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RESEARCH ARTICLE

MOLECULAR STUDY ON THE EXTRACELLULAR CHITINOLYTIC ACTIVITY AND ANTIFUNGAL ACTIVITY OF *Bacillus subtilis* TS22

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ABSTRACT

An extracellular chitinase producing bacterium *Bacillus subtilis* TS22 was isolated from shrimp shell waste. It was identified by as gram positive rod shaped bacteria, Indole, Citrate, Oxidase, Catalase, Nitrate positive and Methyl Red, Vogeus Proskauer negative. The carbohydrate fermentation occurs by producing acid but not gas in Glucose, Maltose and Sucrose. The genomic size of *Bacillus subtilis* TS22 was 870bp confirmed with the help of 16s rRNA primer viz forward primer F5-AGAGTTTGATCMTGGCTCAG-3| and reverse primer 5|AAGGAGGTGWTCCARCC-3|.The obtained gene sequence were submitted and deposited to NCBI under accession number JQ 727436. When plotted for phylogenetic tree showed 98% similarities with Bacillus species. The *Bacillus subtilis* TS22 produced the extracellular chitinase having 127U/ml of chitinolytic activity. The molecular weight of chitinase was 50KDa in 12% sodium dodecyl sulphate polyacrylamide gel electrophoresis. The *Bacillus subtilis* TS22 showed antifungal activity against three fungal pathogens such as *Phytophthora parasitica*, *Alternaria solani* and *Pythium aphanidermatum.*

INTRODUCTION

Chitin is the second abundant after cellulose, linear biopolymer in the nature (Watanabe *et al*., 1990). It is widely distributed in nature as the integument of insects, crustaceans, a component of fungal cell wall and Entamoebal cyst wall (Wang *et al*., 2006). It has been estimated that the annual formation rate and steady-state amount of chitin is on the order 1010–1011 tons (Gooday, 1994). The enormous amounts of chitin and chitosan continuously generated in nature require disposal and recycling on a formidable scale (Younes *et al*., 2012). The complete enzymatic hydrolysis of chitin to free *N*-acetyl glucosamine and of chitosan to free glucosamine is performed by enzyme systems, the actions of which are known to be both synergistic and consecutive (Somashekar and Joseph, 1996). The catabolism of Chitin typically occurs in two steps involving the initial cleavage of the chitin polymer by polysaccharides chitinase into Chitin oligosaccharides and then further cleavage to N-Acetyl glucosamine monomers by chitobiases (Pelletier and Sygusch, 1990). Chitinases are a wide spread group of enzymatic systems that breakdown chitin an abundant insoluble linear polymer of β-1, 4-linked N-Acetyl glucosamine (Lee *et al*., 2007). Chitinase enzymes are found in many organisms and their properties to be closely related to their biological functions (Li *et al*., 2007).

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Chitinases are generally found in organisms such as bacteria, parasites, arthropods, crustaceans, higher plants and mammals (Li *et al*., 2004). Many chitinolytic bacteria show antifungal activity against the plant and animal fungal pathogens (Augustine *et al*., 2005; Muraki *et al*., 2002). The technologies have paid the ways to convert the waste materials into use full products by application of highly enzymatic producing bacteria such as the Bacillus subtilis *that leads the deproteinization of the crustacean wastes Yang et al., 2000).*

MATERIALS AND METHODS

Preparation of Colloidal Chitin

The preparation of colloidal chitin was followed Robert and Selitrennikoff modified method (1986) by weighing the 20gms of Chitin powder and were slowly dissolved into 350ml of concentrated Hydrochloride acid (HCL) then left at 40^0C for overnight incubation on stirring position. After incubation, the mixture was dissolved into the 95% ice cold ethanol with rapid stirring and again kept for overnight incubation at 35^0C. The precipitate was collected by centrifugation the solution at 5000g for 20 minutes at 4^0C. The precipitate was washed with sterile distilled water until the colloidal chitin become neutral (pH 7.0).

