Biocatalysis and Agricultural Biotechnology 20 (2019) 101264

Contents lists available at ScienceDirect

Biocatalysis and Agricultural Biotechnology

journal homepage: www.elsevier.com/locate/bab

Cloning, expression, purification and characterisation of serine alkaline protease from *Bacillus subtilis* RD7

Yewande Suberu[a,b,*], Idowu Akande[a], Titilola Samuel[a], Adekunle Lawal[b], Ademola Olaniran[c]

[a] Department of Biochemistry, College of Medicine, University of Lagos, PMB 12003, Lagos, Nigeria
[b] Department of Biotechnology, Federal Institute of Industrial Research, Oshodi, PMB 21023, Lagos, Nigeria
[c] Department of Microbiology, College of Agriculture, Engineering and Sciences, University of KwaZulu-Natal, Westville Campus, Durban, Private Bag X54001, South Africa

ARTICLE INFO

Keywords:
Cloning
Bacillus
Serine alkaline protease
Phylogenetic analysis
Sequencing

ABSTRACT

Proteases (E.C 3.4) are enzymes that break peptide bonds between amino acid groups of proteins. *Bacillus subtilis* RD7, an extracellular protease producer was genetically characterised, *B. subtilis* genomic DNA was isolated, oligonucleotide primers specific to serine alkaline protease gene of *B. subtilis* were designed and its PCR amplification was done. The purified PCR product and pET15b vector were subjected to restriction digestion with Nde1 and BamH1 and transformed into *Escherichia coli* DH5α competent cells. The recombinant expression of serine alkaline protease gene studied by inducible expression and analysis by SDS-PAGE, established that the serine alkaline protease protein had an estimated molecular size of 43 kDa. Gene sequencing of the insert from selected recombinant clone showed it to be a 1203bp gene encoding a protein of 400 amino acids. The sequence was blasted and aligned with known serine alkaline protease genes for comparison with their nucleotide and amino acid sequences. This identified major matches with closely related subsp. of *B. subtilis*. The insert also showed many substitutions (mutations with other sp. of *Bacillus* which established that serine alkaline protease of *B. subtilis* RD7 is a novel gene. The phylogenetic analysis of serine alkaline protease gene and its predicted amino acid sequences also validated that serine alkaline protease gene is a novel gene and has been accessioned in GenBank with accession number (**MN097797**). When expressed in *E. coli*, the recombinant enzyme was over expressed in the cytoplasm as soluble and active form. The purified enzyme was completely inhibited by PMSF. The enzyme showed maximum activity at pH 10 and 40 °C. It was stable at pH from 6 to 11 and below 70 °C. The aim of this study is to clone serine alkaline protease gene from *B. subtilis* RD7 (**MG255317**), its expression in mesophilic *E. coli* strain BL21, Purification and characterisation of the expressed protein.

1. Introduction

Proteases are one of the most widely used industrial enzymes that catalyses the hydrolysis of peptide bonds in proteins. They are further grouped as hydrolase because they catalyse the cleavage of covalent/chemical bonds using water, they are generally referred to as hydrolytic enzymes with the major role of proteolysis hence they are specifically named proteolytic enzymes.

Currently, the worldwide sales of industrial enzymes are estimated at about $4.2 billion in value (Suberu et al., 2019; Singh, 2016). Proteases represent one of the three largest groups of industrial enzymes and is projected to reach a global market of approximately $ 2.21 billion in terms of value by 2021 at a Compound Annual Growth Rate (CAGR) of 6% from 2016 to 2021. Out of all these proteases produced, proteases from microbial origin accounts for the largest share in the market in terms of value, followed by the animal and plant source respectively (Proteases Market by Source, 2016).

Proteases are a distinct class of enzymes, because they are of immense physiological as well as commercial importance. They also possess both synthetic and degradative properties. Proteases which are the most commercially applicable enzymes occurring in a wide diversity of plants, animals, and microorganisms plays a vital role in both physiological processes, e.g. blood coagulation, zymogen activation by proteolysis, transport of secretory protein across membranes, tumour growth, inflammation, cell growth, tissue arrangement, morphogenesis in development and protein catabolism (Singh, 2016; Souza et al., 2015). Proteases produced from microorganisms are the largest group of industrial enzymes and account for greater than 60% of the total global sale of enzymes (Souza et al., 2015 and Zambare et al., 2011).

Proteases have been the focus of intense research for many decades

* Corresponding author. Department of Biotechnology, Federal Institute of Industrial Research, Oshodi, PMB 21023, Lagos, Nigeria.
E-mail address: talktoyewande@yahoo.com (Y. Suberu).

https://doi.org/10.1016/j.bcab.2019.101264
Received 9 May 2019; Received in revised form 25 June 2019; Accepted 23 July 2019
Available online 24 July 2019
1878-8181/

due to their broad applications in various biotechnological processes. From an industrial point of view, most of the proteases available lack the desired properties; therefore, it is pertinent to continuously search for better and more efficient thermostable alkaline proteases. There is also a great emphasis on developing enzyme based environmentally friendly/green technologies considering the pollution caused by chemical based manufacturing/production technologies. However, enzymes are quite fragile for hostile industrial processes because they are biological molecules; therefore, there is quest to research into robust enzymes capable of functioning well under industrial conditions (Singh et al., 2014).

Utilization spectrum of proteases is limited despite their wide application potential due to lack of industrially desirable characteristics among the available proteases. Proteases intended for industrial application must have stability towards surfactants, solvents, oxidants and stability at high temperature and pH, considering that most of the industrial processes are accomplished under hostile conditions including extremes of temperatures and pH, presence of inhibitors etc. (Subba et al., 2009). Proteases that are thermostable, pH-stable, organic solvent resistant proteases may find novel applications in diagnostic, detergent, pharmaceutical, tannery, effluent treatment, and other industries. Hence attention is focused currently on finding new protease producing microorganisms to meet the requirements of the industry (Singh et al., 2014).

Protein engineering has been used to control and manipulate genes to develop proteases which show unique specificity and enhanced stability. It also helps in the understanding of the structure-function relationships of the enzymes. But research into immense microbial diversity for targeting the producers of novel proteases with industrially desirable characteristics still constitutes a significant research/green area (Singh and Bajaj, 2015). Microbial Proteases have been isolated and characterised from several bacterial (Joshi and Satyanarayana, 2013) and fungal species (Choudhary, 2013.). Enzymes from these *Bacillus* species are known to be poly-extremotolerance and are capable of functioning in adverse ecological conditions (Singh and Bajaj, 2015).

Several *Bacillus* spp. has been reported to produce proteases., *B. subtilis, B. amyloliquefaciens, B. licheniformis, and B. cereus* have become most popular in biotechnological processes for protease production due to their excellent fermentation qualities, high product yields and the lack of toxic by-products (Joshi and Satyanarayana, 2013). In addition, the vast diversity of proteases in contrast to the specificity of their action has attracted worldwide attention in attempt to exploit their physiological and biotechnological applications in various industries (Sanatan et al., 2013). Out of all the proteases, alkaline proteases hold a great potential for application in the detergent and leather industries due to the increasing trend to develop environmentally friendly technologies. The goal of expression cloning is to produce large quantities of specific proteins or to visualize the image of the gene of interest by homology modelling. Gene expression/cloning is a technique that helps researchers to understand the function of proteins. *E. coli* is widely used successfully as a mesophilic host to produce recombinant proteins because of its well-known genetics, cultivation simplicity, high transformation efficiency, inexpensiveness and rapidity.

In this paper, cloning of serine alkaline protease gene from *B. subtilis* and its expression in mesophilic *E. coli*, purification and characterisation of the expressed protein is reported. This serine protease has many important industrial applications with prowess in detergent making.

2. Materials and methods

2.1. Materials

The *Bacillus subtilis* RD7 (assertion number - **MG255317**) was obtained from our previous study (Suberu et al., 2019). *E. coli* DH5α used as the competent host cells were prepared in the laboratory. Oligonucleotide primers with sites for restriction enzymes were designed and synthesized by Inqaba biotech, South Africa. T7 promoter and terminator were also synthesized by Inqaba biotech, South Africa. Taq polymerase was purchased from Southern Cross Biotech., Cape Town, South Africa. The vector pET15b was isolated and prepared using plasmid DNA extraction kits. The DNA ligase, DNA molecular size marker, all restriction enzymes, gel extraction kit, PCR and plasmid purification kit were purchased from thermo scientific (Thermo Fisher Scientific, Massachusetts, USA).

