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Screening and Strain Improvement of Protease-Producing *Bacillus* spp. From Various Environmental Samples with Kinetic Characterization

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ABSTRACT

Background and Objective: Proteolytic enzymes account for about 60% of total enzyme sales worldwide, making them of major industrial importance. This study aimed to isolate, screen, and enhance protease-producing *Bacillus* spp. from four environmental sources.

Methods: Twenty-one proteolytic isolates were obtained in primary screening from various food sources, the maximum of which (8 isolates) was obtained from the spoiled pulses. *Bacillus* spp was determined as the most active isolate (casein hydrolysis zone measuring 17 mm) by gram staining, biochemical tests and Vitek 2 BCL card (more than 90 % confidence level). Strain improvement applied: UV irradiation (15 min); chemical mutagens (acridine orange, ethidium bromide (25 – 100µg/ml)). The production of enzymes was carried out at SMB at a temperature of 37°C for 72 h (150 rpm) and the amount of protein was determined by the Folin-Lowry method. The enzyme was partially purified by ammonium sulphate precipitation (80% sat.) & its kinetic parameters determined.

Results: UV mutagenesis produced a significantly greater increase in protease production, giving a 38 mm clearance zone compared with the chemical mutagens tested. Maximum enzyme activity after partial purification was 121.03 µM/min. The optimum temperature and pH for enzyme activity were 30°C and pH 6, respectively, indicating a mesophilic acid protease.

Conclusions: UV mutagenesis is an effective strategy for improving protease production in *Bacillus* spp., with promising applications in food processing and the dairy industry.

Introduction

Proteases (EC 3.4) hydrolyse peptide links in proteins to break them down into smaller peptides or free amino acids. They have been found in every type of organism and play a vital role in their physiology, including in cellular communication, protein recycling, and nutrient uptake[1]. Protease is the most commercially important industrial enzyme accounting for about 60-65% of the global enzyme market, the major application of which is in the detergent industry, food industry, pharmaceutical industry and bioremediation of proteinous wastes from different sources and silver recovery from X-ray film[2,3,8]. Acidic proteases, neutral proteases, and alkaline proteases are the three primary categories of proteases according to their ideal ph. Serine proteases, cysteine proteases, aspartate proteases, threonine proteases, and metalloproteases are the groups into which they can be divided based on their catalytic processes. They can be further classified as exopeptidases, which target the polypeptide's N or C termini, and endopeptidases, which target peptide bonds in the center of the polypeptide chain[4]. Considering this, microbial sources are preferred over plant or animal sources for production of proteases[5-6].In the microbial producers, *Bacillus* sp. bacteria are most predominant accounting for around 35% of the global sales of microbial enzymes, as they are highly utilized in industries due to their potent extracellular protease activity and high tolerance to diverse physico-chemical conditions [7].

Isolation of proteinase-producing microorganisms from protein-rich solution like spoiled milk products, decaying pulses and soil from the poultry farms is a good approach to find high enzyme yielding strains. Primary screening is still done on skimmed milk agar since proteolytic activity can be easily seen in the clear halos (zones of clearance) around the colonies resulting from casein hydrolysis [10]. Enzymes are often boosted using mutagenesis to produce more of the enzyme than the wild-type strain can naturally. UV radiation causes thymine dimers to form, which affect the shape of the DNA helix, and could cause beneficial mutations that increase the production of protease. There are a number of compounds called intercalators which are known to cause frameshift mutations, including ethidium bromide and Acridine orange [11].

The protease produced is usually partially purified by dialysis and ammonium sulphate salting out to get the concentrated portion of the enzyme for kinetic studies [12]. The kinetic characterization such as determination of optimum temperature and pH is significant to define the operating range of the enzyme and to know whether the enzyme is suitable for specific industrial use. The activity of enzymes tends to rise as temperature increases until the optimum value is reached, and then it decreases above the optimum value, due to the irreversible thermal denaturation of the enzymes. Similarly, the pH level influences the ionization of active site residues and binding of a substrate, so that each enzyme shows activity at a particular pH [13].The present study was aimed to isolate and screen the protease-producing *Bacillus* spp. from different environmental sources and increase the production of protease by using mutagenesis techniques like UV, Acridine orange and Ethidium bromide and then to evaluate the effects of the mutants on the production of protease and induction of over-producing strains. Enzyme was also partially purified and its kinetic characteristics were determined. The potential of the identified promising mutant strains of this study for their successive genetic improvement, and in various food, detergent, textile and pharmaceutical industries is noteworthy.

Materials and Methods

Collection of Samples

A total of 21 samples were collected from different sources (7 samples of curd, 8 samples of spoiled pulses, 2 samples of soil from poultry farm & 4 samples of spoiled milk) for the purpose of isolation of protease producing microorganisms. These samples were further used for isolation of protease producing microorganism to produce protease.

prepared Skimmed Milk Agar

To isolate the protease producing *Bacillus* spp., Skimmed Milk Agar (SMA) was used and prepared by incorporating the following (per litre of distilled water):peptic digest of animal tissue (5.0 g), yeast extract (5.0 g), bacteriological agar (15.0g) and sodium chloride (5.0 g), the pH was adjusted to7.4 ± 0.2.The medium was autoclaved at121°C for 15 minutes at 15 psi then supplemented with 10 ml of filter-sterilized skimmed milk (10% w/v) before pouring plates; skimmed milk was the only proteinaceous substrate used for extracellular proteolytic activity detection[30].

