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Research Article

Isolation and Partial Characterization of Antifungal Protein from *Bacillus subtilis*

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Abstract

Bacillus subtilis was isolated and identified from soil samples of The Nilgiris district of Tamilnadu. From *B.subtilis* antifungal protein was precipitated by using ammonium sulfate precipitation method and desalted by using desalting column. Desalted protein was screened for antifungal activity against *Aspergillus flavus, Aspergillus fumigatus, Candida albicans, Fusarium solani, Penicillium notatum* and *Rhizopus oryzae* . The partially purified antifungal protein was characterized by SDS PAGE and also effect of proteolytic enzymes, heat and pH on anti fungal protein was studied.

Keywords: Antifungal Protein, *B.subtilis*, Antimicrobial Peptide

Introduction

The incidences of fungal infections have increased significantly in past twenty years due to increase in number of people whose immune system is compromised by AIDS, organ transplantation and cancer therapy. This phenomenon is aggravated by the rapid development of drug resistance against most of currently used antifungal drugs such as fluconozole, itraconozole and amphotericin B. The toxicity of few available antifungal agents and development of resistance during treatment in others are becoming a major problem in the management of current antifungal treatment. The development of new antifungal agent with different mechanism of action and acceptable toxicity is thus urgently needed (Monica Benincasa *et al*., 2006) Antimicrobial peptides (AMPs) have become as a potential antibiotics due to their killing ability against a wide spectrum of microorganisms. Unlike currently available conventional antibiotics, which typically interact with a specific target protein, most of these cationic AMPs target the cell membrane of invading microorganisms leading to cell lyses and death (Chan *et al.*, 2006; Durr *et al*., 2006) Thus, AMPs offer the possibility of a new class of therapeutic agents which are complementary to existing antibiotics and to which bacteria may not be able to develop resistance. AMPs are important components of innate defenses of all species of life (Boman, 1995).With this background the present study has been designed for the isolation and characterization of antifungal protein from *Bacillus subtilis* for its antifungal potentials.

Materials and Methods

Sample Collection

Soil samples from the district of The Nilgiris, Tamil Nadu was collected randomly from different areas of the land in sterilized poly bags sealed properly and brought to the laboratory for further analysis. The samples were then stored at 4˚C until used, to minimize the metabolic activities of microorganism

and to keep them in their exact qualitative and quantitative level of population.

Isolation of Bacteria

Serial dilution agar plating method (or viable plate counting method) was followed to isolate bacteria from sample. One gram of soil sample was added to 100 ml of sterile distilled water and serially diluted up to 10^{-8} dilution. Then 0.1mL of each of the dilution was transferred to sterile nutrient agar plates and spreaded using L-rod. The plates were then incubated at 34˚C for 24 hours to obtain colonies. The individual colonies were picked upon the basis of their characteristics such as size, shape, surface appearance, texture and color. Then streaked onto fresh nutrient agar plates for purification. This process was repeated several times until pure cultures of the isolates were obtained. The purified colonies were sub cultured on to nutrient agar slants and stored at 4˚C for further studies and screened for its antimicrobial activity.

Collection of Clinical Pathogens

The clinical fungal pathogens such as *Aspergillus flavus, Aspergillus fumigates, Candida albicans, Fusarium solani, Penicillium notatum* and *Rhizopus oryzae* were collected from Kovai Medical Center and Hospitals, Coimbatore. The clinical fungal pathogens were sub cultured in potato dextrose agar plates.

Screening of Soil Bacteria for Its Antimicrobial Activity

The overnight grown culture of bacterial isolate in nutrient broth was centrifuged at 6000 rpm for 10 minutes and the supernatant was collected. The grown culture of fungal pathogens was uniformly swabbed on the surface of sterile potato dextrose agar plate using cotton swab. Wells of 3mm size were made with sterile cork borer on the seeded plate and 50µL of supernatant was added to the well aseptically. The plates were incubated without inverting for 48 hours at 37°C and the zone of inhibition was observed and recorded.

