INT. J. BIOL. BIOTECH., 12 (3): 347-353, 2015.

CLONING AND EXPRESSION OF SERINE ALKALINE PROTEASE FROM A LOCALLY ISOLATED *BACILLUS SUBTILIS*

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ABSTRACT

Serine Alkaline Protease (SAP) gene was isolated from a locally isolated strain of *Bacillus subtilis*. The gene was amplified and the size of the gene was found to be 1162bp. It was cloned in chemically competent *E coli* DH_5 α cells and was transformed on plate for blue white selection of colonies. Plasmid was isolated after picking the white colonies. Double digestion was done by *Hind III* and *Nde I* and the result was confirmed by running the sample on agarose gel. Colonies showing positive results were transformed in expression vector pET 21 a. Expression was checked by running the sample on SDS PAGE that confirmed the gene was fully expressed.

Key words: Proteolytic, cloning, gene, plasmid, *E. coli*, fermentation.

INTRODUCTION

Proteases represent the major commercially exploited group of enzymes which accounts almost 60% of total global enzyme production (Herbert, 1992). Among them serine alkaline proteases are most widely exploited enzymes because in past recent years there is much increase of alkaline protease as industrial catalysts and there is expected upward increase of this enzyme in future (Kumar *et al.*, 1999). They are advantageous over chemical catalysis due to many reasons i.e. high catalytic activity, substrate specificity and production in increased quantities and also cost effective. They are produced by yeast, molds, fungi, bacteria, plants and animals. Bacterial proteases are characterized by working at alkaline pH, high substrate specificity and high optimum temperature (Rao *et al.*, 1998). The most dominant species producing microbial proteases is *Bacillus* species. A group of different species of *Bacillus* commencing different environments have been studied for production of protease but prospective source of protease producing species are *Bacillus subtilis* and like *B. cereus*, *B. lichniformus*, *B. amyloliquefaciens* and *B. majovensis* (Gupta *et. al.*, 2002). Enzymes from *Bacillus* species can withstand high temperature, pH, non-aqueous environment and chemical denaturing agents (Johnvesly and Naik, 2001). The enzyme obtained from these species have predominant share in the worldwide market of industrial enzymes (Beg *et. al.*, 2003, Wang *et. al.*, 2006).

Alkaline *Bacillus* protease have good properties that meet the requirements of detergent industries, a significant share among them is of protease produced by *Bacillus* species (Horikoshi, 1992). The serine alkaline protease from *B. subtilis* is also used in the degradation of waste produced by feathers of poultry industry (Dalev, 1994). Another important application is use of alkaline protease on X-ray film which helps in degrading the gelatinous coat thus recover the silver without producing waste material (Horikoshi, 1999).

Random mutagenesis has been used to increase the production of the proteases. But this technique is hazardous and time consuming because many cells are crudely mutagenized and then many thousands of them are analyzed to isolate the correct one (Brannigan and Wilkinson 2002). Cloning is done as an alternative to this method and expression is done for getting protease with required quantities and qualities. By this method new enzymes with suitable characters are produced and then used for different purposes (Rao *et al.*, 1998). One major advantage of cloning is that the desired gene can be isolated from different sources such as thermophilic or hyper thermophilic strains and then cloned in mesophilic host for over expression. Thus enzyme can be produced easily at laboratory conditions. *E. coli* is widely used and successful mesophilic host to produce recombinant proteins. The features that contribute to make *E. coli* as a successful host include well-known genetics, cultivation simplicity, high transformation efficiency, inexpensiveness and rapidity. Suitable strains of *E. coli* such as JM109, DH5 α, K12 and Nova Blue have been developed. They are convenient hosts for initial cloning of target DNA into different cloning and expression vectors, for maintaining plasmids, possess high transformation efficiencies and also well known for good plasmid yields. The vector used is often PTZ57R/T because it contains TA overhangs and gives one step PCR cloning product. It has ddT over hangs at its two ends and the PCR product containing ddA is ligated with it a circular molecule having two nicks is generated. The advantage of this process is that it prevents recircularization of plasmid vector in the process of ligation. The DNA can be excised from vector and can be sub cloned in other vector (Clark, 1988).

