ASIAN JOURNAL OF PHARMACEUTICAL AND CLINICAL RESEARCH

INNOVARE ACADEMIC SCIENCES
Knowledge to Innovation

Vol 10, Issue 10, 2017

Online - 2455-3891
Print - 0974-2441

Research Article

PARTIAL CHARACTERIZATION AND CLONING OF PROTEASE FROM *BACILLUS*

VALYA RAMAKRISHNAN[1], YUVARANI THAMBIDURAI[2], SATISH KUMAR RAJASEKHARAN[3], SUCHARITHA KANNAPPAN MOHANVEL[2]*

[1]Moores Cancer Center, University of California, San Diego, USA. [2]Department of Biotechnology, D. G. Vaishnav College, Arumbakkam, Chennai - 600 106, Tamil Nadu, India. [3]Department of Lifescience, Center for Research and Development, PRIST University, Thanjavur - 614 904, Tamil Nadu, India. Email: charusunder@gmail.com

Received: 16 April 2017, Revised and Accepted: 23 June 2017

ABSTRACT

Objective: The present research focused on amplification of protease gene from *Bacillus* strain which was then assessed for maximal enzyme activity.

Methods: A putative *Bacillus* strain was isolated from soil, inoculated into protease production media, and optimized with appropriate pH and temperature conditions for maximal enzyme activity. Genomic DNA was isolated from the strain and amplified the fragment by polymerase chain reaction (PCR) using gene-specific primers for protease. The fragment is then ligated into a T/A cloning vector and transformed into calcium chloride-treated competent *Escherichia coli* DH5α cells. The plasmids were then isolated and confirmed the presence of the gene.

Results: A specific amplification of 1.1 kb was observed following PCR. The amplified product includes the coding sequence and a signal peptide sequence of the protease gene. After cloning with T/A cloning vector pTZ57R/T and transformed into *E. coli* DH5α competent cells, the recombinant plasmid was selected using blue-white selection. Plasmid DNA isolated from the recombinant strains and confirmed the presence of a gene of interest using PCR and quantified by an assay for maximal protease activity. The optimum pH was found to be 10.1 and giving an activity of 21.566 international unit (IU)/ml, and the optimum temperature was found to be on 60°C giving an activity of 38.708 IU/ml.

Conclusion: Amplification of protease gene by PCR isolated from *Bacillus* strain and optimization of pH and temperature conditions for the assessment of subtilisin Carlsberg produced by it. Subtilisin which is protein engineered can be used in commercial products such as stain cutter, dishwashing detergents, cosmetics and food processing, and contact lens cleaner.

Keywords: *Bacillus subtilis,* Gene amplification, Polymerase chain reaction, Protease, Subtilisin Carlsberg.

 DOI: http://dx.doi.org/10.22159/ajpcr.2017.v10i10.19206

INTRODUCTION

Microorganisms play an important role in industrial scale for the production of intracellular and extracellular enzymes [1,2]. Enzyme reactions are a crucial process in living cells, which act as catalysts are essential for life [3-5].

Numerous strains of *Bacillus* produce extracellular proteolytic enzyme which has broad specificity and an alkaline pH optimum. These enzymes now called as subtilisin Carlsberg was discovered by Linderstrom-Lang and Ottesen [6] and purified by Gtintelberg and Ottesen [7]. This enzyme can be called as subtilisin A and subtilopeptidase A, and an alkali was considered to be responsible for the transformation of ovalbumin to plakalbumin [6-8] and used for the transformation of ribonuclease to ribonuclease S [9].

Bacillus subtilis which was often used for the production of most chemicals and industrial enzymes [1,10,11]. Proteases act in developing biopharmaceutical products which aid in cleaning contact lens and to get rid of debris [12]. It also supports in the natural healing process in skin ulceration by efficiently removing necrotic material [13].