2.2. Methods

2.2.1. Bacterial strains and plasmids

The *Bacillus subtilis* RD7 were grown in 10 ml Luria Bertani (LB) broth overnight at 37 °C and 200 rpm to obtain inoculum culture. *Escherichia coli* DH5α competent host cells were prepared by chemical method using $CaCl_2$ and stored in the bio freezer at −80 °C for further use. The pET15b vector was inoculated in 10 ml LB broth containing ampicillin, incubated overnight at 37 °C and 200 rpm to obtain inoculum, 2 ml of inoculum was inoculated into 100 ml LB broth containing ampicillin, incubated overnight at 37 °C and 200 rpm. The plasmid was isolated and prepared using plasmid DNA purification kits following manufacturer's instructions (Thermo Fisher Scientific, Massachusetts, USA).

2.2.2. Primer design

The gene sequence of *Bacillus subtilis* was used to design primers for PCR using software primers. The various features (melting temperature and GC contents) of the selected primers were then found by using the Oligonucleotide Properties Calculator (Oligo Calculator version 3.27). Primers used for polymerase chain reaction were 5^1-ATT CAT ATG ATG GTG GAT TAC GAA CGT GAG G-3^1 (R_5F primer) with melting temperature (T_m) 60.4 °C and GC content 42% as forward primer and 5^1-AAA GGA TCC TTA ACT GCC TAA TTG GTC TGC-3^1 (R_5R primer) with melting temperature (T_m) 60.3 °C and GC content 43% as reverse primer. The appropriate primer pairs were selected in the identified conserved sequence region for amplification of the structural gene region of serine alkaline protease (RD7) gene. The sequences in this primer were also manipulated using NEB CUTTER V 2.0 to include suitable restriction sites; Nde1 (CATATG) in *Bacillus subtilis* RD7 forward primer and BamH1 (GGATCC) in reverse primer. The designed primers were synthesized by Inqaba biotech (South Africa).

2.2.3. Amplification of the gene by PCR

After the overnight culture of *Bacillus subtilis* RD7 in LB broth, the genome of the bacterium was extracted by DNA extraction using Quick-DNA Fungal/Bacterial Miniprep kit following manufacturer's instructions (Zymo Research Corp. USA). PCR was performed using the R_5F primer as forward primer and the R_5R primer as reverse primer. 2 μL of DNA extracted from *Bacillus subtilis* RD7 (8.9 ng) was amplified in a 50 μL reaction mixture containing 2.5 μM of each primer, 0.5 mM each of deoxyribonucleotide triphosphate (DNTPs), 10X PCR buffer, 2 mM $MgCl_2$. and 2U of Supertherm Taq polymerase (Southern Cross Biotech., Cape Town, South Africa). Thermal cycling was done in a T100 thermal cycler (Bio-Rad, Hercules, California, USA) at the following cycling conditions: Initial denaturation at 95 °C for 5 min, 34 repeated cycles of 95 °C for 1 min, 58 °C for 1 min, 72 °C for 1 min and a final elongation at 72 °C for 10 min. The amplified DNA (5 μl) was electrophoresed in a 1% agarose gel at 70 V for 60 min. Thereafter, the gel was stained in Ethidium bromide for 10 min and visualised under a UV light (Syngene, Cambridge. UK).

2.2.4. Digestion of vector and gene insert with restriction enzymes

Digestion of plasmid vector (pET15b) and gene insert (R5) was done using appropriate Fast Digest restriction enzymes: Nde1 and BamH1 in the presence of a compatible buffer 10X Fast Digest buffer (Thermo Scientific Fast Digest, Massachusetts, USA) at 37 °C for 30 min.

2.2.5. Construction of the recombinant cloning vector containing serine protease gene

The PCR product was extracted from the 1% agarose gel using DNA extraction kit. The amplified gene was inserted into pET15b cloning vector by ligation method. The gene insert (98.2 ng/μl) and vector (191.2 ng/μl) (molar ration of 3:1 respectively), DNA ligase (1 μl), 10x ligase buffer (1 μl) were added at final volume of 10 μl and incubated at 22 °C for 30 min. Transformation was carried out by heat shocking the *E. coli* DH5α competent cells prepared by $CaCl_2$ method at 42 °C for 1 min (Sambrook and Maniatis, 1989). After the screening of transformed colonies on LB agar ampicillin culture medium by colony PCR, plasmid preparation of clones showing prominent bands was done using plasmid DNA extraction kit. The recombinant plasmids were confirmed by colony PCR and restriction digest using Fast Digest (Xho1 and Hind III) restriction enzymes.

2.2.6. Analysis of sequence data

The recombinant clones were sequenced at a biotechnology lab (Inqaba Biotech, Pretoria, South Africa) and the sequences edited using Chromas Ver. 2.2.4 (Technelysium Pty Ltd, Brisbane, Australia). The analysis of the sequenced data and sequence similarity searches were performed using basic local alignment search tool (BLAST N) program of NCBI. Homology alignment was done with CLUSTAL W program and phylogenetic tree constructed using Mega 7 version 5.2 software (Tamura et al. 2011; Kumar et al., 2016).

2.2.7. Construction of expression plasmids

Serine protease gene was amplified by PCR using oligonucleotide specific primers: forward primer that contained a unique CATATG restriction site, 5^1 extension (ATT), N-terminal His tag and an ATG initiation codon and reverse primer that contained a unique GGATCC restriction site, 5^1 extension (AAA) and a TAG stop codon. The pET15b carries an N-terminal His-Tag sequence followed by a thrombin site and three cloning sites. The Nde1/BamH1 digested fragments of pET15b-gene was introduced into the BL21 expression host by heat shock transformation of the *E. coli* BL21 competent cells prepared by $CaCl_2$ method at 42 °C for 1 min (Sambrook and Maniatis, 1989).

2.2.8. Expression of serine alkaline protease gene in E. coli

E. coli BL21 cells transformed with recombinant clones (pET15b-gene) were grown in 100 ml of LB medium containing 50 μg/ml of ampicillin at 37 °C with shaking until the absorbance at 600 nm was approximately 0.6. Subsequently, expression of the serine protease gene was induced by adding 100 μL of 100 mM IPTG and incubating at 37 °C for 4 h. After centrifugation at 5000 x g for 30 min at 4 °C, pellets were suspended in 50 mM sodium phosphate buffer ($NaHPO_4$) pH 9.0 and then disrupted by sonication for 5 min with a 30-sec pulse. The cell components were separated into soluble and insoluble fractions by centrifugation at 12000 x g for 20 min.

2.3. Purification of recombinant enzyme (serine alkaline protease)

After induction for 4 h, cells were harvested in buffer (0.1 M Glycine NaOH buffer pH 9.0) containing 5 mM $CaCl_2$ and disrupted by sonication. The supernatant obtained by centrifugation was purified by affinity chromatography using the AKTA protein purification systems; FPLC P-900 UV-900 Frac-950 AKTA soft (GE Amersham Pharmacia Explorer Purifier, California, USA) and a sephacryl 200 column. Immobilized metal affinity chromatography (IMAC) was used to interact the amino acid residues and the divalent metal ions (cobalt) immobilized on the resins. Binding buffer (imidazole) was used to bind the his-tagged proteins and then washed un bound proteins using the wash buffer. The elution buffer was used to elute the purified his-tagged proteins. The active fraction with single peak was collected and dialyzed against the buffer. The recombinant enzyme was finally identified as a single band on SDS-PAGE and used for further biochemical characterisation.

2.4. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS PAGE)

Homogeneity and molecular mass of the purified protease was estimated by SDS polyacrylamide gel electrophoresis by the method described by Laemmli 1970 using mini Bio-Rad Sub Cell GT electrophoresis system (Bio-Rad Laboratories, California, USA). The enzyme samples were loaded on the gel along with 100 kDa Page Ruler prestained protein marker SMOBio PM2500 (Green Bio Research, California, USA) and the resolved proteins were visualised by Coomassie staining with Coomassie brilliant blue R-250 dye (Sigma Aldrich USA). SDS-PAGE was performed on 10% running gel as described by Laemmli, and the resolved proteins were visualised by Coomassie staining. 100 kDa Protein standard was used as molecular marker.

2.5. Characterisation of serine alkaline protease

2.5.1. Serine alkaline protease assay

Proteolytic activity was measured following the method of Kole et al., 1988 by using azocasein (Sigma) as a substrate. The reaction mixture contains 120 μL of enzyme extract, 480 μL of azocasein (1% w/v) dissolved in 0.1 M Glycine NaOH buffer (pH 10), this was incubated at 37 °C in a heating block for 30 min. The reaction was terminated by addition of 600 μL of Trichloro acetic acid (TCA), the samples were kept on ice for 30 min and centrifuged at 13,200 rpm for 15 min. 800 μL of the supernatant were neutralised by addition of 200 μL of 1.8N NaOH, mixed properly and absorbance was read at 440 nm using a Carry 60 UV–Vis Spectrophotometer (Agilent Technologies, California, USA). One unit of enzyme activity was defined as the amount which yielded an increase in A_{440} of 0.01 in 30 min at 37 °C.