The vector used for sub cloning is pET which is bacterial plasmid specifically designed enabling rapid production of genetically modified protein in increased quantities. pET plasmid has important features like *lac*I gene coding for *lac* repressor protein, a T7 promoter which is specific for T7 RNA polymerase only; it is not found in any other genome of prokaryote, *lac* operator which helps to block transcription, an ampicilin resistance gene and *col* E1 origin of replication (Blaber, 1998). To develop T7 lac promoter system *lac* promoter in combination with T7 RNA polymerase promoter is used. This system is not only powerful and efficient for the transcription and translation of target proteins but also presents different characters of *lac* promoter to prevent the leaky expression under regulatory system. For the induction of T7 lac promoter system IPTG is used. This chemical is not degraded by the cell and thus, helps in regulation of expression of the desired protein is easy to control **(**Kilikian, 2000).

Once protein is expressed, the next step is its production. This process is done by fermentation. The protein can be produced in large quantities by different methods of fermentation in relatively short times by these genetically modified microorganisms. As a result, they produce an abundant and regular supply of product (Gupta *et al.,* 2002). In any successful fermentation the overall cost of enzyme production along with down streaming process is major focusing step in enzyme industry (Gupta *et al.,* 2002). Commonly protease production has been practiced using submerged fermentation. Submerged fermentation is done using a substrate that is dissolved or remain suspended in the medium containing water (Sumantha *et al.,* 2006). The extracellular protease production is affected by media used and different conditions of the system i.e. glucose concentration, carbon and nitrogen ratio and metal ions (Verala *et al.,* 1996; Beg *et. al.,* 2002). Different other physical factors such as inoculum density, pH, temperature, aeration and agitation and time of incubation should also be kept in mind (Gupta *et al.,* 2002).

MATERIALS AND METHODS

Bacterial Strains and Vector Selection

Escherichia coli DH5α harboring the recombinant cloning vector pTZ57R/T was obtained from Fermentas Inc., Hanover, Germany. *Bacillus subtilis* and *E .coli* strain BL21 codon plus were obtained from the Institute of Industrial Biotechnology, GC University Lahore. pTZ57R/T cloning vector was selected due to its versatile characters yielding recombinants as high as 90%. This vector does not require many procedures for the modification of PCR products and is a ready to use vector. The expression vector used in the present study was pET.

Primer Designing

From gene sequence of *Bacillus subtilis* primers for PCR were designed using software primers 3.0 and Fast PCR. Different features (melting temp. and GC contents) of the selected primers were then found by Oligonucleotide Properties Calculator. Primers used for polymerase chain reaction were 5' GCCATATGGAGAGGATAAAGAGTGAGAGGCAAA 3' with melting temperature 63°C and GC content 44% as forward primer and 5' GCGAATTCTTACTGAGCTGCCGCCTGTAC 3' with melting temperature 66°C and GC content 57% as reverse primer.

Isolation of Chromosomal DNA

10 mL culture of *B. subtilis* was used to isolate of genomic DNA. The broth was centrifuged at 6500 *g* for ten min at room temp. The pellet was separated and dissolved in 100 microlitre of TEN buffers (10mM Tris-HCl, 1mM EDTA, 10mM NaCl) and centrifuged at 6500 rpm for 5 min. After centrifugation, the supernatant discarded and pellet was dehydrated. Then freshly prepared 100µl SET buffer was mixed with the pellets and later on 50µL of lysozyme (10mg/mL) was also added. After gentle mixing, 500µL of TEN buffers and 100µL of 5Molar NaCl was added. It was centrifuged at 6500 *g* for 10 min after adding equal volume of phenol: chloroform (1:1) mixture. The viscous upper layer was collected and chloroform was added in it. It was centrifuged again. The precipitation of DNA was done by adding chilled absolute ethanol. DNA could be stored at 20 °C. The DNA was air dried and finally dissolved in either, double distilled sterilized water or TE buffer. The extracted deoxyribonucleic acid was analyzed by agarose gel containing ethidium bromide. The DNA band in the gel was checked by using UV transilluminator and picture was taken using DOC gel documentation system (SynGene) using GeneSnap software.