Of all the alkalophilic microorganisms that had been screened for use in various industrial applications, members of the genus *Bacillus* had been found to be predominant and a prolific source of alkaline proteases. These proteases are of great importance in detergent industry due to their high thermostability and pH stability.

Genetic manipulation of the host bacteria had been undertaken to maximize the industrial production of the enzyme. Genes encoding protease had been cloned and sequenced from a variety of organisms, some of which are used commercially. Cloning the gene in a plasmid is one of the options available for genetic manipulation, but the more stable option seems to be chromosomal integration and expression.

Genetic manipulation of some *B. subtilis* is easier due to the relative ease in their transformation. On the other hand, industrially important organisms are not amenable to genetic transformations and are difficult to transform efficiently. Therefore, there was an increasing interest in developing methods for amplification of genes, especially those encoding protease in industrially important bacilli.

In this research, the polymerase chain reaction (PCR) amplification of putative protease genes from a putative *Bacillus* strain isolated from slaughterhouse soil and the optimization of the pH and temperature conditions for the assessment of subtilisin Carlsberg produced by it.

METHODS

Screening for protease producers

Soil sample was collected from slaughterhouse and was serially diluted, 100 µl of 10^{-3} dilution of each sample was spread plated on Luria-Bertani agar plates. Milk powder and agar were dissolved in about 60 ml and in 40 ml of distilled water, respectively, autoclaved at 15lbs pressure, 121°C for 15-20 minutes. The two solutions were then mixed well, added to Petri plates, and allowed to cool under sterile conditions. The plates were then incubated at 37°C overnight. Colonies resembling *Bacillus* were inoculated into LB broth and subjected to heat shock treatment at 80°C for 10 minutes. A loopful of culture suspension was streaked onto LB agar plates and incubated at 37°C overnight. A volume

of 50 µl of culture suspension was drop plated onto milk agar plates, incubated for 18 hrs at 37°C and observed in halo formation (indicative of milk protein consumption by the bacteria).

Protease assay

Protease activity was measured by tyrosine residue, released by the digestion of casein, which was used as the substrate. The tyrosine or other aromatic residues react with Folin–Ciocalteau's phenol reagent under alkaline conditions to give blue color. The absorbance was measured at 660 nm [14].

Culture

A volume of 1 ml of the overnight grown culture of DGVP2 was inoculated into 50 ml of protease production broth.

Effect of pH on protease production

The effect of pH on alkaline protease production from *B. subtilis* under the study was carried out using different pH like 9-10.5. The optimization media with the above pH was inoculated with the test sample and the protease assay was done after 24 hrs. The best pH was concluded by reading the absorbance at 660 nm.

Effect of temperature on protease production

The effect of temperature on protease production was studied by taking various temperatures such as 16°C, 37°C, 50°C, 60°C, and 90°C. The optimization media was inoculated with the test sample at different temperatures and the protease assay was done after 24 hrs. The solutions were centrifuged individually at 3000 rpm at room temperature for 10 minutes and the supernatant was used for color development.

Activity per ml=Abs (test)−Abs (EB)*120/0.276 *1/1000*V*Dilution factor

where Abs (test)=Absorbance of test sample at 660 nm
Abs (EB)=Absorbance of enzyme blank at 660 nm
120/0.276=120 µg of tyrosine give approximately 0.276 OD
V=The total volume of the reaction mixture
1/1000=The conversion factor for the microgram to milligram of tyrosine.
DF=Dilution factor.

Isolation of genomic DNA

For cells with rigid cell wall, disruption usually requires extraction buffer which contains detergent. CTAB is commonly used for this purpose. The extraction buffer also contains reducing agent (β-mercaptoethanol) and a chelating agent (ethylenediaminetetraacetic acid). This helps to inactivate nucleases that are released from the cell and can cause serious degradation of the genomic DNA. Keeping the reactions cold, when possible can minimize their effects. Phenolic compounds may also be released on disruption of tissues and these may interfere with subsequent uses of the DNA (e.g., if it is to be used in the PCR). Polyvinylpyrrolidone can be added to the extraction buffer to remove phenolic compounds. Phenol extraction can be used to remove any traces of proteins and the genomic DNA can be precipitated using either ethanol or isopropanol. Precipitated DNA can be hooked out of the solution or collected by centrifugation. The DNA isolation was carried out following the methodology of Saghai-Maroof *et al.* [15].

