ISSN Print: 2394-7500
ISSN Online: 2394-5869
Impact Factor: 5.2
IJAR 2018; 4(7): 141-146
www.allresearchjournal.com
Received: 05-05-2018
Accepted: 10-06-2018

Shreyasi Chakrabarti
Department of Biotechnology, B.K. Birla College, Kalyan. Dist. Thane

Nidhi Tiwari
Department of Biotechnology, B.K. Birla College, Kalyan. Dist. Thane

Pratik Khule
Department of Biotechnology, B.K. Birla College, Kalyan. Dist. Thane

Shreya Marar
Department of Biotechnology, B.K. Birla College, Kalyan. Dist. Thane

Shivani Kakkar
Department of Biotechnology, B.K. Birla College, Kalyan. Dist Thane

Annika Durve Gupta
Department of Biotechnology, B.K.Birla College, Kalyan. Dist Thane

Correspondence
Annika Durve Gupta
Department of Biotechnology, B.K.Birla College, Kalyan. Dist Thane

Isolation of protease producing bacteria from spoiled food and extraction of protease enzyme

Shreyasi Chakrabarti, Nidhi Tiwari, Pratik Khule, Shreya Marar, Shivani Kakkar and Annika Durve Gupta

Abstract
Proteolytic enzymes are omnipresent and found in almost all living organisms. The protease enzyme producing bacteria are explored to achieve high quality industrial grade enzyme to produce various products. A total of five bacterial isolates, having protease producing ability, were isolated from different fermented food samples. Of these, three bacterial isolates showing optimum proteolytic activity were selected for further characterization by quantitative enzymatic assay method. Various growth parameters like effect of pH, temperature, etc on enzyme activity were studied. These isolates were identified on the basis of their morphological and biochemical features. The protease enzyme was extracted from the bacterial isolates and their enzymatic activity was estimated which were found to be maximum at neutral and alkaline pH. The crude protease enzyme was used for various application *viz*. digestion of milk and egg proteins, removal of stains etc. These protease extracts could be used for various profitable commercial applications.

Keywords: protease enzyme, bacteria, fermented food samples, digestion of milk, stain removal

Introduction
Proteases are highly demanded and economically valued group of enzymes with various industrial applications. Proteases are the biocatalyst that hydrolyze peptide bonds present in proteins and hence classified as hydrolyses (Ellaiah *et al*., 2002). Protease are the class of enzymes, which occupy key position with respect to their applications in both physiological and commercial fields. They represent one of the three largest groups of industrial enzymes. These enzymes are ubiquitously found in all living organisms essential for cell growth differentiation (Tiwari *et al.*, 2015; Gaur and Wadhwa, 2008; Gaur *et al*., 2010) [2, 7, 8]. These enzymes account for about 60% total worldwide sale of enzymes (Rao *et al*., 1998). There is renewed interest in the study of proteolytic enzymes, mainly due to the recognition that they not only play a critical role in cellular metabolic processes but have also gained considerable attention in the industrial community. The extracellular proteases are of commercial value and find multiple applications in various industrial sectors. They have got wide range of commercial usage in detergents, leather, food and pharmaceutical industries (Bhaskar *et al*., 2007; Jellouli *et al.*, 2009) [9, 10]. Among all the proteases, alkaline proteases are primarily used as detergent additives (Gupta *et al*., 2002) [11].
Microorganisms are known to be highly versatile in producing a wide range of enzymes, with varied patterns of activity. Due to the physiological and biochemical properties of microorganisms, they are considered as the most common source of commercial enzymes (Tiwari *et al.*, 2015) [2]. Several microbial strains including fungi (*Aspergillus flavus*, *Aspergillus miller*, *Aspergillus niger* and *Penicillium griseofulvin*) and Bacterial (*Bacillus licheniformis*, *Bacillus firmus*, *Bacillus alcalo*, *Bacillus subtilis* and *Bacillus thuringiensis*) have reported to produce proteases.
Production of these biocatalyst using agro-biotech substrate under solid state fermentation and conditions provide several advantages in producing, cost effectiveness in labour, time and medium components in addition to environmental advantages like less effluent production, waste minimization (Pandey *et al.*, 2000). Bacterial species are either isolated from effluents of industries or been bought from institutes like MTCC.
.

