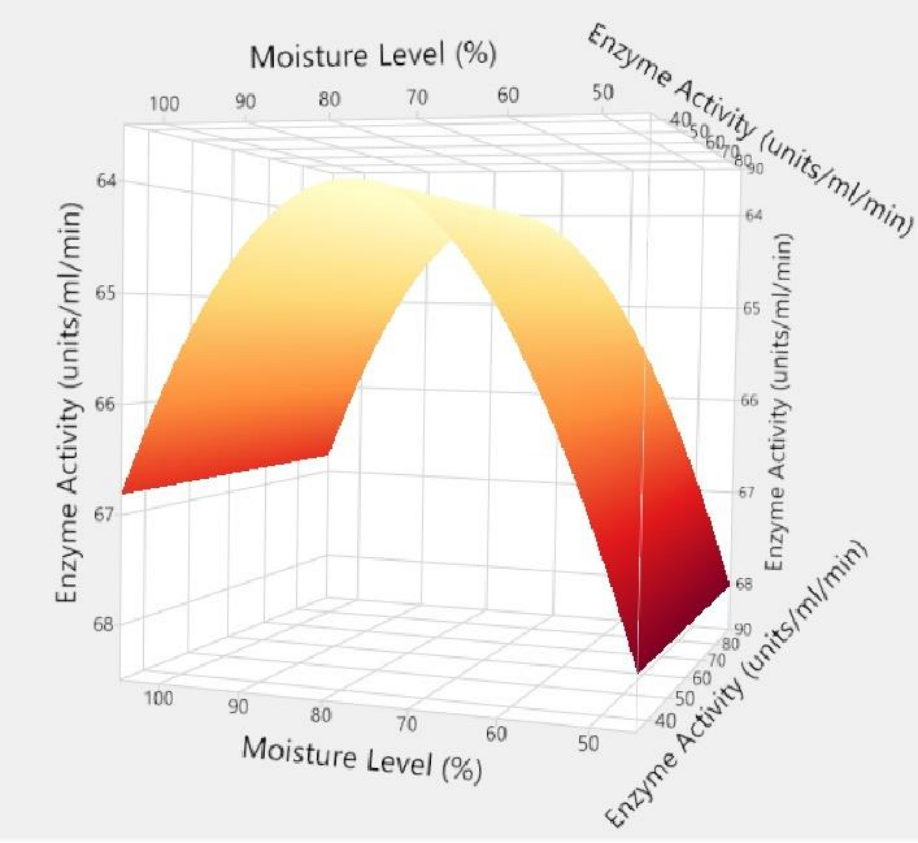

Figure 6. Effect of moisture to substrate ratio on protease production.

Substrate optimization

Different substrates were tested for optimized protease production. Maximum protease production of 70.21 U/mL from *Bacillus subtilis* K-5 was noticed with wheat bran and minimum protease production of 17.73 U/mL was observed with wheat straw (Table 1). Comparison with different substrates is given in Figure 7.

It has been reported in literature that a low cost substrate like wheat flour, wheat bean, rice husk and molasses are suitably effective for the growth of microorganism and enzyme production [22, 33]. Results of current study were in agreement with [43] who used wheat bran as substrate for protease production from PD-4 isolate of *Bacillus subtilis* and reported that maximum protease activity was obtained by using wheat bran as substrate.

Table 1. Effect of different substrates on protease production

Type of substrate	Optical density (660 nm)	Tyrosine Equivalent Concentration (µg/mL)	Enzyme Activity (U/mL)
Wheat Bran	1.70 ± 0.02	127.65 ± 1.42	70.21 ± 0.78
Corn Cob	0.62 ± 0.02	45.21 ± 1.35	24.87 ± 0.74
Rice Polish	1.07 ± 0.03	79.82 ± 1.92	43.90 ± 1.06
Waste Bread	0.90 ± 0.02	66.84 ± 1.59	36.76 ± 0.87
Wheat Straw	0.45 ± 0.02	32.23 ± 1.78	17.73 ± 0.98