PCR Amplification and Gene Clean

PCR amplification of Serine Alkaline Protease gene was done. The PCR product was analyzed using 0.8% agarose gel. The fragments of PCR product were separated from gel by using QIA quick PCR purification kit (Qiagen, Hilden, Germany). The QIA quick spin column was kept in a provided 2mL collection tube and the gel solution was placed in it. It was centrifuged at high speed for 30 mins. About 0.5 mL of QG buffer was placed to the column and centrifuged again for 1 min. Then 0.75mL of PE buffer (wash buffer) was added containing ethanol and

centrifuged at high speed for 1 min. The supernatant was discarded from the reservoir tube. To elute DNA, 38µl of pre warmed (50°C) EB buffer (10mM Tris-Cl, pH 8.5) was added and incubated at 50°C for 3 min. Then mixture was centrifuged at high speed for 1 min. After that the second elution was done stored at -20°C.

Ligation and Preparation of Competent Cells

Ligation was done using QIAGEN ligation kit using the reagents. Competent cells were prepared by using 1mL of overnight culture of *E. coli* DH5α, which was inoculated into 100ml of LB medium and incubated with vigorous shaking in a shaker until O.D reaches to 0.6. The cells were then collected in 50ml falcon tube, cooled for 15 min and centrifuged in a pre-cooled centrifuge at 6000 rpm for 10 min at 4°C. The pellet obtained was suspended in 20 mL ice cold 50mM $CaCl_2$ and incubated on ice for 40 min and centrifuged again at 6000 rpm for 10 min at 4°C. The pellet was suspended in 2 ml ice cold 50mM $CaCl_2$ and kept on ice for 30 min. 200µl of these cells were streaked on LB-Ampicillin plate to check the contamination of the cells.

Transformation

The competent cells were thawed and 200µL of competent cells were mixed rapidly with 15µL ligation mixture and incubated on ice for 40 mins. Transferred to a preheated (42°C) water bath for 2 min and were quickly put on ice for 5 min. This is done for destruction of membrane of cells. Then cells were mixed with 800µL LB broth and regenerated at 37°C for 1 hour in an incubator. Bacterial cells were then plated on LB plates containing the required antibiotic ampicillin (100mg/ml), IPTG (0.1M) and X-Gal (40mg/ml) for selection of recombinants i.e. by blue white colonies.

Preparation of X-gal, IPTG Plates

135µL of X-gal (40mg/mL), 800µL of Ampicillin (100mg/mL) and 130µL of 0.1 M IPTG was added in 100mL LB agar medium then poured into petri plates. The plates were left for at least 30 min before spreading at 37°C. The 100µL of transformed and regenerated culture was spread on to the LB agar plates. Plates were incubated overnight at 37°C.

Expression of gene in *E. coli* BL 21 Codon Plus

Plasmid isolation was done by alkaline lysis method (Birnboim and Doly, 1979) and analysed by agarose 0.8 % gel electrophoresis. From the positive clones, a single colony was picked and inoculated in 10 mL of starter culture having ampicillin (100mg/mL) in 50 mL of flask at 37°C, 200 rpm for overnight. Culture was refreshed by inoculation of 50 mL LB ampicillin media with 1 mL of overnight culture. The flasks were placed in shaker at 37°C, 200 rpm until O. D reached to 0.6. Then all the flasks were given heat shock in shaking incubator at 42°C, 150rpm for 1 hour. For the process of induction, 250 µL IPTG (100mM) was added and kept in a shaker at 37°C and200 rpm. After 24 hours, cells were harvested from 10 mL of culture and centrifuged at 6000 rpm for 15 min. The supernatant was stored at 4°C, to check the enzyme that was secreted extracellularly. The pallet was suspended in 500 µL 50 mM Tris-Cl pH 8.0. The sample was centrifuged at 6000 rpm for 15 min after sonication. Pallet was discarded and supernatant was saved to check the activity of enzyme. Same procedure was done after harvesting from 48 and 72 hours culture. SDS-PAGE was done to observe the different bands for the expression of the gene. Image was saved by the gel documentation system (Syngene) containing different bands.