Protease production conditions are optimized and highest protease producing environments with optimized nutrient sources, pH and temperature were determined to get maximum protease from each bacterium.

In the present study, highest alkaline and neutral producing strains were isolated from various and identified. Further the proteases were extracted and tested for various commercial applications.

Materials and Methods

Test chemicals

The media for bacterial growth was obtained from Himedia, India. The chemical solutions for were prepared in distilled water. Milk powder (Everyday) was obtained from the local market.

Sample Collection

The sample sources for isolation of the protease producing bacteria - spoiled foods (Bread, Curd) condiments (Pickles) and preserved foods (Ketchup, Mayonnaise) - were obtained from household kitchen in Kalyan.

Screening of protease producing microbes

The samples were streaked on skimmed milk agar medium (5% milk powder and 3% agar) and incubated at 37°C for 72hrs (Tiwari *et.al,* 2015) [2]. Proteolytic activity *i.e*. the hydrolysis of casein, is seen by a zone of clearance around the colony.

The isolates were subjected to morphological, cultural and biochemical studies. Standard Biochemical tests included Indole, Methyl red, Voges Proskauer and Citrate Test, TSI slant, Nitrate Reductase, Urease and 1% sugar solutions of Sucrose, Glucose, Lactose, Xylose, maltose and Mannitol with Andrade's indicator. The bacterial isolates were studied for the effect of temperature, pH and osmotic pressure on their growth.

Inoculum preparation for enzymatic assay

A loopful culture of bacterial isolates was inoculated into 10ml of Luria Bertani Broth medium till a culture density (OD_{600}) of 0.8 was obtained. These 24 hrs old culture suspensions were inoculated in 200ml of Production medium (Glucose-0.20gm, Peptone-0.30gm, $MgSO_4$-0.5gm, KH_2PO_4-0.2gm, $FeSO_4$-0.004gm, Distilled water-200ml) (Yasser Hussein, 2015) [4]. The flasks were incubated at 37°C for 72 hrs. It was further centrifuged at 10,000 rpm for 10 minutes. The supernatant (crude enzyme) was used for further protease assay.

Purification of crude enzyme by Ammonium Sulphate precipitation

The crude enzyme extract was purified by ammonium sulfate precipitation. The supernatant obtained after the centrifugation was collected in a flask and finely powdered ammonium sulphate (20-80%) was added slowly to the clear supernatant with constant stirring. The solution was centrifuged at 10000 rpm for 10 min and the supernatant and pellet was collected. The pellet was dissolved in phosphate buffer (pH 7.2). This was used as sample for protease and protein estimations using Lowry method (Lowry *et al*., 1951).

Protein Determination

The total protein content of the crude extract and the purified sample was determined by Folin-Lowry method. The protein concentration was determined based on the standard curve obtained with bovine serum albumin (1.0 mg/ml) as standard at 660 nm (Lowry *et al*., 1951).

Protease enzyme assay

Protease activity was determined by Anson method (Yasser Hussein, 2015) [4]. The casein solution (0.65% casein) was incubated with crude enzyme in alkaline phosphate buffer (pH 10) at 37°C for 10min. The reaction was stopped by adding 110 mM TCA. An enzyme blank was prepared. After incubation at room temperature for 30min both test and blank solutions were centrifuged at 10,000rpm for 10min. 0.4 ml of supernatant, 1.0ml 50mM Na_2CO_3 and 0.5ml Folin-Ciocalteau's reagent was incubated at room temperature for 30 min and the absorbance was measured at 660nm.