Each value is the mean of four replicates

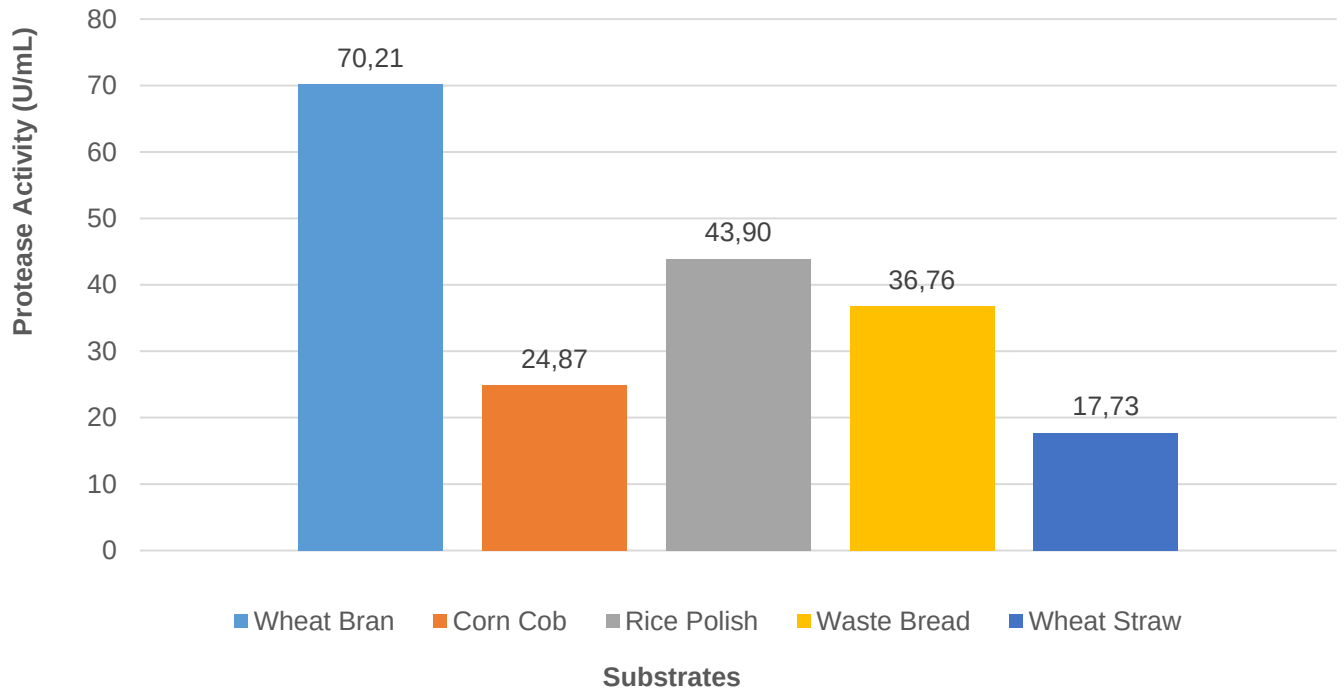

Figure 7. Effect of different substrates on protease production

Solid State Fermentation on Optimized Conditions

Four different batches of protease were produced on optimized culture conditions to validate these condition. Protease activity of 71.18, 68.67, 70.76 and 69.93 U/mL were noted in batch 1, 2, 3 and 4 respectively (Table 2).

Table 2. Alkaline protease production at optimized conditions through solid state fermentation

Batches	Optical Density (660 nm)	Tyrosine Equivalent Concentration (µg/mL)	Enzyme activity (U/mL)
Batch 1	1.72	129.43	71.18
Batch 2	1.66	124.85	68.67
Batch 3	1.71	128.67	70.76
Batch 4	1.69	127.14	69.93
SEM	0.01	1.01	0.56

Optimized conditions: Temp = 35ºC, pH = 9, Incubation time = 36 hours and moisture level = 75%

Characterization of Protease Enzyme

Effect of temperature, pH and substrate concentration was studied on activity and stability of protease to determine its optimum temperature, pH, K_m and V_{max}.