Isolation of Protease Producing *Bacillus* spp

All collected soil samples underwent a heat-treatment process (80°C for 10 minutes in a water bath) to diminish non-spore-forming pollutants and to encourage the proliferation of thermally resistant endospores typical of the species Bacillus. Then serial tenfold dilutions were made up to 10^{-5} by transferring 1 ml from each dilution at the 10-fold steps into9 ml sterile saline (0.9% NaCl, w/v) and vortexing thoroughly at each step. Using the standard 4 quadrant streak plate technique, a loopful from the 10-5 dilution was streaked onto the surface of pre-prepared SMA plates to get well isolated colonies. Aseptically, using a class II laminar airflow cabinet, sterile Petri dishes (100 × 15 mm), inoculation wire loops, sterile micropipettes and test tubes were used for all plating procedures. The inoculated plates were then inverted and incubated for 24 to 48 hours at 37°C with the atmosphere being aerobic [31].

Screening and Selection of Potent Isolates

After 24 hours of primary incubation colonies appearing on SMA plates were macroscopically inspected to see if they had clear transparent halos (zones of clearance) around the colonies, which would be a sign of extracellular casein hydrolysis by the proteolytic enzymes. The colonies with discernable areas of clearing were chosen as presumptive protease producers and then retested with spot inoculations on fresh sterile SMA plates. Spot-inoculation was done on the marked places of each selected colony and the plates were re-incubated at37°C for 24-48hours under the same condition, but on an aerobically inclined surface of the agar. Plates were inspected after the second incubation for the reappearance of zones of clearance around the colonies of double spotted bacteria, indicative of proteolytic activity. The diameter of zone of clearance and the colony was measured in millimeters using a standard ruler and Proteolytic Index(PI)was calculated for each isolate as follows:

PI = Zone of hydrolysis (mm) / Colony diameter(mm).

To compare the proteolytic activity among isolates, a quantitative criterion was used based on the PI value, where a value > 2.0 was considered as strong production of proteases. The highest PI isolates were streaked out on fresh Nutrient Agar plates for purification; then they were transferred to 4°C of Nutrient Agar slant for short-term storage until further morphological, biochemical and molecular identification [32].

Morphological and Biochemical Identification of *Bacillus* spp.

Morphological examination of the isolated colonies was carried out on Nutrient Agar including shape, color, margin and surface texture of the colonies. The cell morphology and arrangement were determined by Gram staining and the presence of heat-resistant endospores typical of Bacillus spp. was confirmed by Schaeffer-Fulton staining. Biochemical characterization was performed by conventional tests such as catalase, oxidase, Voges-Proskauer, nitrate reduction, indole production, hydrolysis of starch, liquefaction of gelatin and fermentation of glucose according to Bergey's Manual of Systematic Bacteriology. Rapid and accurate species level identification was done with the Vitek 2 automated system (bioMérieux, France) using the GP card, which uses biochemical reaction profiles for identification.

Strain Improvement by Mutations

After observing the colony and its area, the chosen colony was inoculated into four sterile skimmed milk agar plates and two broth tubes. The plates and tubes containing the protease producer were used for UV and chemical mutation, respectively [33].

UV Mutation

Selected isolate was grown into an overnight culture in Nutrient Broth at 37°C shaking (150 rpm) for 18–24 hours and then adjusted to 1.5 × 10^8 CFU/ml (0.5 McFarland standard). Plates were exposed to UV irradiation (254 nm, 15 W) at a set distance (30 cm) from the agar surface for 5, 10, 15 and 20 minutes with an unexposed plate as negative control. All procedures were done in complete darkness to avoid photo reactivation and plates were left for 24–48 hours at 37°C. Colonies were grown on SMA for screening for casein hydrolysis zones and PI was calculated from surviving colonies:

Survival rate (%) = (Number of colonies on the UV treated plate / Number of colonies on the control plate) × 100

Mutants having a PI value much higher than the parent strain were purified on Nutrient Agar and kept as glycerol stocks (20% v/v) at −80°C [18].

Chemical Mutation (by Acridine Orange and Ethidium Bromide)

Acridine Orange (AO) and Ethidium Bromide (EtBr) were serially diluted to 25, 50, 75 and 100 µg/ml from their stock solutions in 1mg/ml sterile distilled water in amber tubes. Handling was

carried out in a chemical safety cabinet and using appropriate PPE. An overnight culture (1.5×10^8 CFU/ml (0.5 McFarland standard)) was added (1 ml) to 9 ml Nutrient Broth supplemented with each mutagen concentration, together with an unsupplemented control. A loopful of each tube was spot-inoculated onto SMA and then incubated at 37°C for 24–48 hours at regular intervals of 5, 10, 15 and 20 minutes. Casein hydrolysis zones were evaluated for colonies and PI was calculated. Survival rates of 70-80% were deemed to be optimum. A PI value significantly higher than the parent was obtained from the mutants; these mutants were purified and stored at −80°C in 20% (v/v) glycerol[33,35].

