

Isolation of a fibrinolytic enzyme BKII gene from local isolate of *Bacillus*

Zahid Mushtaq* and Amer Jamil

Molecular Biochemistry Lab, Department of Chemistry & Biochemistry, University of Agriculture, Faisalabad-38040-Pakistan

Abstract

Regulation and production of Fibrinolytic enzymes from bacterial sources especially from *Bacillus* strains has taken a leading role in the medical sciences for the treatment of cardiovascular disorders as it removes thrombus or clots adding to its significant role in curing human health issues saving millions. Significant progress has been made during the last few years on the studies of fibrinolytic enzymes in identifying, cloning, purification, characterization and overproduction of these for commercialization in medical sciences and in fields like detergents development. In our present research an act was made to isolate and clone a metalloprotease fibrinolytic enzyme Bacillokinase (BKII) gene from a local isolate of *Bacillus spp.* This enzyme has strong fibrinolytic activity and also reported to be plasminogen activator. Doubos salts media with a pH of 7.2 at 40 °C was employed for the maintenance of growth of locally isolated specie of *Bacillus*. PCR amplification of fibrinolytic enzyme gene was done using specifically designed primers from genomic DNA isolated from the bacterial cell culture under optimized conditions. The amplified product of 1381 base pairs including the peptide signal sequence along with ORF was then ligated into pTZ 57 R/T cloning vector and transformed in *E. coli* Top10 cells. The clone was then confirmed by restriction analysis. This study can further help in the study of genetic diversity of these enzymes, their characteristics, regulation and hyper production for its different applications.

Key words: *Bacillus*, Bacillokinase gene, fibrinolytic enzyme, cloning, PCR

Full length article Received: 29-06-2012

Revised: 15-07-2012

Accepted: 23-07-2012

Available online: 31-07-2012

*Corresponding Author, e-mail: zahidbiochemist@yahoo.com ; amerjamil@yahoo.com

1. Introduction

Thrombosis, that is the formation or presence of a thrombus (a clot of coagulated blood attached at the site of its formation), in a blood vessel is one of the most widely occurring diseases in modern life, which often causes disturbance in haemostasis a leading cause of cardiovascular diseases causing disability and death. According to world health organization (WHO), 17 million people die of such cardiovascular diseases (CVDs) every year [18]. Consequences like imbalance in the haemostasis i.e. balance between coagulation and anticoagulation, may result in excessive bleeding or formation of a thrombus that can adhere to the unbroken wall of the blood vessels. Fibrin can accumulate in the blood vessels which can interfere with blood flow and lead to myocardial infarction and other serious cardiovascular diseases. Unless the blockage is removed promptly, the tissue that is normally supplied with oxygen by the vessel will die or be severely damaged [15]. If damaged tissue or region is large, the normal conduction of electrical signals through the ventricle will be disturbed, leading to irregular heartbeat, cardiac arrest or even death [14].

In earlier times treatment of thromboembolic vascular disease was dependent on the use of anticoagulants, such as heparin and warfarin (coumarin) to inhibit the formation of fibrin clots. However, recognition that lysis of preformed fibrin could be accomplished *in vivo* by a process involving the conversion of inactive plasminogen to an active enzyme plasmin which led to an alternative enzyme based approach [3].

There are twenty enzymes that assist in clotting of blood in human body, while only one can break it down, called plasmin or any other plasmin like protease [1]. Fibrinolytic enzymes dissolve the blood clots. Further insoluble fibrin fiber is hydrolyzed into fibrin degradation products by plasmin, which is generated from plasminogen by plasminogen activators such as the tissue plasminogen activator (t-PA), vascular plasminogen activator, blood plasminogen activator, Hageman factor, urokinase and streptokinase plasminogen complex [20]. Failure of fibrin / clot degradation leads to disorders that require medications using fibrinolytic enzymes as effective remedy. There are many fibrinolytic or thrombolytic agents that convert plasminogen to plasmin. Currently, several thrombolytic

agents such as streptokinase, urokinase, reteplase (r-PA), prourokinase, alteplase (t-PA), reptilase, brinase and anisoylated purified streptokinase activator complex (APSAC) are available for clinical use. Several other agents derived from different microbial and non-microbial sources are also an important dimension of today's research. All these thrombolytic agents still suffer significant shortcomings like requiring large therapeutic doses, short plasma half-life, limited fibrin specificity, reocclusion and bleeding complications [3].