2.5.2. Protein determination of serine alkaline protease

Protein contents of the alkaline protease enzyme extracts were determined by following the method of Lowry et al., 1951 with Bovine serum albumin (BSA) as standard. Protein extract, 20 μl was measured in two tubes and 80 μl distilled water was added to it. Distilled water was used as blank while BSA standard curve was equally set up, (200 μg) 2 mg/ml, 1000 μl of alkaline solution was added to all the tubes, mix thoroughly and allowed to stand for 10 min, 100 μl of Folin- C solution was added to all the test tubes and left for 30 min after which the optical density was read at 600 nm wavelength in a Carry 60 UV–Vis Spectrophotometer (Agilent Technologies, California, USA). The protein concentration was estimated using values extrapolated from the standard graph of protein.

Calculation:

Protein concentration (mg/ml) = Absorbance value/ Gradient

2.5.3. Determination of specific activity of serine alkaline protease

The Specific activity of an enzyme gives the measurement of the activity of the enzyme. This is the activity of an enzyme per milligram of total protein (expressed in units/mg). It is the amount of product formed by an enzyme in a given amount of time under given conditions per milligram of protein.

Specific activity of the serine alkaline protease was determined using the formula below.

Calculation:

Specific activity = Enzyme activity (units/ml)/Protein concentration (mg/ml)

(units/mg)

2.5.4. Effect of pH and temperature on enzyme activity and stability of serine alkaline protease

The following buffers were used to investigate the effect of pH on protease activity: 0.1 M Tris HCl (pH 3.0–7.0) and 0.1 M Glycine NaOH buffer (pH 8.0–11.0) containing 5 mM $CaCl_2$. The effect of pH on protease stability was determined by incubating aliquots of the purified enzyme in buffers with different pH values for 30 min at 37 °C. The optimum temperature for protease activity was determined over the range of 20–80 °C by the protease assay. Assay mixtures were equilibrated at the required temperature before adding the enzyme. The effect of temperature on protease stability was determined by incubating aliquots of the purified enzyme for 30 min in 0.1 M Glycine NaOH buffer (pH 10) containing 5 mM $CaCl_2$ at various temperature. The relative activity was then determined under standard assay conditions.

2.5.5. Effect of inhibitors on serine alkaline protease activity

The activity of the purified protease was determined following incubation with various concentrations (5, 10) mM of Phenyl methane sulfonyl fluoride (PMSF), Ethylene diamine tetra acetic acid (EDTA), Dichlorodiphenyltrichloroethane (DDT) and Mercaptoethanol at 37 °C for 1 h. The relative activity was determined spectrophotometrically following protease enzyme assay under standard conditions and the enzyme activity of the control (without inhibitors) was taken as 100%.

2.5.6. Effect of metal ions on serine alkaline protease activity

The effect of different metal ions (Cu, Fe, Zn, Mg, Mn, Ca, Na and Co) with varying concentrations of 5 and 10 mM respectively on the stability of the serine alkaline protease was studied. The assay was carried out by pre-incubating the enzyme and metal ions for 10 min at 40 °C then buffer and substrate were added and incubated for 30 min at 40 °C. The residual activity was calculated and the enzyme activity of the control (without metal ions) was taken as 100%.

2.5.7. Effect of solvents on serine alkaline protease activity

The effect of solvents (Acetone, Chloroform, Ethanol, Hexane and Propanol) on the stability of serine alkaline protease was studied by pre-incubating the enzyme with each solvent for 10 min at 40 °C and protease assay was done as earlier described. The relative activity was calculated and the enzyme activity of the control (without solvents) was taken as 100%.

2.5.8. Effect of surfactant on serine alkaline protease activity

The effect of Surfactants (Tween 20, Tween 80, Triton 100 and Sodium Dodecyl Sulphate) with varying concentrations of 0.5 and 1% on the stability of alkaline protease was carried out by pre-incubating the enzyme with each surfactant for 10 min at 40 °C and protease assay was done as earlier described. The residual activity was calculated and the enzyme activity of the control (without surfactants) was taken as 100%.

2.5.9. Effect of substrate concentration on serine alkaline protease activity

The effect of different level of substrate (azocasein) concentrations ranging from (1–40 mM) on serine alkaline protease activity was studied to determine the maximum rate of reaction (velocity) of the enzyme. The vmax and Km of the enzymes were determined using the Michaelis-menten curve.

2.5.10. Effect of substrate specificity on serine alkaline protease activity

The effect of different substrates (Casein, Bovine Serum Albumin, Azocasein and Gelatin) on serine alkaline protease activity was studied following the protease enzyme assay as described earlier.

2.5.11. Stability of serine alkaline protease towards commercial detergents

The stability of serine alkaline protease towards commercial detergent was done by preparing detergent solutions (Sunlight, Omo, Surf, Tide and Ariel) in 0.1 M Glycine NaOH buffer pH 10.0 to get a final concentration of 1, 10 and 20 mg/ml of each detergent. Prior to assay the detergent solutions were heated at 100 °C for 1 h to deactivate the endogenous protease. The enzyme was pre-incubated with each detergent with different concentrations for 10 min at 40 °C and protease assay was carried out. The residual activity was calculated and the enzyme activity of the control (without detergents) was taken as 100%.

2.5.12. Effect of serine alkaline protease on protein stain removal

The test fabrics (cotton) pieces (1.5 × 1.5) cm stained with egg yolk (5 µl) were taken in 250 ml Erlenmeyer flasks and subjected to different temperature (30, 40, 55 °C) treatments in 50 ml of reaction mixture under different sets as (i) Control (water only), (ii) Detergent (Ariel) 1% (v/v of 15 mg/ml) in water, (iii) Enzyme (100 U/ml) in water and (iv) Detergent 1% (v/v of 15 mg/ml) + Enzyme (100 U/ml) in water. Finally, water was replaced with buffer (50 mM Glycine NaOH, pH 10.0) to determine the effect of protease on stain removal. Stain removal was checked qualitatively by visualization.

2.6. Nucleotide sequence accession number

The nucleotide sequence of serine protease gene from *Bacillus subtilis* RD7 reported in this study has been deposited in the GenBank database under accession number **MN097797**.

2.7. Statistical analysis

All the experiments and enzyme assays were carried out in triplicate and the standard deviation for each test was calculated. The standard deviations (n = 3) are indicated as error bars. Statistical analysis of all results was carried out using Duncan multiple range test, Standard deviation and ANOVA accordingly using IBM SPSS Statistics Ver.22.0 (IBM Corporation, United States of America.). Statistically significant at ($p \leq 0.05$).

3. Results

3.1. Expression and purification of recombinant enzyme (serine alkaline protease)

The SDS PAGE result of the crude serine alkaline protease fractions from *Bacillus subtilis* RD7 shown in Fig. 1 indicates that the molecular weight of the expressed recombinant product was about 43 kDa which corresponds with the prediction by gene sequence. Fig. 1 also shows the result of the highest concentrated protease active fraction after purification retaining a single band at approximately 43 kDa. The obtained sequences were deposited in the GenBank database with accession number: (**MN097797**). The phylogenetic tree of the cloned gene R5 is shown in Fig. 2.

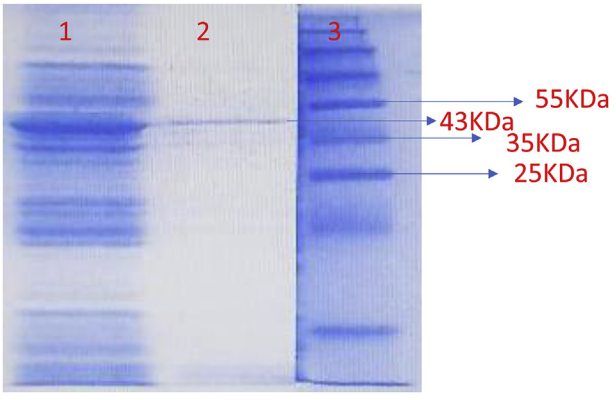

Fig. 1. SDS page image of r5 protein after purification. Lane 1 = Crude R5 protein; Lane 2 = Purified R5 protein; Lane 3 = Protein marker.