Production & Extraction of Protease Enzyme

Protease production was done in Skimmed Milk Broth (SMB) which consists of peptic digest of animal tissue (5.0 g), yeast extract (5.0 g) and sodium chloride (5.0 g) and adjusted to pH 7.4 ± 0.2 per litre of distilled water. The medium (500 ml in 1000 ml conical flasks), was autoclaved at 121°C for 15 minutes at 15 psi and cooled to room temperature. The highest PI isolate was pre-cultured in Nutrient Broth at 37°C and 150 rpm for 18 hours before inoculating into the production medium at 1% (v/v) (approx. 1.5×10^8 CFU/ml). Bacteria was allowed to grow by incubating fermentation at 37°C, 150 rpm for 72 hours and was checked daily by measuring OD_{600}. The cells were centrifuged out from the culture broth at 10,000 rpm/4 °C for 10 minutes. The supernatant was obtained as crude enzyme extract and the total protein estimated by using the Folin-Lowry method with bovine serum albumin (BSA) as standard. Under standard assay conditions, the activity of protease was determined by calculating the amount of tyrosine released from casein per ml of enzyme per minute [10].

Biuret Test

Copper sulphate ($CuSO_4 \cdot 5H_2O$, 1.5 g), sodium potassium tartrate (4.5 g) was dissolved in 0.2 N NaOH (250 ml) then potassium iodide (KI, 2.5 g) was added to prevent auto-reduction of Cu (II) ions and then filled to 500 ml with 0.2 N NaOH. The reagent was kept in an amber bottle in the refrigerator at 4°C. A stock solution of BSA (5 mg/ml) was made in distilled water and frozen at −20°C. Standard curve was made using BSA concentration of 0.2, 0.4, 0.6, 0.8, 1.0, 1.2, 1.4, 1.6, 1.8 and 2.0 mg ml. To this 1 ml of each standard or sample was added with 3 ml of Biuret reagent, vortex mixed and incubated at 37°C for a period of 10 minutes. The absorbance was recorded at 540 nm using a reagent blank. Protein concentrations of samples of unknown concentration were calculated using the linear regression equation from the standard curve [37].

Protease Assay

Casein was used as a substrate and Folin-Ciocalteu method was used to determine the activity of crud protease. The reaction mixture was prepared by mixing 1 ml of 1% casein in 0.05 M sodium phosphate buffer (pH 7.0) with 1 ml buffer and 1 ml enzyme preparation and then incubated for 20 minutes at 37°C. After 10 minutes 1 ml of 5% Trichloroacetic acid (TCA) was added to terminate the reaction and after filtration on Whatman No. 1 filter paper the filtrate was used for the determination of protein. The clear filtrate (1ml) was then reacted with 5ml of 0.4M Na_2CO_3 and 0.5ml of diluted Folin-Ciocalteu reagent (1:2 v/v) and allowed to stand in the dark at 37°C for 20 minutes. The absorbance was measured at 660 nm using a reagent blank. A standard calibration curve (0-200 µg/ml) was used to determine tyrosine. The amount of protease which released 1µg of tyrosine in one minute under the conditions of the assay was taken as one unit of enzyme activity. Each assay was done three times [38].

Folin Lowry Method

Protein concentration was measured using a Lowry colorimetric method. Solution A was 20 g/L Na_2CO_3 in 0.1 N NaOH solution while Solution B was 5 g/L $CuSO_4 \cdot 5H_2O$ and 1 g/L sodium potassium tartrate. Solution C (Working reagent) was freshly-made daily by the mixing of solutions A and B in a 50:1 (v/v) ratio, and was used within 2 hours. The Folin-Ciocalteu was used at 1:2 (v/v) diluted with distilled water. The BSA standard solutions were prepared in triplicate with 1 ml of solutions, 5 ml of Solution C were added to each tube followed by immediate addition of 0.5 ml of diluted Folin-Ciocalteu reagent, mixed thoroughly and allowed to stand for 10 minutes at room temperature. Tubes were placed in the dark at room temperature for 30 minutes and then absorbance was read at 750 nm with a reagent blank. A calibration curve was generated and protein concentrations of unknown samples were determined by using the linear regression equation. Triplicate were carried out for all assays [36].

Partial Purification by Salting-Out Method

Crude enzyme extract obtained by centrifugation at 10,000 rpm, 4°C for 10 minutes of the fermentation broth. The total protein concentration and baseline protease activity were measured before purification. The partial purification was done by precipitating the enzyme

with ammonium sulphate at 20, 40, 60, and 80% saturation (w/v) as calculated by Green and Hughes (1955). The crude extract was gently stirred at 4°C and gradually made to ammonium sulphate concentration and equilibrated overnight. Precipitates were recovered by centrifugation at 10000 rpm at 4 °C for 20 minutes, washed and redissolved in 0.1 M sodium phosphate buffer (pH 7.0) and dialyzed against three changes of this buffer (3 L each) at 4 ° C for 6 hours per change to remove ammonium sulphate completely [34]. The activity of protease and total protein was measured at each purification step, then the following parameters were calculated:

Specific Activity (U/mg) = Total Activity (U) / Total Protein (mg)

Specific Activity of crude extract = 1000 * Purification Fold * Enzyme Activity of each step / volume of each step

Characterization of partially purified Protease Enzyme

Effect of Temperature

The effect of temperature on the activity of the protease was studied by incubating the reaction mixture (enzyme preparation + 1% of casein in 0.05M sodium phosphate buffer, pH 7.0) at 20, 30, 40, 50, 60, 70 and 80° C for 20 minutes. Blank tubes were set up exactly the same as the test tubes, but TCA was added before the substrate was added to the tubes to inhibit enzyme activity. Reactions were stopped by adding 1 ml of 5% TCA, left at room temperature for 10 minutes and then filtered through Whatman No.1 filter paper. The tyrosine concentration in clear filtrate was measured by Folin-Lowry method at 750nm and the activity of protease was expressed in U/ml. Relative activity (%) was expressed as a percentage of the maximum activity. Each experiment was repeated three times [25].