Bacteria Identification

The isolated strain was identified according to the 16S rDNA sequence of bacteria. Genomic DNA was extracted and purified (by enzymatic method) according to Sambrook and Russell and its purity was assessed as the OD260/OD280. The 16S rDNA gene was selectively amplified with the 16S partial PCR forward (5'- CAGCAGCCGCGGTAATAC-3') and reverse (5'-TACGGCTACCT TGTTACG-3') primers.

A thermal cycler (MJ Research) was used for amplification and programmed as follows: 95^{o}C for 5 min, followed by 30 cycles of 95^{o}C for 1min (denaturation), 59^{o}C for 50 sec (annealing),72^{o}C for 1 min (extension), and a final extension of 5 min to allow for extension of any incomplete products. The amplification products were purified by using gel extraction kits and then DNA sequencing on both strands were directly performed by Genei Pvt. Ltd. Banglore (India).The 16S rDNA sequences were submitted to Genebank .The BLAST algorithm was used to search for homologous sequences in Genebank. The sequence was reverse aligned and compared to similar database sequences, Distance Matrix based on Nucleotide Sequence Homology was studied Using Kimura-2 Parameter. The phylogenetic tree was drawn by using MEGA 3.1 software using Neighbor Joining method.

Production of antimicrobial protein

For the production of antimicrobial protein the bacterial isolates *Bacillus subtilis* was grown in 500 ml TSB medium at 37^{o}C in a rotary shaker at 125 cycles per minute for desired times. Determination of the number of viable cells were (CFU ml^{-1}) carried out by viable plate count method. After cultivation for 72 hours the CFS was collected by centrifugation at 10,000 g for 15 minutes.

Protein Precipitation

Ammonium Sulphate precipitation Method

To the cell free supernatant, ammonium sulphate was added to achieve 30% saturation. It was stirred well and incubated at 4°C for overnight. After incubation period, the precipitate was collected by centrifugation at 6000rpm for 15 minutes at 4°C.The precipitates were dissolved in sterile distilled water. Antifungal activity of the precipitate was tested using agar well diffusion assay and the results were recorded.

Desalting of Precipitated Proteins

The desalting column and buffer (25mM Sodium chloride) was brought to room temperature before use. The top cap of the column was removed followed by the bottom cap. Care should be taken to prevent the air bubbles in the column. The column was washed 5 times with 25mM sodium chloride buffer and was

allowed to flow under gravitational force. The sample was loaded gently and allowed to drain completely. Antimicrobial activity of the desalted protein was tested by agar well diffusion assay and the result was recorded

Screening For Antifungal Activity of Antimicrobial Proteins

The antifungal activity of protein against clinical fungal pathogens was tested by agar well diffusion method. Potato Dextrose Agar plates were prepared. Clinical fungal pathogens were uniformly swabbed on surface of PDA plates using sterile cotton swab moistured with saline. 20µg of protein was added to well aseptically. Then the plates were incubated without inverting at 25º-28ºC. The zone of inhibition was observed for every 24 hours interval. The entire antifungal activity assay was carried in triplicates and mean of the inhibition zone was calculated.

Characterization of antimicrobial proteins

Effects of proteolytic enzymes, heat and pH on anti microbial proteins

Effect of Proteolytic Enzymes on antimicrobial proteins

The effects of proteolytic enzymes were tested on antimicrobial protein. Aliquots of antimicrobial proteins were treated with papain, trypsin or pronase E at final concentration of 2 and 10 mg ml^{-1} at 37°C for one hour. An untreated protein and the enzyme in the buffer alone served as control. After this treatment antimicrobial proteins were checked antibacterial activity.

Thermal stability analysis of antimicrobial proteins

To analyze the thermal stability, aliquots (50 µg) of proteins were exposed to temperature ranging from 30 to 100 °C for 30 minutes, 100 °C for 5- 60 minutes,121 °C for 15 minutes and frozen for up to 30 days. After this treatment antimicrobial proteins were checked antibacterial activity.

Analysis for pH stability

Samples of the antimicrobial proteins were incubated at different pH values (pH 3-11) for 2 hours at 25°C, neutralized to pH7 and tested for antimicrobial activity.