Chitinolytic Activity of *Bacillus subtilis* TS22

The *Bacillus subtilis* TS22 is isolated from shrimp shell waste for analysis of chitinolytic activity (Younes *et al*., 2012). The

medium was prepared by taking 1% of colloidal chitin in 1% K_2HPO_4, 0.05%$MgSO_4$ were mixed uniformly then 2% of agar was added and sterilized. After sterilization the isolated bacterium was inoculated by streaking and stabbing into the medium then followed by incubation at 28^0C for 7 days to screen out the chitinolytic activity. The highest zone of hydrolysis producing bacterial strain was selected which revealed that strain secretes more extracellular chitinolytic enzyme then proceeded for the further study.

Identification of *Bacillus subtilis*

The morphological and biochemical characterization of the isolated bacterial strain *Bacillus subtilis* TS22 was performed according to method of Garrity *et al*. (2005).

Microscopic morphology

The microscopic identification of bacterial strain TS22 was followed the Gram's staining, Capsule staining, Endospore, flagella staining.

Biochemical Tests

The biochemical identification of bacterial strain TS22 was done by carrying out the tests such as Iodole, Methyl red, Voges-Proskauer, Citrate, Oxidase, Catalase, Nitrate, Gelatin liquefaction, Starch hydrolysis. The carbohydrate fermentation tests such as Glucose, Maltose, Ribose, Rhamnose, Sucrose and Xylose.

Molecular confirmation by 16s rDNA

Extraction of bacterial DNA

The DNA was extracted from the bacterial isolate by following the method of Sambrook *et al*. (2001). The single colony of bacterial strain *Bacillus subtilis* TS22 was inoculated into the 50ml of Luria Bertani broth and incubated at 20°C on rotatory shaker, overnight. Then, 1.5ml of culture was transferred into eppendroff tubes and spun at 8,000 rpm for 10 min. The supernatant were discarded and drained on tissue paper. The pellet was resuspended into 400µl of TE buffer and 32µl of lysosome was added then incubated at 37°C for 30 min. After incubation at 37°C for 30 min, 100µl of 0.5mM EDTA was added followed by 60µl of 10% SDS and 1.5µl of proteinase K (50µl/1ml) respectively then incubated at 50°C for 60 minutes. After incubation at 50°C for 60 minutes, tubes were brought at room temperature and 250µl of phenol: chloroform: isoamylalcohol in the ratio of 25:24:1 were added and centrifuged at 10, 000rpm for 10 minutes. The aqueous phase was transferred to another eppendroff tubes and RNAase was added at final concentration of 50µl/ml and mixture was incubated at 60°C for 1 hour. After incubation, DNA was precipitated with ice cold ethanol and precipitated DNA was collected by centrifugation at 5,000rpm for 10 minutes. Finally, the pellet was washed with 70% ethanol and completely air dried and resuspended in l00µl of TE buffer at pH 8.0.

Polymerase chain reaction

The polymerase chain reaction was carried out by following a method of Sambrook and Russel (2007). The 16s rDNA forward primer F5- AGAGTTTGATCMTGGCTC AG-3| and reverse primer 5|AAGGAGGTGWTCCARCC-3| was used to obtain amplification. The 50µl reaction mixture was transferred into 0.5ml microfuge amplification tube containing mixture in the following order: 10×amplification buffer (5µl), 20mM solution of four dNTPs, pH 8.0 (1µl), 20 µM forward primer (2 µl), 20 µM reverse primer (2 µl), Taq DNA polymerase (2 µl), nuclease free water (33 µl), DNA sample (5µl), and total volume (50µl). The reaction mixture was centrifuged at 4,000 rpm for 5 minutes then placed in the thermal cycler fitted with heated lid. The nucleic acids amplified by setting denaturation at 94°C for 1 minute, annealing at 55°C for 30 seconds and extension at 72°C for 1 minute in the thermal cycler and number of denaturation cycles repeated to 30 cycles. After polymerase chain reaction, 15µl of amplified DNA product was run in an agarose gel electrophoreses by preparing 1.5% agarose gel and DNA bands were viewed under the UV transilluminator.

