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# SERRAPEPTIDASE GENE OF SERRATIA MARCESCENS FROM PLANT ORIGIN EXPRESSED BY *PICHIA PASTORIS* HAS PROTEASE ACTIVITY

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***Keywords:***
Serratia marcescens, Pichia pastoris, Serratiopeptidase, cloning, purification, Mass spectroscopy.

***Abstract***
Serrapeptidase (Spep) produced from Serratia marcescens is a secretary protein devoid of signal peptide. Spep was over expressed in E.Coli as inclusion bodies however the purified fusion protein was enzymatically inactive. To achieve the production of active Spep, in this study, the gene corresponding to Spep (Accession No. KP869847) from the genomic DNA of Serratia marcescens MTCC 8707 was cloned into Pichia pastoris using a modified transfer vector. The expression was induced with methanol and the secreted protein was affinity purified. The yield of the recombinant protein was 0.06 mg/ml with a maximum activity of 30 U/ml. An optimum activity of recombinant Spep was observed at 30oC and pH 8.0. The molecular weight of the purified mature active recombinant protein was 52 kDa. The secreted protein was characterized by mass spectrometric analyses using LC/MS-MS. To the best of our knowledge this is the first report on the production of active form of Spep from S.marcescens of plant origin. This protein may be exploited for the pharmaceutical and insecticidal application.

## Introduction

Few species of entrobacteria secrets protease extracellular, one such species is *Serratia marcescans* [(1)]. Previous studies examined *S.marcescens* isolates of different origins and characterized various extracellular proteases secreted by them. *S. marcescens* kums 3958, from human corneal ulcer produce more than four proteases[(2)]. Out of these four proteases, serrapeptidase (Spep) is major metalloprotease widely used as anti-inflammatory drug. It was first isolated from *Serratia E-15* [(3)]. Subsequently this enzyme was also studied in *Serratia marcescens ATCC 25419*, isolated from *Insect* hemolymph [(4,5)]and few clinical strains such as *S. marcescens* BG, from damaged tissue[(6)]. Also many soil samples origin of *Serratia marcescens* had been studied for the major metalloprotease production [(7)]. *S. marcescens* strain SRM (MTCC 8708) used in this study was isolated from the flowers of summer squash plants, showing no apparent symptoms of yellow vine disease. Application of Spep as pharmaceutical and insecticidal agent was well studied [(8,9,10)]. Therefore, the large scale production of Spep is significant for the commercial purpose. Purification to homogeneity is not possible by conventional methods of isolation from the cultured organism, and homogenous preparation is needed for above applications and also for conducting further studies on its characterization. Thus the well known rDNA approach is ideal, need based and will have no problem for getting regulatory approval for subsequent application. Spep from different sources has been expressed with prokaryotic expression systems using *E.coli* as host. Initially, Spep gene from *Serratia* E-15 was cloned into pTSP26 and expressed in *E. coli*-JM 103, the expressed enzyme was detected inside the cells but not in the culture medium [(11)]. Also, Braunagel et al., [(12)] observed that Spep from *S.marcescens* strain SM6 expressed in *E.coli* using lac promoter was secreted into medium and immuniologically detectable but inactive protein of slightly higher

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molecular weight. As well as expression of *S.marcescens* HR-3 Spep in *E.coli* (DE3)/pLysS strain using expression vector pET32a (+) was tried, wherein the enzyme was highly expressed as inclusion bodies and purified fusion protein was enzymatically inactive form [13]. Thus it is clear that the problem of secreting Spep into the medium by *E.coli* may be due to either the *S.marcescens* secretion genes are not clustered near the Spep structural gene or incomplete or incorrect processing of the protease zymogen, which are not functional in *E. coli* [14]. Importantly, Spep is secreted by N-terminal signal peptide independent pathway and require secretory protein on the membrane of host cell. Hence, Yeast can be the favored alternative host for the expression of this secretary protein. In this study the gene corresponding to Spep (Accession No. KP869847) from the genomic DNA of *Serratia marcescens* MTCC 8707 was cloned into *Pichia pastoris* using a modified transfer vector.