Effect of pH

The activity of protease was determined by Folin-Lowry method against the pH range of 5.0 - 9.0. Casein substrate (1% w/v) was prepared in 0.05 M sodium phosphate buffer (pH 5.0–7.0) and 0.05 M Tris-HCl buffer (pH 8.0–9.0). The enzyme mixture (1 ml) was mixed with casein substrate (1 ml) at each pH level and incubated for 20 min. at the optimum temperature found before. Blank tubes were prepared in the same way as tubes, except that the TCA was added before the substrate to deactivate enzyme activity. Reactions were stopped with 1 ml of 5% Trichloroacetic acid) TCA) , incubated for 10 minutes at room temperature and filtered through Whatman No. 1 filter paper. The activity of the protease was measured in the clear filtrate at 750 nm using Folin-Lowry method and was expressed as U/ ml. Relative activity (%) was worked out as a percentage of the maximum recorded activity. All assays were repeated in triplicate [9].

Results and Discussion

A total of 21 isolates recovered from the four tested sources, morphological and biochemical identification showed that all isolates were members of the genus *Bacillus*, with Gram positive rod-shaped appearance, catalase positive and endospore positive. The following were identified as species: *Bacillus subtilis*, *Bacillus cereus*, *Bacillus licheniformis* etc. The greatest number of protease producing isolates was recovered from spoiled pulses 8 followed by curd 7, spoiled milk 4 and soil of poultry farm 2. The abundance of *Bacillus* spp. was as follows:(Table 1).

Table1: Number of protease producer microorganism isolated from different sources

Sources	No. of isolated protease producer
Spoiled milk	4
Spoiled pulses	8
Soil of poultry farm	2
Curd	7

These isolates isolated from the above-mentioned sources yielded zones of clearance 17 mm around the colony due to the hydrolysis of protein of milk by producing the protease enzyme is similar to previous works. Pulses and dairy products are known protein-rich environments which are selectively enriched with microorganisms that are able to hydrolyze casein [14].

Qualitative Assay of Protease for Isolation and Screening of Producer

The ability to produce protease was assessed by measuring diameter of the clear zones on 48 hours' incubation at 37°C of Skimmed Milk Agar (SMA) plate for all *Bacillus* spp. recovered from different sources tested. These findings are summarized in the table2 below and are shown figure1 below.

There was a large variation proteolytic activity between the various sources, of all isolates tested. The *Bacillus* spp. isolated from the spoiled milk showed the maximum proteolytic activity with a maximum zone of clearance of 17 mm followed by the isolate from the spoiled pulse (12 mm), curd (9 mm) and poultry farm soil (7 mm), respectively. The hydrolysis zone diameter was calculated for every isolate based on the Proteolytic Index (PI) and this value was divided by the diameter of the colony. The value of PI obtained for the isolate which spoiled the milk was the highest, indicating that the isolate has a

high capacity in secreting extracellular proteases than all the other isolates tested.

Table 2. Protease production activity of efficient isolate sources after 48 hours respectively.

Sr No.	Bacteria and source	Protease production after 48-hour incubation (Zone of clearance in mm)
1	*B.spp.*-spoiled milk	17 mm
2	*B.*spp.-Spoiled pulses	12mm
3	*B.* spp. - Curd	9mm
4	*B.*spp.-Poultry farm soil	7mm

Photo plate 1

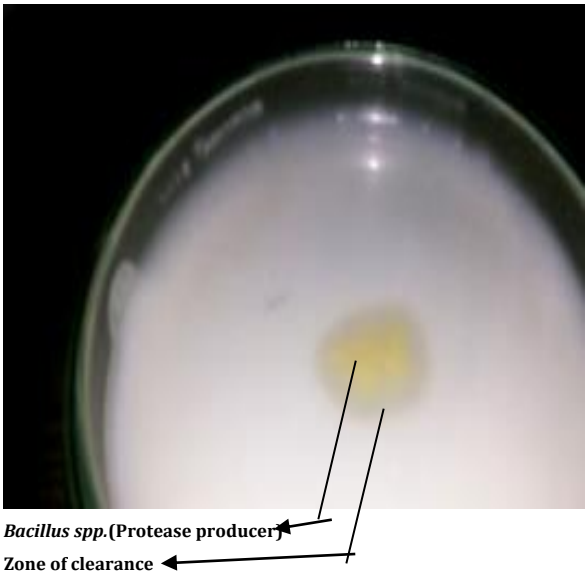


Figure1:Protease production activity of isolate (recovered from spoiled milk) after 48 hours of incubation on Skimmed milk agar

The zone of clearance obtained from *Bacillus* spp. isolated from spoiled milk (17 mm) after 48 hours on skimmed milk agar indicated that *Bacillus* spp. had the greatest proteolytic activity of all the bacteria tested. Results are in agreement with earlier reports of *Bacillus* spp. as the prevalent extracellular protease producers as well as the significant caseinolytic activity that had been reported [15]. The alkaline proteases consistently produced by isolates from dairy sources have high casein-hydrolyzing activity as evidenced by high clearance zones of >15 mm after 48 hours' incubation.