Analysis of antimicrobial proteins by SDS PAGE

Glass plates, spacers and comb were cleaned thoroughly and dried.Glass plates were sealed using vaseline and assembled using clips.12% separating gel was prepared and poured inside the chamber between the glass plates without any air bubbles and allowed to polymerize for 30-60 minutes.After polymerization, 3% stacking gel was prepared and poured over the resolving gel.Comb was inserted between the glass plate and the gel was allowed for polymerization.After polymerization of stacking gel, comb was removed gently without destroying the shape of the wells.The spacer at the bottom of the glass plate was removed and the plate was fixed in the electrophoretic apparatus.Running buffer was poured into the electrophoretic tank without any air bubbles.The sample to be loaded was prepared by mixing sample and loading dye in 1:2 concentrations. Marker was also prepared in the same way.It was then denatured for 3 minutes and loaded in the wells. Marker was added in the first well and power was supplied. Once the bromophenol blue dye reaches 1cm above the bottom of the gel, current was put off.The gel was then carefully removed for staining.

Protein visualization by silver staining of gels

The gel was kept in fixation solution for 1 hour with gentle shaking.It was then rinsed with 50% methanol for 12 minutes.The gel was rinsed with double distilled water twice for 5 minutes.The gel was then immersed in 0.02% sodium thiosulphate solution for 60 seconds exactly with gently shaking.It was rinsed with double distilled water twice for a minute.The gel was immersed in chilled 0.1% silver nitrate solution. It was covered with foil paper and incubated for 25 minutes with gentle shaking in the cold room.The gel was rinsed with double distilled water twice for 20 seconds.It was then immersed in developing solution and was shaken in room temperature until bands appear.The reaction was stopped by removing developer and swirling in fix solution for 5 minutes. Finally, the gel was rinsed with double distilled water thrice for 1 minute.

Results and Discussion

Totally 12 different bacteria were isolated from soil samples, based on their appearance, size, shape and color. These 12 bacterial isolates were screened for antifungal activity against *Aspergillus flavus, Aspergillus fumigates, Candida albicans, Fusarium solani, Penicillium notatum* and *Rhizopus oryzae* Out

of these 12 bacterial isolates the isolate which has shown effective growth inhibition activity against fungal pathogens was identified as *Bacillus subtilis* by 16S rDNA sequencing. The isolated *B.subtilis* has the closest homolog with the *Bacillus sp*.M307, *Bacillus sp*.M306 and *Bacillus subtilis* subsp.strain SB3130. Motta *et al*., (2007) have reported that 16s rDNA based identification of bacterium P34 as *Bacillus* species which producing a bacteriocin like substance. The cell free supernatant (CFS) of *B.subtilis* was collected and it was observed to inhibit the growth of clinically significant fungal pathogens. These findings clearly explain the presence of extracellular antimicrobial substance in the culture supernatant. In order to isolate the antimicrobial substance the cell free supernatant was collected by centrifugation of culture broth and it was sterilized by 0.25µm membrane filter. The filter sterilized CFS from *B.subtilis* was treated with ammonium sulphate (30% Saturation) to precipitate the protein present in the CFS. The precipitated protein was desalted by using readily available packed desalting column purchased from Bangalore Genei.

During the preliminary small scale production (500ml) without pH control at the beginning pH was 7.4± 0.2, at the end of 72 hours it was observed as 6.8± 0.4. The spent broth i.e, cell free supernatant of 500ml yields 2.2gm of protein after 30% saturated ammonium sulphate precipitation. After purification with desalting column, 0.8gm of protein was obtained from *B.subtilis.*

The antimicrobial peptide of *Pseudomonas aeruginosa* 4B was precipitated by ammonium sulphate. After cultivation for 108 hours the cells were harvested by centrifugation at 10,000 g for 15 minutes. Ammonium sulphate precipitate was dissolved in 10mm Tris buffer pH-7.5. (Motto and Brandelli, 2002).As described above in the present study also the antimicrobial proteins were isolated by ammonium sulphate precipitation method from the cell free supernatant of *B.subtilis* .