## Materials and Methods

**Strain, plasmids, enzymes, reagents and growth media**

The *Serratia marcescens* MTCC 8707 used in this study was procured from Microbial Type Culture Collection and Gene Bank (MTCC), Chandigarh, India. It was isolated from flowers of summer squash (*Cucubita pepa*) [15] and grown in specific medium. The genomic DNA was isolated from the organism using Tri-Xtract (G-Biosciences St.Leuis, Mo,USA) [16]. The expression vector pPIC- ZαA, *E.Coli* DH5α and *Pichia pastoris* were obtained from Invitrogen (USA). Luria–Bertani (LB) medium (Hi-media, India) was prepared for the cultivation of *E. coli* as described in the Manual of Molecular Cloning [17]. Medium for transformation and expression studies (Invitrogen, USA) were prepared according to the instruction of Easy select *Pichia* Expression Kit [18]. Ni-NTA column purchased from Qiagen, Germany.

**Modification of expression vector**:

In order to make the production cost effective Zeiocin restriciton gene was replaced by Kanamycin restriction gene in pPIC- ZαA expression vector. In brief, the expression vector pPICZαA was restriction digested at *Nco*I and *Stu*I site, which removed zeocin coding sequence and subsequently cloned with $Kan^R$ gene at specific site. This modification was accomplished by using gene specific primers.

**Construction of expression vector**

Sequence analyzed Spep Gene (Accession No. KP869847) of 1.5Kb from *Serratia marcescens* MTCC 8707 was cloned into pUC 18 at *Eco*RI site (pUSpep). The recombinant cloning vector was named as pUSpep, was restriction digested at *Eco*RI site and released Spep gene was sub-cloned into pPIC- ZαA (modified vector). The final construct was named as pPIC-Spep (Fig 1). The vector was transformed into *E.Coli* DH5α cells and subsequently, positive colonies were screened from LB agar plate with Kanamycin. Successful cloning was confirmed by bacterial colony PCR using specific primers (5'AOX: 5'dGACTGGTTCCAATTGACAAGC-OH3' & 3'AOX: 5'dGCAAATGGCATTCTGACATCC-OH3').

**Orientation conformation and transformation in Pichia pastoris GS115**

Orientation of the insert was confirmed by digesting with *SmaI*, the restriction enzyme which is located in vector as well as gene of interest. Hence single enzyme cuts two site and excised the gene of interest. The digested products were loaded on agarose gel and the recombinant plasmid with correct orientation of the insert (pPIC-Spep) was linearized with *Bgl II* and electrotransformed into 50µl *Pichia pastoris* GS115 with field strength of 1500 V/cm using Gene Pulsar II (Bio-Rad, USA). Transformants were streaked on minimal methanol with Histidine (MMH) agar plates. Plates were incubated at 30°C for minimum 3 days and growth of isolated colonies was monitored.

**Screening of positive transformants**

After successful transformation of construct into *Pichia pastoris* GS115 host cells, screening of positive transformants were done. The chromosomal DNA of *P.pastoris* was isolated from the spheroplast followed by phenol: chloroform extraction. The chromosomal integration of the plasmid DNA and right orientation of the expression cassette containing Spep gene were characterized by PCR analysis using vector specific primers (5'AOX: 5'dGACTGGTTCCAATTGACAAGC-OH3' & 3'AOX: 5'dGCAAATGGCATTCTGACATCC-OH3').

**Induction of protein expression**

Induction of the protein expression was carried out by standard procedures [18]. Single colony of positive clone of *Pichia* was inoculated in 25 mL MGYH kept in two 250 mL flasks and incubated at 30°C in a shaking incubator (250-300 rpm) to reach an $A_{600}$ of 6. The cells were harvested by centrifugation at 3,000g for 5 min at room

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temperature. The pellet was suspended in MMH to an $A_{600}$ of 1.0 (about 100-200 mL medium) and incubated at 30°C. The supernatant was analyzed for expressed protein was labeled as before induction. Methanol was added to a final concentration of 0.5% at every 24 h interval up to 96 h.
The expressed Spep in culture supernatants was monitored at 48, 72 and 96 h were analyzed by loaded on 12% SDS-PAGE.