Gram's Nature of Isolated Protease Producer (*Bacillus spp.*)

The isolate from the most efficient site of getting the protease was subjected to a thorough identification procedure which included morphological and biochemical studies. Morphological features of the genus *Bacillus* were confirmed by gram staining, which showed Gram-positive large, straight bacilli present singly, in pairs or in short chains. The presence of oval endospores was confirmed through staining of the endospores using the Schaeffer-Fulton method, which did not cause much cell swelling, which is characteristic of *Bacillus* spp.

The isolate was biochemical positive for catalase, oxidase, hydrolyzed starch and Voges-Proskauer (VP) test and showed positive nitrate reducing activity, which are typical biochemical characteristics of *Bacillus* spp. The VITEK® 2 BCL Card (bioMérieux, France) for Gram-positive spore-forming bacilli gave an excellent probability of identification, > 90% and the isolate was identified as Bacillus species, with reliable identification of the species level *Bacillus subtilis* or *Bacillus cereus* based on the automated biochemical profile and rod shaped bacteria as shown in following fig. 2.

Photo Plate 2

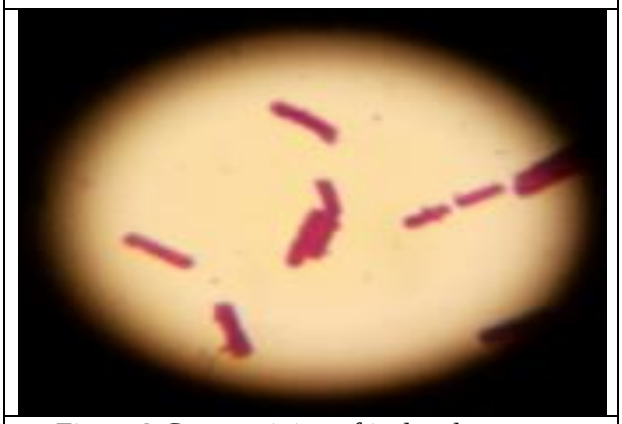


Figure 2:Gram staining of isolated protease producer from spoiled milk

Moreover, the presence of Gram-positive large rod-shaped bacilli with central endospores is characteristic of the genus *Bacillus* and VITEK® 2 BCL card was found to deliver very good reliability of over 90% with very good accuracy over conventional biochemical methods in the identification of Gram-positive spore-forming bacilli, including *B. cereus* and *B. subtilis* [16].

Strain Improvement by UV Radiation

The *Bacillus* spp. isolated from spoiled milk were subjected to UV irradiation, Ethidium-bromide (EB)and Acridine- orange (AO) for the

intervals of times (5, 10, 15 and 20 min) and the result obtained is presented in table 3.

Table 3. Zone of clearance in mm after 48 hours after UV, EtBr and Acridine orange exposure

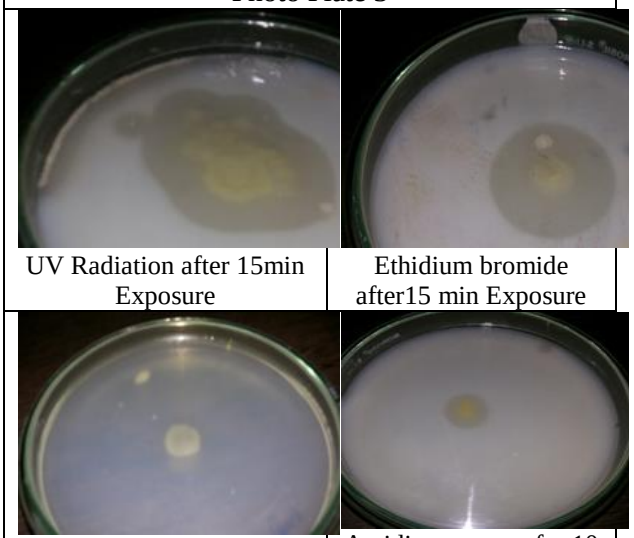
Sr. No.	Time of Exposure	Zone of clearance in mm		
		UV	Ethidium Bromide	Acridine orange
1	5 min	19	18	17
2	10 min	25	20	18
3	15 min	38	22	-
4	20 min	-	-	-
5	Control	18		

The effect of UV, ethidium bromide and Acridine orange mutations on the synthesis of protease by separated potential strains is shown. Bacillus spp. showed growth readings of 19 mm, 18 mm and 17 mm, respectively after a 5-minute exposure to the three mutagens after 48 hours' incubation. The size of the zones of clearance for the three different mutagens after 10 min of exposure was shown as 25 mm, 20 mm and 18 mm size. For 15 minutes of exposure to UV and Ethidium bromide 38 and 22 mm of zone of clearance was observed respectively. No zone around the colony was observed after 15 or 20 minutes' exposure to Acridine orange. Likewise, after 20 minutes of exposure to UV and Ethidium bromide no zone was formed around the colony this can be seen in the figure3.

