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Characterization of extracellular protease from *Bacillus licheniformis*

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Abstract

Extracellular proteases have a fundamental position with respect to their physiological roles as well as their commercial applications. *Bacillus licheniformis* RT7P1, was evaluated in this study for production of extracellular protease activity. The culture was maintained on 1% w/v casein plates. In this study, the strain was found to produce maximum enzyme at pH 7 and temperature 37°C after 72 h. The optimum assay pH and the temperature was 10 and 50°C, respectively. The enzyme was stable between pH 9-11and thermal stability data showed that enzyme was stable at 100°C. For cloning of protease gene from *Bacillus licheniformis*, primers were designed to pick their full-length sequences from the genomic DNA obtained from different *Bacillus* species. Genomic DNA was isolated from *Bacillus licheniformis* strain RT7P1 and then the protease gene was amplified from it by using RT7P1 specific primers. This amplified product (1725bp) was then cloned in PTZ57R/T vector. The clone was confirmed by restriction analysis with *EcoR1* and *BamH1*, which showed two fragments of (2886 bp) and (1725 bp), which showed that insert was cloned in the right orientation. The study indicates that *Bacillus licheniformis* RT7P1 is a good source of commercial thermostable alkaline extracellular protease.

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Introduction

Alkaline proteases from extremophilic microorganisms are able to withstand harsh conditions in industrial processes. Heat-stable and solvent-tolerant biocatalysts are valuable tools for processes in which hardly decomposable polymers need to be liquefied and degraded, while cold-active enzymes are of relevance for food and detergent industries. Extremophilic microorganisms are a rich source of naturally tailored enzymes, which are more superior to their mesophilic counterparts for applications at extreme conditions. Bacterial alkaline proteases show high activity due to their alkaline pH, and their high substrate specificity. That is the reason that bacterial alkaline proteases are used for industrial purposes. Approximately 300 enzymes are used for industrial and biotechnological applications (Singhal *et al.*, 2012). Protease is produced commercially about 60%. They are produced from the plant, animal and microbial sources (Boominadhan *et al.*, 2009). They constitute a complex and large group of enzymes which vary in properties like catalytic mechanism, active site, and pH, temperature, and substrate-specific (Morya and Yadav, 2009). In the worldwide synthesis of enzymes, approximately 45% proteases are obtained from microbial sources (Lagzian and Asoodeh, 2012). The studies showed that nutritional factors (carbon and nitrogen) and physical factors (pH, incubation time, temperature and inoculum concentration) also effect on the production of protease (Boominadhan *et al.*, 2009). Alkaline proteases are used in mostly leather, detergent, medical purposes, chemical industry, food processing and silver recovery (Cotarleţ *et al.*, 2009). For new promising strains, isolation and characterization of protease enzyme by using cheap nitrogen and carbon sources are continuing process for industrial uses (Suganthi *et al.*, 2013). A new trend is developed in which wastes are converted into useful biomass by using microorganisms (Pant *et al.*, 2014). Alkaline proteases show slow activity and stability in the detergent industry so new alkalophilic microorganisms are isolated and screened which have potential in detergent industries (Mathew and Gunathilaka, 2015).

The isolation of environmental DNA Libraries for beneficial activities can provide a new tool of new molecules and enzymes. Protease is studied in protein engineering and protein chemistry as well as in food additives, dehairing and cleansing agent (Wilson and Remigio, 2012). In modern science, enzymes with high stability at elevated temperature in the wide range of pH and resistance to detergents, chelators, organic solvents are of great importance. This study focused on the *Bacillus licheniformis* RT7P1, for the rmostable alkaline protease production, optimization of culture conditions, protease characterization and cloning of protease gene.

Materials and methods

Culture and Growth Conditions

The indigenous *Bacillus licheniformis* RT7P1 strain supplied by the molecular biology Lab., Industrial Biotechnology Division, NIBGE, Faisalabad was used in this study, which had previously been isolated from a peat sample. The culture was maintained on casein (1%w/v) plates containing composition of medium, 1 g/L K_2HPO_4, 1 g/L $(NH4)_2HPO_4$, 0.5 g/L $MgCl_2$, 10 g/L Yeast extract, 10 g/L Casein and 3%agar. The pH of the medium was adjusted to 7 with 1N HCl/NaOH and then autoclaved. Plates were incubated at 37°C for 24h and observed for zones of clearance, which indicate proteolytic activity.

