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Screening and Production of Peptidase Producing *Bacillus* Species Isolated from Soil

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Authors' contributions

This work was carried out in collaboration between all authors. Author SR performed the study and wrote the first draft of the manuscript. Authors SAM and SBH managed the analyses of the study. Author RK managed the literature searches. All authors read and approved the final manuscript.

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Original Research Article

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ABSTRACT

Aims: Proteases are the most deliberate microbial enzyme at mercantile, industrial, health professional, analytic, symptomatic, outgoing interruption and other sectors.
Methodology: About 8 isolates were recovered from different soil samples gathered from various fields of Abdul Hakeem District Khanewal of Pakistan. Bacterial identification was performed by Gram's staining, endospore staining and hang drop motility test. Samples after serial dilution plated on gelatin agar plates which have composition, Nutrient agar 7.5 gram, gelatin 21 gram, NaCl 0.15 gram, Agar,1.5 gram and 300 ml of distilled water. On these plates zone of inhibition appeared after incubation of 48 hours. The enzyme was extracted by ammonium sulphate precipitation technique. Protease enzyme was characterized by Fourier Transform Infrared Spectroscopy. Enzyme producing activity of bacterial isolate was studied by using spectrophotometer at 280 nm.
Results: Among these, three isolates (SA1, SA2, SA3) showed good proteolysis activity, two isolates display moderate activity (SA7, SA8) while the rest were inactive. The plates with clear zone of inhibition were selected for quantitative tests of protease and colonies of bacteria were

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shifted onto new substrate media plates by wire loop for further testing. Protease assay was performed on enzyme solution at pH 8. The spectral peaks were observed in the region of 1000-3500 cm^{-1} are 3227.03, 1636.55, 1445.50 and 1061.13 cm^{-1}. The C-H group appeared in the region 3227.03 cm^{-1}.

Conclusion: These proteases producing Bacillus species has positive impact in biotechnology and can be conveniently considered for further industrial applications.

Keywords: Bacillus species; protease; isolation and characterization.

1. INTRODUCTION

Enzymes are mainly produced by living cells but they can also be extracted from organisms and used for pharmaceutical, industrial and biotechnological applications. Overall, enzymes provide green and economically sustainable alternative strategies in industrial transformations by saving energy and avoiding pollution [1]. Microbial proteases are degradative enzymes, which catalyze the total hydrolysis of proteins. The molecular weight of proteases ranges from 18–90 k Da. These enzymes are found in a wide diversity of sources such as plants, animals and microorganisms but they are mainly produced by bacteria and fungi. Microbial proteases are predominantly extra cellular and can be secreted in the fermentation medium. Proteases are classified according to their structure and properties of the active sites. Protease can be obtained from all origins of life that is animals, plants and micro-organisms. Neutral proteases are of non-mammalian animal origin and due to their mild proteolytic action, they are especially suitable for the preparation of primary cells and secondary sub cultivation in cell culture since it is gentle on cell membranes. They are frequently obtained from Bacillus polymyxa and have molecular weight in range of 30-40 Daltons and pH optima in range of 6-7. One of the most widely used bacteria *Bacillus subtilus* can produce peculiar chemicals and major source of industrial enzymes including protease and amylase. With the arrival of new frontiers in biotechnology, the protease application spectrum has extended into many new fields, such as medicinal, clinical, and analytical chemistry. A number of physiologic abilities exhibited by Bacilli allow them to live in a wide range of habitats, including many intense habitats such as hot springs, desert sands and Arctic soils. Bacilli are an extremely diverse group of bacteria that include both the causative agent of anthrax (Bacillus anthracis) as well as several species that synthesize important antibiotics [2]. The genus Bacillus contains a number of industrially important species and approximately half of the present commercial production of bulk enzymes derives from the strains of Bacillus sp. These strains are specific producers of extracellular proteases and can be cultivated under extreme temperature and pH conditions to give rise to products that are, in turn, stable in a wide range of harsh environments [3]. Microbial proteases account for approximately 40% of the total worldwide enzyme sales [4]. Achromopeptidase from *Achromobarter lyticus*, Calpain from rabbit-skeletal muscle, Carboxypeptidase A,B,C from pancreas, Chymotrypsin from bovine pancreas, Clostripain from *Clostridium histolyticum*, Dipeptidyl-peptidase from beef spleen, thrombin from human plasma, Trypsin from bovine pancreas, subtilisin from *Bacillus subtilis* Gelatinase from human, Glutamyl endopeptidase from *Staphylococcus aureus* V8 and Papain from *Carica papaya* are some commonly used commercially available protease [5]. Mode of cultivation greatly influences the production of metabolites by microorganisms. Batch and continuous are two main methods for cultivation of micro-organisms. Variation in the C/N ratio can regulate the fermentation of proteases and process can be scaled-up by prolonging the stationary phase of the culture using continuous or chemostat and fed-batch approaches.