**Purification of expressed protein using Nickel-NTA based affinity column**

One milliliter of 50% Ni-NTA slurry was added to 4 mL of diluted culture supernatant and mixed gently by shaking at 200 rpm on a rotary shaker at 4ºC for one hour and 20mM lysate buffer (50 mM NaH2PO4, 300 mM NaCl, 10 mM imidazole, pH to 8.0) was added. Lysate-Ni-NTA mixture was then loaded into a column with the bottom outlet cap. Bottom cap was removed after the slurry with protein packed properly into the column. The obtained elute was labeled as flow through and was stored for SDS-PAGE analysis. Then the column was run with 4 ml of wash buffer (50 mM NaH2PO4, 300 mM NaCl, 20 mM imidazole, pH to 8.0) and the protein was eluted step-wise with 250mM, 500mM, 750mM, 1000mM with required concentration of imidazole, pH to 8.0 and the imidazole gradient elution was were loaded on 12% SDS-PAGE & analyzed using protein molecular weight markers.

**Assay of the recombinant Spep activity**

Qualitative analysis of the recombinant Spep was carried out by loading the culture supernatant into the zymogram. The zymogram was prepared by mixing 0.2% Casein with 12% PAGE as per standard procedure [19]. The casein gels were pre run with a buffer containing 25mM Tris-base, 192mM glycine, 1mM EGTA and 1mM DTT (pH 8.3) for 15 minutes at 4ºC. The samples were loaded into wells and run at 125V for 3hrs at 4ºC. The gels was then removed and incubated in 20mM Tris-Hcl (pH 7.4 at room temperature), 10mM DTT, 1-4 mM CaCl2 with slow shaking for 60minutes. The gel was then further incubated overnight (20 to 24hrs) at ambient temperature in the same buffer. Finally the gel was stained with Coomassie blue.

Quantitative analysis of the recombinant Spep activity was performed as follows: 0.1 mL of suitably diluted purified Spep solution (1:10) was added to pre-warmed substrate (casein 1%) and incubated at 37˚C for 10 minutes for the enzyme to react with substrate. The reaction was stopped by addition of 3 mL of trichloroacetic acid and the non-hydrolyzed casein was precipitated by centrifugation at 3,000 rpm for 15 min. Peptide concentration of the supernatant was determined by measuring absorbance at 280 nm using tyrosine as a standard [20]. One unit of protease activity was defined as the amount of the enzyme required to liberate 1 µmol of tyrosine per minute. The amount of protein present in purified culture supernatant was analyzed by Bradford method using BSA as standard [21].

**Biochemical characterization of recombinant Spep**

Protease activity of purified Spep was studied at various pH and temperature conditions with casein 1% (w/v) as substrate and the pH stability of the protease was determined by incubating enzyme preparation in buffers of different pH in the range of 2.0- 10.0 using the following buffer systems: Sodium acetate buffer of pH 3.0 -5.0, potassium phosphate buffer of pH 6.0-8.0; Tris-HCl buffer (100 mM) of pH 8.0-9.0 and glycine–NaOH buffer of pH 9.0-12.0. Similarly, to determine thermal stability, the enzyme was pre-incubated at different temperatures (10-50ºC) for 1 hr and then the enzyme activity was assayed under standard assay conditions as mentioned earlier.

**In situ digestion and Mass Spectrometry analysis**

The Coomassie blue stained protein band of Spep was excised from the gel and *in situ* digested with trypsin. The resulting peptide mixtures were analyzed by LC/ MS-MS (HCT Ultra PTM Discovery System, Bruker Daltonics) and the proteins were identified in the NCBI database using MASCOT software.

## Results and discussion

**Cloning of Spep gene in Yeast Transfer Vector**

The Spep gene segment (1.5kb) was released from pU-Spep by *ECo*RI restriction digestion and ligated into corresponding site of pPICZαA (modified vector). The positive *E.coli* transformats were selected on LB agar ($Kan^R$) and the presence of insert was analyzed from isolated plasmid by PCR amplification. The resulting PCR amplicon was loaded on agarose gel and the gel band confirmed the presence of 1.5kb Spep gene (~1483bp) in the pPIC-Spep (Fig 2).
Further, the orientation of gene inserts in the recombinant plasmids was also confirmed by *SmaI* digestion. The *Sma*I restriction digested clone (pPIC-Spep) products were nearly 2.0 kb (Fig 3 lanes 2,4 ) showed that the insert is in 5'-

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3' correct orientation whereas nearly 1.5 kb product indicated the 3'-5' wrong orientation (Fig 3, lane 3 ) and 4.5 kb product indicated vector self ligated (Fig 3, lane 3). The plasmids with correct orientation were used for further studies.