Optimization of Protease Production and Assay for Protease Activity

For the production of crude enzyme, effect of time course, temperature effect, pH effect and effect of different substrate concentration was studied at 37 ^{o}C in10 mM K_2HPO_4 solution (pH 10) after regular time intervals of (24, 48, 72, 96 and120 h), temperature ranges (30, 35, 37, 40, 45 and 50°C), pH ranges 4-9 and varying concentrations of casein (0.5, 1, 1.5, 2, 2.5 and 3%) respectively.

Protease activity assay was performed by some Lowry method using tyrosine as a standard. The activity of protease was measured on the basis of liberated tyrosine residues. One unit of protease activity will be defined as the amount of enzyme that liberated 1 µg of tyrosine residues under assay conditions.

Characterization of Protease and Zymography

The optimum temperature for protease activity was carried out at a temperature (20 - 90°C) for 20 min. The thermostability of protease was examined by incubating the enzyme in 10mM potassium phosphate solution (pH 10) at 100°C. The enzyme samples were taken after (1-10) min of incubation for the assay of enzyme activity. The crude enzyme was incubated with 10mM potassium phosphate solutions of varying pH (8-12) at 50°C for 20 min. The pH stability was measured by incubating the enzyme at pH 6 to 12 in different buffers (0.1M) such as KH_2PO_4-K_2HPO_4 (6.0-7.5), Tris–HCl (8.0-9.0), 10mM potassium phosphate buffer (pH 10.0) ,Glycine-NaOH (9.0 – 13.0) and Na_2HPO_4-NaOH (11.0-12.0) at 50°C for 20 min. Zymography is a technique in which substrate copolymerized with the polyacrylamide gel, for the detection of enzyme activity as well as molecular weight (Talebi *et al.*, 2013). Samples were prepared in the standard SDS-PAGE treatment buffer (5% sodium dodecyl sulfate [SDS], 2% sucrose, 0.005% bromphenol blue in 0.5 M Tris-HCl, pH 6.8, containing 0.4% SDS stacking gel buffer). The sample was subjected to electrophoresis in Tris-glycine buffer, pH 8.3, at 12.5 mA per gel. Following electrophoresis, the SDS was removed by incubation with 2.5% Triton X-100 for 2 h at 25°C, and the gel was then incubated in neutral buffer (0.1 M Tris-HCl, pH 7.8 containing 1 mM CaCl2) at 37°C for 24 h. The zymogram was subsequently stained Coomassie Brilliant Blue and areas of digestion appear as clear bands against a darkly stained background where the substrate has been degraded by the enzyme. Gelatin is the most commonly used substrate and is useful for demonstrating the activity of gelatin-degrading proteases.

Polymerase Chain Reaction and Cloning

The DNA isolated from the strain was used as template in PCR amplification of protease gene (Gärtner, *et al.*, 1988). The sequence of protease gene was retrieved from the gene bank. Primers were designed to pick their full-length sequences. Specific primers of *Bacillus licheniformis* RT7P1 forward primer of the following sequence 5′ATGGCCAACAGACAGAAGATC3′ and RT7P1 reverse primer 5 TCAGGGGCTGGTTCTTCTGTT3′ were used in this study for the amplification of protease gene from B*acillus licheniformis*.

Fresh PCR amplified products were used for cloning into Fermentas (pTZ57R) cloning vector (2886bp). The cloned plasmids were confirmed through restriction with *BamHI* and *EcoRI*.

Results and discussion

Protease is one of the most important groups of industrial enzymes which occupy a pivotal position with respect to their physiological roles as well as commercial applications.