Fibrinolytic enzymes have been purified, cloned and studied from many plants, animals and microbial sources [3]. Microorganisms are important source for thrombolytic agents. staphylokinase from *Streptococcus aureus* and Streptokinase from *Streptococcus hemolyticus* were earlier proved to be effective in thrombolytic therapy [6]. Streptokinase (SKC), a conventional fibrinolytic drug that is produced by some strains of beta-hemolytic streptococci is included in the world health organization's (WHO) model list of essential medicines (WHO, 2005). It is used in life threatening deep-vein thrombosis, acute myocardial infarction, pulmonary embolism and thromboembolism like disorders. Fibrinolytic enzymes from microbial sources have been reported from various species of *Bacillus*, *Pseudomonas*, *Staphylococcus*, *Alteromonas*, *Coryneform* bacteria, *Penicillium*, *Aspergillus*, *Fusarium*, *Trichotecium*, *Actinomyces*, *Streptomyces* and *Esherichia coli* [3,4,25].

The fibrinolytic enzymes from bacterial strains have great importance regarding agents especially from *Bacillus* sp. because of their efficiency in fibrinolytic process including plasmin activation. They also have an industrial application as detergents [9]. *Bacillus* species produces a variety of fibrinolytic enzymes including nattokinase (NK) from *Bacillus natto* [12], substilin DFE [16] and substilin DJ-4 from *Bacillus amyloliquifaciens* [21]. A fibrinolytic enzyme (FE) from *Bacillus substilis* LD-8547 has also been reported by Wang [24]. The genus *Bacillus* from fermented foods is also an important source to produce fibrinolytic enzymes [18]. Other microbes like *Streptomyces* sp. [23] and *Enterococcus faecalis* [27] are strong producers of fibrinolytic enzymes. Regarding bacterial pathogenesis, plasmin and the system of fibrinolysis is very important as it facilitates tissue invasion by dissolution of the fibrin that acts as barrier at the site of infection, by hydrolysis of extracellular matrix proteins such as lamin or fibronectin, and by the activation of latent collagenases and other zymogen forms of metalloproteinases [5]. Evidences showed that patients with pulmonary embolism when treated with streptokinase and urokinase are three times more likely to show clot resolution than those patients taking heparin alone [15]. However, it has a short half-life and it needs continuous infusion to achieve its greatest efficacy [22]. There are two different types of thrombolytic agents depending upon the mechanism of their action. One is activator of plasminogen, such as tissue-type plasminogen activator (t-PA) [7], and urokinase [8], which activate plasminogen into active plasmin to degrade fibrin. The other type is plasmin-like proteins which directly degrades fibrin in blood clots, thereby digesting the thrombi rapidly and completely [9].

Almost all fibrinolytic enzymes that is subtilisin of *Bacillus* origin belong to serine type. All these fibrinolytic enzymes have high substrate specificity to fibrin, different from other proteases with broad substrate specificity. Furthermore, protease genes from several fungi, bacteria and viruses have been cloned and sequenced with the prime aims of overproduction of the enzyme, delineation of the role of the enzyme in pathogenicity, and alteration in enzyme properties to suit its commercial application. Despite the extensive research on several aspects of proteases, there is a paucity of knowledge about the genetic analysis of fibrinolytic enzymes. Understanding the role and contribution of each fibrinolytic enzyme to the overall fibrinolysis by an organism is important when selecting *Bacillus* strains for commercial production of fibrinolytic agents. Although different fibrinolytic enzymes are still widely used in thrombolytic therapy, researchers are still looking for more cheaper and safer means of fibrinolytic enzyme sources. *Bacillus* specie has been recognized as being safe for humans [29]. Accordingly, the isolation of diverse *Bacillus* strains and characterization of their fibrinolytic enzymes are important, as the ability to compare many different fibrinolytic enzymes will generate useful information towards developing strains with enhanced fibrinolytic capabilities. Considering these an attempt was made to isolate Bacillokinase (BKII) gene from *Bacillus* of our local environment. BKII which belongs to extracellular metalloprotease family having strong proteolytic, fibrinolytic and amidolytic activity showing optimal activity at pH 7.0 and at 50 °C favourable for use of human, it also showed clear preference for the plasminogen for fibrinolysis (plasminogen activator type) [9].

Owing to these properties and functions the present project was therefore designed to isolate a gene encoding a fibrinolytic enzyme BKII from a locally isolated strain of *Bacillus* sp. that could be used in pharmacological and clinical research in future.

2. Materials and methods

2.1. Selection of bacterial strain

A locally isolated strain of *Bacillus* sp. was employed for the isolation of a fibrinolytic enzyme gene. The strain identification as *Bacillus* sp. was confirmed by the Institute of microbiology, University of Agriculture, Faisalabad.