Activity assay of serine alkaline protease

The method of McDonald and Chen (1965) was used to find the activity of protease in which casein was used as a substrate. 1 m1 of the enzyme extract was taken in the test tube and 4.0 ml of 1.0% casein was added in it. Enzyme sample was then incubated for 1 hour at 35°C. The residual protein was then precipitated by addition of 5ml of 5% trichloroacetic acid. Then the precipitates were permitted to settle for 30 min and then centrifuged for 5 min at 5000 rpm. One ml of supernatant was mixed with 1 ml of NaOH and 5ml of alkaline reagent. 0.5ml of Folin and Ciocaltaue reagent was added after 10 min as a result, blue colour appeared. Then the optical density of the mixture was read on spectrophotometer at 700 nm (Cecil-CE7200-series, Aquarius, UK).One unit of protease activity is defined as the amount of enzyme required to produce an increase of 0.1 in optical density under defined conditions

RESULTS AND DISCUSSION

Genomic DNA was isolated using method by Kronstad *et al.*, (1983) and was run on agarose gel electrophoresis which showed the presence of required band (Fig1). The band resides above the 10 kb marker as it is total DNA of

the *Bacillus subtilis.* Priest and his fellows (1994) used the same procedure of DNA isolation and isolated the DNA of *Bacillus thuringensis.*

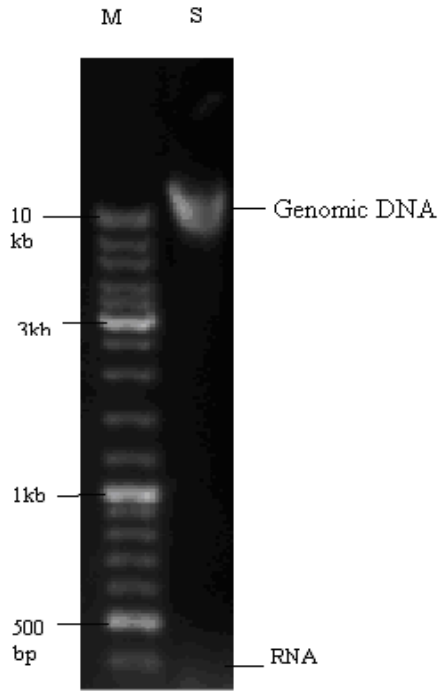


Fig. 1. Genomic DNA isolated from *Bacillus subtilis*.

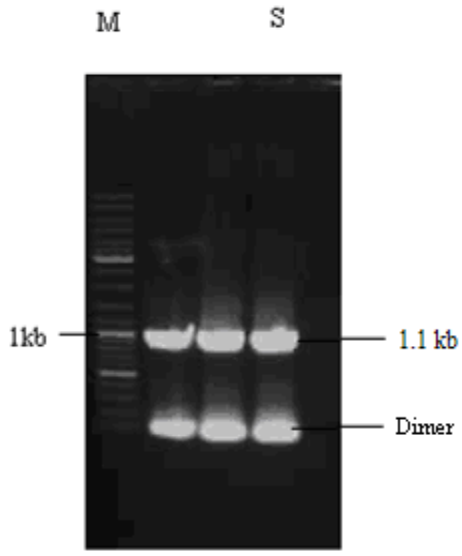


Fig. 2. PCR product of the desired gene (M: Marker; S: Sample)