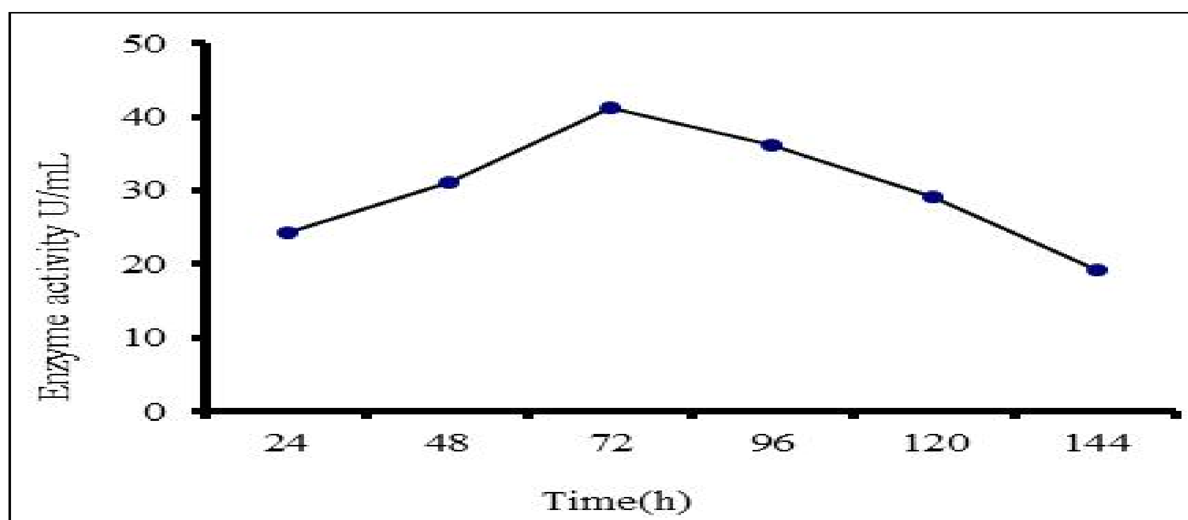

Fig. 1. Effect of time course on enzyme production.

The study of time course revealed that *Bacillus licheniformis* RT7PI gradually show an increase in enzyme production, and its activity was found (24.3 U/mL) after 24h, (31.2 U/mL) after 48h and maximum level of protease activity 41.2 U/mL after 72h (Fig.1). whereas in contrast to alkaline protease production which was enhanced after 39 h in *B. licheniformis* (Sayem *et al.*, 2006). These findings were in association with the observations of (Bernlohr and Clark, 1971).

The decline in protease activity after 96 and 120 h in the present investigation indicated less reproduction and highest death rate of *B. licheniformis* (Fig. 1). Temperature is an important environmental factor affecting the growth and production of metabolites by microorganisms. The optimum temperature for protease production was observed at 37°C and protease activity was 44.2U/mL (Fig. 2).

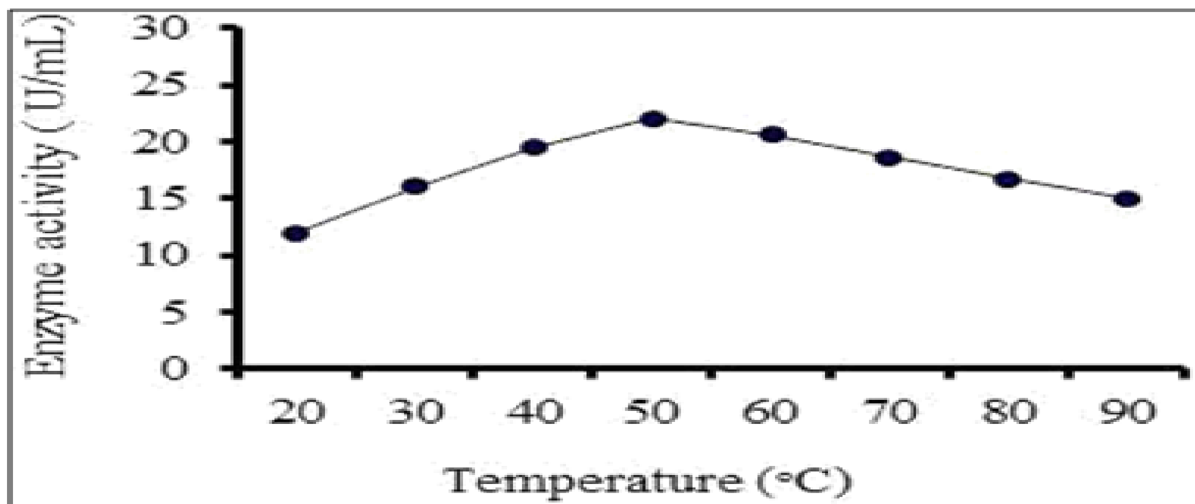

Fig. 2. Effect of Temperature on enzyme production.

These findings were in association with the observation of (Sayem, *et al.*, 2006). The study conducted on protease production from *Bacillus horikoshii* showed maximum growth at 37°C (Joo and Choi, 2012). The effect of initial pH of the medium on crude enzyme production was examined by varying the pH of the culture medium and a maximum activity of the enzymes was found on pH7 as shown in (Fig.3). Similar results were recorded by (Rozs *et al.*, 20010. Study on the substrate concentration indicated that increasing concentration of casein enhanced the enzyme production (Fig. 4). Ferro *et al*, observed that casein was the best source for production of alkaline protease (Ferrero *et al.*, 1996).