**Transformation of pPIC-Spep into Pichia pastoris**

In order to express the Spep gene, recombinant plasmid (pPIC-Spep) was linearized with *Bgl II* and transferred into GS115 strain of *Pichia pastoris* and grown on MMH agar plates. This linearized construct upon transformation will integrates into *Pichia pastoris* genome by homologous recombination at AOX1 promoter locus thus providing stability to insert Spep gene.

The resulting positive clones which were amplified from Spep expression cassette flanked by AOX1 sequences showed 2.0 kb band (corresponding Spep gene with 1464 bp and rest is from the vector) in agarose gel electrophoresis (Figure 4).

**Expression of Spep gene in Pichia pastoris**

The PCR positive *Pichia* clones were grown separately and the expression was induced with 0.5% methanol. Since the pPIC-Spep was composed of the sequence encoding Spep located at downstream to the highly inducible AOX promoter, which permitted high-level expression and α-factor secretion signal helps in secretion of the recombinant protein into the medium. The culture supernatants were analyzed by SDS-PAGE. The molecular weight of the protein was deduced from the standard graph drawn between log molecular weight of the standard protein markers against their mobility. A protein band of 52 kDa was seen in the gel (Fig 5) corresponding to Spep and the expression of recombinant Spep reached maximum at 72 h. The translated product was present in the form of a fusion protein, which gets secreted out after cleavage of the signal sequences. Further the secreted proteins were effectively eluted at 500 - 750 mM in Ni-NTA column was detected by SDS-PAGE (Fig 6); hence the $His_6$ tag fusion helps in purification of secreted protein by single step affinity chromatography.

**Enzyme Characterization**

The zymogram result confirms that the secreted protein could digest casein and it was supported by the clearance zone shown in zymography (Fig 7). Further the enzyme assay evident that the secreted recombinant Spep has 30U/ml proteolytic activity. The protein yield was found to be 0.6 mg/ml of purified culture supernatant. Hence, the specific activity was calculated as 50U/mg. The optimum pH and temperature was found to be 8 and 30$^{o}$C respectively (Fig 8 a, b). Hence the enzyme characterization shows that the Spep secreted has protease activity which could effectively digest the protein, casein. Therefore, it is concluded that *Pichia pastoris* could be an effective expression system producing active Spep enzyme with good yield, whereas the other prokaryotic system fails to secrete active enzyme.

**Protein sequence of recombinant Spep**

The 52kDa Spep excised from polyacrylamide gels were digested with trypsin and the resulting thirteen peptide sequence were analyzed by LC/MS-MS (HCT Ultra PTM Discovery System, Bruker Daltonics) (Fig 9 a) and the proteins were identified in the NCBInr database using the MASCOT software. The peptide sequence obtained has specifically covering 46% of sequence of insecticidal protein from *Serratia marcescens*. The total score obtained was 789, total mass of 52.2 kDa and calculated pI of 4.64. This result indicating that the expressed protein corresponds to Serralysin family with consensus sequence for zinc binding motif (HEXXH...) and glycine-rich region associated with calcium binding (Fig 9 b).

## Conclusion

Most of the bacterial metalloprotease are toxic and causes pathogenesis to insects such as, thermolysin, neurotoxin families. Serrapeptidase is described under serralysin family wherein, the consensus sequence contain unique zinc binding motif '**H**EXX**H**XXGXX**H**…SXMSY' with methionine turn. It is used as anti-inflammatory drug for many decades and it appears to act on the inflammatory peptides such as bradykinin, histamine, hence analgesic. It has more importance in pharmaceutical industries and production by normal conventional methods from *Serratia E-15* or *Serratia marcescens* is followed. The new Genetic engineering approaches have helped to express gene of interest in heterologous host resulting in overproduction of the gene product. Serrapeptidase expression through prokaryotic vector fails to secrete the enzyme into medium and forms inactive insoluble inclusion bodies. The yeast has emerged as a powerful heterologous expression system for the production of high levels of functionally active recombinant proteins. To expedite such approaches that circumvent several problems encountered in expressing the Serrapeptidase, we expressed it in yeast *P. pastoris* system.