The results of the present work are in accord with previous studies showing that exposure of *B. subtilis* to UV for an optimal period of 15 minutes increased the synthesis of the protease by 40% while longer exposure caused damage to the DNA resulting in the complete suppression of growth [17]. Reported that chemical mutagens such as Ethidium bromide stimulated the production of protease at low concentrations and completely inhibited the activity at higher concentrations in *Bacillus* spp. [18]. Moreover, confirmed that Acridine orange as an intercalating agent was the least mutagenic agent in comparison to UV and Ethidium bromide with complete inhibition at higher exposure time of 15-20 minutes as in the current study, where no clearance zone was observed with Acridine orange at prolonged exposure time of 15 and 20 minutes [19]. Depending on mutagenesis and selection, the enhancement (or improvement) of protease producing strains has been done. Most commonly used method is inducing mutations in parent strains using mutagens (Such mutants of Bacillus are used for commercial production). One of the most prominent physical mutagens is UV-radiation have often used [28]. One possible strategy for developing hyper producer strains is the frequent application of UV treatment in conjunction with chemical mutagens, such as Ethidium bromide and Acridine orange.

Since lack of UV light was identified as the major limiting factor for uneven production, microbial UV mutation has been carried out to enhance its production. One is UV rays; they are a crucial strain mutation as well. Pyrimidines (thymine and cytosine) are especially vulnerable to alterations induced by ultraviolet (UV) radiation absorption. This may result in the formation of thymine dimers that can distort the DNA and inhibit the replication of other DNA molecules. Mutations by UV are often detrimental, but sometimes it creates an organism which has been better adapted to the surrounding environmental conditions with superior bio catalytic activity. One of the most important properties that DNA confers on a microorganism is its ability to mutate, which can introduce new genotypes into a gene pool [29]. Similarly-through chemical mutagens such as, Ethidium bromide and Acridine orange the strain quality can be improved in terms of superior protease activity.

Photo Plate 3



UV Radiation after 15min Exposure | Ethidium bromide after15 min Exposure

Acridine orange after15 min Exposure | Acridine orange after10 min Exposure

Figure 3: Strain improvement by UV Radiation, Ethidium bromide and Acridine orange after exposure

Enzyme Activity

The table 4 below show the activity of crude enzyme extract was first assessed using the casein-Folin test at 750 nm, which was then used to measure the activity of the purified enzyme extract. The crude extract had an optical density of 0.132 at 750 nm on the standard tyrosine calibration curve which corresponds to an enzyme activity of 18.97 µM/min. The enzyme activity of

the crude extract was found to be 4530 U with remaining volume of 239 ml. The activity of an enzyme is expressed as the amount of enzyme which releases 1 µg of tyrosine per minute per mL at the given conditions.

•**Enzyme Activity**(U/ml): =18.97µM/min/ml

•**Total Activity** (U): = Activity (U/ml) × Total volume (ml)

•**Specific Activity** (U/mg): =Total Activity /Total Protein (mg)

Table4.Enzyme activity during partial purification of crude enzyme by salting out method.

Enzyme activity of crude enzyme	**O.D. at 750 nm - 0.132**		**Enzyme Unit – 18.97 uM/min**	
Concentration of Ammonium sulfate $(NH_4)_2SO_4$	O.D. ppt. Protein (pellet)	Enzyme Unit in uM/min	O.D. at 750nm of Supernatant	Enzyme Unit in uM/min
40%	0.221	31.78	0.634	91.13
60%	0.437	62.81	0.132	18.97
80%	0.842	121.03	- 0.102	-

80% precipitation with ammonium sulfate was done, but supernatant was not showing any enzymatic activity, so no further precipitation with concentrated ammonium sulfate was done see fig 4. This partially purified enzyme was further used for kinetic study.

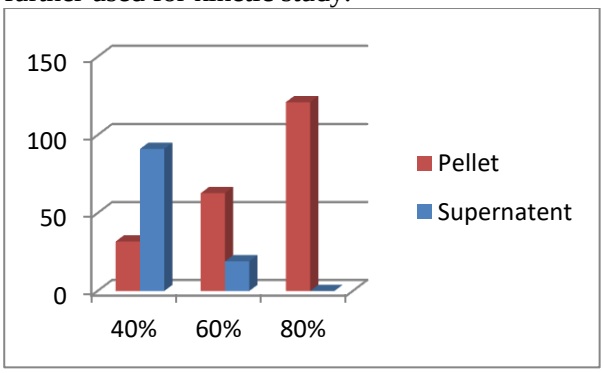


Figure 4: Partial purification of protease by Salting-Out method of ammonium sulfate

The crude extract had an activity of 18.97 U/ml of the protease activity which was found to increase with the increase in the ammonium sulphate saturation up to 80% saturation (121.03 U/ml) and after that no activity was observed in the supernatant. The Casein-Folin (Anson) method for the quantitation of tyrosine released has been well established for the characterization of bacterial proteases, and was used to evaluate the protease activity during purification. The results are in line with the previously reported study of recovery of protease from Bacillus spp. which suggested that the optimum concentration for maximum recovery of protease was 80% ammonium sulphate saturation and the precipitation with ammonium sulphate was proved to be cost effective for initial purification [20,21].