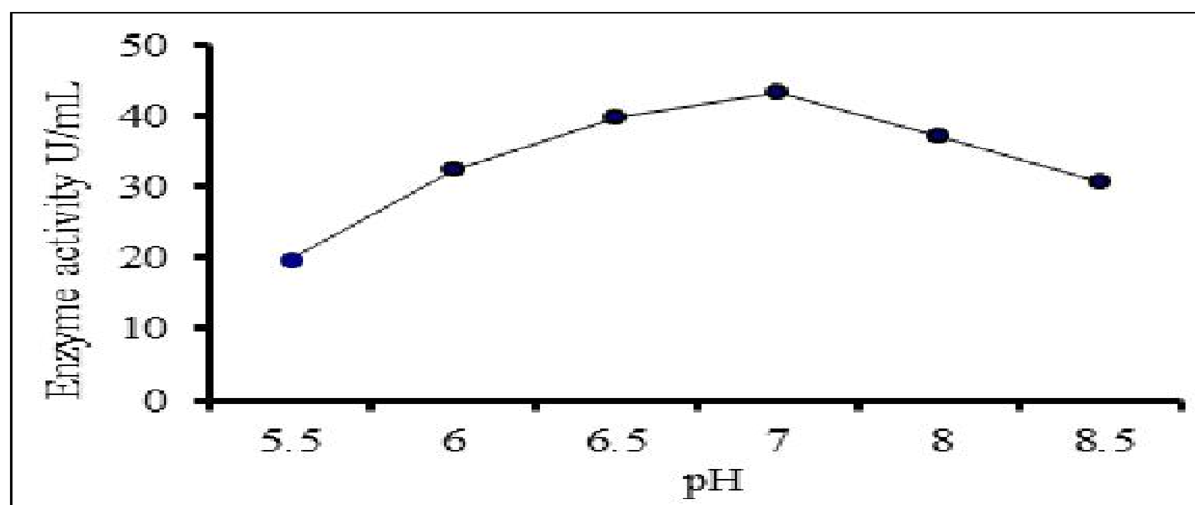

Fig. 3. Effect of pH on enzyme production.

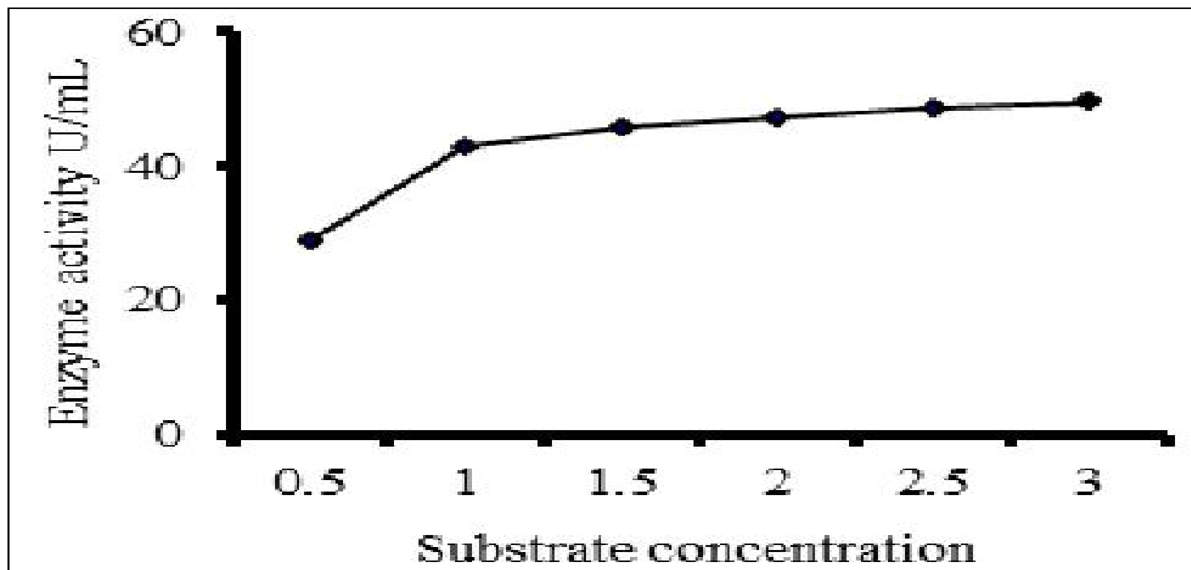

Fig. 4. Effect of Substrate Concentration.

Bacillus licheniformis RT7PI produce a thermostable alkaline extracellular protease

The optimum temperature for protease activity was found to be 50°C (Fig.5).

The enzyme was stable at 100°C for 3 min afterward a gradual decrease in activity was observed after 6 minutes at 100°C (Fig. 6). These results explained thermostability of protease. It was reported that the thermophilic neutral protease from thermophilic *Bacillus* strain showed that protease remained stable at 50°C during 1 h incubation (Guangrong *et al.*, 2006). Stability study indicated that this enzyme retained about 95% and 74% of its maximum activity after 1h at 60°C.