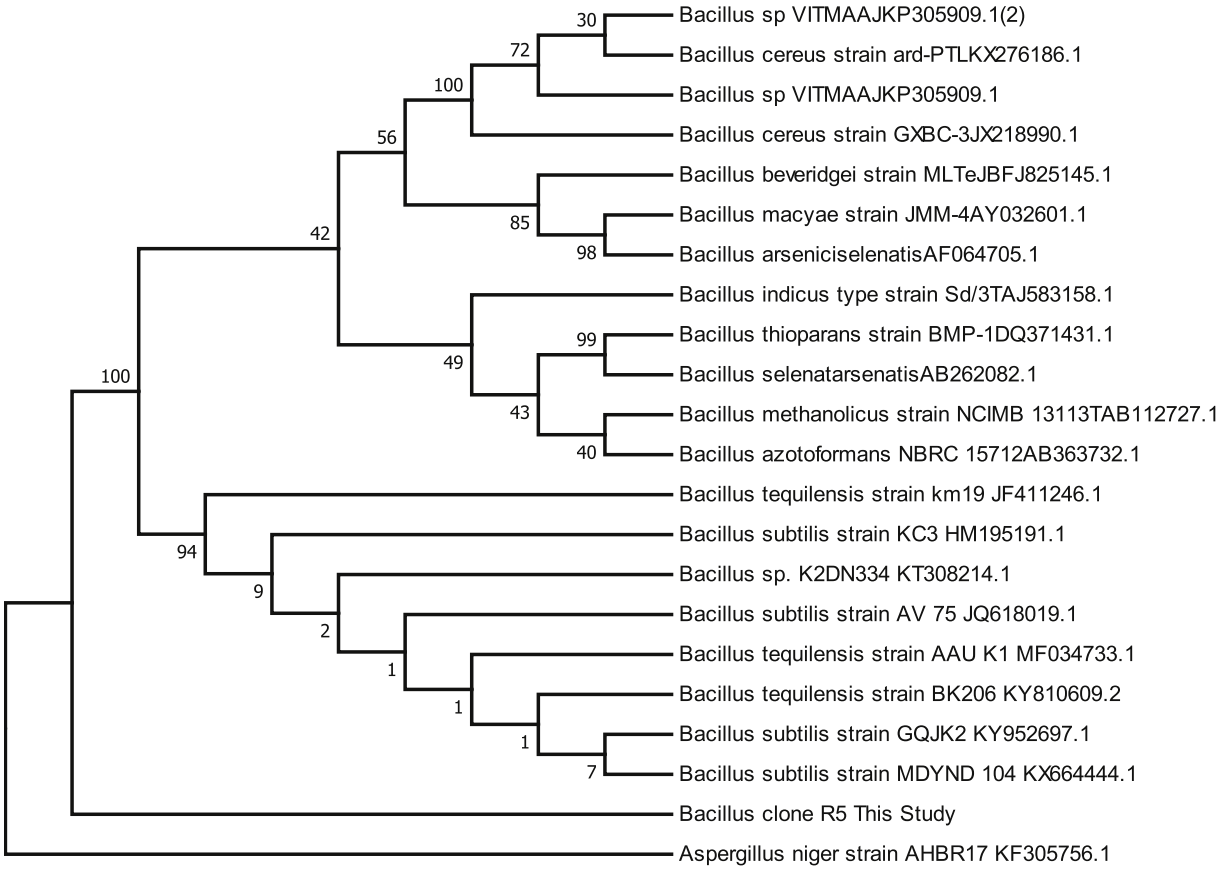

Fig. 2. Phylogenetic dendrogram based on the sequence of the cloned R5 gene from *Bacillus subtilis* strain RD7. Number in parenthesis are accession numbers of published sequences. Bootstrap values were based on 1000 replicates. (Saitou and Nei, 1987).

3.2. Characterisation of serine alkaline protease

The optimum serine alkaline protease was observed at pH 10 at 40 °C. (Figs. 3A and 4A). The enzyme activity and specific activity was found to be 606.73 U/ml and 6.24 u/mg/ml of protein respectively for the serine protease (Tables 1 and 2). Fig. 5 shows that the protease was inhibited by only PMSF as compared to the other inhibitors (EDTA, DDT and mercaptoethanol). Fig. 7 shows that Azocasein was the preferred substrate for protease enzyme production. Figs. 6, 8 and 9 shows the effect of metal ions, solvents and surfactants on protease activity. The vmax and Km of the protease was observed to be 4.248 and 2.80 respectively. Fig. 10 shows the effect of detergent solubility and stability on protease activity.

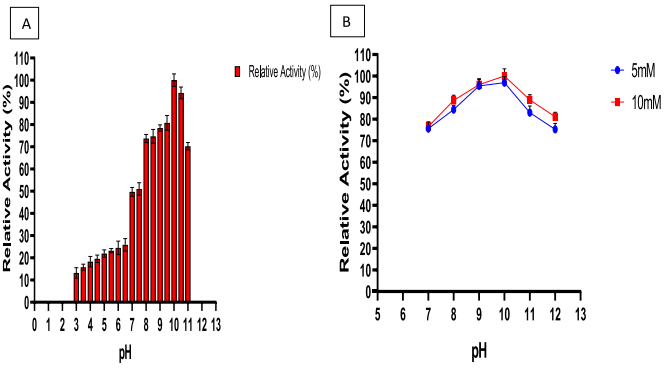

Fig. 4. Effect of pH and pH stability on protease activity.

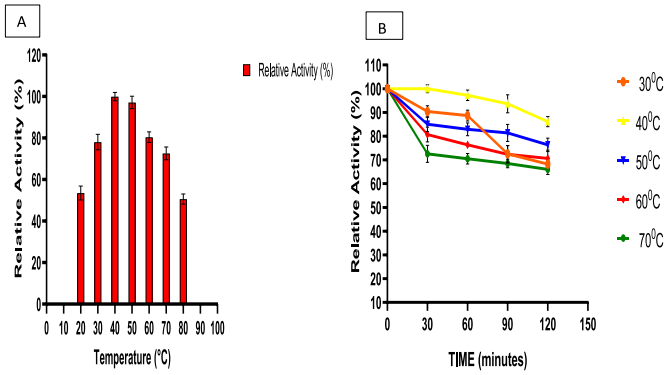

Fig. 3. Effect of temperature and temperature stability on protease activity.

Table 1
Serine alkaline protease enzyme profile.

	1	2	3	MEAN ± SD
Enzyme activity (U/ml)	615.03	600.92	604.24	606.73 ± 6.02
Protein content (mg/ml)	95.00	98.75	98.13	97.29 ± 0.7

Table 2
Specific activity of serine alkaline protease.

Enzyme activity (U/ml)	Protein content (mg/ml)	Specific activity (U/mg of protein)
606.73	97.29	6.24

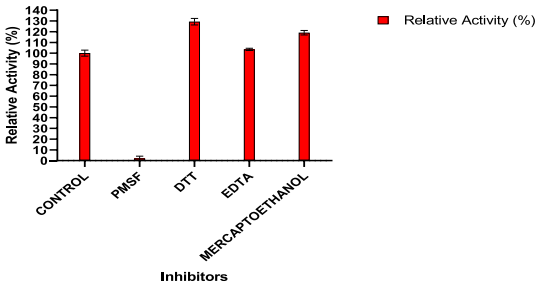

Fig. 5. Effect of inhibitors on protease activity.

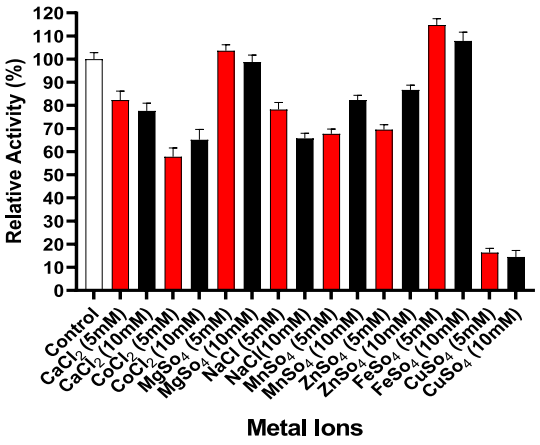

Fig. 6. Effect of metal ions on protease activity.

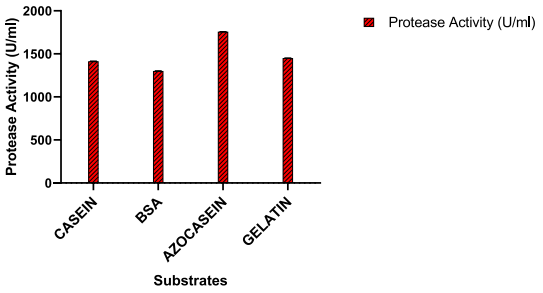

Fig. 7. Effect of substrate specificity on protease activity.