Protease activity

The protease enzyme activity of the bacterial isolates was determined with the moles of tyrosine released. One unit (U) of proteolytic enzyme activity was defined as the amount of enzyme that liberated 1µg tyrosine per ml per minute from casein under specified assay conditions. Enzyme units were measured using slope obtained from tyrosine as standard (Sharma *et al.,* 2015) [5].

$$\text{Enzyme activity (U/ml)} = \frac{\mu \text{ mol tyrosine equivalent releases} * \text{Total volume of assay}}{\text{Volume of enzyme taken} * \text{Incubation time}}$$

Applications

Digestion of natural proteins

Milk samples (5 ml) were taken in sterile test tubes and were treated with the extracted crude enzymes individually. The tubes were incubated at 37°C for 24 hrs. The tubes were checked for milk clotting after 24 hrs.

Egg whites were taken in sterile test tubes and were treated with the extracted crude enzymes. The tubes were incubated at 37°C for 24- 48 hrs. The tubes were checked for coagulation after 24-48 hrs.

Removal of Stains

Pieces of cotton fabric were stained with blood, saffranin dye and turmeric paste. Each cotton fabric was soaked with the combination of detergent and crude enzyme extract at room temperature for few hours. After incubation, the cotton fabrics were rinsed with water and air dried. The same procedure was done with control stained cotton fabric piece which was soaked only with sample detergent.

Results and Discussion

Five morphologically distinguished bacterial isolates were isolated on Skimmed milk agar plate. A zone of proteolysis was seen surrounding the bacterial colony. Out of five isolates, three bacterial isolates showed proteolytic activities at both alkaline and neutral pH (Figure 1).

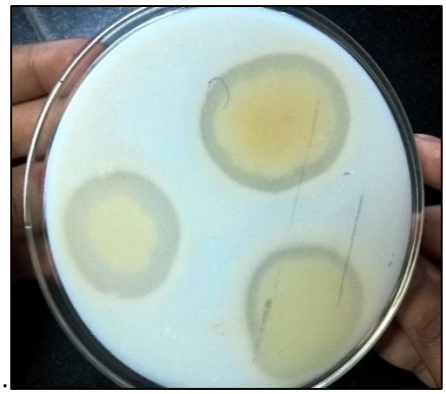

Fig 1: Bacterial isolates showing zone of proteolysis around the colony on Skimmed milk agar plate

Identification of the bacterial isolates

Colony characters of the organisms were recorded by observing the colonies grown on sterile Nutrient agar plates. Gram staining (Gram, 1884) was carried out to determine the Gram nature of the organisms. Morphological, cultural and biochemical studies of the bacterial isolates were carried out. The colony characters of the bacterial isolates were studied on the basis of size, shape, colour, margin, opacity, elevation, consistence and motility. Identification of Bacteria on the basis of morphological characteristics is not reliable for all groups of organisms, including bacteria which possess limited morphological differentiation (Entis *et al.,* 2001) [14] and can be relied on the basis of biochemical tests and assimilation assays (Reva *et al.,* 2001) [15]. Biochemical tests were performed to identify the organisms (Harley and Prescott, 2002) [13]. The results obtained were compared using Bergey's Manual of Systematic Bacteriology (Volume I and II) (Table 1). The isolates obtained may belong to the *Bacillus* sp, *Acinetobacter* sp. and *Gluconobacter* sp.

Table 1: Biochemical Test for bacterial identification

Biochemical tests	PS	KS	BS
Indole test	-	-	-
Methyl red test	+	+	+
Voges proskauer test	-	-	-
Citrate test	-	-	+
Triple sugar iron test			
Slant	pink	yellow	pink
Butt	pink	pink	pink
H_2S	-	-	-
Gas	-	-	-
Sugar fermentation test			
Glucose	A	A	A
Lactose	A	A	A
Maltose	A	-	A
Sucrose	-	A	A
Mannitol	A	A	A
Oxidase	+	+	+
Catalase	+	+	+
	Gram negative rods	Gram negative coccobacilli	Gram negative rods

Das and Prasad (2010) studied the isolation, purification and mass production of protease enzyme from *Bacillus subtilis*. They isolated the dominant protease producing strains by using the skim milk agar.