The basic purpose of this study is to isolate the peptidase producing Bacilli from soil followed by the production and characterization of these metabolites.

2. MATERIALS AND METHODS

2.1 Sample Collection

Three fertile soil samples were collected from fields of Abdul Hakeem district Khanewal. Two oil waste samples were collected from oil deposit of Sher Shah Multan from different sites. Three sewerage samples were collected from main hole surrounding of Jinnah colony Abdul Hakeem District Khanewal.

2.2 Isolation and Screening

These samples were serially diluted and then plated on gelatin agar plates. Gelatin agar plates prepared with following composition i.e; Nutrient agar 7.5 gram, gelatin 21 gram, NaCl 0.15 gram, Agar 1.5 gram and mixed all these chemicals in 300 ml of distilled water [6]. Whole media was autoclaved and poured under laminar air flow (streamline EN1822-1 classH13). Then plates were placed into incubator (MMM-Ecocell.55) for 24 hours at 37℃. Diluted samples were spread on plates and incubated for 48 hours at 37℃. The plates with clear zone of inhibition were selected for quantitative tests of protease and colonies of bacteria were shifted onto new substrate media plates by wire loop for further testing.

2.3 Identification of Bacterial Strains

Bacterial identification was done by various tests that were Gram's staining, endospore staining and hang drop motility test. Firstly, the slide was heat fixed with the specimen by passing it on a burner various times by forceps. Slide was flooded with crystal violet solution in staining tray and allowed to stay for 1 minute. The stain was rinsed with tap water and flooded with solution of iodine for one minute. Slide was flooded with decolorizer for almost five seconds and washed with water. After washing, slide was suspended in safranin for 30 seconds and then cleaned with water. Now dried the slide on absorptive paper and placed it in an upright orientation and microscopically examined under a 100X objective [7]. Smear of organisms was prepared on a clean microscopic slide, air dried it and fixed by heating it. Small piece of blotting paper was placed over the smear and put the slide on wire gauze on a ring stand. Gently heated the slide via Bunsen burner until it started to evaporate. The slide was heated for 3-5 min after starting evaporation. As the paper begun to dry, added two drops of malachite green to keep it moist. Using a clothes peg removed the slide carefully after 5 minutes from the rack. After 5 min of heating, blotting paper was removed and allowed the slide to cool up to room temperature for 2 minutes. Then, slide was washed with water to clear the malachite green. Now smear was stained with safranin for 2 minutes and rinsed slide from each side. The slide was observed under high power of microscope. Firstly, took a cover slip and put Vaseline dot on each corner from backside. Then took a full loop of broth by sterilized wire loop and placed it in the center of cover slip. Now by taking a cavity slide inverted it on the cover slip in that way the drop hangs freely from the cover slip in the space of depression and microscopically examined [8].

2.4 Production of Protease Enzyme

Media containing Casein 2.0%, dextrose 1.0%, peptone 10%, MgSO4 0.02%, KH2PO4 0.04%, NaCl 0.2%, CaCl2 0.002% and 200 ml water was prepared for enzyme production with pH 7.00 at 370C [9]. Autoclaved the media and placed it in rotatory flask incubator (GFL.3005) for 24 hours at 150 rpm. For inoculum development, isolate from potent bacterial strains was taken in nutrient broth and incubated for 24 hours. 10 ml inoculum was added to production media to make it 200 ml then incubated the material in rotatory shaker. Samples were withdrawn at various intervals after 24 hours and 48 hours respectively and centrifuged (Scilogex-D3024R) at 10,000 rpm at 100C for 10 minutes. Supernatant was collected about 100 ml which was used for further testing.

2.5 Purification by Ammonium Sulphate Precipitation Technique

Purification of enzyme was done by ammonium sulphate precipitation method. Supernatant was fractionated with ammonium sulphate between 50% and 70% of saturation. All precipitation steps were carried out at 4℃. The precipitates were re suspended in the 0.1M buffer at pH 7 [10].