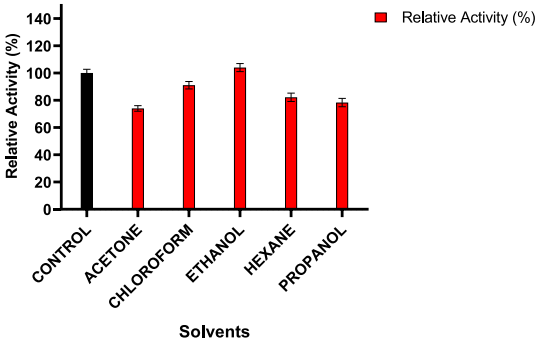

Fig. 8. Effect of solvents on protease activity.

3.3. Effect of serine alkaline protease on protein stain removal

The efficiency of serine alkaline protease (100 U/ml) in protein stain (egg yolk) removal was examined at 30, 40, 55 °C with 1% Ariel (v/v of 15 mg/ml). There was a little stain (egg yolk) removal with detergent and enzyme when used independently (Fig. 11). It took 2 h to completely remove the egg yolk stain at 30 °C whereas at 40 and 55 °C,

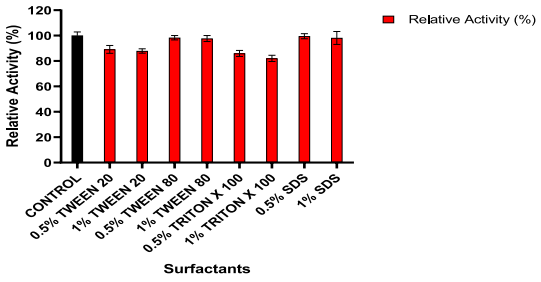

Fig. 9. Effect of surfactants on protease activity.

the stains were removed in 1 h and 30 min respectively. However, in the absence of enzyme, the time taken to remove stain was very long. As water was replaced with buffer (50 mM Glycine NaOH, pH 10.0), the stains were removed completely within 10 min at 40 °C with 100 U/ml enzyme and 1% (v/v of 15 mg/ml) detergent (Fig. 11).

4. Discussion

The aim of this study was to clone the serine alkaline protease gene from *Bacillus subtilis strain* RD7. In this work, new genes named (R5 gene) was cloned from *Bacillus subtilis strain* RD7. The R5 gene was expressed in BL21 expression host successfully and the expressed products had an approximate molecular weight of 43 KDa. The size of the PCR product (amplified R5 gene) conformed with the expected gene size of 1203bp. The results obtained from the digestion of the PCR product with the appropriate restriction enzymes confirmed the fidelity of gene amplification. After gene cloning, restriction of plasmid product with appropriate enzymes (Xho1 and Hind III) confirmed the integrity of the cloning process. Isolation of the gene insert from vector using restriction digest further confirmed the authenticity of the cloned product. Further confirmation of the identity of the cloned gene revealed 100% matching of the gene of interest with the translated protein sequence.

Some studies have been done on cloning alkaline protease gene from *Bacillus species*. The cloning of alkaline protease gene from *Bacillus subtilis* 168 by (Mohammad Sadeghi et al., 2009); molecular cloning and nucleotide sequence of the gene for alkaline protease from *Bacillus circulans* MTCC 7906 (Kaur et al., 2012); purification and characterisation of cloned alkaline protease gene of *Geobacillus stearothermophilus* (Iqbal and Ul-Haq1, 2014). However, in this study the R5 gene was cloned from *Bacillus subtilis* RD7 in pET15b cloning vector transformed in *E. coli* DH5α competent cell and expressed in *E. coli* BL21 expression host. This cloned gene was translated to a protein of the serine protease family with a 1203bp ORF encoding 400 amino acid protein.

The purity of the serine alkaline protease enzyme was confirmed by SDS PAGE and the molecular weight of the serine protease was found to be approximately 43 KDa (Fig. 1). A 35 KDa serine protease from *Bacillus pumilus* CBS and a 35 KDa manganese-dependent alkaline serine protease from *Bacillus pumilus* TMS55 have been previously reported (Jaouadi et al., 2008; Ibrahim et al., 2011). Similarly, a 38 KDa organic solvent and detergent stable protease was also reported from *Bacillus* specie RKY3 (Reddy et al., 2008). In addition, a 39 KDa purified protease was reported by Iqbal and Ul-Haq1, 2014 where they purified and characterised cloned alkaline protease gene of *Geobacillus stearothermophilus*. Therefore, the molecular weight of the cloned serine protease enzyme reported in this study was found to be in the range of some reported *Bacillus* proteases.

The expressed serine protease enzyme showed maximum protease activity of 606.73 U/ml (Table 1) which further confirms that the cloned gene was highly expressed in active form, therefore fulfilling the goal of cloning to produce large quantities of specific protein (serine alkaline protease) with high enzyme activity. The vmax and Km of the enzyme was observed to be 4.248 and 2.80 respectively.

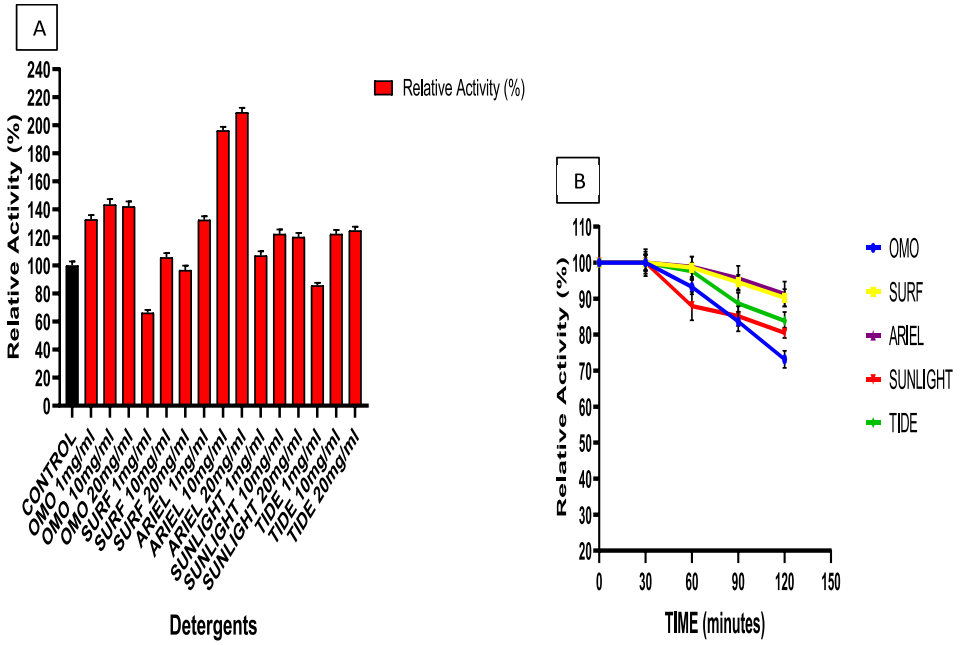

Fig. 10. Effect of detergent solubility and stability on protease activity.

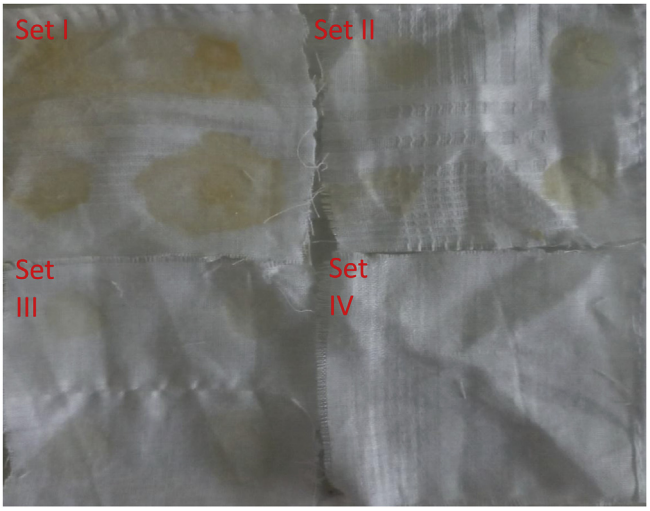

Fig. 11. Effect of serine alkaline protease expressed from *Bacillus subtilis* RD7 on removing egg yolk stains at 40 °C and pH 10.
Set I Control (Glycine NaOH buffer, pH 10)
Set II Detergent 1% (v/v of 15 mg/ml) in buffer (Glycine NaOH buffer, pH 10)
Set III Enzyme only (100 U/ml) in buffer (Glycine NaOH buffer, pH 10)
Set IV Detergent 1% (v/v of 15 mg/ml) + Enzyme (100 U/ml) in buffer (Glycine NaOH buffer, pH 10).