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Serrapeptidase was expressed as a secretory component using *P. pastoris.* The secreted protein is enzymatically active with maximum activity of 30 U/ml was confirmed by casein digestion. Also the secreted 52 kDa protein was sequence analyzed, by LC/MS-MS, could confirm that the recombinant protein correspond to serralysin family. This is the first report of producing active Serrapeptidase from *S.marcescens* of plant origin using *P. pastoris* as host. Hence it is concluded that *P. pastoris* could be an effective host system to secret active Serrapeptidase. The expressed protein has potentiality of being used as pharmaceutical and insecticidal agent that needs to be evaluated.

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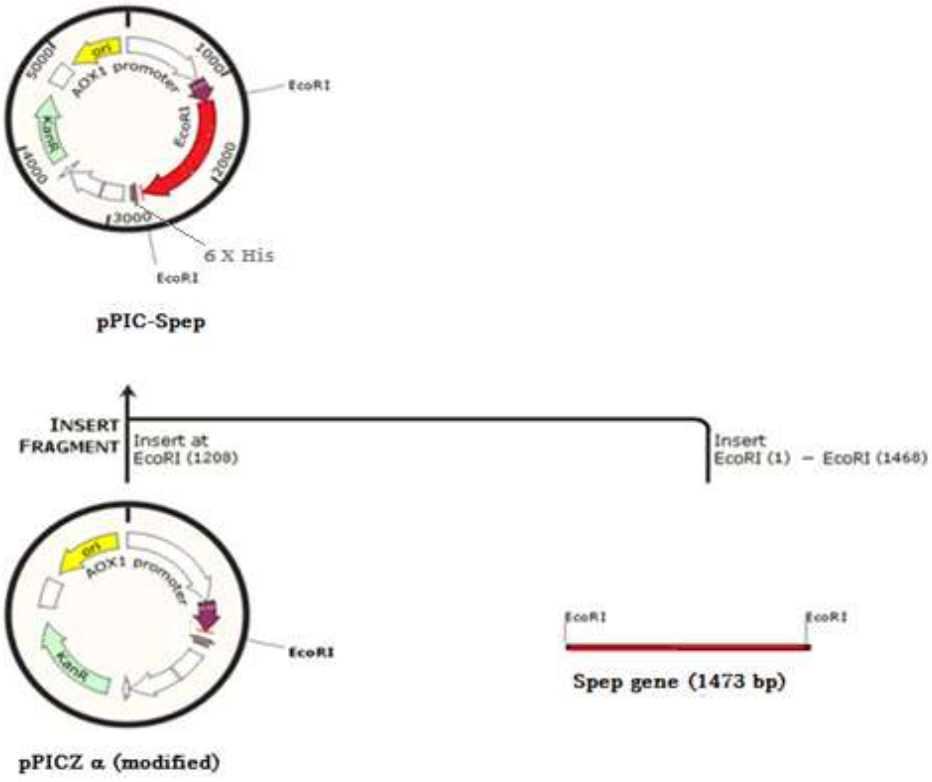


***Figure 1.Construction of the expression vector (pPIC / Spep). Spep gene released form pU-Spep by EcoRI digestion. pPICZ α (modified with Kanamycin resistance site).***

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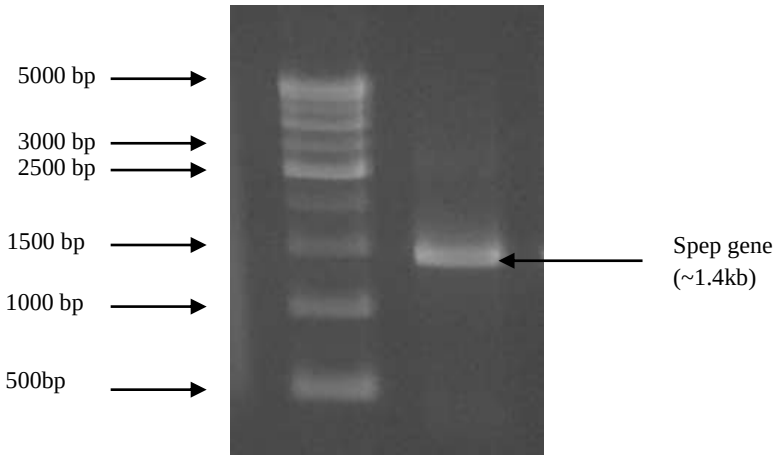


***Figure 2: Agarose gel electrophoresis of PCR amplicon from pPIC-Spep; lane 1, Standard DNA molecular weight marker (500bp ladder, Invitrogen, USA); lane 2, PCR Amplified Spep gene (~1483) amplicon from pPIC-Spep.***