Effect of temperature on protease activity

The activity of crude protease was studied at different temperatures ranging from 25ºC to 75ºC and it was observed that protease produced from *Bacillus subtilis* K-5 showed 96.32% activity at 60ºC (Figure 8). The enzyme activity decreased rapidly when temperature was increased above 60ºC. The activity of enzyme remained stable between 50-60ºC. It can be concluded that *Bacillus subtilis* K-5 is thermophilic strain for production of protease. Results of current study were in agreement with [1] who observed maximum proteolytic activity of enzyme from *Bacillus subtilis* at 60ºC, while, [40] found maximum enzyme activity of alkaline protease at 55ºC by *Alcaligenes faecalis.* Similar results were observed by other investigators where a maximum temperature of 55ºC was recorded for an alkaline protease from *B. stearothermophilus* Ap-4 [10] and 60ºC for protease [11] derived from *Bacillus sp.* B21-2. [34] also reported that maximum activity of crude protease produced by *Bacillus subtilis* was between 55ºC to 60ºC.

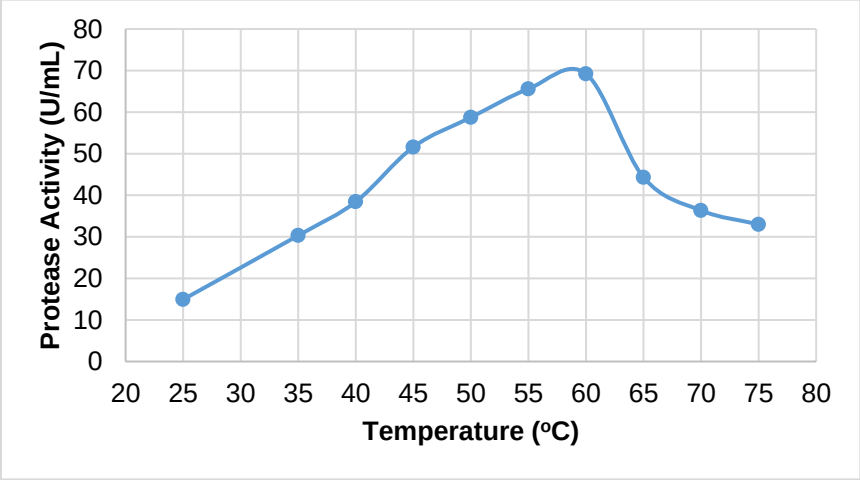

Figure 8. Effect of temperature on protease activity and stability

Effect of pH on protease activity

The activity of crude protease was studied at different pH ranging from 6 to 11 and it was found that protease produced from *Bacillus subtilis* K-5 remained active in pH range 9-10 with maximum activity of 72.17 U/mL at pH 10.0 when casein was used as a substrate (Figure 9). It can be concluded that selected bacterial strain was alkalophilic and enzyme produced by this strain was alkaline protease. Our findings were in agreement with [34] who reported maximum protease activity of crude enzyme at pH of 9.5. [1] also reported maximum protease activity at pH 10 which was produced by *Bacillus subtilis.* Our findings were also in accordance with [25] who reported optimum activity of protease at pH 9.0 produced from *Bacillus subtilis.* Findings of our study were in contrast with [44] and [18] who reported maximum activity and stability of protease at pH 8.0 when casein was used as a substrate under standard assay conditions.