PCR

The PCR master mix was prepared and 50 µl of the reaction mix was aliquoted in 2 PCR tubes (1 experiment tube and 1 negative control). Then, 1 µl of the template DNA was added at the end of the experiment tube alone. It was vortexed briefly and spun down for 3 seconds. The amplification reaction was carried out in a thermal cycler. A volume of 30-35 L of the amplified DNA from the reaction mix was removed and analyzed on 1% agarose gel. 1 kb DNA molecular marker was also loaded in one of the wells to ensure the size of the amplified PCR product. The entire product was repeated for the second time using high-fidelity Taq polymerase and the respective ×10 assay buffer to ensure avoidance of mutations.

Agarose gel electrophoresis

To clone the gene into the vector, PCR product was separated and purified in preparative agarose gel electrophoresis following the method described by Sambrook [16].

Gel elution

The gel portion containing the amplified PCR product was cut carefully using a sterile blade and stored in a microfuge tube at −20°C. Care was taken not to switch on the UV transilluminator for long, to prevent mutations. The gel was then crushed well with a 1000 µl tip. The volume of the gel contents was assumed to be 40 µl. To this, 360 µl of sterile Milli-Q water was added followed by 400 µl of tris-saturated phenol. The tubes were then stored for 10 minutes at −80°C. The tubes were then removed, thawed, and spun in a centrifuge for 5 minutes at 10,000 rpm. The supernatant was transferred to a fresh microfuge tube and to this 400 µl of chloroform was added. The contents were centrifuged at 10,000 rpm for 5 minutes. 250-300 µl of the supernatant was taken in a fresh microfuge tube, to this about 25-30 µl of 3 M sodium acetate was added, followed by 625-800 µl cold 100 ethanol. Tubes were stored at −20°C for 30 minutes. The contents were centrifuged at 12,000 rpm for 5 minutes. The pellet was dried and resuspended in 25 µl of sterile Milli-Q water. The eluted DNA was stored at −20°C. The amount of DNA obtained was quantified either visually by running an aliquot on an agarose gel or by UV absorbance using the following equation [16].

DNA (µg/ml) = OD. (A260 nm) × Dilution factor × 50

Ligation using T/A cloning vector

The T/A cloning method allows convenient ligation of PCR amplified products, which possess excess Adenines at their ends, to a vector provided with dT overhangs on both ends. The vector pTZ57 R/T has been precleaved with Eco321 and treated with terminal deoxynucleotidyl transferase to create 3' ddT overhangs at both ends. Ligation with PCR amplified fragment produces a circular molecule with two nicks and this can be transformed with high efficiency.

The ligation mix was set up as follows. The mixture was incubated at 37°C for 5 minutes and this was used for the transformation reaction.

Competent cell preparation

DH5α strain of *Escherichia coli* was revived from −80°C by plating on LB agar plate without antibiotics and incubated at 37°C overnight. A single colony from this plate was picked and inoculated into 5 ml of LB broth and incubated overnight at 37°C with agitation. A volume of 500 µl of the overnight culture was inoculated into 50 ml of LB broth and incubated at 37°C with agitation until the OD was 0.4. The cells were chilled on ice for 20 minutes and transferred to pre-chilled sterile centrifuge tubes. The tubes were centrifuged at 4°C, 6000 rpm for 5 minutes to pellet the cells. The cells were resuspended gently in 20 ml of 100 mM calcium chloride incubated on ice for 45 minutes. The cells were resuspended gently in 20 ml of 100 mM calcium chloride incubated on ice for 45 minutes. The cells were centrifuged at 6000 rpm at 4°C, for 10 minutes, The supernatant was discarded and the cells were resuspended in 1700 µl 37°C with agitation of 100 mM calcium chloride incubated on ice for 45 minutes. The cells were centrifuged at 6000 rpm at 4°C, for 10 minutes. The supernatant was discarded and the cells were resuspended in 1700 µl 37°C with agitation of 100 mM calcium chloride + 300 µl of sterile glycerol solution. 100 of the cells were aliquoted into cryotubes and stored at −80°C.