2.2. Growth conditions for bacterial culture

Dubous salts medium [19] was used for obtaining bacterial growth of locally isolated *Bacillus* strain obtained from the department of Microbiology, University of Agriculture, Faisalabad. Dubous salts medium K₂HPO₄ (1%), NaNO₃ (2%), KCl (1%), MgSO₄ (1%), Yeast extract (0.2%) as w/v and Sterilized glucose (as carbon source) as medium ingredients were employed to obtain growth of bacterial cells. The pH of the medium was maintained at 7.2 with 1.0 N HCl / NaOH before sterilization. The medium was autoclaved at 121 °C for 20 minutes at 15 psi pressure. Bacterial culture was transferred to sporulation medium slants and plates aseptically. The slants and plates were incubated at 40 °C for 24 hours for sporulation.

2.3. DNA isolation

The microbial pellet of the *Bacillus* specie was employed for Genomic DNA isolation. The isolation of the nucleic acid avoiding RNA by use of RNases was according to the protocols defined by Yamada [26]. The DNA isolation was confirmed on 1% agarose gel (w/v in 1X TAE) and stained with ethidium bromide. The DNA samples were stored at -20 °C till further step. Further quantification of DNA was done by spectrophotometric analysis. The purity of the DNA was checked by finding A260/280 ratio. Quantity was determined in ng/mL.

2.4. Polymerase chain reaction (PCR)

Fibrinolytic enzyme's gene (FE) was amplified by PCR in 50 µL reaction mixture from the genomic DNA of locally isolated *Bacillus* sp. using specifically designed primer according to the reported sequence [9] with specific restriction sites using online bioinformatics software (www.JustBio.com & <http://simgene.com/Primer3>). The optimized conditions of the reaction mixture were 50 ng of Genomic DNA, 10X PCR buffer (Fermentas), 2.5mM dNTPs (Mixture), Forward and reverse primers 0.1-1µM each, 1-5 mM MgCl₂ & 1.25 U/50 µL reaction of *Taq* DNA Polymerase using Gene Amp PCR System 2400 as thermocycler (Perkin Elmer, USA). The amplification was done using 1 minute hold for initial denaturation at 95 °C followed by 35 cycles of repeated denaturation (94 °C for 1 min), annealing (56 °C for 1 min) and extension/polymerization (72 °C for 2 min) followed by extended polymerization of 10 min at 72 °C. The primers utilized were P1:5'-GGGATCCAACCTGCTGCAGCGGCG-3', P2:5'-GGATCCGTTCCGGCGTGATATCGGCCAGCG-3' with *Bam*H I cleavage sites were included at the 5'-ends. The amplicons were confirmed on 1% agarose gel electrophoresis in 1X TAE. For further cloning step increased volume of the product was eluted and recovered/extracted from agarose gel by the help of Gel Extraction Kit following the manufacturer's protocol (Fermentas) which was also confirmed electrophoretically.

2.5. Cloning of Fibrinolytic enzyme's gene

2.5.1. Competent cells

Glycerol stock (stored at -80°C) of manually prepared TOP10 strain of *E. coli* with 0.1M MgCl₂ & 0.1M CaCl₂ was used for the transformation of the recombinant vector [33].

2.5.2. Ligation

The PCR amplified gene was cloned in into cloning vector PTZ57 R/T (Fermentas). The ligation reaction was set up as given in Table 1;

Table 1. Ligation mixture setup for ligation of *BKII* gene into vector pTZ 57 R/T

Serial No.	Components	Quantity
1	Plasmid vector pTZ57 R/T	2 µL
2	Purified PCR fragment	6 µL
3	5 x ligation buffer	4 µL
4	T4 DNA ligase, 5 U	1 µL
5	Deionized nuclease free water	Up to 20 µL

A control ligation was performed using 4.0 µL of control PCR fragment provided with the kit. The reaction mixture was incubated overnight at 16 °C for maximum yield of positive recombinants.

2.5.3. Transformation

For cloning the gene the ligation mixture was transformed by heat shock method at 42 °C for 2 minutes in *E-coli* top10 competent cells. It was allowed to grow in LB broth for an hour and then spread on the LB-Ampicillin-Agar plates (yeast extract 0.5% w/v, Bacto tryptone 1%, NaCl 1% and agar 1.75% w/v alongwith a final concentration of 100 µg/mL Ampicillin) in a sterilized environment. The positive clones on Blue-white screening using IPTG & X-GAL were confirmed by either restriction digestion with *Bam*HI/ *Eco*RI restriction enzymes or by colony PCR using recombinant cells as template for the reaction and already specific primers of gene P1 & P2. For digestion the miniprep plasmid DNA isolation was done following the protocol of Sambrook [33]. Control reactions accompanied at each step of the experiments conducted. The products at each stage were visualized on UV-illuminator after agarose gel electrophoresis.