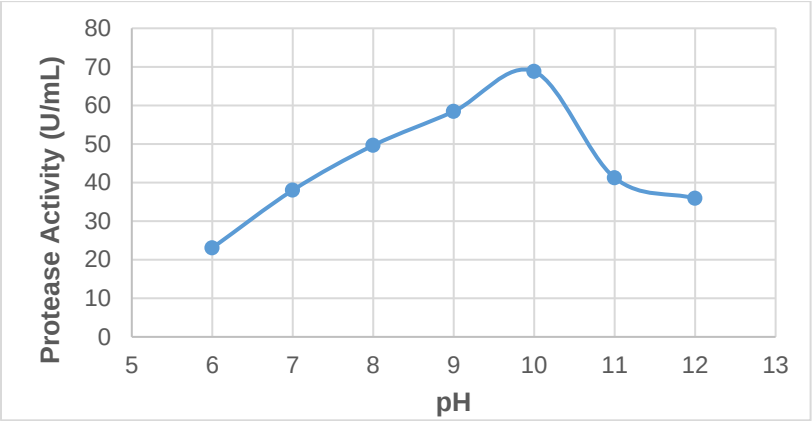

Figure 9. Effect of pH on protease activity and stability

Bulk production of enzyme through solid state fermentation

After completing characterization of protease produced from *Bacillus subtilis* K-5, bulk protease was produced in 500 and 1000 mL flasks under optimized and validated culture conditions of pH, temperature and incubation time. This enzyme was produced as per requirement to be used for performance trials in broilers.

Partial Purification of Crude Protease Enzyme

The crude protease was subjected to partial purification with ammonium sulphate precipitation method and Sephadex Gel-100 gel filtration chromatography.

Ammonium sulphate purification

Partial purification of protease was carried out at different percentages of ammonium sulphate (10-85%) with 20 mL volume of crude enzyme for each percentage. Maximum enzyme precipitation was observed at 70% saturation level with protease activity of 71.09 U/mL. The pellet showed higher alkaline protease activity compared to supernatant indicating the precipitation of enzyme by salting out (Figure 10). Protein concentration 0.57 mg/mL and specific activity of 124.72 U/mg was noted after ammonium sulphate precipitation (Table 3).

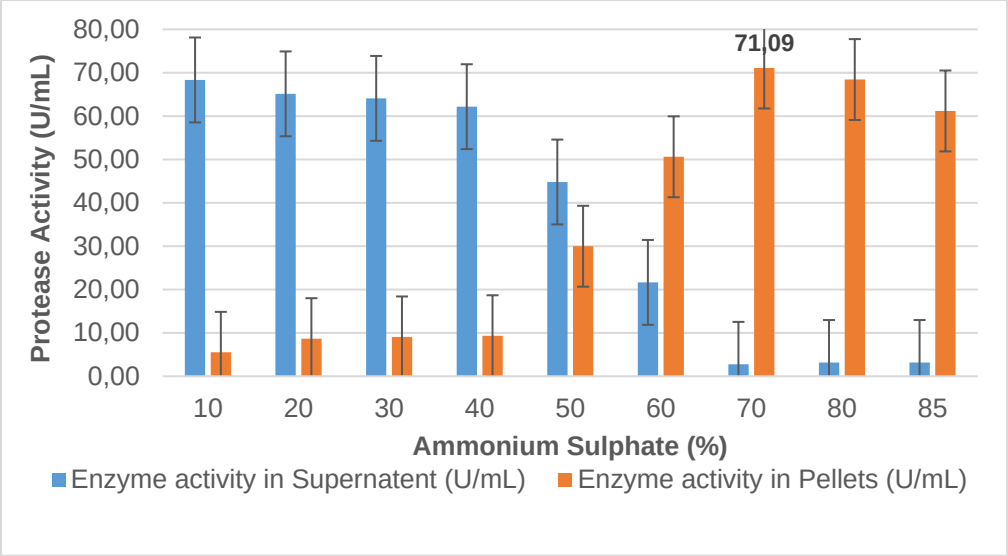

Figure 10. Protease purification by ammonium sulphate precipitation

Gel filtration chromatography

Sephadex G-100 (Sigma Aldrich, St. Louis, USA) was used to prepare chromatographic column. The protein pellets obtained after 70% saturation with ammonium sulphate were dissolved in 0.1M Tris-HCl buffer (pH 8.5)

and loaded on column of Sephadex G-100 equilibrated with same buffer. Five (5) mL enzyme from ammonium sulphate precipitation purification was used. Thirty eluted samples each of 1 mL were taken and first 5 elution samples were discarded. Next were labelled as elution samples 1-25 and were tested for protease activity. Elusion with highest protease activity of 63.21 U/mL was selected for further characteristics (Figure 11). The protein contents 0.44 mg/mL and specific activity of 143.65 was observed in purified protease after gel chromatography (Table 3).