Transformation of competent cells with the ligation mix

Transformation of competent bacterial cells was carried out as described by Hanahan [17]. Competent cells were thawed from −80°C deep freeze. A volume of 5 µl of the ligation mix was added to the 100 µl aliquot of the competent cells. The vials were tapped gently and incubated on ice for 30 minutes. The cells were given a heat shock by placing them for

90 seconds in a water bath set at 42°C. The tubes were immediately placed on ice for 5 minutes. A volume of 100 µl of the cells were then plated on LB agar plates that had been pre-incubated with 80 µl of X-Gal and 8 µl of IPTG and contained ampicillin. The plates were incubated at 37°C overnight and observed for the appearance of colonies pUC19 was transformed into *E. coli* DH5α cells to determine the transformation efficiency (TE) of competent cells. Transformation efficiently of the competent cells is calculated using the following formula:

TE of competent cells:

TE = Number of colonies/ml/µg of plasmid DNA

Identification of recombinants using blue-white selection

α-complementation

Many of the vectors in current use (e.g., the pUC series) carry a short segment of *E. coli* DNA that contains the regulatory sequences and t-coding information for the first 146 amino acids of the beta-galactosidase gene (lacZ). Embedded in this coding region is a polycloning site that does not disrupt the reading frame but results in the harmless interpolation of a small number of amino acids into the amino-terminal fragment of beta-galactosidase. Vectors are these types are used in host cells (e.g., DH5α) that code for the carboxy terminal portion of beta-galactosidase. Although neither the host-encoded nor the plasmid-encoded fragments are themselves active, they can associate to from an enzymatic active protein. This type of complementation in which deletion mutants of the operator proximal segment of the lacZ are complemented by beta-galactosidase – negative mutants that have the operator – proximal region intact, is called α-complementation.

The lac + bacteria that result from α-complementation is easily recognized because they form blue colonies in the presence of chromogenic substrate X-Gal (also abbreviated BCIG for 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside). However, insertion of a fragment of a foreign DNA into the polycloning site almost invariably results in the production of an amino-terminal fragment that is capable of α-complementation. Bacteria carrying recombinant plasmids therefore form white colonies. This method identification therefore known as blue-white selection of recombinants. This development of this simple color test has greatly simplified the identification of recombinants constructed in plasmid vectors of this type. It is easily possible to screen many thousands of colonies that carry putative recombinant plasmids. The structure of these plasmids is then verified by restriction analysis of mini preparation of plasmid DNA.

The recombinant clones, after transformation, were thus identified by blue-white. Blue colonies indicated the absence of the desired gene. White colonies indicated the presence of insert DNA and 10 of them were selected and streaked into LB ampicillin plates to obtain pure cultures. A single colony from each of the recombinants was inoculated into 5 ml of LB broth containing ampicillin (100 µg/ml) and incubated at 37°C with agitation, overnight. A volume of 5 ml of the overnight grown culture was used for plasmid preparation.

PCR amplification to confirm the presence of the gene

The isolated plasmid DNA was used as a template to set up a PCR reaction for the confirmation of the presence of the gene. PCR using Sig F and ProtR primers to confirm the presence of the gene. The reactions were set up in PCR tubes and given a short spin before placing them in the thermal cycler. The conditions of PCR reaction are the same as mentioned previously. The samples were subjected to agarose gel electrophoresis and the gel was visualized under UV transilluminator to check for amplification.