Effects of pH

The pH of the culture medium strongly affects many enzymatic processes and transport of compounds across the cell membrane. Investigation on the proteolytic activity for high enzyme production in the strains grown in acidic, neutral and alkaline medium revealed that highest enzyme activity was found in bacterial isolates grown in alkaline medium. pH of the culture medium is important for cell growth (Li *et al.,* 2001; Singh and Das, 2011) [2], perhaps relating to its influences on nutrient solubility and uptake, enzymatic activity, cell membrane morphology, by-product formation and redox reactions (Bajaj *et al.,* 2009). Bacterial isolates were studied at different pH 4.0, 7.0 and 10.0.The bacterial isolates showed maximum growth at alkaline pH - 10, less growth at neutral pH and no growth at acidic pH.

Effects of Temperature

As the temperature increases, so does the rate of reaction. But very high temperature denatures enzymes. The bacterial isolates were inoculated in skim milk agar plates and incubated at different temperature. After 24 hrs of incubation the isolates showed maximum growth at a range of 28 to 37°C while it showed minimum growth at 4°C and no growth at 60°C.

Growth Curve

It is very essential to detect the optimum incubation time at which an organism exhibit highest enzyme activity since organisms show considerable variation at different proteolytic zone and hence, it was selected as the best incubation periods (Yossan *et al.,* 2006). The isolated strains were studied for their growth characteristics. They were grown in the presence and absence of casein. These isolates showed exponential growth phase within 24 hrs. Generation time was found to be 301 min of PS, 462 min of KS and 301 min of BS (Figure 2).

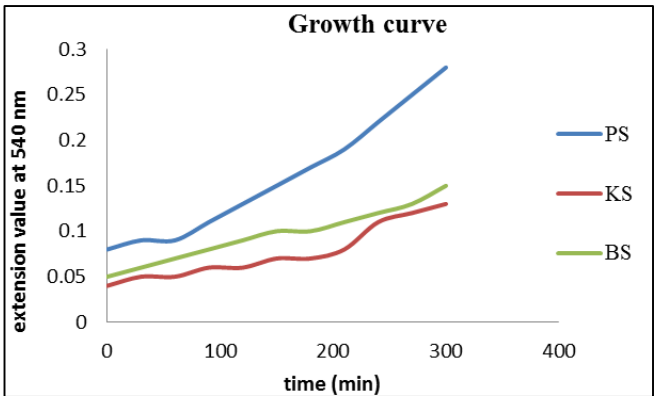

Fig 2: Growth curve of the isolated bacterial stains

Protease enzyme concentration and enzyme activity

The protease enzyme was extracted and the enzyme was purified using Ammonium sulphate precipitation method. Protein concentration of the crude and purified was estimated using Lowry method (Lowry *et al*., 1951). It was observed that the amount of enzyme produced by PS was maximum in compared to other isolates. But the enzyme activity for the enzyme produced by KS was found to be more (28.67 U/mg) (Figure 3; Table: 2)