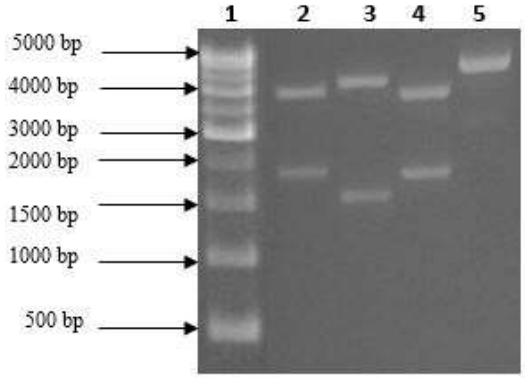


***Figure 3: Agarose gel electrophoresis of restriction Digestion of pPIC- Spep clones for orientation check: lane 1, Standard DNA molecular weight marker (500bp ladder, Invitrogen, USA), lane 2,4, Digested clone (pPIC- Spep) in correct orientation, lane 3, Digested Clone (pPIC-Spep) in wrong orientation and lane 4, Vector self ligated.***

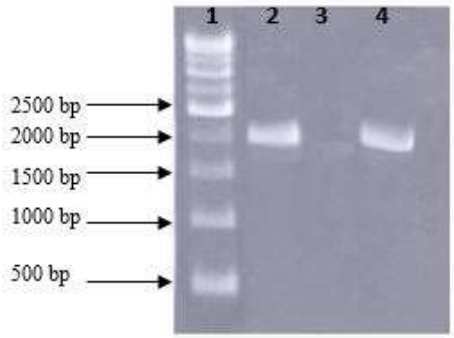


***Figure 4: Agarose gel electrophoresis of PCR amplicon using AOX1 primers from recombinant P.pastoris genome. lane 1, Standard DNA molecular weight marker (500bp ladder, Invitrogen, USA), lane 2 & 4 belong to positive clones showing 2.0 kb band was amplified from Spep expression cassette flanked by AOX1 sequences, lane 3, negative clone without gene of interest.***

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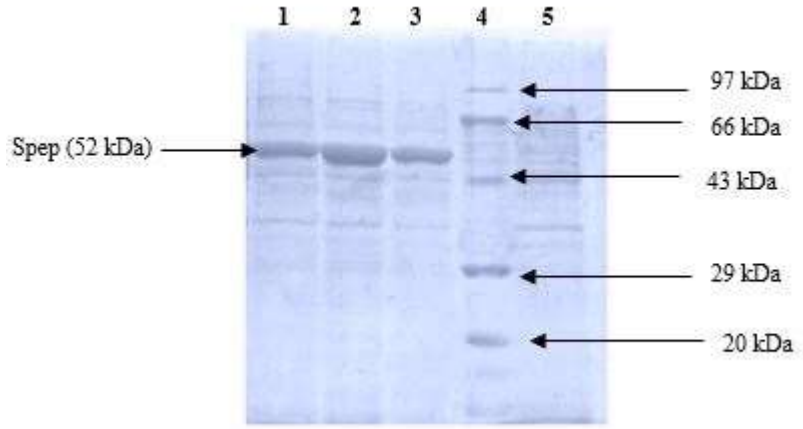


***Figure 5: SDS-PAGE Analysis of Expressed Spep: lane1, Proteins from induced culture supernatant of Pichia cells (GS115) 48hrs after induction; lane2, 72hrs after induction; lane 3, 96hrs after induction, lane 4, Standard protein molecular weight marker (PMW-L, Bangalore, Genei, Bangalore), lane 5, before induction with 0.5% methanol.***