RESULTS

Screening for protease producers

The three soil sample isolated were serially diluted and plated on LB agar plates. Five of the colonies that morphologically resembled bacilli were subjected to heat shock treatment. The LB broth inoculated with these five colonies were incubated at 80°C for ten minutes and plated on LB agar plates. Colonies were observed only in two of the five LB agar plates indicating that they could be bacilli. Overnight broth cultures of these five strains of DGVP2 were used for plating on milk agar plates. One of these plates showed a significant halo formation indicating protease production.

Protease assays

Optimization of pH

The assays were performed over a range of pH, provided by the buffers (pH 9.0, 9.5, 10.1, and 10.6) at 37°C represented in Tables 1 and 2 (Graph 1).

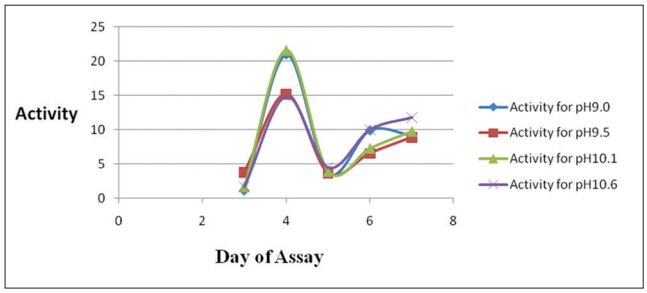
Optimization of temperature

The assays were performed over a range of temperatures (16°C, 37°C, 50°C, 60°C, and 90°C) at pH 10.1 represented in Tables 3 and 4 (Graph 2).

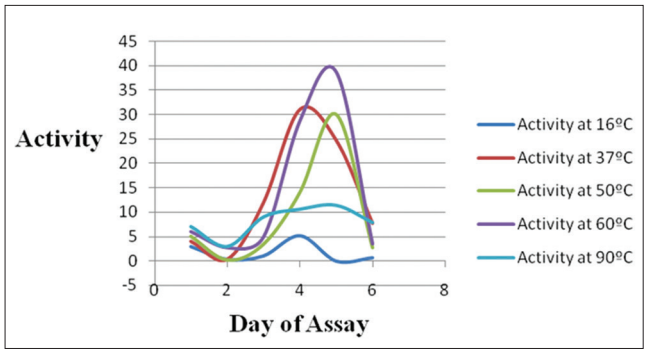
The maximal activity was when the enzyme was subjected to incubation at the temperature of 60°C. On the basis of the assays, it is evident that the maximal activity was attained on day 6, while utilizing a buffer at pH 10.1 and incubation temperature at 37°C.

Table 1: OD value at different pH

Day of assay	3	4	5	6	7
Dilution factor	7	11	11	16	7
OD					
pH 9					
Enzyme blank	0.387	0.177	0.161	0.207	0.249
Test	0.508	1.553	0.404	0.653	1.195
pH 9.5					
Enzyme blank	0.220	0.257	0.163	0.265	0.28
Test	0.615	1.256	0.406	0.562	1.208
pH 10.1					
Enzyme blank	0.322	0.193	0.177	0.161	0.204
Test	0.498	1.608	0.438	0.491	1.211
pH 10.5					
Enzyme blank	0.296	0.198	0.175	0.160	0.251
Test	0.472	1.177	0.472	0.613	1.470



Graph 1: Optimization of pH



Graph 2: Optimization of temperature

Table 2: Calculations for enzyme activity

Day of assay	Activity for pH 9.0	Activity for pH 9.5	Activity for pH 10.1	Activity for pH 10.6
3	1.173	3.831	1.707	1.707
4	20.972	15.226	21.566	14.92
5	3.703	3.705	3.97	4.526
6	9.909	6.584	7.31	10.04
7	9.175	8.913	9.767	11.8233