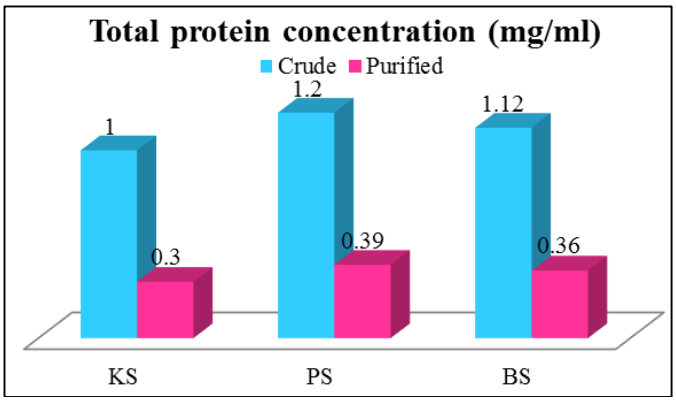

Fig 3: Estimation of protein by Folin-Lowry method

Table 2: Enzyme Activity

Sample	Total Protein (mg)	Total enzyme activity (U)	Specific activity (U/mg)
Crude			
KS	1.0	0.05	0.05
PS	1.2	0.06	0.05
BS	1.12	0.056	0.05
Ammonium sulphate fraction (70%)			
KS	0.3	8.6	28.67
PS	0.39	10.6	27.18
BS	0.36	10.0	27.78

The protease activity was minimum as reported by Arun Kumar Sharma *et al*., (2015) [5] who reported the enzyme activity of 243 U/mL using casein as a substrate

Applications

Proteases are well known enzymes for their wide range of application in food industry, detergent industry and pharmaceuticals industry for the preparation of ointments and medicine. They are also widely used in leather industry for dehairing and bating of hides as an alternative for toxic chemicals which in turn hamper the environment. Extreme environments are important sources for isolation of micro organisms for novel industrial enzymes production (Kumar and Takagi, 1999) [17].

Digestion of natural proteins

Bacteria secrete proteases to hydrolyze the peptide bonds in proteins and therefore break the proteins down into their constituent amino acids (Sims and Wander, 2002) [6]. After 24 hrs of incubation, the milk samples were found to be clotted. All the 3 enzymes samples showed clotting in the milk. Proteases are a highly demanded and valuable group of enzymes with various industrial applications, covering a market share of 60% of the total enzyme market (Gaur and Wadhwa 2008; Gaur *et al.*, 2010) [7, 8]. There is a wide and considerable use of protease enzymes in the food industry nowadays, particularly in meat tenderization, milk curdling and wine and beer turbidity clearance.

Milk samples which were treated with the extracted crude enzymes showed the presence of clotting after incubation at 37°C for 24 hrs. Egg whites also showed the presence of coagulation after incubation at 37°C for 24 hrs (Figure 4; figure 5)

Fig 4: Effect of protease on clotting of milk

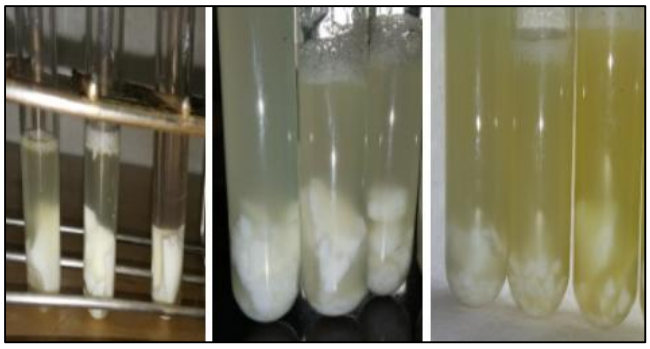

Fig 5: Effect of protease on digestion of egg white

Removal of Stains

Protease enzyme cleaves the blood stains and as a detergent surfactant removes the stain completely from cloth. Washing performance of blood stained cloth was maximum with the detergent and enzyme combination. Blood stains were partially removed in the detergents treated cloth

After using the crude protease enzyme along with the detergent on the stained cloth, it was observed that considerable amount of stain (turmeric paste, blood stain, safranin stain) was removed (figure 6).