Antifungal spectrum of *B.subtilis* antimicrobial protein was studied (Table1). Briefly the *B.subtilis* antimicrobial protein has the ability to inhibit the growth of all fungi tested i.e: *A.flavus* (16mm), *A.fumigates* (18mm); *Candida albicans* (16mm), *Fusarium solani* (22mm), *Rhizopus oryzae*(19mm) and *Penicillium notatum* (21mm). Jesper Magnusson *et al.,* (2003) have described the antifungal activity of lactic acid bacteria against fungal pathogens like *Aspegillus fumigates, Aspegillus nidulans, Penicillium commune* and *Fusarium sporotrichorides*.

The antimicrobial protein isolated from *B.subtilis* was tested for sensitivity to several proteases like papain, trypsin, protienase K and pronase E. The residual activity of 0% was observed after the enzyme treatment. At the same time while the substances were treated with lipase. It has produced the 96% of residual activity. This clearly demonstrates the protein nature of the antimicrobial substance. The antimicrobial protein obtained from *B.subtilis* was active over a pH range of 4-10 remaining about 100% residual activity. Further the antimicrobial protein was stable till 80ºC. After autoclaving 65% residual activity was observed. Remarkably their activity was not lost after 30 days of freezing temperature storage.

The isolated peptides from *B.subtilis* were analyzed for enzyme treatment, heat treatment and pH effects. The study report explains the ability of protein to retain the activity upto 80C. Further it was active in wide pH range from 4-9. And also it retains 100% residual activity after the freezing temperature storage. Similarly thuricin isolated from *B.thurin genesis* exhibits a narrow range of inhibitory activity. The peptide shows no loss of activity on heat treatment up to 80°C and over a pH range (Melissa *et al*., 2003) .

SDS-PAGE electrophoresis of *B.subtilis* protein showed 2 major bands with molecular weight of 47 kDa and a band between 21 to 31 kDa. Parret *et al*., (2005) reported that 30 kDa protein isolated from *Bacillus cereus* have significant antibacterial activity.

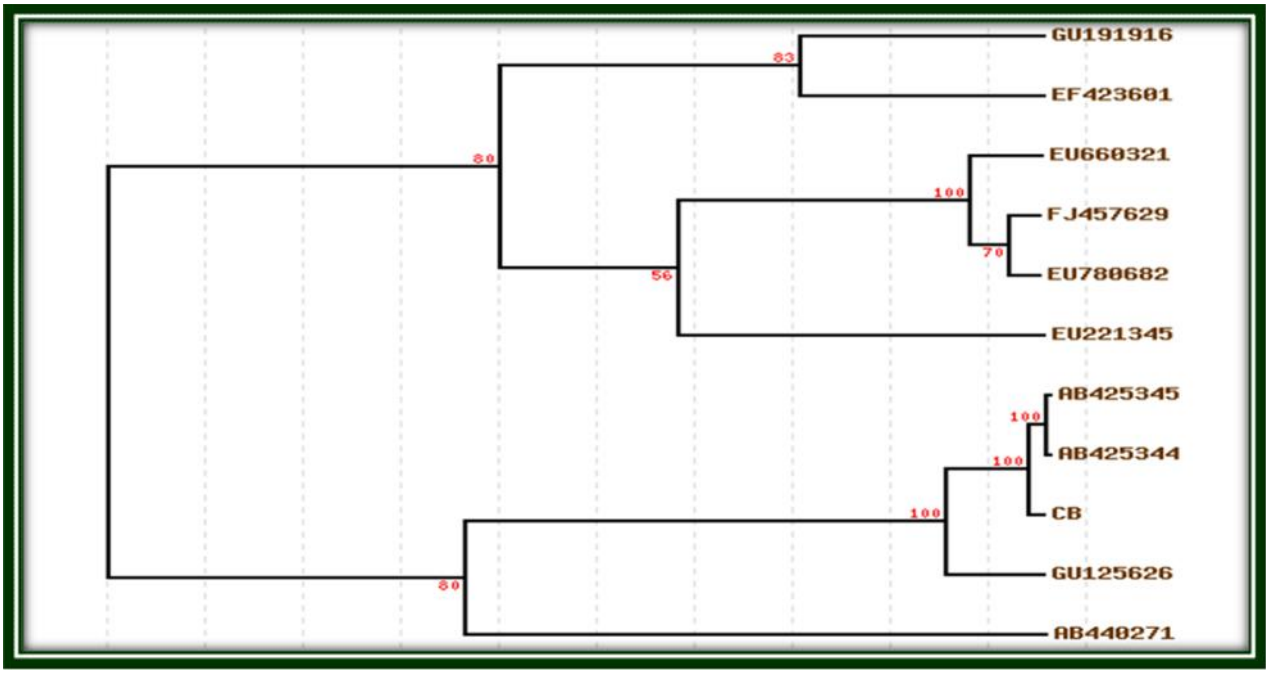
The result clearly demonstrates the antifungal potentials of the antimicrobial protein isolated from *B.subtilis*. Further characterization of this antimicrobial proteins will pave the way for novel protein based drug development to treat multi drug resistant organisms In future this protein may be a good candidate in the field of fungal treatment.