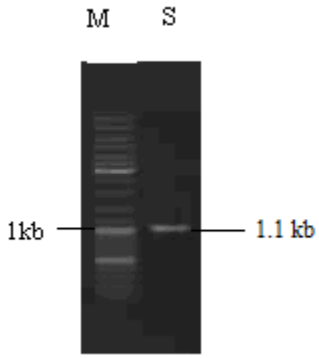


Fig. 3. Blue white colonies selection

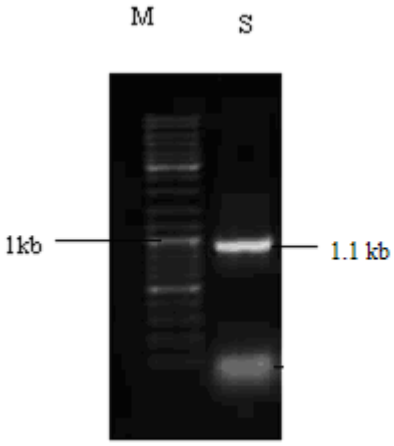


Fig. 4. Colony PCR of white colony (M: Marker; S: Sample)

The primers for the SAP were designed as described in material and method section. The serine alkaline protease gene was amplified by PCR following initial denaturation, annealing, extension and final extension steps. Agarose gel electrophoresis was employed for the analysis and purification of PCR product. The size of PCR product appeared on the gel was 1.1 kb (Fig.2). Sun and his co workers (2004) performed PCR with specific primers and conditions to isolate the serine alkaline protease gene from *Bacillus* sp WRD-2.

The gene was cleaned by using QIAGEN Quick gel Extraction Kit. Figure 3 shows the result when agarose gel electrophoresis of the gene was done. The PCR product was then ligated with pTZ57R/T vector and transformed into a chemically competent *E. coli* $DH_5\alpha$ cells. Figure 4 shows the growth of productively transformed cells on plate having X-gal and IPTG with ampicillin. The blue and white colonies appeared on the plates. The white colonies were of transformed cells. Tsujibo and his fellows (1997) ligated the amplified DNA by using Ligation

Pack (Nippon Gene, Inc., Tokyo, Japan). This ligation mixture was incubated at 4°C overnight. Aftab *et al.*, *(*2012) also used the same procedure of ligated DNA into pTZ57/RT checked it by blue white colonies.

Fig. 5. Bands of isolated plasmids from white colonies (M: Marker; S: Sample)

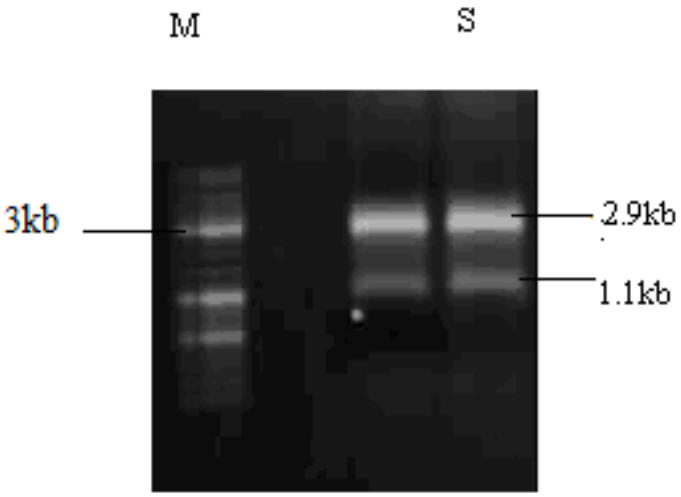


Fig. 6. Double digestion with *Nde*I and *Hind*III (M: Marker; S: Sample)