Table 3: OD value at different pH

Day of assay	3	4	5	6	7
Dilution factor	5	13	11	16	7
OD					
16°C					
Enzyme blank	0.344	0.328	0.307	0.153	0.331
Test	0.397	0.347	0.315	0.275	0.641
37°C					
Enzyme blank	0.453	0.230	0.293	0.330	0.257
Test	0.610	0.893	0.515	0.551	1.181
50°C					
Enzyme blank	0.388	0.216	0.388	0.151	0.303
Test	1.142	1.936	1.308	1.444	1.387
60°C					
Enzyme blank	0.529	0.251	0.255	0.150	0.219
Test	1.845	1.621	2.197	1.896	1.386
90°C					
Enzyme blank	0.485	0.196	0.243	0.167	0.296
Test	0.587	0.623	0.417	0.324	1.102

PCR amplification

Genomic DNA isolated from the given strain was used as a template for PCR for amplification protease gene. An amplified DNA fragment corresponding to 1.1 kb, as expected, was observed on the agarose gel. This DNA fragment was gel eluted, purified, and reserved for the subsequent cloning experiment.

T/A cloning of the protease gene

The 1.1 kb PCR amplified product was ligated into the T/A cloning vector and transformed into *E. coli* DH5α whose TE using pUC19 was found to be 10^5 cells/ml. The *E. coli* DH5α used for competent cell preparation were initially checked in its growth on LB agar medium containing ampicillin. As there was no growth, this indicates that the strain was not contaminated with ampicillin-resistant strains.

Two white colonies were streaked onto LB agar plates containing ampicillin, X-Gal, and IPTG.

Plasmids isolated from the two white colonies were subjected to PCR causing signal forward and protease-reverse primers to confirm the presence of the gene in the recombinants; specific amplification was observed in both cases.

DISCUSSION

Bacillus species are the dominant bacterial workhouses in microbial fermentation. The capacity of selected *Bacillus* strains to produce and secrete large quantities (20-25 g/L) of extracellular enzymes has placed them among the most important industrial enzyme producers. Of all proteases, alkaline protease produced by bacilli is of great importance due to their high thermostability and pH stability [18]. In this study, slaughterhouse soil samples were screened for protease producers and a putative *Bacillus* strain was identified.

Primers were designed based on the nucleotide sequence of *Bacillus licheniformis* (Accession number X91261) available in the National Center for Biotechnology Information (NCBI) database. An expected size of 1.1 kb DNA fragment was amplified with the primers using the isolated genomic DNA from the selected putative *Bacillus* strain. The technique of PCR has been used widely to clone the gene encoding protease from a variety of bacteria. The amplified product was cloned into a T/A cloning vector and transformed into *E. coli*. The present state of knowledge of protein export in *Bacillus* species is still relatively poor. Therefore, the most thoroughly characterized export systems of *E. coli* [19] are utilized for the expression of industrially important enzymes such as alkaline proteases. The recombinants of *E. coli* harboring the protease gene from *Erwinia chrysanthemi* and from *Pseudomonas aeurginosa* [20] have been shown to secrete recombinant proteins.

Bacteriocin release proteins (BRPs) can be used for the release of heterologous protein from the *E. coli* cytoplasm into the culture medium. The gene for a highly thermostable alkaline protease was cloned from *Bacillus stearothermphilus* F1 by the PCR; The recombinant F1 protease was efficiently excreted into the culture medium using E. coli XL1-Blue harboring two vectors: pTrcHis bearing the protease gene and pJL3 containing the BRPs [21]. It was reported the cloning and sequencing of a serine protease gene from a thermophillic *Bacillus* species and its expression in *E. coli* [22].