2.6 Protease Assay

Protease assay was performed according to Anson method. For measuring protease activity, enzyme solution 0.2 ml was mixed with 2.5 ml of 1% casein in 0.01M phosphate buffer (pH 7) and incubated for 10 minutes at 37℃. Phosphate buffer was prepared by taking 1.75 g of K_2HPO_4 and 1.37 g of KH_2PO_4 in 100 ml of water separately, then 61.5 ml from first solution and 38.5 ml from second solution were taken to make it 100 ml buffer. The reaction was terminated by adding 5 ml of 0.1 M trichloro acetic acid (TCA). A suitable blank was run simultaneously in which TCA was added to the enzyme solution followed by substrate addition. After incubation at room temperature for 30 minutes, both samples were centrifuged at 10,000 rpm for 10 minutes [9]. Enzyme producing activity of bacterial isolate was studied by using spectrophotometer at 660 nm wavelength.

2.7 Effect of Parameters on Enzyme Production

The effect of pH on enzyme activity was checked out using different pH ranges 6-9. The sample was inoculated with optimized production media and after passing 24 hours protease assay performed. By reading the absorbance at 660nm the best pH was concluded [11]. The effect of temperature on enzyme production was calculated by taking different temperatures like 0℃, 25℃, 37℃ and 40℃. The test sample was inoculated with optimized production media and after passing 24 hours protease assay performed [11].

2.8 Characterization of Protease Enzyme

Protease enzyme was characterized by Fourier Transform Infrared Spectroscopy. The liquid membrane method was adopted for this spectroscopy. In this method, several drops of the sample were dropped onto NaCl aperture plate by sandwiching it under another aperture plate in such a way that no gas bubbles were trapped. The thickness was adjusted according to the sample absorbance by inserting spacers between the aperture plates. A thin liquid membrane was formed between the two aperture plates by the measured sample and it easily formed the graph on the screen [9].

3. RESULTS

3.1 Isolation and Screening

Zone of inhibition appeared on plates which showed presence of bacteria in one sewerage sample and two fertile soil samples. These samples were coded as SA1-10. The proteolytic activity has been shown in Fig. 1.

3.2 Identification of Bacterial Strains

Results of Gram's staining, spore staining and motility test was checked according to Bergey's manual of systematic bacteriology. Slide prepared by endospore staining showed that bacteria were spore forming as they attained the green color. Microscopic results showed that it was a gram-positive bacterium because it showed violet color after staining. Slide showed that it was motile bacterium as it showed apparent, directional and place to place locomotion (Table 1).

Table 1. Morphological characterization of isolates

Gram's staining	+ ve
Shape	Rods
Size	Long
Motility	+ve
Spores	+ ve
Arrangement	Independent

3.3 Purification by Ammonium Sulphate Precipitation Technique

Supernatant before and after precipitation shows absorbance at wavelength between 280 nm (Fig. 2). The protease assay was assessed on gelatin agar plates (Fig. 3).

3.4 Effect of Parameters on Enzyme Production

We observed proteolytic activity at pH 8.0 was considered to best at temperature 37℃ (Fig. 4).

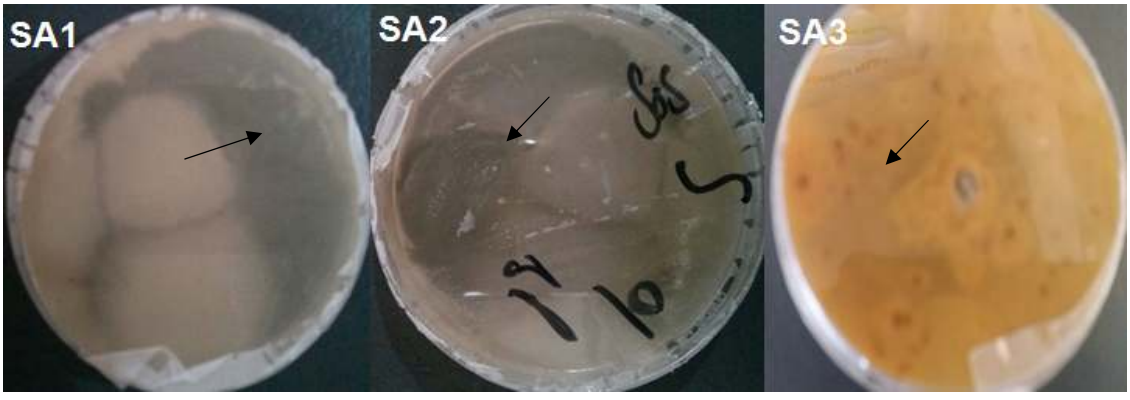

Fig. 1. Proteolytic activity has been shown by Bacillus species SA1-3. Arrows indicate the enzymatic activity