Agent	Pathogens	Amount	Zone of inhibition mm(±SD)
B.subtilis Antifungal protein(Column purified	*Aspergillus flavus*	50µg	16(0.3)
	Aspergillus fumigates	50µg	18(0.3)
	Candida albicans	50µg	16(0.3)
	Fusarium solani	50µg	28(0.2)
	Rhizopus oryzae	50µg	19(0.3)
	Penicillium notatum	50µg	21(0.3)

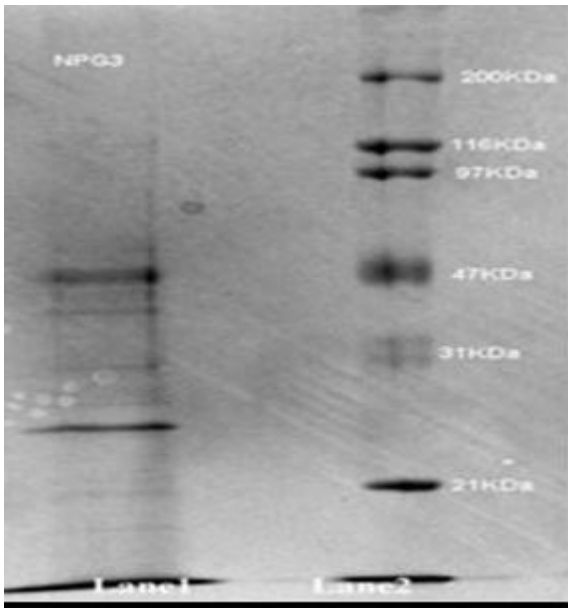
Antifungal activity study of antimicrobial protein isolated from *B.subtilis*