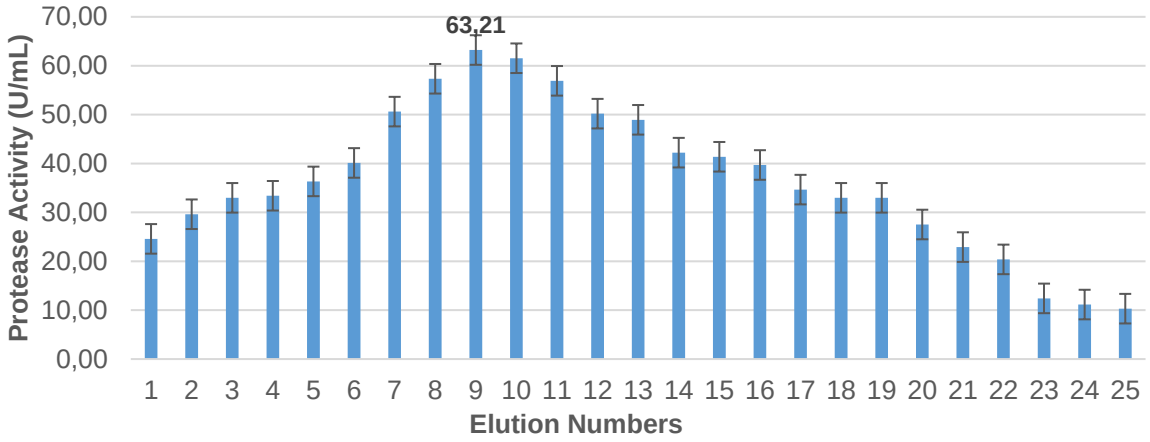

Figure 11. Protease activity in eluted samples after gel filtration chromatography

Estimation of protein and specific activity of protease enzyme

The protein contents in different samples were calculated by using BSA as standard. The total protein in crude enzyme was found to be 0.633 mg/mL and specific activity was 111.56 U/mg (Table 3). Proteins contents and specific activity were calculated after each purification step. There was increase in specific activity and decrease in protein contents indicated the purification of alkaline protease after each step. The results of current study were in agreement with [24] who reported total protein 0.68 mg/mL and 0.64 mg/mL, specific activity 127.34 U/mg and 121.67 U/mg of crude protease produced from *Bacillus subtilis* PCSIR-5 at culture conditions of 48 hrs incubation time and pH 8.0, respectively. It was further reported that protein contents were decreased with the increase in incubation time above 48 hours because protease production was decreased. [6] also reported 0.35 mg/mL total protein and specific activity 116.28 U/mg in crude protease produced from *Bacillus subtilis* isolated from degraded abattoir waste. Our results were in contrast with the study of [42] who reported total protein 1.37 mg/mL and specific activity 33.23 U/mg in crude protease produced from *Bacillus cereus* SU12. [34] reported protein concentration 3.28 mg/mL in crude protease produced from *Bacillus subtilis*. Current study was also in contrast with [17] who reported specific activity 7.71 U/mg of crude protease produced from *Bacillus* SNR01. Total protein contents and specific activity of protease produced from *Bacillus subtilis* K-5 is given in Table 3.

Table 3. Summary of purification steps of protease enzyme from *Bacillus subtilis* K-5

Purification Steps	Enzyme Volume (mL)	Protease Activity (U/mL)	Total Activity (U/mL)	Protein contents (mg/mL)	Total Protein (mg/mL)	Specific Activity (U/mg)
Crude Enzyme	100	70.28	7028	0.63	63	111.56
$(NH_4)_2SO_4$ Precipitation	20	71.09	1422	0.57	11.4	124.72
Sephadex G-100	5	63.21	316.05	0.44	2.2	143.65

Effect of Substrate Concentration on Protease Activity

Different concentrations of substrate (Casein) were used to perform enzymatic activity to find the effect of varying concentration. The results were used to draw the double reciprocal plot and values of V_{max} and K_m were calculated from the equation obtained from plot (Figure 12). The enzyme has V_{max}= 344. 83 mg/mL/min and K_m= 100.04 mg which shows that its very active enzyme with good affinity towards substrate. [1] reported that protease have a high level of hydrolytic activity against casein as substrate and poor to moderate hydrolysis of BSA and egg albumin, respectively. An alkaline protease is highly substrate specific and exhibit maximum activity towards casein as substrate [4,19].