Effect of Temperature

The impact of different temperatures on the protease enzyme activity of Bacillus sp. As presented in Table 5. The optimal temperature for the enzyme was determined to be 30°C, yielding a maximum rate of 156.16 µM/min and an optical density of 1.092 at 750 nm at this temperature. The enhancement of enzyme activity at optimal temperature results from the elevation of molecular kinetic energy, which amplifies the frequency of collisions between the enzyme and substrate, facilitating the formation of the enzyme-substrate complex. In addition to the optimum temperature, a gradual decrease in the protease activity was found as the temperature was raised, reaching 139.29, 120.31 and 121.03 µM/min at 40, 70 and 80 °C respectively. This decrease is probably due to the heat denaturation of the enzyme protein and conformational changes to the three-dimensional structure of the active site that affects how the enzyme will bind to the substrate, and subsequently its catalytic activity.

Table5.Effect of temperature on activity of protease

Temperature in ^{O}C	**O.D. at 750 nm**	**Enzyme unit in µM/min**
20	0.920	132.24
30	1.092	156.16
40	0.969	139.29
50	0.940	135.12
60	0.915	131.53
70	0.837	120.31
80	0.842	121.03

Similar to that of chemical catalysts, enzymatic activity depends on temperature show in figure 5. However, there is a maximum temperature for enzyme reaction, above that activity declines due to denaturation of the enzyme protein. These 2 processes are averaged and it is from this averaging that the apparent temperature "optimum" arises. What is not observed, when increasing temperature increases the rate of reaction? Pinpoint the wrong statement about rising reaction rate at high temperature. 1) The normal increase in reaction rate with temperature

2) The increased thermal denaturation of enzyme above a critical temperature.

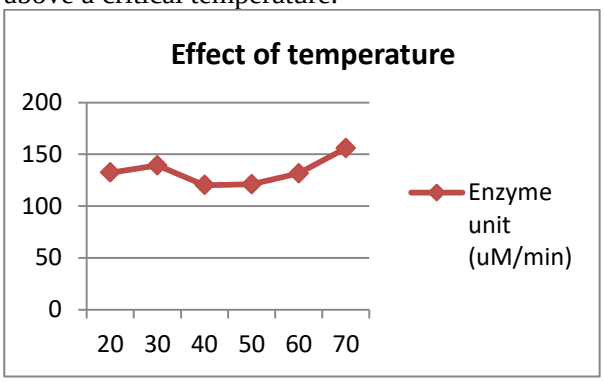


Figure 5. Effect of temperature on the activity of protease

Previous studies conducted on the *Bacillus* proteases reported the optimal activity of the Bacillus proteases around 30-40°C, which is the mesophilic temperature range as they are adapted to moderate temperature environment [22]. Moreover, alkaline proteases produced by Bacillus subtilis exhibited their highest caseinolytic activity at 30°C and the 3-D structure of the enzyme was gradually denatured above 40°C, which explains the decrease in activity [23]. Preservation of the partial activity at higher temperatures (80°C, 121.03µM/min) during the present study indicates that the Bacillus protease is moderately thermostable, which is related to presence of stabilizing disulfide bond and hydrophobic interactions in molecular structure of Bacillus proteases [24].

Effect of pH

Table (6) presents the results on the influence of varying pH levels on the protease activity generated by *Bacillus* sp. The findings indicated that enzyme activity peaked at pH 6.0, exhibiting a maximum enzymatic activity of 91.42 µM/min with an optical density of 0.636 at 750 nm. The protease generated by *Bacillus* sp. is classified as an acid protease, functioning well in mildly acidic environments. The optimal activity at acidic pH may be associated with the ionization state of the amino acid residues in the active site, which are crucial for the enzyme's appropriate conformation and catalytic function.

As the pH values are lower or higher than optimal, the activity of the protease reduces. Under very acidic conditions, the activity slightly decreased to 77.48 µM/min at pH 5.0 and further to 71.58 µM/min at pH 4.0, indicating that the activity of the enzyme was decreased by protonation of certain residues of the active site. At the same time, the activity was significantly reduced at the alkaline pH, yielding the activity value of 80.78 µM/min at pH 8.0 and 62.53 µM/min at pH 9.0, possibly because of deprotonation and changes in the structure of the enzyme at higher ph.

Table6. Effect of pH on activity of protease enzyme.

pH of Enzyme reaction	O.D. at 750 nm	Enzyme unit in µM/min
4.0	0.498	71.58
5.0	0.539	77.48
6.0	0.636	91.42
7.0	0.473	69.99
8.0	0.562	80.78
9.0	0.435	62.53

The figure 6 show that most of the enzymes have optimum pH at which the enzyme activity is greatest; enzyme activity decreases when pH is not at the optimum. The acid base property of the enzyme & substrate is responsible for pH activity relationship of enzyme. Generally, the pH – activity profile will depend on the substrate concentration because the KM of most enzymes will change with ph.

This optimal pH is not necessarily the same as the normal intracellular pH of the enzyme, and may be either a rising or falling pH-activity curve.