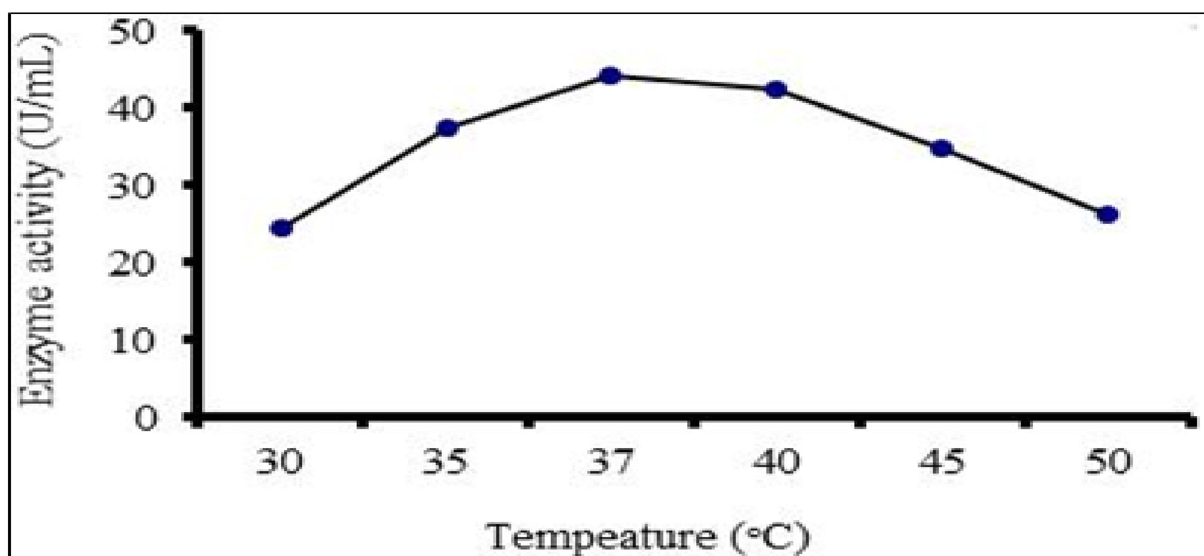

Fig. 5. Effect of Temperature on Protease Activity.

The protease retained more than 80% and 65% of its activity after 30 min of incubation at 60°C (Nascimento and Martin, 2006). Maximum enzyme activity 43.1 U/mL was at pH10 (Fig.7). Enzyme activity was found to be stable at pH range of (9 -11).

The study implicated alkaline nature of protease. Thermostable alkaline protease showed maximum activity at pH 10 and it was found to be stable between pH 8-10 (Adinarayana *et al.*, 2003).

Zymographic demonstration of protease, clear bands represented protease that has degraded the substrate in the gel (Talebi *et al.*, 2013). The molecular weight of protease was found to be 33 kDa (Fig: 8).

PCR Amplification and Cloning

Gene cloning is a rapidly progressing technology that has been improving understanding of the structure-function relationship of genetic systems. The genomic DNA of *Bacillus licheniformis* strain RT7P1 was used as a template in PCR.