3. Results and Discussion

Fibrinolytic enzymes are now-a-days the major fibrinolytic (thrombolytic) agents used widely for therapeutical purposes for the treatment of thrombosis that is responsible for various cardiovascular diseases. Fibrinolytic enzymes therefore produced by different microorganisms have been reported [9]. Among them, *Streptococcus haemolyticus* produces streptokinase [13] and staphylokinase produced by *Staphylococcus aureus* [2] have been studied most extensively. Presently this enzyme being approved by WHO and is extensively being used in the treatment of cardiovascular disorders. The fibrinolytic enzymes from *Bacillus* sp. have great interest as thrombolytic agents because of their use being safer and efficiency in fibrinolysis process including plasmin activation. They also have an industrial application in detergent production as they produce a variety of both extracellular and intracellular proteases [9]. Fibrinolytic enzymes have been previously reported from a number of *Bacillus* species like Bacillokinase II (BKII) from *Bacillus subtilis* Strain A1 [9], an enzyme from *Bacillus* sp. BK-17 [10], fibrinolytic enzymes (QK-1 and QK-2) from *Bacillus subtilis* QK02 [11], aprE86-1 gene of mature fibrinolytic enzyme from *Bacillus amyloliquefaciens* CH86-1 [31], a strong fibrinolytic enzyme (FE) from *Bacillus subtilis* LD-8 [24] and a similar effort cloning fibrinolytic enzyme (subtilisin gene) from *Bacillus subtilis* PTCC1023 [30], still many more fibrinolytic enzymes are being reported from different species of *Bacillus*. We amplified the fibrinolytic enzyme gene from *Bacillus* species, and cloned the gene in pTZ57 R/T vector. The growth condition for the *Bacillus* that was locally isolated was similar to the conditions followed by Rajoka & Bashir [19] employing dubos salts medium. The cellular mass of this local isolate was then used DNA isolation following the method of Yamada [26]. RNA contamination was subjected to RNases treatment for its removal. The isolated DNA was separated by gel

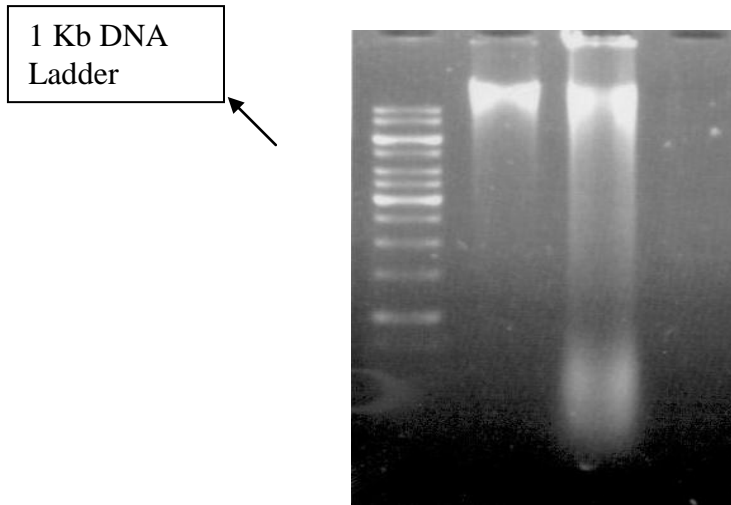


Fig. 1. DNA samples extracted from locally isolated *Bacillus* spp. Lane 2 and 3 shows DNA samples S1& S2 respectively and lane 1 shows the marker (M) 1 kb DNA Ladder (Fermentas).