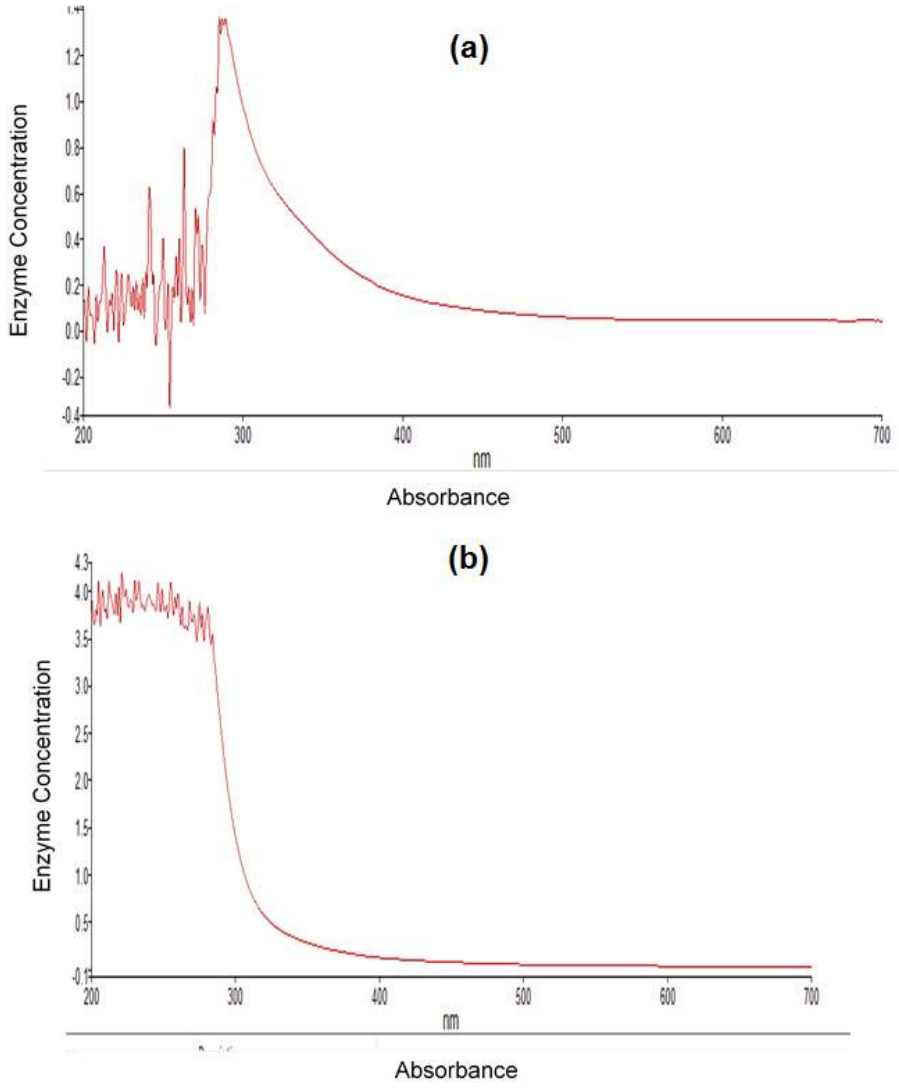

Fig. 2. Protease assay (a) supernatant (b) precipitates after ammonium sulphate precipitation technique

3.5 Characterization of Protease Enzyme

Bacillus strain showed higher potential for alkaline protease activity. Characterization was performed using FTIR to identify the functional groups of the components based on the peak value in the region of IR radiation. Fig. 5 showed spectral bands in the region of 1000-3500 cm^{-1} 3227.03, 1636.55, 1445.50 and 1061.13 cm^{-1}. The spectrum of protease (sample) confirmed C-H group (alkanes) in region of 3227.03 cm^{-1} and showed the strong band, broad intensity and sp^3 hybridization at this range. Presence of carboxylic acid group was also observed in the region 1636.55 cm^{-1}. Strong band stretching appeared at 1445.50 cm^{-1} due to (C=C) bond which are aromatic and two stretching bands at 1061-13 cm^{-1} was observed which belongs to (C-O) ester group (Fig. 5).