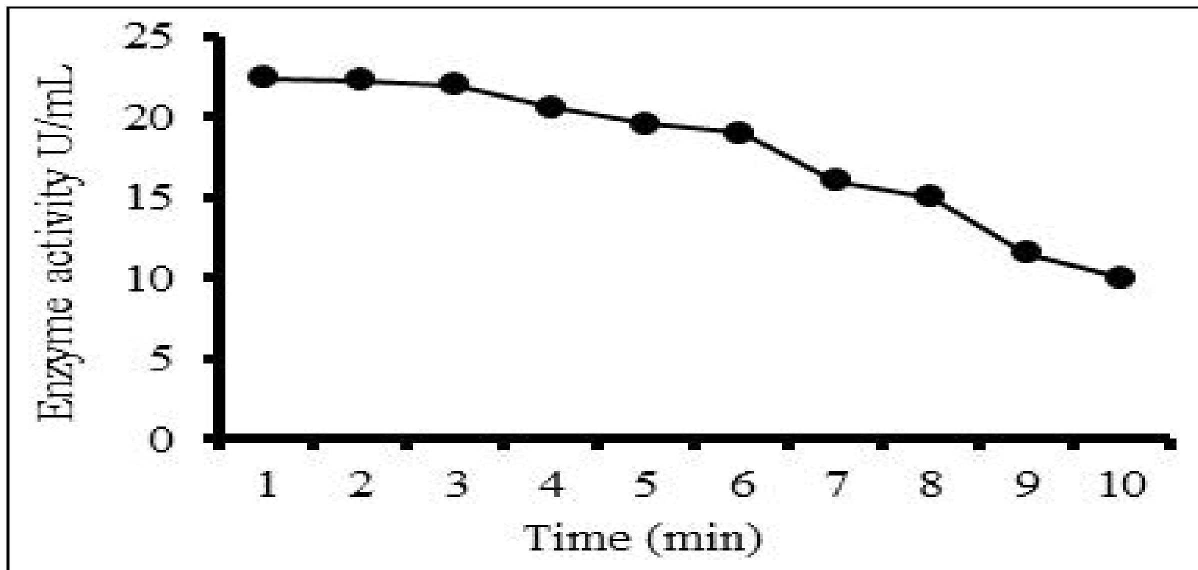

Fig. 6. Thermal stability on enzyme activity.

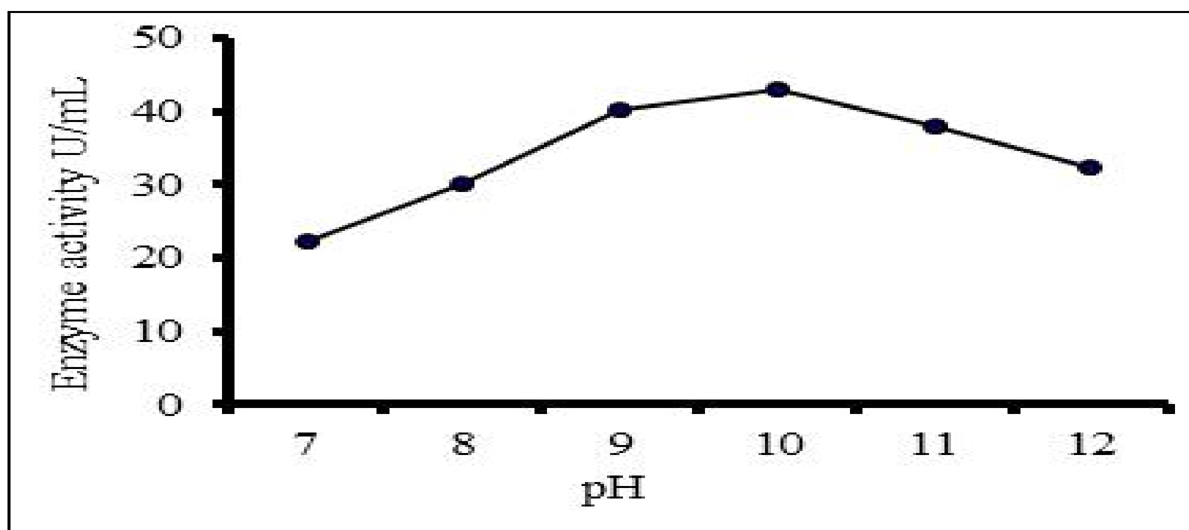

Fig. 7. Effect of pH on enzyme activity.

The PCR products were run on 1 % agarose gel along with 1Kb Fermentas DNA ladder to estimate the size of amplicon showed the amplification of (1725bp) gene fragment of protease from *Bacillus licheniformis* (Fig.9). PCR amplified fragment (1725bp) was cloned into a cloning vector (pTZ57R/T) and the development of white colonies represented recombinant colonies.

The plasmid was isolated and the clone was confirmed through restriction with *EcoRI* and *BamHI*. The two fragments of 2886bp and 1725bp appeared on the gel which showed that the insert was cloned in right orientation (Fig. 10).

The TA cloning method is especially suitable for cloning of PCR fragments amplified with primers.

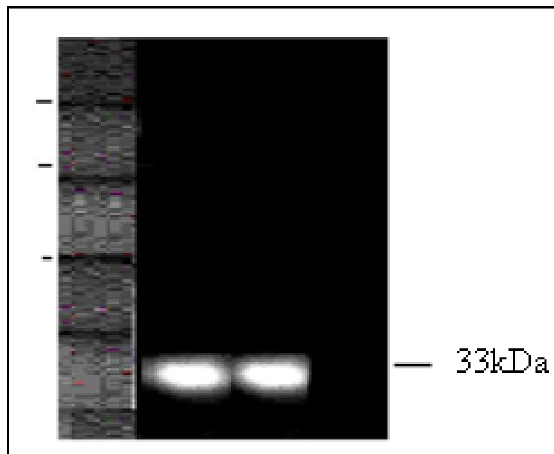

Fig. 8. Zymogram: Lane M Fermentas proteinase marker of size (18-118 kDa), Lane A and sample of *B. licheniformis* R7P1

The high efficiency of this method is based on the use of a specifically designed cloning vector, pTZ57R/T. The vector has been pre-cleaved with *Eco32* and treated with terminal deoxynucleotidyl transferase to create 3"-ddt overhang at both ends. PCR fragment is ligated into the vector, a circular molecule with two nicks, the product can be used directly to transform *E. coli* cells with high efficiency, An additional advantage of this approach is that the T-overhang prevent recirculization of the vector during the ligation procedure.