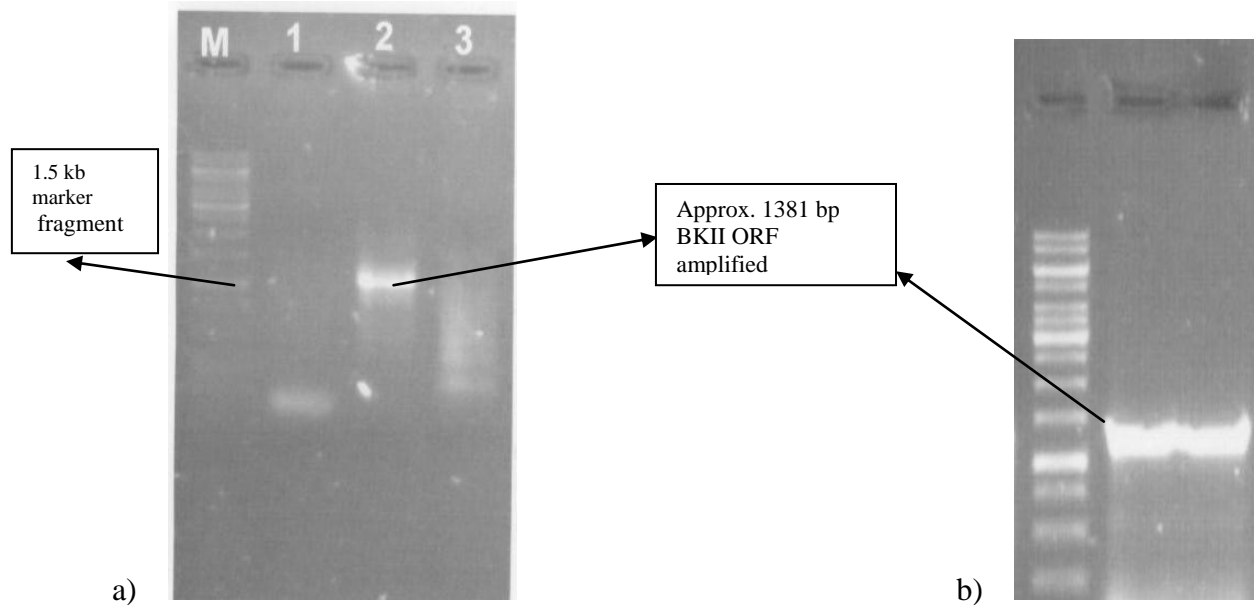


Fig. 2. Fibrinolytic enzyme Bacillokinase gene (BKII) amplification from *Bacillus* sp. (A) Lane 3 shows unsuccessful amplification (Smear) with 10 pmol primer. A control reaction without DNA was run in lane 1 alongwith 1 kbp ladder (Fermentas) (B) the figure shows 30 μ L elution on 0.8% agarose gel for gel extraction.

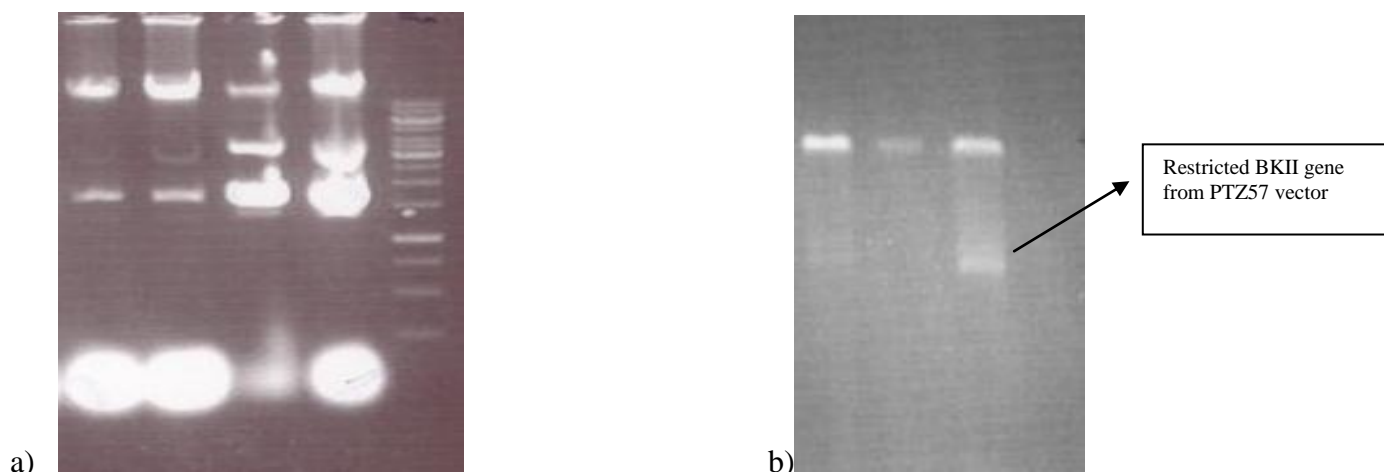


Fig. 3. Results of Mniprep plasmid DNA isolation and restriction digestion of recombinants of BKII: a) Lane 1-4 shows the results of miniprep from recombinants of BKII plate along with 1 Kb Fermentas marker in lane 5. (b) The figure shows result of double digestion shown by the release of insert (1.38 kb) in lane 3 alongwith PTZ57 (2.8kbp) vector preceding above it (Fermentas).

electrophoresis and quantified to be 110 (S₁) & 119 (S₂) nanograms spectrophotometrically at 260 nm [32].