Automated sequencing

The sequencing of the genomic DNA amplicon coding for bacterial strain *Bacillus subtilis* TS22 was carried out at scientific synergy company, Canal Bank Road, Chennai, India using an instrument ABI3130, Amersham Biosciences United kingdom by following method of Sambrook *et al*. (2001).

Chitinase Assay

The chitinase assay was followed by method of Imoto and Yagashitha (1971) using colloidal chitin as the substrate. The enzyme solution (0.2ml) was added to 1ml substrate solution, which contained purified 0.3% colloidal chitin in sodium phosphate buffer (50mM, pH 6.0). The mixture was incubated for three hour at 55^0C then centrifuged at 10000g for 10 min. After centrifugation the reaction was stopped by boiling the 450µl of supernatant, 650µl of 0.5M sodium carbonate and 0.05% potassium ferricyanide reagent for 15 minutes. The Reducing sugar level were determined relative by keeping to (10-200µl/ml) N-Acetyl-β-D glucosamine as standard (Miller 1959). The substrate and enzyme blanks were prepared and the absorbance was measured at 420nm in ultra violet visible spectrophotometer. One unit of chitinase activity is defined as the amount of enzyme producing 1µmol reducing sugar per minute. The formula applied to calculate the enzyme activity was used as Enzyme Activity (U/ml) is equal to change in absorbance per minute x 1000 x total volume of reaction mixture divided by Molar extinction co-efficient x Volume of enzyme in Assay

Molecular weight determination by SDS-PAGE

The molecular weight of the protein in bacterial strain *Bacillus subtilis* TS22 was determined by the method of Laemmli *et al*., (1970) staining the protein with 10% methanol, 7% acetic acid and 0.2% coomassie brilliant blue for 4 hours and destained with 10% methanol, 25% acetic acid solution for 12 hours.

Analyzing Antifungal Activity of Bacterial strain *Bacillus subtilis* TS22

The spot inoculation method for antifungal activity of bacterial strain *Bacillus subtilis* TS22 was followed by Ajar *et al*. (2009). The nutrient agar medium was prepared, sterilized at

121^0C and poured into the sterilized peteriplates and allowed to solidify under aseptic conditions. After solidification bacterial strain *Bacillus subtilis* TS22 was spot inoculated on nutrient agar medium and incubated at 37^0C for 48 hours. After incubation the molten potato dextrose agar medium containing the spores of test fungus, was spread on the same plate and reincubated at 27^0 C for 3 days.

RESULTS

Analysis of Chitinolytic Activity of *Bacillus subtilis* TS22

After the incubation for 7 days the bacterial strain *Bacillus subtilis* TS22 showed a clear zone around the colonies in the colloidal chitin medium thus indicates that it had the ability to produce extracellular chitinase which utilizes the chitin as sole source of nitrogen and leads the formation of zone around the colonies.

Identification of *Bacillus subtilis*

The isolated bacterial strain TS22 observed as rod shape, Gram positive bacilli, centrally located oval shaped spore, capsulated, motile bacteria.

Biochemical Tests

The isolated bacterial strain TS22 was biochemically identified as *Bacillus subtilis* by showing various reaction interpretations are represented in Table 1.