4. DISCUSSION

Enzyme synthesis is an emerging dynamic area of academic and most conspicuous, more considerable "application research" in biotechnology. Many methods are reported in literature for enzyme synthesis specifically for protease enzyme. In spite of various procedures are available for synthesis of protease enzyme, there is essential need to explore more

Fig. 3. Proteolytic activity analysis on gelatin agar plates. Zone of hydrolysis by proteases has been shown

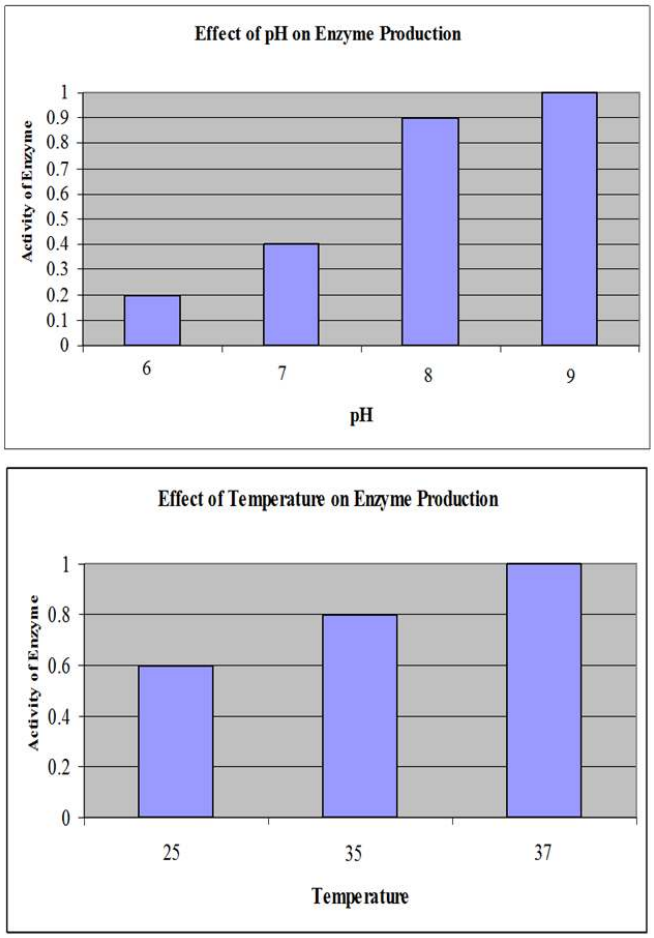

Fig. 4. Evaluation of pH and temperature on protease production

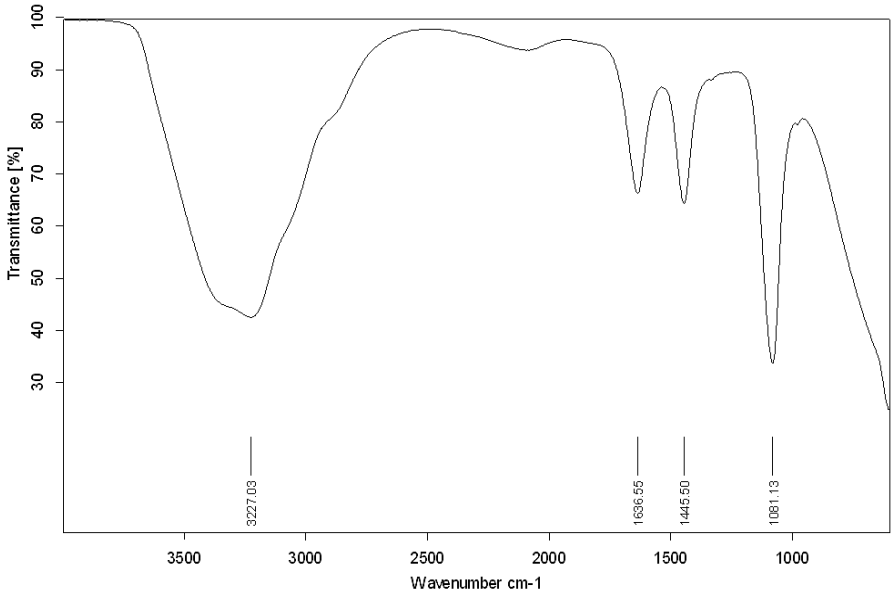

Fig. 5. Protease characterization by FTIR

appropriate techniques, procedures or methods that are more considerable and environmentally suitable for synthesis of protease enzyme because available methods and techniques have advantages but also have some serious issues and drawbacks which cannot be neglected anymore such as cost, complications of reactions, health and safety considerations associated with these methods. Methods used for protease enzyme synthesis, reported in literature are not satisfactory because these are fraught with various problems and complications such as high energy consumption, toxic chemicals, unwanted Concisely list of methods used for protease synthesis have some extraordinary issues that cannot be manage by little efforts. So, simple, uncomplicated and safe method must be employed for protease synthesis. Here we adopted a single step, easy and rapid procedure for enzyme synthesis that is the most considered method and is facile approach for protease synthesis. This biological synthesis serves advancement over many chemical methods due to its simplicity, convenience in single step procedure and performance at normal condition by products.

Peptidase enzyme was successfully bio synthesized by rapid, simple and cost-effective method from Bacillus species which was isolated from soil sample, had been characterized by morphological tests and FTIR spectroscopy. Bacterial strain was gram positive, rod shape, motile and colonies were independent. Peptidase has shown maximum activity at 8 pH and 37℃. Resultant Peptidase enzyme has excellent work in detergent and tanning industry. As introducing this biological method for production of peptidase we are going to except certain aspects which are associated with this peptidase enzyme.