The serine protease enzyme showed activity in the wide range of pH from 6 to 11 with maximum protease activity at pH10 ($P \leq 0.05$), which reveals that the cloned enzyme is alkaline in nature. Some of the *Bacillus* derived proteases reported by Hongxia et al. (2015) showed high activity in a broad pH range of 8.0–11.0 and Asokan and Jayanthi, 2010 also recorded high activity at pH 9.0. Similarly, a halophilic bacterium isolated from sea water catalysed reactions in the pH range of 8–11 and performed optimally at pH 10 (Raval et al., 2014). The protease from *Bacillus subtilis* VSG-4 reported by Giri et al. (2011) had the maximum activity at pH 9 with a sharp decline in the activity in pH value lower than pH 9. This is in accordance with this study that revealed that the optimum activity of the serine protease was found to be pH 10. Further characterisation of the enzyme reveals that the protease was active at varied temperature. However, the optimum temperature was found to be 40 °C ($P \leq 0.05$). This agrees with other studies which reported a serine protease by *Bacillus subtilis* DR8806 showing highest activity of 45 °C and was stable at that temperature up to 70 °C (Farhadian et al., 2015). Similarly, Cha et al., 2005 reported that the protease from *Bacillus subtilis* SS103 was active at 37 °C and the alkaline protease from *Bacillus subtilis* VSG-4 was active over a range of temperature from 40 to 60 °C with an optimum at 50 °C. The enzyme retained 99.99 and 100.00% of its residual enzyme activities (Figs. 3 and 4) at 40 °C and pH 10 respectively.

One of the significant outcomes of the present study was the expression of R5 gene of *Bacillus subtilis* RD7 that showed stability after subjecting it to a range of temperatures (30–70 °C) under alkaline pH 7–11 for 2 h preincubation period. The pH and temperature stability of serine alkaline protease discussed in this study agrees with several findings of other purified proteases from *Bacillus mojavensis, Bacillus licheniformis, Bacillus linens, Bacillus subtilis* under similar conditions but only for 1 h of preincubation (Rattray et al., 1994; Durham et al., 1987; Beg and Gupta, 2003; Gödde et al., 2005). It is known that thermostable enzymes have rigid structure and higher temperature introduces flexibility and fluidity in their structural features (Khajeh et al., 2001; Sundaram et al., 1980). The temperature stability of the proteases makes them enzymes of choice for industrial uses. The pH tolerance of proteases has also been extensively studied due to their potential industrial applications. (Iqbal et al., 2015). The pH and temperature are the most crucial factors that allow proteases to be utilized in detergent industries (Sanatan et al., 2013; Verma et al., 2013).

R5 proteases retaining high activity and stability at high alkaline pH and temperature makes this enzyme a potential candidate to be explored for their use in the detergent and other related industries (Iqbal et al., 2015) Kumar and Takagi 1999; Horikoshii, 1999; Takami et al. 2000).

The serine protease enzyme was completely inhibited by serine protease inhibitor; Phenyl methane sulfonyl fluoride (PMSF) at both 5 and 10 mM concentration suggesting it is serine in nature (Adinarayana et al., 2003). The other tested inhibitors such as Dichlorodiphenyltrichloroethane (DDT), Ethylene diamine tetra acetic acid (EDTA) and Mercaptoethanol had little or no effect on the stability and activity of the protease which further confirms that the enzyme is not likely to be a metallo or cysteine protease. The DDT, mercaptoethanol and EDTA

showed an increase relative activity of 129.36, 119.09 and 103.52% (Fig. 5) respectively which means it enhanced the protease activity. This agrees with the work of Mothe and Sultanpuram (2016) that reported the tested alkaline protease was almost undisturbed in the presence of EDTA showing an increased relative activity of 96% and least activity of 30% in the presence of Na^{2+}.

The enzyme is very sensitive to PMSF indicating that the purified alkaline protease is a serine protease. PMSF is known to sulfonate the essential serine residue at the active site of the protease which result in a total loss of the enzyme activity (Deng et al., 2010). The serine protease described in this study has shown stability in the presence of chelating agents such as EDTA. These characteristics enhance the prospect of *Bacillus subtilis* R5 serine alkaline protease to be used as an additive in detergent industry. This is in accordance with the study of Kozlov et al., 2013 and Hirata et al. (2013) showing stability of protease by an addition of EDTA and extraction of thermophilic proteases in the presence of EDTA. In addition, Iqbal et al. (2015) also reported stability of *stearothermophilic* alkaline serine protease by EDTA. It is known that detergents containing high number of chelating agents such as EDTA functions as water softeners and assist in stain removal (Walsh, 2002).

Among the metal ions tested on the enzyme activity, Fe^{2+} and Mg^{2+} ions increased relative activity up to 114.77%, 103.65% and 107.89%, 98.70% (Fig. 6) at 5- and 10-mM concentrations respectively. A serine protease from *Bacillus subtilis* DR8806 was stimulated by K^{+}, Ca^{2+}, Mg^{2+} and Fe^{2+} at 10 mM concentration up to 134, 129, 128 and 112% respectively. Whereas, Na^{+} ions had no significant effect on enzyme activity (Farhadian et al., 2015). Metal-decreasing activity was observed in the presence of Co^{2+}, Ni^{2+} by Farhadian et al. (2015), Ibrahim et al. (2011), Jain et al. (2012), Priya et al. (2014) and Shah et al. (2010). In contrast, Mothe and Sultanpuram (2016) showed less activity in the presence of Fe^{2+} and Cu^{2+} ions.

The proteases were inhibited in the presence of heavy metal Cu^{2+} due to its toxicity for the protease with relative activity of 16.40 and 14.47% at 5 and 10 mM respectively. Heavy metals are known to chelate enzymes causing their precipitation and deactivation (Pandey et al., 2013). Enzymes are known to consist of several metals and the substitution or displacement of one of the metal ions with the same charge or size results in inhibition of enzyme activity. Fe^{2+} is the most powerful prooxidant among metal ions. (Parasad, 2004).

Azocasein was found to be the preferred substrate for protease produced by *Bacillus subtilis*. (Fig. 7). However, the enzyme could hydrolyze several other proteins like Casein, Bovine serum albumin (BSA), Gelatin etc which is an important characteristic of this alkaline protease (Mothe and Sultanpuram, 2016; Adinarayana et al., 2003) reported that casein was a good substrate for protease produced by *Bacillus subtilis*. Also, proteases produced by *Bacillus haloduran* S373 CAS6 (Annamalai et al., 2013). *Bacillus cereus* TK U006 (Wang et al., 2009) and *Bacillus subtilis* DR8806 (Farhadian et al., 2015) showed the most activity towards casein as a substrate.

It has been noted that different substrates show different specificity for protease, however in this study proteases from *Bacillus subtilis* is highly specific for azocasein because it showed maximum enzymatic activity which reveals its higher specificity for this substrate as compared with others. This could be due to maximization of the binding energy of the substrate for the enzyme which is used to determine the substrate affinity (Weiner and Williams, 1995). This is in accordance with (Freeman et al. 1993; Phadatare et al. 1993) showing high activity towards native and modified proteins including azocasein, keratin, elastin etc.

Different organic solvents exerted their effects on the stability of the enzyme activity with Ethanol having 104% where moderate effect was found with chloroform (91.02%) and hexane (82.17%) while propanol (78.36%) and acetone (74.04%) (Fig. 8) lowered the alkaline protease activity. This is in accordance with Farhadian et al. (2015), showing that ethanol and methanol at all tested concentrations strongly enhanced enzyme activity as compared to the control. In contrast Jain et al. (2012) showed that the protease was activated with hexane. In addition, Rai and Mukheerje demonstrated subtilisin-like specie isolated from *Bacillus subtilis* DM04 increased its activity with hexane, methanol and ethanol (Rai and Mukherjee, 2010).

The increased enzyme activity or stability in the presence of organic solvent is also an important factor to produce chiral compounds in non-aqueous solvents (Zaks and Klibanov 1988). Organic solvent such as Ethanol, methanol etc induces the protease activity and cause structural changes in the enzyme which in turn results in increased catalytic activity (Peek et al., 1992).

According to literature, a good detergent protease must be compatible and stable with all commonly used detergent compounds such as surfactants, bleaches, oxidizing agents etc which might be in the formulation (Gupta et al., 2005).

The serine proteases from *Bacillus subtilis* in the study shows that the presence of the anionic surfactant SDS improved the interaction of the enzyme with substrate and this may result in increased relative activity (99.46 and 98.15) % at 0.5% and 1% SDS respectively as compared to other surfactants (Fig. 9). This is in accordance with the work of Mothe and Sultanpuram (2016) that reported increased relative activity with SDS than Tween 20 (Cheng et al., 2010). also reported that the protease produced from *Bacillus alcalophilus* TCCC11004 was stable in 0.5% SDS and retained 70.3% of its initial activity after 1 h of incubation. A decrease in relative activity (85.95 and 82.05) was seen in 0.5% and 1% of the non-ionic surfactant Triton X 100. This inhibition may be because of a reduction in hydrophobic interactions and protein-protein contacts that play a role in stabilizing the protein tertiary structure (Traut et al., 1989).