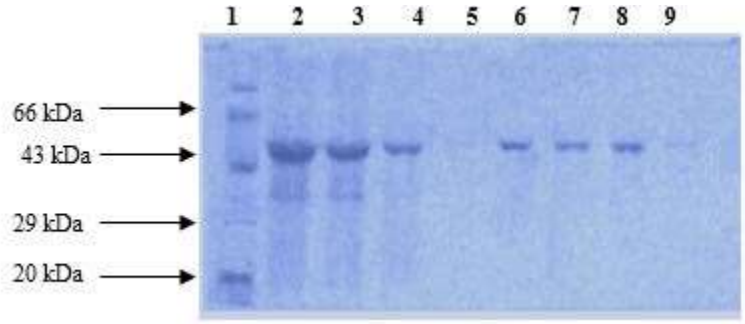


***Figure 6: SDS-PAGE Analysis of Ni-NTA Purified Spep: lane 1 - Standard protein molecular weight marker (Bangalore Genei, Bangalore), lane 2, Supernatant (Load); lane 3, flow through; lane 4, wash; lane 5, elution (250 mM Imidazole); lane 6, elution (500 mM Imidazole); lane 7, elution (500 mM Imidazole); lane 8, elution (750 mM Imidazole); lane 9, elution (1 mM Imidazole).***

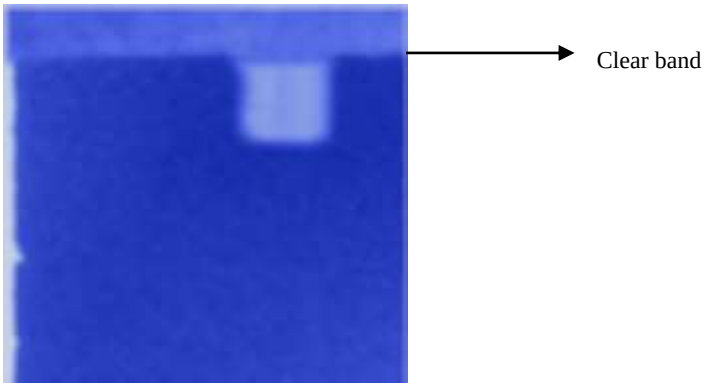


***Figure 7: Zymogram of recombinant Spep shows clear zone.***

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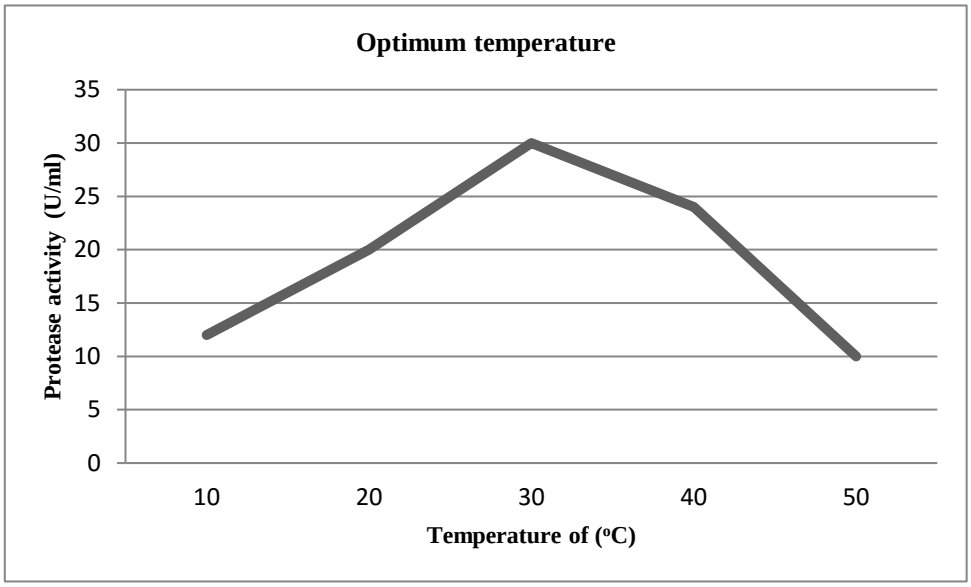
**(a)**