Spectrophotometer graph showed the protease enzyme precipitation peaks at the absorbance of 200_700 nm and maximum concentration of enzyme was shown at 280 nm. Protease assay results are attained at 200_600 nm absorbance and maximum activity with broad peeks was shown at 250_300 nm absorbance. Supernatant precipitation with ammonium sulfate showed broad peeks at the 200_250 nm wavelength. In literature when protease assay was performed in presence of tris buffer results attained at 400-900nm wavelength and maximum activity with broad peak was shown at 600nm [12]. Fourier transform infrared spectroscopy (FTIR) is used for identification of the functional groups of the components based on the peak value in the region of IR radiation. Esters group are very useful in structure determination as they not only estimate the type of group but also tell the number of carbonyl groups.

5. CONCLUSION

We isolated protease from Bacillus species collected from soil. Three species SA1-3 showed

effective proteolytic activity. These strains can be effectively considered in industrial and health applications for production of these enzymes at large scale level.

CONSENT

It is not applicable.

ETHICAL APPROVAL

It is not applicable.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

REFERENCES

1. Sevinc N, Demirkan E. Production of protease by *Bacillus* sp. N-40 isolated from soil and its enzymatic properties. J. Biol. Environ. Sci. 2011;5:95-103.
2. Perreten V, Vorlet-Fawer L, Slickers P. Microarray-based detection of 90 antibiotic resistance genes of gram-positive bacteria. J. Clin. Microbiol. 2005;43:2291-2302.
3. Siddalingeshwara K, Uday J, Huchesh C. Screening and characterization of protease from *Bacillus* sp. Int. J. Appl. Biol. Pharm. Tech. 2010;1:575-581.
4. Ayaz NO. Formation of proteases from newly isolated strain isolated from Saudi Arabia. J. Appl. Pharm. Sci. 2012;2:190-193.
5. Bankus J, Bond J. Some commercially available proteases. Proteolytic Enzymes. 2001;295-316.
6. Shaikh N, Patel A, Mehta S. Isolation and screening of cellulolytic bacteria inhabiting different environment and optimization of cellulase production. Univers. J. Environ. Res Technol. 2013;3:39-49.
7. Beveridge TJ. Structures of gram-negative cell walls and their derived membrane vesicles. J. Bacteriol. 1999;181:4725-4733.
8. Holm C, Jespersen L. A flow-cytometric gram-staining technique for milk-associated bacteria. Appl. Environ. Microbiol. 2003;69:2857-2863.
9. Khan MA, Ahmad N, Zafar AU. Isolation and screening of alkaline protease producing bacteria and physio-chemical characterization of the enzyme. Afr. J. Biotechnol. 2011;10:6203-6212.
10. Pant G, Prakash A, Pavani J. Production, optimization and partial purification of protease from *Bacillus subtilis*. J. Taibah Univ. Sci. 2015;9:50-55.
11. Das G, Prasad M. Isolation, purification & mass production of protease enzyme from *Bacillus subtilis*. Int. Res. J. Microbiol. 2010;1:26-31.
12. Martins F, Ramires EC. Adaptation and validation of a proteolytic activity methodology using dye-crosslinked substrate tablets. Adv. Enzyme Res. 2014;2:113.

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