In general, alkaline protease production is found to be maximum at pH 9-13 [14]. The protease from *Alcaligenes* sp. was optimized for its maximum production and the effect of metal ions on its activity was also studied. The enzyme was purified using ammonium sulfate precipitation and it has been effectively used with the commercial detergents [23]. The 3D structure of serine protease from *B. subtilis* sp. was evaluated using different bioinformatics tools and implied that this enzyme is highly thermostable as well as having an excellent solubility in water [24]. *Bacillus* which is an important species for enzyme production, especially protease. Hence, the enzyme is optimized and studied in immobilization and fermentor conditions to yield maximum production. The enzyme is highly stable in entrapment and fermentation method [25]. It is reported that *Bacillus* spp. has shown antifungal activity in particular against *Candida albicans.* The maximum antifungal activity was observed on the production of *Bacillus* on Yeast Mannitol Agar (YMA) culture medium [26]. Both temperature and pH plays an important role in deciding the utility of the protease in detergents. Therefore, a temperature range of 16-90°C was chosen to assay the activity of the protease. The highest activity was recorded as 38.708 international units (IU)/ml, at the temperature of 60°C. This indicates the thermal stability of the enzyme. The overall maximal activity was recorded as 38.708 IU/ml, at the temperature of 60°C, while using a buffer at pH 10.1.

CONCLUSION

A putative *Bacillus* strain showing a halo on protease-screening medium was isolated from one of the slaughterhouse samples. Primers were designed based on the coding region of the protease gene of *B. licheniformis* (NCBI Accession No. X91261). A specific amplification of 1.1 kb was observed following PCR. The amplified product included the coding sequence and the signal peptide sequence of the protease gene. The amplified product was cloned into T/A cloning vector pTZ57R/T and transformed into *E. coli* DH5α competent cell. *E. coli* strains containing the recombinant plasmid were selected using blue-white selection. Plasmid DNA isolated from the recombinant strains was diluted and used as a template for PCR to confirm the presence of gene of interest. Protease production of the strain was quantified by an

Table 4: Calculations for enzyme activity

Day of Assay	Activity at 16°C	Activity at 37°C	Activity at 50°C	Activity at 60°C	Activity at 90°C
3	0.367	1.087	5.22	9.117.	0.70
4	0.342	11.942	30.982	24.67	7.69
5	0.122	3.38	14.02	30.05	2.65
6	2.704	4.899	28.665	38.708	3.480
7	2.942	8.962	10.513	11.318	7.817

assay. The pH and temperature conditions of the assay were optimized for maximal protease activity. The optimum pH was found to be 10.1 giving an activity of 21.566 IU/ml; and the optimum temperature was found to be at 60°C giving an activity of 38.708 IU/ml.