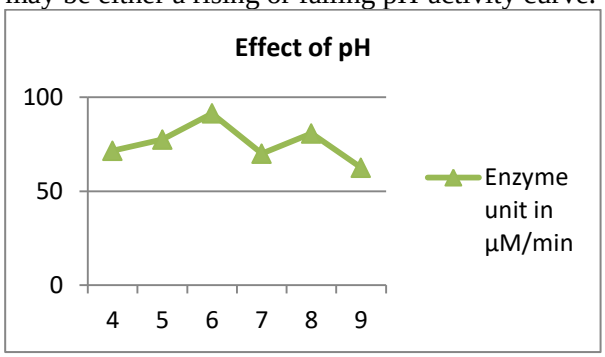


Figure 6. Effect of pH on the activity of protease

Optimization of pH for *Bacillus* spp. acidic proteases (activity optimum at pH 6.0) suggested that they are adapted to slightly acidic conditions such as spoiled milk [15]. showed that some of the Bacillus strains isolated from dairy environment have the optimum activity at acidic to neutral pH range (5.0–6.0), which is the pH of their environment of origin [26]. These decreases in activity with increasing pH (pH 7.0–9.0) at the alkaline end in the current study further supports the acidic nature stated above, which is similar to that reported by [27]. who classified such enzyme as acid proteases which can be used in food and dairy industries?

Conclusion

The present study successfully isolated 21 proteolytic *Bacillus* spp. from four environmental sources with highest number of isolates (8 isolates) from spoiled pulses. Bacillus spp. isolated from spoiled milk, which showed the greatest zone of casein hydrolysis (17 mm) and the highest Proteolytic Index, during morphological, biochemical and confidence >90% identification by Vitek® 2 analysis, is the most potent producer of protease. The physical mutagenesis, namely, UV for 15 minutes' treatment, had the highest efficiency in the strain improvement with protease production (38 mm clearance zone) compared to chemical mutagens, Acridine orange and Ethidium bromide, hence, UV is a reliable and efficient method for strain improvement by inducing overproducing strains. Partial purification of the enzyme at 80% Ammonium sulfate saturation provided the highest enzyme activity (121.03 µM/min) and the enzyme's kinetic parameters optimal activity at (pH 6 and 30°C) indicated that it's a mesophilic acidic protease with moderate thermostability. The above physicochemical properties, along with the increased production capacity of the mutant strain produced by UV, suggest the strain to be a good candidate for further genetic improvement and scale-up fermentation studies, and have strong biotechnological potential for its application in food processing, dairy and related industries.

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Declaration of interests

The authors declare that they have no conflict of interest.

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Publication consent

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Data and material availability:

All data would be provided based on request.

Author contribution:

Jawad N. K. Makassees: Conceptualization, methodology, investigation, data curation, formal analysis, visualization, writing—original draft preparation, writing—review and editing, and supervision.

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عزل وتحسين سلالات بكتيريا *Bacillus* spp المنتجة للبروتييز من عينات بيئية متنوعة مع توصيف حركي

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المستخلص

الخلفية العلمية والاهداف: تُمثل الإنزيمات المحللة للبروتين حوالي 60% من إجمالي مبيعات الإنزيمات عالميًا، مما يجعلها ذات أهمية صناعية بالغة. هدفت هذه الدراسة إلى عزل وفحص وتعزيز سلالات بكتيريا العصوية المنتجة للبروتياز من أربعة مصادر بيئية.

طرائق العمل: تم الحصول على واحد وعشرين عزلة بروتينية في الفحص الأولي من مصادر غذائية متنوعة، وكان أكبرها (8 عزلات) من البقوليات الفاسدة. تم تحديد بكتيريا *Bacillus* spp كأكثر العزلات نشاطًا (منطقة تحلل الكازين بقياس 17 ملم) عن طريق صبغة اكرام، والاختبارات البيوكيميائية، والفحص بكارت Vitek 2 BCL (بمستوى ثقة يزيد عن 90%). تم تطبيق تحسين السلالة من خلال: التشعيع بالأشعة فوق البنفسجية (15 دقيقة)؛ والمطفرات الكيميائية (الأكريدين البرتقالي، وبروميد الإيثيديوم (25-100 ميكروغرام/مل)). تم إنتاج الإنزيمات في SMB عند درجة حرارة 37 درجة مئوية لمدة 72 ساعة (150 دورة في الدقيقة)، وتم تحديد كمية البروتين بطريقة فولين-لوري. تم تنقية الإنزيم جزئيًا عن طريق الترسيب بكبريتات الأمونيوم (80% تشبيع)، وتم تحديد معاييره الحركية.

النتائج: أدى التعرض الضوئي بالأشعة فوق البنفسجية إلى زيادة ملحوظة في إنتاج البروتياز، مما أسفر عن منطقة تثبيط قطرها 38 ملم مقارنةً بالمطفرات الكيميائية التي تم اختبارها. بلغ الحد الأقصى لنشاط الإنزيم بعد التنقية الجزئية 121.03 ميكرومول/دقيقة. وكانت درجة الحرارة المثلى ودرجة الحموضة المثلى لنشاط الإنزيم 30 درجة مئوية و6 على التوالي، مما يشير إلى بروتياز حامضي محب للحرارة المعتدلة .

الاستنتاجات: يعد التعرض الضوئي بالأشعة فوق البنفسجية استراتيجية فعالة لتحسين إنتاج البروتياز في أنواع البكتيريا العصوية، مع تطبيقات واعدة في معالجة الأغذية وصناعة الألبان.

الكلمات المفتاحية: أنواع البكتيريا العصوية، البروتياز، الطفرات، كبريتات الأمونيوم، التنقية