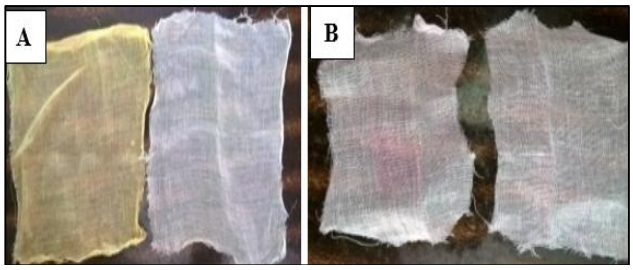

Fig 6: Stain removal effect of crude protease extract (A) Turmeric (B) Saffranin

Krishnaveni *et al* (2012) [19] studied the production and optimization of extracellular Alkaline Protease from *Bacillus subtilis* isolated from dairy effluent. Proteolytic bacteria are most important for industries such as food and fermentation. Kebabcý Özgür and Cihangir Nilüfer (2010) studied the isolation of protease producing novel *Bacillus cereus* and detection of optimal conditions. Srinivasan *et al* (2009) [20] studied the isolation and characterization of thermostable protease producing bacteria from tannery industry effluent. Setsuo Fujimura and Takeshi Nakamura (1986) [22] studied isolation and characterization of a protease from *Bacteroides gingivalis*. A protease was purified from *Bacteroides gingivalis* ATCC 33277 culture fluid by sequential procedures including ammonium sulfate precipitation, ion-exchange chromatography, and isoelectric focusing. Gerze *et al.,* (2005) [23] studied partial purification and characterization of protease enzyme from *Bacillus subtilis megatherium*. A comparative analysis of protease producing microbes isolated from tannery effluent was studied by Siva Muthuprakash and Jayanthi Abraham (2011) [24].

Microbial proteases have a number of commercial applications in industries such as in food, leather, meat processing and cheese making. Enzyme production by micro-organisms is greatly influenced by media components, especially carbon and nitrogen sources, and physical factors such as temperature, pH, and incubation time and inoculums density (Muthulakshmi *et al.,* 2011). It is important to produce the enzyme in inexpensive and optimized media on a large scale for the process to be commercially viable (Muthulakshmi *et al.,* 2011).

Conclusion

Protease is being produced in large scale, preferably bacteria proteases are used in industries. Even though commercial enzymes obtained from bacteria have demerits, to overcome with these demerits, newer organisms from newer environment may be helpful in finding a bacteria. In the pharmaceutical industry, varying and specific proteases are used in developing effective therapeutic agents. Besides the alkaline proteases are used in leather industry for the removal of hairs and parts which are present on the animal skin.

Screening of bacteria that produced protease was carried out on milk agar. The clear zone of casein hydrolysis of bacteria milk protein was an indication of protease secretion. Similar, the clear zone of casein hydrolysis was an indication of protease secretion as reported by Folasade *et al*. (2005).

Further characterization is needed to explore these selected isolates as industrial protease producing strain.