REFERENCES

1. Gupta R, Beg QK, Lorenz P. Bacterial alkaline proteases: Molecular approaches and industrial applications. Appl Microbiol Biotechnol 2002;59:15-32.
2. Moo-Young M, Chisti Y. Biochemical engineering in biotechnology. Pure Appl Chem 1994;66:117-36.
3. Sierecka JK. Purification and partial characterization of a neutral protease from a virulent strain of *Bacillus cereus*. Int J Biochem Cell Biol 1998;30(5):579-95.
4. Johnson L. Symmetry at the molecular level in biology. Eur Rev 2005;13:77-95.
5. Pingoud A, Fuxreiter M, Pingoud V, Wende W. Type II restriction endonucleases: Structure and mechanism. Cell Mol Life Sci 2005;62(6):685-707.
6. Linderstrom-Land K, Ottesen M. A new protein from ovalbumin. Nature 1947;159(4050):807.
7. Guntelberg AV, Ottesen M. Comptes-rendus Des Travaux Du Laboratoire Carlsberg journal 1960;32:63.
8. Linderstrom-Lang K, Schellman JA. Protein structure and enzymatic activity. In: Lardy H, Myrback K ed. The Enzymes 2nd ed. New York: Academic Press 1959;443-510.
9. Richards FM. On an active intermediate produced during the digestion of ribonuclease by subtilisin. C R Trav Lab Carlsberg Chim 1955;29(17-19):329-46.
10. Esakkiraj P, Immanuel G, Sowmya SM, Iyapparaj P, Palavesam A. Evaluation of protease-producing ability of fish gut isolate *Bacillus cereus* for aqua feed. Food Bioprocess Technol 2009;2:383-90.
11. Chu WH. Optimization of extracellular alkaline protease production from species of *Bacillus*. J Ind Microbiol Biotechnol 2007;34(3):241-5.
12. Anwar A, Saleemuddin M. Alkaline protease from *Spilosoma obliqua*: Potential applications in bio-formulations. Biotechnol Appl Biochem 2000;31:85-9.
13. Sjodahl J, Emmer A, Vincent J, Roeraade J. Characterization of proteinases from antarctic krill (*Euphausia superba*). Protein Exp Purif 2002;26:153-61.
14. Saghai-Maroof MA, Soliman KM, Jorgensen RA, Allard RW. Ribosomal DNA spacer-length polymorphisms in barley: Mendelian inheritance, chromosomal location, and population dynamics. Proc Natl Acad Sci U S A 1984;81(24):8014-8.
15. Sambrook J, Michael R. Green, Molecular Cloning - A Laboratory Manual. New York: Cold Spring Harbor Laboratory Press; 1989.
16. Hanahan D, Jessee J, Bloom FR. Plasmid transformation of *Escherichia coli* and other bacteria. Methods Enzymol 1991;204:63-113.
17. Naidu KS, Devi KL. Optimization of thermostable alkaline protease from species of Bacillus using rice bran. Afr J Biotechnol 2005;4(7):724-6.
18. Simonen M, Palva I. Protein secretion in *Bacillue* species. Microbiol Rev 1993;57(1):109-37.
19. Atsumi Y, Yamamoto S, Morihara S, Fukushima J, Takeuchi H, Mizuki N, *et al.* Cloning and expression of the alkaline proteinase gene from *Pseudomona aeruginosa* IFO 3455. J Bacteriol 1989;171(9):5173-5.
20. Fu Z, Hamid SB, Razak CN, Basri M, Salleh AB, Rahman RN. Secretory expression in *Escherichia coli* and single-step purification of a heat-stable alkaline protease. Protein Expr Purif 2003;28(1):63-8.
21. Saul DJ, Bryce M, Ronnie HM, Peter LB. Cloning and sequencing of a serine proteinase gene from a thermophillic *Bacillus* species and its expression in *E. coli.* Appl Environ Microbiol 1994;60(11):3981-8.
22. Sellami-Kamoun A, Haddar A, El-Hadj Ali N, Ghorbel-Frikha B, Kanoun S, Nasri M. Stability of thermostable alkaline protease from *Bacillus licheniformis* RPI in commercial solid laundry detergent formulations. Elsevier Microb Res 2006;163:299-306.
23. Aishwarya M, Kumari S, Sivasubramanian A, Muthuraman MS. Production, characterization and purification of alkaline protease from *Alcaligenes* sp., and its application in detergent industry. Asian J Pharm Clin Res 2013;6(4):151-5.
24. Irajie C, Mohkam M, Nezafat N, Mohammadi F, Ghasemi Y. *In silico* analysis of nattokinase from *Bacillus subtilis* sp natto. Int J Pharm Clin Res 2017;9(4):286-92.
25. Vijayalakshmi S, Ranjitha J, Rajeswari SV. Enzyme production ability by *Bacillus subtilis* and *Bacillus licheniformis* - A comparative study. Asian J Pharm Clin Res 2013;6(4):29-32.
26. Balouiri M, Bouhdid S, Harki EH, Sadiki M, Ouedrhiri W, Ibnsouda SK. Antifungal activity of *Bacillus* spp. Isolated from *Calotropis procera* ait. Rhizosphere against *Candida albicans*. Asian J Pharm Clin Res 2015;8(2):213-7.