GACGAACGCTGGCGGCGTGCCTAATACATGCAAGTCGAGCGGACAGATGGGAGCTTGCTCCCTGATGTTAGCGGCGGAC
GG
GTGAGTAACACGTGGGTAACCTGCCTGTAAGACTGGGATAACTCCGGGAAACCGGGGCTAATACCGGATGGTTGTTTGA
AC
CGCATGGTTCAAACATAAAAGGTGGCTTCGGCTACCACTTACAGATGGACCCGCGGCGCATTAGCTAGTTGGTGAGGTAA
C
GGCTCACCAAGGCRACGATGCGTAGCCGACCTGAGAGGGTGATCGGCCACACTGGGACTGAGACACGGCCCAGACTCCT
AC
GGGAGGCAGCAGTAGGGAATCTTCCGCAATGGACGAAAGTCTGACGGAGCAACGCCGCGTGAGTGATGAAGGTTTTCGG
AT
CGTAAAGCTCTGTTGTTAGGGAAGAACAAGTACCGTTCGAATAGGGCGGTACCTTGACGGTACCTAACCAGAAAGCCAC
GG
CTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGAATTATTGGGCGTAAAGGGCTCGCAGG
CG
GTTTCTTAAGTCTGATGTGAAAGCCCCCGGCTCAACCGGGGAGGGTCATTGGAAACTGGGGAACTTGAGTGCAGAAGAG
GA
GAGTGGAATTCCACGTGTAGCGGTGAAATGCGTAGAGATGTGGAGGAACACCAGTGGCGAAGGCGACTCTCTGGTCTGT
AA
CTGACGCTGAGGAGCGAAAGCGTGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAGTGCTA
AG
TGTTAGGGGGTTTCCGCCCCTTAGTGCTGCAGCTAACGCATTAAGCACTCCGCCTGGGGAGTACGGTCGCAAGACTGAAA
C
TCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAGGT
CT
TGACATCCTCTGACAATCCTAGAGATAGGACGTCCCCTTCGGGGGCAGAGTGACAGGTGGTGCATGGTTGTCGTCAGCTC
G
TGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGATCTTAGTTGCCAGCATTCAGTTGGGCACTCTAAG
G
TGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCCTTATGACCTGGGCTACACACGTG
CT
ACAATGGACAGAACAAAGGGCAGCGAAACCGCGAGGTTAAGCCAATCCCACAAATCTGTTCTCAGTTCGGATCGCAGTC
TG
CAACTCGACTGCGTGAAGCTGGAATCGCTAGTAATCGCGGATCAGCATGCCGCGGTGAATACGTTCCCGGGCCTTGTACA
C
ACCGCCCGTCACACCACGAGAGTTTGTAACACCCGAAGTCGGTGAGGTAACCTTTTAGGAGCCAGCCGCCGAAGGTGGG
AC
AGATGATTGGGGTGAAG

Data showing aligned sequence data (1475bp) of *Bacillus subtilis*



Phylogenetic Tree made in MEGA 3.1 software using Neighbor joining method (*B.subtilis*)



SDS PAGE Analysis of *B.subtilis* and *B.pumilus* antimicrobial protein

Lane.1.*B.subtilis* antimicrobial protein. Lane.2.Marker protein

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References

Boman, H. G. (1995). Peptide antibiotics and their role in innate immunity. Annu. Rev. Immunol, **13:** 61-92.

Chan D. I., Prenner E. J., and Vogel H. J, (2006). Tryptophan and arginine rich antimicrobial peptides: structures and mechanism of action. BBA Biomembranes, **1758:** 1184- 1202.

Durr U. H. N., Sudheendra U. S., Ramamoorthy A. (2006). LL-37, the only human member of the cathelicidin family of antimicrobial peptides. BBA Biomembranes, **1758:** 1408-1425

Jesper Magnusson., Katrin Strom., Stefan Roos., Jorgen S jogren and John Schnurer.(2003). Broad and complex antifungal activity among environmental isolates of lactic acid bacteria. FEMS microbiology,**219:**129-135.

Melissa Ahern., Saskia Verschueren., Douwe Van Sinderen. (2003). Isolation and characterization of a novel bacteriocin produced by *Bacillus thuringiensis* strain B439. FEMS Microbiology Letters, **220:** 127- 131.

Monica Benincasa., Marco Scocchi., Sabrina Pacor., Alessandro Tossi., Donatella Nobili., Giancarlo Basaglia., Marina Busetti and Renato Gennaro.(2006). Fungicidal activity of five cathelicidin peptides against clinically isolated yeasts. Journal of antimicrobial chemotherapy,**58:** 950-959

Motta, A.S.,Brandelli. (2002). Characterization of an antimicrobial peptide produced by *Brevibacterium linens*. J. Appl. Microbiol, **92:**63-71.

Motta, A.S.,Lorenzini, D.M., and Brandelli.(2007).Purification and partial characterization of antimicrobial peptide produced by a novel *Bacillus sps*. Isolated from Amazan basin.Curr Microbiol,**54:**282-286.

Parret, A.H.A., Temmermam,K., and De Mot,R. (2005). Novel lectin-like bacteriocin of biocontrol strain *Pseudomonas fluroscens Pf-5*. Appl Environ Microbial, **71:** 5197-5207.