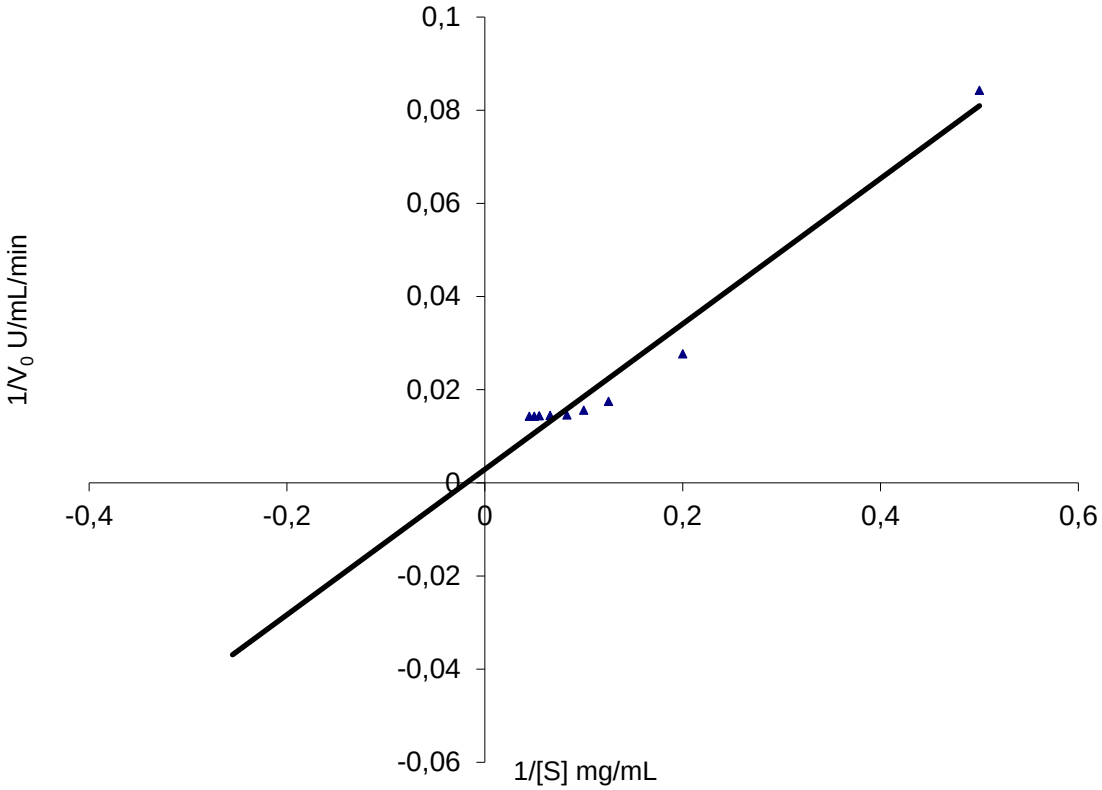

Figure 12. Double reciprocal plot (Line-Weaver Burk) to find V_{max} and K_m of alkaline protease with casein

CONCLUSION

Bacillus subtilis K-5 was identified as potential microorganism for the production of alkaline protease. Culture conditions optimization through Response Surface Methodology and characterization of protease indicates that protease produced from *Bacillus subtilis* K-5 is thermophilic and alkaliphilic in nature which is a prerequisite for poultry feed applications. This protease has high affinity towards substrate so it can be used in poultry diets by reducing dietary protein levels which will save feed cost and lower nitrogen excretion and protein waste into the environment.

Funding: This research received no external funding.
Acknowledgments: The authors are gratefully to University Institute of Biochemistry and Biotechnology & National Center of Indutrsial Biotechnology, PMAS Arid Agriculture Univerisity, Rawalpindi, Pakistan for providing all facilities and equipments to carry out this research work.
Conflict of Interest: The authors declare no conflict of interest

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