**(b)**

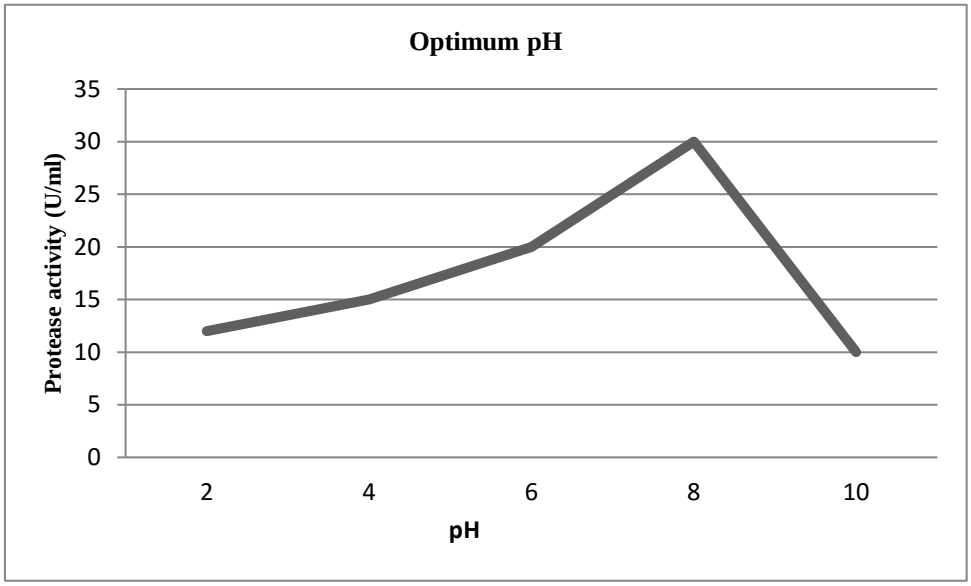



***Figure 8: Determination of (a) optimum temperature (b) optimum pH of recombinant Spep.***

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**(a)**

| Peptide | Observed mass (Da) | Predicted mass (Da) | Score |
|---|---|---|---|
| 1-LTFSFPDYK | 1116.5454 | 1116.5492 | 40 |
| 2-HPATEDYGR | 1044.4527 | 1044.4625 | 27 |
| 3-DVTYAEDTR | 1068.4427 | 1068.4724 | 34 |
| 4-GIDKIDLSFFNK | 1396.6254 | 1395.7398 | 39 |
| 5-IVGQVDVATDFIV | 1374.7854 | 1374.7395 | 52 |
| 6-DSFSNEQAGLFITR | 1584.4854 | 1583.758 | 76 |
| 7-TGDTVYGFNSNTGR | 1488.6854 | 1487.6641 | 53 |
| 8-DIFVFSAASDSAPGASDWIR | 2111.9182 | 2110.996 | 88 |
| 9-LSLQSWADVANITFTEVAAGQK | 2349.2182 | 2348.2012 | 47 |
| 10-GGAGNDVLFGGGGADELWGGAGK | 2061.6454 | 2060.9552 | 89 |
| 11-VIFAAWDAGGNDTFDFSGYTANQR | 2623.0582 | 2622.1776 | 82 |
| 12-AIEITESSLAAATTGYDAVDDLLHYHER | 3061.5382 | 3060.4676 | 128 |
| 13-QTFTHEIGHALGLSHPGDYNAGEGNPTYR | 3139.4482 | 3138.4544 | 34 |

**(b)**

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  1 MQSIKKAIEI TESSLAAATT GYDAVDDLLH YHERGNGIQI NGKDSFSNEQ
 51 AGLFITRENQ TWNGYKVFGQ PVKLTFSFPD YKFSSTNVAG DTGLSKFSAE
101 QQQQAKLSLQ SWADVANITF TEVAAGQKAN ITFGNYSQDR PGHYDYGTQA
151 YAFLPNTIWQ GQDLGGQTWY NVNQSNVKHP ATEDYGRQTF THEIGHALGL
201 SHPGDYNAGE GNPTYRDVTY AEDTRQFSLM SYWSETNTGG DNGGHYAAAP
251 LLDDIAAIQH LYGANLSTRT GDTVYGFNSN TGRDFLSTTS NSQKVIFAAW
301 DAGGNDTFDF SGYTANQRIN LNEKSFSDVG GLKGNVSIAA GVTIENAIGG
351 SGNDVIVGNA ANNVLKGGAG NDVLFGGGGA DELWGGAGKD IFVFSAASDS
401 APGASDWIRD FQKGIDKIDL SFFNKEAQSS DFIHFVDHFS GTAGEALLSY
451 NASSNVTDLS VNIGGHQAPD FLVKIVGQVD VATDFIV
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***Figure 9: (a) Peptide sequence obtained through mass spectrometry of trypsin digest of secreted Spep. (b) Amino acid sequence of Spep. Boldface amino acid residues: sequences of Spep enzyme matching with insecticidal protein of Serratia marcescens; black shadow: zinc binding motif; gray shadow: glycine-rich region associated with calcium binding.***