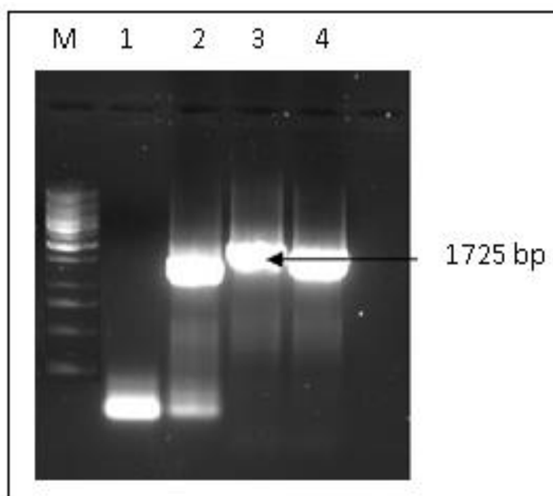

Fig. 9. Lane M, 1kb Fermentas DNA ladder # SM1303. Lane 1 represents negative control; Lane 2 positive control of another *B. licheniformis* strain Lanes 3 and 4; PCR amplification of RT7P1 gene (1725bp).

As a result, the yield of the recombinants as high as 90%. Amplified gene product used in cloning is more sensitive and reliable method as compared to random cloning method. In Random cloning undesired gene can also be cloned and their expression can also interfere with the expression of the desired gene. *Bacillus lentus* and *Bacillus subtilis* 168 alkaline protease genes have been cloned and sequence (Johnston *et al.*, 2009). Subtilisin gene 1.5 kb has been cloned in pTZ57R/T that have three ORFs (*Tk1689*, *Tk1675* and *Tk0076*) in encoding three subtilisin-like serine protease precursors (Rasool *et al.*, 2011).

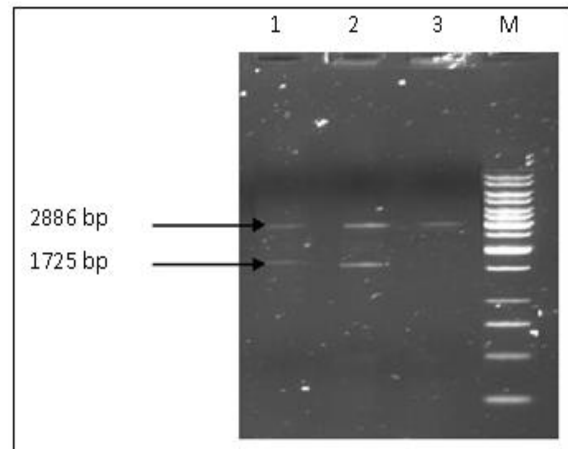

Fig. 10. Restriction digestion of pTZ57R/T with *EcoR1* and *Bam HI to excise the cloned fragment*, Lane M, 1kb Fermentas ladder, SM#0313, Lanes 1 and 2 represent RT7P1 gene (1725 bp), Vector cloned into pTZ57R/T vector, Lane 3 control.

Conclusion

Bacillus licheniformis RT7P1 has shown to produce thermostable extracellular protease. This protease displayed high activity in a broad range of pH and revealed high substrate specificity. The enzyme showed optimum activity at pH 10 and temperature 50°C, respectively. The culture was stable between pH 9-11and thermal stability data showed that enzyme was stable up to 100°C. The study indicates that *Bacillus licheniformis* RT7P1 is a good source of commercially thermostable alkaline extracellular protease.

References

Singhal P, Nigam VK, Vidyarthi AS. 2012. Studies on production, characterization, and application of microbial alkaline proteases. Nature, **3,** 653-669.

Boominadhan U, Rajakumar R, Karpaga P, Sivakumaar V, Joe MM. 2009. Optimization of protease enzyme production using *Bacillus* Sp. isolated from different wastes. International Journal of Botany and Research **2,** 83-87 p.

Morya V, Yadav D. 2009. Production and partial characterization of neutral protease by an indigenously isolated strain of *Aspergillus tubingensis* NIICC-08155. International Journal of Microbiology **8,** 1-6.