3.1. Amplification of the gene

To isolate the fibrinolytic enzyme (FE) gene of locally isolated *Bacillus* sp. a set of oligodeoxyribonucleotide primer was designed from the genes sequence available from GeneBank NCBI based on deduced nucleotide sequence of *BKII* gene from *B. subtilis* A1 alongwith the signal peptide sequence including shine-dalgarno region [9] encoding fibrinolytic gene. The primer was designed using Primer3 online primer designing tool (<http://simgene.com/Primer3>) for the proper PCR amplification of the genomic region encoding BKII that was not practiced. Similarly Jeong and his co-workers [9] reported that 1381 bp gene fragment of fibrinolytic enzyme Bacillokinase II (BK-II) consisted of an open reading frame of 1023 basepairs encoding 341 amino acids and showed that the mature enzyme consisted of 288 amino acid. The deduced amino acid sequence showed significant homology with *Erwinia carotovora* metalloprotease by 65% and the biochemical properties of the purified enzyme suggested it to be a fibrinolytic metalloprotease with optimum activity at pH 7.0 and 50 °C. The sequences with *Bam*H I cleavage sites were included at the 5'-ends of the primers to facilitate cloning by use of functional primer pair ; 5'-GGGATCCAAGTCTGCAGCGGCG-3' (Forward) & 5'-GGATCCGTTCCGGCGTGATATCGGCCAGCG-3' (Reverse) were according to the published sequence available by Jeong and his co-workers [9] but they did not use it for PCR amplifications.

Therefore much work in optimizing the amplification of the gene was done regarding the optimization of MgCl₂, Primers & DNA concentrations to be used for amplification alongwith PCR thermal cycling conditions as we optimized before in our work for the amplification of *creI* gene [32].

The optimum concentrations for the amplification of BKII 1381bp gene were MgCl₂ 3.0 mM, Primers 20 picomoles each (diluted with TE buffer) & DNA 50 ng, where as the thermal cycling conditions are already mentioned in the previous section 2.4. Followed by extended polymerization of 10 min at 72 °C to add poly A tail that helps in T/A cloning.

PCR was performed on both the DNA samples S₁ and S₂ isolated from bacterial culture after several tries the product achieved was electrophoretically viewed on 1% agarose at 50 volts for 2 hours as shown in figure 2.

3.2. Cloning into PTZ57 R/T plasmid

After successful amplifications of the gene encoding fibrinolytic enzyme the PCR product was purified from agarose gel with the help of Gel Extraction Kit (Fermentas) following the manufacturer's protocol. The purified fibrinolytic enzyme gene was then ligated in PTZ57 R/T plasmids by T₄ DNA ligases using the protocol of Ins T/A clone™ kit (Fermentas) in *E-coli* top10 competent cells (that were chemically made). Control reaction was also set containing bacteria having no PTZ57 R/T plasmids. After incubation (overnight), recombinant bacteria containing PTZ57 R/T plasmids showed growth on ampicillin LB-plates and others were killed. Control plates did not show growth, as expected.

White colonies after successful transformations by heat shock method from plates grown in LB media with ampicillin added ensuring the growth of recombinants were then subjected to miniprep plasmid DNA isolation by following the protocols given in Molecular cloning Laboratory Manual [33]. Plasmids DNA was finally dissolved after in TE buffer for long term storage and saving it from nucleases. The plasmid DNA samples were run on 1% agarose gel as shown in Figure 3a. the results of confirmation of clones of BKII gene after restriction with

EcoRI & *BamHI* at 37 °C overnight in 20uL total reaction mixture following instructions at www.fermentas.com for double digest. The restriction site was introduced through primer set used. The results were run on 0.8% agarose gel as shown in figure 3b. Further similar sized insert was also confirmed by specific primers through colony PCR.

The results shown in the Figure will be further used for sequencing analysis and expression studies. Similar results of cloning and identification of *BKII* fibrinolytic enzyme's gene were reported with different strategies from *Bacillus* strains by Jeong & co-workers [9] but without PCR based cloning that is practice in this article. The DNA fragment encoding the intact fibrinolytic enzyme (FE) was also amplified from total DNA of *Bacillus subtilis* LD-8547 by PCR and cloned into BI-21 *E. coli* using pET32 expression vector [24].

Peng, [17] also carried out the purification, characterization and cloning of a fibrinolytic enzyme produced by *Bacillus amyloliquefaciens* DC-4 screened from douchi (traditional Chinese soybean food). Similarly, Zhang & his co-workers [28] reported the DNA fragments encoding pro-subtilisin DFE cloned after amplification into the vector pET32a to obtain N-terminal Trx fusion expression plasmid. In the same way till present similar to our work genomic DNA was isolated and used for PCR amplification of the subtilisin gene of approximately 1100 bp by means of the specific primers by Ghasemi and his colleagues [30].