References

1. Masichandran M, Fazilahmed N, Parthasarathi A. Comparative study on the protease producing Bacteria isolated from dairy effluents of Chennai Region, identification, characterization and Application of enzyme in detergent formulation. Asian Jr. of Microbiol. Biotech. Env. Sc. 2014; 16(1):41-46.
2. Tiwari O, Sarabati K, Bidyababy T, Singh K, Singh O, Oinam G, *et al*. Isolation, Identification and Characterization of Protease Producing Bacillus spp. from Unexplored Ecosystems of Indo-Burma Biodiversity Hotspots, Int.J. Curr. Microbiol. App. Sci. 2015; 4(11):611-622.
3. Vishnu Priya VG, Preethi S, Karthikeyan S, Ramesh Babu NG. Isolation and Identification of protease producing bacteria from soil. 2016; 03(08).
4. Yasser Hussein Issa Mohammed. Isolation of protease producing microorganisms from food waste. J.Bio.Innov. 2015; 4(6):322-346.
5. Sharma AK, Sharma V, Saxena J, Yadav B, Alam A, Prakash A. Optimization of protease production from bacteria from soil. Applied Research Journal. 2015; 1:388-394.
6. Sims GK. Wander MM. Proteolytic activity under nitrogen or sulfur limitation. Appl. Soil Ecol. 2002; 568:1-5.
7. Gaur S, Wadhwa N. Alkaline protease from senesced leaves of invasive weed Lantana camara. African Journal of Biotechnology. 2008; 7(24):4602-4608.
8. Gaur S, Agrahari S, Wadhwa N. Purification of protease from Pseudomonas thermaerum GW1 isolated from poultry waste site. The Open Microbiology Journal. 2010; 4:67-74.
9. Bhaskar N, Sudeepa ES, Rashmi HN, Selvi AT. Partial purification and characterization of protease of Bacillus proteolyticus CFR3001 isolated from fish processing waste and its antibacterial activities. Bioresour. Technol. 2007; 98:2758-2764.
10. Jellouli K, Bougatef A, Manni L, Agrebi R, Siala R, Y ounes I, Nasri M. Molecular and biochemical characterization of an extracellular serine-protease from Vibrio etschnikovii. Microbiol. Biotechnol. 2009; 36:939-948.
11. Gupta R, Beg QK, Lorenz P. Bacterial alkaline proteases: molecular approaches and industrial applications. Appl. Microbiol. Biotechnol. 2002; 59:15-20.
12. Pandey A, Soccol CR, Mitchell D. New developments in solid state fermentation. Bioprocesses and products. Proc. Biochem. 2000; 35:1153-1169.
13. Harley JP, Prescott LM. Laboratory exercises in microbiology. Fifth edition. The McGraw –Hill publishing companies, 2002.
14. Entis P, Fung DFC, Griffiths MW, Mclntyre L, Russell S, Sharpe AN. Tortorello ML. American Public Health Association. USA, 2001.
15. Reva ON, Sorokulova BI, Smironov VV. Simplified technique for identification of the aerobic spore-forming bacteria by phenotype. Int. J. Syst. Evol. Microbiol. 2001; 51:1361-1371.
16. Yossan QY, Peng Y, Wang H, Zhang Y. Purification and characterization of an extracellular alkaline serine protease. Microbiol. 2006; 46:169-173.

17. Kumar CG, Takagi H. Microbial alkaline proteases: From a bioindustrial viewpoint. Biotechnol. Adv. 1999; 17:561-594.
18. Sharma AK, Sharma V, Saxena J, Yadav B, Alam A, Prakash A. Optimization of protease production from bacteria from soil. Applied Reseach Journal. 2015; 1:388-394.
19. Krishnaveni K, Mukesh Kumar DJ, Balakumaran MD, Ramesh S, Kalaichelvan PT. Production and optimization of extracellular Alkaline Protease from Bacillus subtilis isolated from dairy effluent. Der Pharmacia Lettre. 2012; 4 (1):98-109.
20. Kebabcý Ö, Cihangir N. Isolation of protease producing novel Bacillus cereus and detection of optimal conditions. African Journal of Biotechnology. 2011; 10(7):1160-1164.
21. Srinivasan TR, Das S, Balakrishnan V, Philip R, Kannan N.. Isolation and characterization of thermos table protease producing bacteria from tannery industry effluent. Research in Science and Technology. 2009; 1 (2):063-066.
22. Fujimura S, Nakamura T. Isolation and Characterization of a Protease from Bacteroides gingivalis. Infection and immunity, 1987, 716-720.
23. Gerze A, Omay D, Guvenilir Y. Partial purification and characterization of protease enzyme from Bacillus subtilis megatherium. Spring. 2005; 121:335-345.
24. Siva Muthuprakash KM, Abraham J. A Comparative analysis of protease producing microbes isolated from tannery effluents. International Journal of Science and Nature. 2011; 2 (1):110-113.
25. Das G, Prasad MP. Isolation, purification & mass production of protease enzyme from Bacillus subtilis, Int. Res. J. Microbiol. 2010; 1:(2):26-31.