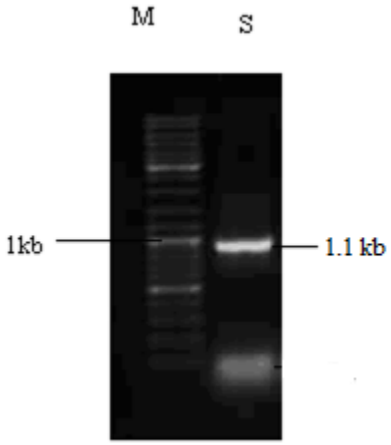


Fig. 7. Colony PCR of Transformed *E. coli* $DH_5\alpha$

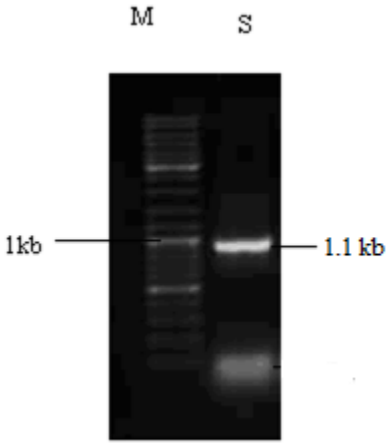
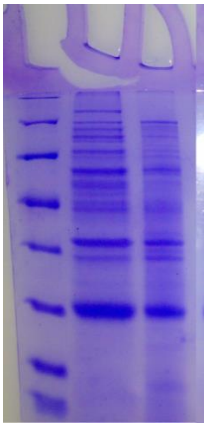


Fig. 8. Colony PCR (M: Marker; S; Sample)



Lane 1: marker, Lane 2: control (pET 21), Lane 3: heat treated serine alkaline protease.

Fig. 9. SDS PAGE Gel showing production of recombinant serine alkaline protease in cell lysate.

A white colony was taken from IPTG X-gal plate and colony PCR was done. The colony PCR product was analyzed on agarose gel. It showed a product of 1.1 kb figure 5. Colony PCR is useful as it is quick and easy method to identify the right clones without labor of growing and then isolating genes from them.

White colonies were selected for the plasmid isolation by mini-prep method in order to confirm transformation. Isolated plasmid was run on agarose gel elctrophoresis along with ladder (Fermentas gene ruler DNA Ladder Mix). The plasmid appeared as bans on the gel as shown in Figure 6. Different workers used different methods to isolate plasmids. For example, plasmids have been retrieved and then purified with the Magic Miniprep DNA Purification System (Promega Corp) (Li and Ujungdahl, 1994). Plasmid DNA from *E. coli* had also been purified with the Qiagen kit (Qiagen Inc., Chatsworth, Calif.) (Tsujibo *et al.,* 1997).

The plasmid DNA was treated with *Nde* I and *HindIII* enzymes in order to carry out double digestion as shown in Fig. 7. Aftab *et al.,* (2012) used same enzymes i.e *Hind III* and *NdeI* to double digest the plasmid DNA and to get the specific gene band at agarose gel.

Genetically modified pET 21(a)/gene were distorted in *E. coli* $DH_5\alpha$. Colony PCR was done to screen the positive clones. Transformed Colony with positive results is shown in Fig. 8.Plasmids isolated from different colonies was used to get transformation in the expression host cells that is *E. coli* BL21 Codon Plus. Colony PCR of positive colonies was done. It showed a band of 1.1 kb as in Fig 9.

The activity assay of enzyme was done by the method of McDonald and Chen (1965) and it showed the activity of 6U/ml. To get expression, recombinant gene was induced with 0.5mM IPTG and serine alkaline protease was successfully expressed in cell lysate using SDS PAGE that was stained with coomassie brilliant blue. The band of protein was observed at 40 kDa in the cloned sample as shown in fig 10. Many workers used SDS PAGE for checking the protein expression and deduced proteins of predicted molecular weights (Pan *et al.,* 2004; Aftab *et al.,* 2012). Tang *et al., (*2004) predicted the weight of 30.5 kDa of extracellular protease using SDS PAGE.

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(*Accepted for publication June 2015*)