To confirm the compatibility of protease with detergent, the data showed that the enzyme is extremely stable in the presence of Ariel and Surf retaining about 91.31 and 90.29% of its initial activity (Fig. 10). However, the enzyme was found to be least stable in the presence of Omo retaining only about 73.15% of its initial activity. According to literature excellent detergent enzymes are expected to be active in the presence of laundry detergents during washing conditions.

Serine alkaline proteases have been incorporated as biobuilders into detergents to elicit their hydrolytic effect by removing proteineaceous materials in stained clothes (Ito et al., 1998; Kobayashi et al., 1995). These enzymes remove not only the obvious stains, such as blood, but also other less obvious materials including proteins from body secretion and skin particles, and food such as egg yolk (Fig. 11), fish, meat and milk. In the absence of proteases, proteinaceous dirt coagulates on the fabric as a result of washing conditions. The high temperature, pH and action of surfactants and sequestering agents used in washing processes dissolve or disperse most of the dirt components, and the bleaching agents decomposes the undissolved dye. This process causes the protein material to precipitate on to the fabric and failure to remove the proteinaceous dirt results in a grey and unclean appearance of the fabric after several washings (Kalisz, 1988).

5. Conclusion

In conclusion, research and development activities involved in developing bacteria species for overexpression of protease will provide information as to the type of genetic alteration/mutation that occurs causing the organism to over express the protease. This study has cloned and characterised protease from a locally isolated *Bacillus subtilis* RD7. Its desirable characters such as stability at high pH, stability at high temperature, broad substrate specificity are all significant characteristics of any enzymes for industrial applications.

Data availability

All sequencing data have been deposited in National Centre for Biotechnology Information (NCBI) under assertion number **MG255317 and MN097797**.

Declaration of interest

The authors declare no conflicts of interest.

Funding

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Acknowledgments

We thank Mr S.O.A. Olatope, Dr Ajit Kumar and Dr Collins Odjadjare for their skillfull technical assistance. We would like to appreciate reviewers for helpful suggestions and comments to improve our manuscript.

References

Adinarayana, K., Ellaiah, P., Prasad, D.S., 2003. Purification and partial characterization of thermostable serine alkaline protease from a newly isolated Bacillus subtilis PE-11. AAPS PharmSciTech 4, 56–63.

Annamalai, N., Rajeswari, M.V., Thavasi, R., Vijayalakshmi, S., Balasubramanian, T., 2013. Optimization, purification and characterization of novel thermostable, haloalkaline, solvent stable protease from *Bacillus halodurans* CAS6 using marine shellfish wastes: a potential additive for detergent and antioxidant synthesis. Bioproc. Biosyst. Eng. 36, 873–883.

Asokan, S., Jayanthi, C., 2010. Alkaline protease production by Bacillus licheniformis and Bacillus coagulans. J. Cell. Tissue Res. 10 (1), 2119–2123.

Beg, Q.K., Gupta, R., 2003. Purification and characterization of an oxidation-stable, thiol-dependent serine alkaline protease from Bacillus mojavensis. Enzym. Microb. Technol. 32, 294–304.

Cha, M., Park, J.R., Yoon, K.N., 2005. Purification and characterization of an alkaline serine protease producing angiotensin I-converting enzyme inhibitory peptide from *Bacillus* sp. SS103. J. Med. Food 8, 462–468.

Cheng, K., Lu, F.P., Li, M., Liu, L.L., Liang, X.M., 2010. Purification and biochemical characterization of a serine alkaline protease TC4 from a new isolated *Bacillus alcalophilus* TCCC11004 in detergent formulations. Afr. J. Biotechnol. 9, 4942–4953.

Choudhary, V.J., 2013. Recovery of silver from used X-ray films by *Aspergillus versicolor* protease. J. Acad. Ind. Res. 2, 39–41.

Deng, A., Jie, W., Yun, Z., Guogiang, Z., Tingyi, W., 2010. Purification and characterization of a surfactant-stable high alkaline protease from *Bacillus sp*. B001. Bioresour. Technol. 101, 7100–7106.

Durham, D.R., Stewart, D.B., Stellwag, E.J., 1987. Novel alkaline- and heat-stable serine proteases from alkalophilic *Bacillus sp*. strain GX6638. J. Bacteriol. 169, 2762–2768.

Farhadian, S., Asoodeh, A., Lagzian, M., 2015. Purification, biochemical characterization and structural modeling of a potential htrA-like serine protease from *Bacillus subtilis* DR8806. J. Mol. Catal. B Enzym. https://doi.org/10.1016/j.molcatb.2015.02.001.

Freeman, S.A., Peek, K., Prescott, M., Daniel, R., 1993. Characterization of a chelator-resistant proteinase from Thermus strain Rt4A2. Biochem. J. 295, 463–469.

Giri, S.S., Sukumaran, V., Sen, S.S., Oviya, M., Banu, B.N., Jena, P.K., 2011. Purification and partial characterization of a detergent and oxidizing agent stable alkaline protease from a newly isolated Bacillus subtilis VSG-4 of tropical soil. J. Microbiol. 49, 455–461.

Gödde, C., Sahm, K., Brouns, S.J., Kluskens, L.D., 2005. Cloning and expression of islandisin, a new thermostable subtilisin from *Fervidobacterium islandicum* in *Escherichia coli*. Appl. Environ. Microbiol. 71, 3951–3958.

Gupta, A., Roy, I., Patel, R.K., Singh, S.P., Kahre, S.K., Gupta, M.N., 2005. One-step purification and characterization of an alkaline protease from haloalkaliphilic *Bacillus* sp. J. Chromatogr. A 1–2, 103–108.

Hirata, A., Hori, Y., Koga, Y., Okada, J., et al., 2013. Enzymatic activity of a subtilisin homolog, Tk-SP, from *Thermococcus kodakarensis* in detergents and its ability to degrade the abnormal prion protein. BMC Biotechnol. 13, 1–7.

Hongxia, C., Liping, W., Yang, Y., 2015. Production and characterization of alkaline protease from a high yielding and moderately halophilic strain of SD11 Marine Bacteria. J. Chem 8, 798304.

Horikoshii, K., 1999. Alkalophiles: some applications of their products for biotechnology. Microbiol. Mol. Biol. Rev. 63, 735–750.

Ibrahim, K.S., Muniyandi, J., Karutha Pandian, S., 2011. Purification and characterization of a manganese dependent alkaline serine protease from *Bacillus pumilus* TMS55. J. Microbiol. Biotechnol. 21, 20–27.

Iqbal, Ul-Haq1, Ikram, 2014. Purification and characterization of cloned alkaline protease gene of *Geobacillus stearothermophilus*. J. Basic Microbiol. 54, 1–12.

Iqbal, Irfana, Aftab, Muhammad Nauman, Afzal, Mohammed, Rehman, Asad Ur, Aftab, Saima, Zafar, Asma, Ud-Din, Zia, Khuharo, Ateeque Rahman, Iqbal, Jawad, 2015. Purification and characterization of cloned alkaline protease gene of *Geobacillus stearothermophilus*. J. Basic Microbiol. 55, 160–171.

Ito, S., Kobayashi, T., Ara, K., Ozaki, K., Kawai, S., 1998. Alkaline detergent enzymes from alkaliphiles: enzymatic properties, genetics and structure. Extremophiles 21, 85–190.

Jain, D., Pancha, I., Mishra, S.K., Shrivastav, A., Mishra, S., 2012. Purification and characterization of haloalkaline thermoactive, solvent stable and SDS-induced protease from *Bacillus* sp.: a potential additive for laundry detergents. Bioresour. Technol. 115, 228–236.

Jaouadi, B., Ellouz-Chaabouni, S., Rhimi, M., Bejar, S., 2008. Biochemical and molecular characterization of a detergent-stable serine alkaline protease from *Bacillus pumilus* CBS with high catalytic efficiency. Biochimie 90, 1291–1305.

Joshi, S., Satyanarayana, T., 2013. Characteristics and applications of a recombinant alkaline serine protease from a novel bacterium *Bacillus lehensis*. Bioresour. Technol. 131, 76–85.

Kalisz, H.M., 1988. In: In: Fietcher, A. (Ed.), Microbial Proteinases in Advances in Biochemical Engineering/biotechnology, vol 36. Springer-Verlag, Berlin), pp. 3–61.

Kaur, Inderjeet, Singh Kocher, Gurvinder, Gupta, V.K., 2012. Molecular cloning and nucleotide sequence of the gene for an alkaline protease from *Bacillus circulans* MTCC 7906. Indian J. Microbiol. 52 (4), 630–637.