Lagzian M, Asoodeh A. 2012. An extremely thermotolerant, alkaliphilic subtilisin-like protease from hyperthermophilic *Bacillus* sp. MLA64. International Journal of Biological Macromolecules **51,** 960–967 p.

Cotarleț M, Negoita T, Bahrim G, Stougaard P. 2009. Cold adapted amylase and protease from new *streptomyces* 4 alga antarctic strain. Journal of Innovative Food Science and Emerging Technologies, **5,** 23-30 p

Suganthi C, Mageswari A, Karthikeyan, S, Anbaagan M, Sivakumar A, Gothandam KM. 2013. Screening and optimization of protease production from a halotolerant *Bacillus licheniformis* isolated from saltern sediments. Journal of Genetic Engineering and Biotechnology **11,** p 47-52.

Pant G, Prakash O, Chandra M, Sethi S, Punetha1 H, Dixit S, Pant AK. 2014. Biochemical analysis, pharmacological activity, antifungal activity and mineral analysis in methanolic extracts of *Myrica esculenta* and *Syzygium cumini*: the Indian traditional fruits growing in Uttarakh and Himalaya. Indian Journal of Pharmaceutical and Biological Research, **2(1),** 26-34 p.

Mathew CD, Gunathilaka RMS. 2015. Production purification and characterization of thermostable alkaline serine protease from *Bacillus licheniformis* NMS-1. International Journal of Biotechnology and Molecular Biology Research, **6(3),** 19-27 p.

Wilson P, Remigio Z. 2012. Production and characterization of protease enzyme produced by a novel moderate thermophilic bacterium (EP1001) isolated from an alkaline hot spring, Zimbabwe. African Journal of Microbiology Research, **6(27),** 5542-5551 p.

Talebi M, Emtiazi G, Sepahy AA, Zaghian, S. 2013. Zymogram analysis of alkaline keratinase produced by nitrogen fixing *Bacillus pumilus* ZED17 exhibiting multiprotease enzyme activities. The journal of Microbiology **6(10),** 1-6 p.

Gärtner D, Geissendörfer M, Hillen W. 1988. Expression of *Bacillus subtilis xyl* operon is repressed at the level of transcription and is induced by xylose. Journal of Bacteriology, **170 (7)**, 3102–3109 p.

Sayem SMA, Alam MJ, Hoq MM. 2006. Effect of temperature, pH and metal ions on the activity and stability of alkaline protease from novel *Bacillus licheniformis* MZK03. Proceedings of the Pakistan Academy of Sciences **43(4),** 257-262 p.

Bernlohr RW, Clark V. 1971. Characterization and regulation of protease synthesis and sctivity in *Bacillus licheniformis*. Journal of Bacteriology, **vol 105(1),** 276–283 p.

Joo HS, Choi JW. 2012. Purification and characterization of a novel alkaline protease from *Bacillus horikoshii*", Journal of Microbiology and Biotechnology **22 (1),** 58-68 p.

Rozs M, Manczinger L, Vagvolgyi LC, Kevei, 2001. Secretion of a trypsin-like thiol protease by a new keratinolytic strain of *Bacillus licheniformis*. FEMS Microbiology Letter, **205(2),** 221-4 p.

Ferrero MA, Castro GR, Abate CM, Bagori MD, Sinerizf. 1996. Isolation, production and characterization of thermostable alkaline protease of *Bacillus licheniformis* MIR29. Journal of Applied Microbialogy and Biotechnology **45,** 327-332 p.

Guangrong H, Tiejing Y, Jiaxing HPO. 2006. Purification and characterization of a protease from thermophilic *Bacillus* strain HS08. African Journal of Biotechnology **5,** 2433-2438 p.

Nascimento CAD, Martin MLL. 2006. Studies on the stability of protease from *Bacillus* sp. and its compatibility with commercial detergents. Brazillian Journal of Microbiology**37,** 307-311 p.

Adinarayana K, Ellaiah P, Prasad DS. 2003. Purification and partial characterization of thermostable serine alkaline protease from a newly isolated *Bacillus subtilis* PE-11", AAPS. Pharmaceutical Science and Technology **4,** 440-448 p.

Johnston J, Pippen X, Pivot M., Lichinitser S, Sadeghi V, Dieras HL, Gomez G, Romieu A, Manikhas, Kennedy MJ. 2009. Lapatinib combined with letrozole versus letrozole and placebo as first line therapy for postmenopausal hormone receptor positive metastatic breast cancer. Journal of Clinical Oncology **27,** 5538-5546 p.

Rasool N, Rashid N, Javed MA, Haider MS. 2011. Requirement of pro-peptide in proper folding of Subtilisin-like serine protease TK0076. Pakistan Journal of Botany **43,** 2059-2065.