4. Conclusion

The fibrinolytic enzyme BKII gene from local isolate of *Bacillus* sp was successfully amplified from the genomic DNA and transformed into *E. coli* Top 10. The gene insert was cloned into pTZ57 R/T vector. This may further help in studying genetic diversity of fibrinolytic enzymes for their better understandings. This gene in future will also be cloned in appropriate expression vector and will be used for enzyme characterization, hyper expression and purification to act as a future remedy or drug of CVDs. It is important from pharmacological, industrial and clinical point of view.

Acknowledgements: The authors are highly grateful to the Higher Education Commission (HEC) Pakistan for facilitating in the research work conducted for the article.

References

- [1] U. F. Ali and Z. M. Ibrahim. (2008). Production and some Properties of Fibrinolytic enzyme from *Rhizomucor miehei* (Cooney & Emerson) Schipper. *Journal of Applied Sciences Research*. 4: 892-899.
- [2] K. Arai, J. Mimuro, S. Madoiwa, M. Matsuda, T. Sako and Y. Sakata. (1995). Effect of staphylokinase concentration of plasminogen activation. *Biochimica et Biophysica Acta* 1245: 69-75.
- [3] K. Balaraman and G. Prabakaran. (2007). Production & purification of a fibrinolytic enzyme (thrombinase) from *Bacillus sphaericus*. *Indian Journal of Medical Research*. 126: 459-464.
- [4] B. P. Batomunkueva and N. S. Egorov. (2001). Isolation, purification and resolution of the extracellular proteinase complex of *Aspergillus ochraceus* 513 with fibrinolytic and anticoagulant activities. *Microbiology*. 70:519-522.
- [5] A. R. Caballero, L. Richard and H. J. Kenneth. (1999). Cloning, Expression, Sequence Analysis, and Characterization of Streptokinases Secreted by Porcine and Equine Isolates of *Streptococcus equisimilis*. *Infection and Immunity*. 67: 6478-6486.
- [6] D. Collen and H. R. Lijnen. (1994). Staphylokinase, a fibrin-specific plasminogen activator with therapeutic potential?. *Blood* 84(3): 680-686.
- [7] D. Collen and H. R. Lijnen. (2004). Tissue-type plasminogen activator: A historical perspective and personal account. *Journal of Thrombosis and Haemostasis*. 2:541-546.
- [8] M.J. Duffy. (2002). Urokinase plasminogen activator and its inhibitor, PAI-1, as prognostic markers in breast cancer: from pilot to level 1 evidence studies. *Clinical Chemistry*. 48:1194-1197.
- [9] Y. K. Jeong, H. K. Jae, G. Sang-Wan, K. Ji-Eun, P. Soon-Suk, C. Kyung-Tae, K. Young-Hee, K. Byung-Woo and J. Woo-Hong. (2004). Molecular cloning and characterization of the gene encoding a fibrinolytic enzyme from *Bacillus subtilis* Strain A1. *World Journal of Microbiology & Biotechnology*. 20. 711-717.
- [10] Y. K. Jeong, U. P. Jeong, B. Hyun, H. P. Sung, K. S. In, W. K. Dong and H. J. Woo. (2001). Purification and biochemical characterization of a fibrinolytic enzyme from *Bacillus subtilis* BK-17. *World Journal of Microbiology & Biotechnology*. 17: 89-92.
- [11] H. K. Ju, Y. Jun-Peng, Z. Lei, A. Yi and Q. Peng. (2004). Identification of two novel fibrinolytic enzymes from *Bacillus subtilis* QK02. *Comparative Biochemistry and Physiology*. 137: 65-74.
- [12] S. H. Kim and N. S. Choi. (2000). Purification and characterization of subtilisin DJ-4 secreted by *Bacillus* sp strain DJ-4 screened from Doen-Jang. *Bioscience Biotechnology and Biochemistry*. 64:1722-1725.
- [13] L.V. Medved, D.A. Solovjov and K.C. Ingham. (1966). Domain structure, stability and interactions in streptokinase. *European Journal of Biochemistry*. 239. 333-339.