Khajeh, K., Khezre-Barati, S., Nemat-Gorgani, M., 2001. Proteolysis of mesophilic and thermophilic a-amylase. Appl. Biochem. Biotechnol. 94, 97–109.

Kobayashi, T., Hakamada, Y., Adachi, S., Hitomi, J., Yoshimatsu, T., 1995. Purification and properties of an alkaline protease from alkalophilic *Bacillus* sp. KSM-K16. Appl. Microbiol. Biotechnol. 43, 473–481.

Kole, M.M., Draper, I., Gerson, D.F., 1988. Production of protease by *Bacillus subtilis* using simultaneous control of glucose and ammonium concentrations. J. Chem. Technol. Biotechnol. 41 (3), 197206.

Kozlov, S.N., Nikolaev, V.B., Markov, E., Urbanovich, L., Mironova, L.V., 2013. Membrane-bound proteases of ompTþ and ompT-Vibrio cholerae strains. Zh. Mikrobiol. Epidemiol. Immunobiol. 2, 3–12.

Kumar, C.G., Takagi, H., 1999. Microbial alkaline proteases: from a bioindustrial viewpoint. Biotechnol. Adv. 17, 561–594.

Kumar, S., Stecher, G., Tamura, K., 2016. MEGA7: molecular evolutionary genetics analysis version 7.0 for bigger datasets. Mol. Biol. Evol. 33, 1870–1874.

Laemmli, U.K., 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227 (5259), 680–685.

Lowry, O.H., Rosebrough, N.J., Farr, A.L., Randall, J.R., 1951. Protein measurement with Folin phenol reagent. J. Biol. Chem. 242, 265–275.

Mohammad Sadeghi, M., Rabbani, M., Naghitorabi, 2009. Cloning of alkaline protease gene from *Bacillus subtilis* 168. Res. Pharm. Sci. 4 (1), 43–46.

Mothe, T., Sultanpuram, V., 2016. Production, purification and characterization of a thermotolerant alkaline serine protease from a novel species *Bacillus caseinilyticus*. Biotech 6, 53. https://doi.org/10.1007/s13205-016-0377-y.

Pandey, S., Ghosh, P.K., Ghosh, S., De, T.K., Maiti, T.K., 2013. Role of heavy metal resistant *Ochrobactrum sp*. and *Bacillus spp*. strains in bioremediation of a rice cultivar and their PGPR like activities. J. Microbiol. 51, 11–17.

Parasad, M.N.V., 2004. Heavy Metals Stress in Plants: from Biomolecules in Ecosystem, second ed. Springer-Verlag, Berlin; Heidelberg, pp. 99.

Peek, K., Daniel, R.M., Monk, C., Parker, L., Coolbear, T., 1992. Purification and characterization of thermostable proteinease isolated from Thermos sp. strain Rt41A. Eur. J. Biochem. 207, 1035–1044.

Phadatare, S.U., Deshpande, V.V., Srinivasan, M.C., 1993. High activity alkaline protease from *Conidiobolus coronatus* (NCL 86.8.20): enzyme production and compatibility with commercial detergents. Enzym. Microb. Technol. 15, 72–76.

Priya, J.D., Divakar, K., Prabha, M.S., Selvam, G.P., Gautam, P., 2014. Isolation, purification and characterisation of an organic solvent-tolerant Ca^{2+}-dependent protease from *Bacillus megaterium* AU02. Appl. Biochem. Biotechnol. 172, 910–932.

Proteases Market by Source, 2016. Availablefrom:http://www.marketsandmarkets.com/Market-Reports/proteases-market184780427.html .

Rai, S.K., Mukherjee, A.K., 2010. Statistical optimization of production, purification and industrial application of a laundry detergent and organic solvent-stable subtilisin-like serine protease (Alzwiprase) from *Bacillus subtilis* DM-04. Biochem. Eng. J. 48, 173–180.

Rattray, F.P., Bockelmann, W., Fox, P.F., 1994. Purification and characterization of an extracellular proteinase from Brevibacterium linens ATCC 9174. Appl. Environ. Microbiol. 61, 3454–3456.

Raval, V.H., Pillai, S., Rawal, C.M., Singh, S.P., 2014. Biochemical and structural characterization of a detergent-stable serine alkaline protease from seawater haloalkaliphilic bacteria. Process Biochem. 49, 955–962.

Reddy, L.V.A., Wee, Y.J., Ryu, H.W., 2008. Purification and characterization of an organic solvent and detergent-tolerant novel protease produced by *Bacillus* sp. RKY3. J. Chem. Technol. Biotechnol. 83, 1526–1533.

Saitou, N., Nei, M., 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol. Biol. Evol. 4, 406–425.

Sambrook, J., Maniatis, T., 1989. Molecular Cloning: a Laboratory Manual, second ed. Cold Spring Harbor Laboratory Press, New York, USA.

Sanatan, P.T., Lomate, P.R., Giri, A.P., Hivrale, V.K., 2013. Characterization of a chemostable serine alkaline protease from *Periplaneta americana*. BMC Biochem. 14, 32.

Shah, K., Mody, K., Keshri, J., Jha, B., 2010. Purification and characterization of a solvent, detergent and oxidizing agent tolerant protease from *Bacillus cereus* isolated from the Gulf of Khambhat. J. Mol. Catal. B Enzym. 67, 85–91.

Singh, R., 2016. Microbial enzymes: industrial progress in 21st century. 3 Biotech 6 174. 2.

Singh, S., Bajaj, B.K., 2015. Medium optimization for enhanced production of protease with industrially desirable attributes from *Bacillus subtilis* K-1. Chem. Eng. Commun. 202, 1051–1060.

Singh, S., Gupta, P., Sharma, V., Koul, S., Kour, K., Bajaj, B.K., 2014. Multifarious potential applications of keratinase of *Bacillus subtilis* K-5. Biocatal. Biotransform. 2, 333–342.

Souza, P.M., Bitten court, M.A., Magalhaes, P.O., 2015. A biotechnology perspective of fungal proteases. Braz. J. Microbiol. 46, 337–346.

Subba, R.C., Sathish, T., Ravichandra, P., Prakasham, R., 2009. Characterization of

thermo and detergent stable serine protease from isolated *Bacillus circulans* and evaluation of eco-friendly applications. Process Biochem. 44, 262–268.

Suberu, Y.L., Akande, I., Samuel, T., Lawal, A., Olaniran, A., 2019. Optimization of protease production in indigenous *Bacillus* species isolated from soil samples in lagos, Nigeria using response surface methodology. Biocatalysis Agric. Biotechnol. 18, 101011.

Sundaram, T.K., Wright, I.P., Wilkinson, A.E., 1980. Malate dehydrogenase from thermophilic and mesophilic bacteria. Molecular size, subunit structure, amino acid composition, immunochemical homology, and catalytic activity. Biochemistry 19, 2017–2022.

Takami, H., Nakasone, K., Takaki, Y., Maeno, G., 2000. Complete genome sequence of the alkaliphilic bacterium *Bacillus halodurans* and genomic sequence comparison with Bacillus subtilis. Nucleic Acids Res. 28, 4317–4331.

Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M., Kumar, S., 2011. MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Mol. Biol. Evol. 28, 2731–2739. https://doi.org/10.1093/molbev/msr121.

Traut, R., Casiano, C., Zecherle, N., Creighton, T., 1989. Protein Function: A Practical Approach. IRL Press, Oxford,UK, pp. 101–133.

Verma, A., Ansari, M.W., Anwar, M.S., Agrawal, R., Agrawal, S., 2013. Alkaline protease from Hermoactinomyces sp. RS1mitigates industrial pollution. Protoplasma 251, 711–718.

Walsh, G., 2002. Proteins: Biochemistry and Biotechnology. Wiley and Sons, Chichester, UK, pp. 421–436.

Wang, S.L., Chao, C.H., Liang, T.W., Chen, C.C., 2009. Purification and characterization of protease and chitinase from *Bacillus cereus* TKU006 and conversion of marine wastes by these enzymes. Mar. Biotechnol. 11, 334–344.

Weiner, B.D., Williams, W.V., 1995. Chemical and Structural Approaches to Rational Drug Design. CRC Press, USA, pp. 163–164.

Zaks, A., Klibanov, A.M., 1988. Enzymatic catalysis in nonaqueous solvents. J. Biol. Chem. 263, 3194–3200.

Zambare, V., Nilegaonkar, S., Kanekar, P., 2011. A novel extracellular protease from *Pseudomonas aeruginosa* MCM B-327: enzyme production and its partial characterization. N. Biotech. 28, 173–181.