- [14] H. Mihara, H. Sumi, T. Yoneta, H. Mizumoto, R. Ikeda and M. Seiki. 1991. A novel fibrinolytic enzyme extracted from the earthworm, *Lumbricus rubellus*. *Journal of Physiology*. 41: 461–472.
- [15] Y. Mine, H. K. W. Ada and J. Bo. (2005). Fibrinolytic enzymes in Asian traditional fermented foods. *Food Research International* 38: 243–250.
- [16] Y. Peng and Y. Z. Zhang. (2002). Isolation and characterization of fibrinolytic enzyme producing strain DC-4 from Chinese douche and primary analysis of the enzyme property. *Chinese High Technology Letters*. 12: 30–34.
- [17] Y. Peng, H. Qing, Z. Ren-Huai and Z. Yi-Zheng. (2003). Purification and characterization of a fibrinolytic enzyme produced by *Bacillus amyloliquefaciens* DC-4 screened from *douchi*, a traditional Chinese soybean food. *Biochemistry and Molecular Biology*. 134: 45-52.
- [18] Y. Peng, Y. Xiao-Juan and Z. Yi-Zheng. (2005). Microbial fibrinolytic enzymes: An overview of source, production, properties, and thrombolytic activity in vivo. *Applied Microbiology & Biotechnology*. 69: 126–132.
- [19] M. I. Rajoka and A. Bashir. (1998). Cloning of multiple *Egl* genes conferring production of endoglucanase on *E. coli*. *Pakistan Journal of Biological Sciences*. 1: 179-182.
- [20] M. H. Shen, J. S. Kim, K. Sapkota, S. E. Park, B. S. Choi, S. Kim, H.H. Lee, C. S. Kim, H.S. Chun., C.I. Ryoo and S.J. Kim. (2007). Purification, characterization, and cloning of Fibrinolytic Metalloprotease from *Pleurotus ostreatus* Mycelia. *Journal of Microbiology and Biotechnology*. 17 (8): 1271–1283.
- [21] H. Sumi, H. Hamada, H. Mihara, K. Nakanishi and H. Hiratani. (1989). Fibrinolytic effect of the Japanese traditional food natto (nattokinase). *Thrombosis & Haemostasis*. 62: 549-556.
- [22] A. G. Turpie, B. S. Chin and G. Y. Lip. (2002). Venous thromboembolism: treatment strategies. *British Medical Journal*. 325: 948–950.
- [23] J. Wang, M. Wang and Y. Wang. (1999). Purification and characterization of a novel fibrinolytic enzyme from *Streptomyces spp.* *Chinese Journal of Biotechnology*. 15: 83–89.
- [24] S. H. Wang, D. Miao, Y. Yan-Ling, L. Wei-Zhu and H. Bi-Fang. (2008b). Gene clone and expression of a fibrinolytic enzyme (FE) in *Escherichia coli*. *Annals of Microbiology*. 58. 95-98.
- [25] L. Xiao-Lan, D. Lian-Xiang, L. Fu-Ping, Z. Xi-Qun and X. Jing. (2005). Purification and characterization of a novel fibrinolytic enzyme from *Rhizopus chinensis* 12. *Applied Microbiology & Biotechnology*. 67: 209–214.
- [26] Y. Yamada, M. Koichi, M. Hossain, U. Kumiko, N. Yayoi, Y. Hideyo and O. Masako. (2002). Comparison of different methods for extraction of mitochondrial DNA from human pathogenic yeasts. *Japanese Journal of Infectious Diseases*. 55: 122-125.
- [27] S. J. Yoon, M. A. Yu, G. S. Sim, S. T. Kwon, J. K. Hwang, J. K. Shin, I. H. Yeo and Y. R. Pyun. (2002). Screening and characterization of microorganisms with fibrinolytic activity from fermented foods. *Journal of Microbiology & Biotechnology*. 12: 649–656.
- [28] R. H. Zhang, L. Xiao, Y. Peng, H.Y. Wang, F. Bai and Y.Z. Zhang. (2005). Gene expression and characteristics of a novel fibrinolytic enzyme (subtilisin DFE) in *Escherichia coli*. *Letters in Applied Microbiology*. 41: 190–195.
- [29] K. I. Fayek and S. T. El-Sayed. (1980). Fibrinolytic activity of an enzyme produced by *Bacillus subtilis*. *Zeitschrift fur Ernährungswissenschaft*. 19: 21–23.
- [30] Y. Ghasemi, F. Dabbagh and A. Ghasemian. (2012). Cloning of a Fibrinolytic Enzyme (Subtilisin) Gene from *Bacillus subtilis* in *Escherichia coli*. *Molecular Biotechnology*. 1: 1-7.
- [31] A. R. Lee, G. M. Kim, G. H. Kwon, K.W. Lee, J.Y. Park, J. Chun, J. Cha, Y.S. Song and J.H. Kim. (2010). Cloning of aprE86-1 gene encoding 27 kDa mature fibrinolytic enzyme from *Bacillus amyloliquefaciens* CH86-1. *Journal of Microbiology & Biotechnology*. 20 (2): 370-374.
- [32] Z. Mushtaq, M. Saadia, R. S. Anjum and A. Jamil. (2009). Cloning of an intronless *creI* gene from *Chaetomium thermophilum*. *Annals of Microbiology*. 59 (4): 785-788.
- [33] J. Sambrook and D.W. Russel. (2001). *Molecular cloning Laboratory Manual*. Vol 3. 3rd edition. CSHL press, New York. (www.molecularcloning.com).