Table1. The biochemical test of *Bacillus subtilis* strainTS22

S. No	Test	Result	S. No	Test	Result
1	Indole	Positive	8	Fructose	Ferment A^+/G
2	Methyl Red	Negative	9	Glucose	Ferment A^+/G
3	Vogeus Proskauer	Negative	10	Maltose	Ferment A^+/G
4	Citrate	Positive	11	Ribose	Negative
5	Oxidase	Positive	12	Rhamnose	Negative
6	Nitrate	Positive	13	Sucrose	Ferment A^+/G
7	Catalase	positive	14	Xylose	Negative

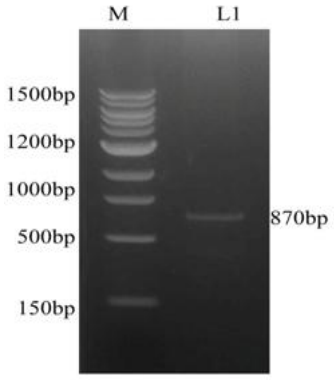

Figure 1. The amplified product of chitin genes of *Bacillus subtilus* TS22 in 0.8% of an agarose gel. Where M-is marker and L1-is amplified DNA showing 870bp.

Confirmation of strain TS22 by 16SrRNA gene

The molecular confirmation of the strain TS22 was identified as *Bacillus subtilis* with the help of 16s rRNA. The genomic DNA was amplified with 16s rDNA primer, Forward primer: 5' TAG GGA AGA TAA TGA CGG3'Reverse primer: 5' CCT CTA TCC TCT TTC CAA CC3'.The amplified PCR product when run in agarose gel electrophoresis, strain TS1 was found having a molecular weight approximately of 870bp when compared with the DNA marker (Figure 1).

16s rRNA Gene sequence

The automated genomic sequence of *Bacillus subtilis* TS22 were obtained are below

AATGCAGTCGAGCGAATGGATTAAGAGCTTGCTCTTA
TGAAGTTAGCGGCGGACGGGTGAGTAACACGTGGGT
AACCTGCCCATAAGACTGGGATAACTCCGGGAAACC
GGGGCTAATACCGGATAACATTTTGAACCGCATGGTT
CGAAATTGAAAGGCGGCTTCGGCTGTCACTTATGGAT
GGACCCGCGTCGCATTAGCTAGTTGGTGAGGTAACG
GCTCACCAAGGCAACGATGCGTAGCCGACCTGAGAG
GGTGATCGGCCACACTGGGACTGAGACACGGCCCAG
ACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCA
ATGGACGAAAGTCTGACGGAGCAACGCCGCGTGAGT
GATGAAGGCTTTCGGGTCGTAAAACTCTGTTGTTAGG
GAAGAACAAGTGCTAGTTGAATAAGCTGGCACCTTG
ACGGTACCTAACCAGAAAGCCACGGCTAACTACGTG
CCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTA
TCCGGAATTATTGGGCGTAAAGCGCGCGCAGGTGGT
TTCTTAAGTCTGATGTGAAAGCCCACGGCTCAACCGT
GGAGGGTCATTGGAAACTGGGAGACTTGAGTGCAGA
AGAGGAAAGTGGAATTCCATGTGTAGCGGTGAAATG
CGTAGAGATATGGAGGAACACCAGTGGCGAAGGCGA
CTTTCTGGTCTGTAACTGACACTGAGGCGCGAAAGCG
TGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCA
CGCCGTAAACGATGAGTGCTAAGTGTTAGAGGGTTTC
CGCCCTTTAGTGCTGAAGTTAACGCATTAAGCACTCC
GCCTGGGGAGTACGGCCGCAAGGCTGAAACTCAAAG
GAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGT
GGTTTAATTCGAAGCAACGCGAAGAACCTTACCAGG
TCTTGACATCTTCTGACAACCCTAGAGATAGGGCTTC
TCCTTCGGGAGCAGAGTGACAGGTGGTGCATGGTTGT
CGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCG
CAACGAGCGCAACCCTTGATCTTAGTTGCCATCATTA
AGTTGGGCACTTTAAGGTGACTGCCGGTGACAAACC
GGAGGAAGGTGGGGATGACGTCAAATCATCATGCCC
CTTATGACCTGGGCTACACACGTGCTACAATGGACGG
TACAAAGAGCTGCAAGACCGCGAGGTGGAGCTAATC
TCATAAAACCGTTCTCAGTTCGGATTGTAGGCTGCAA
CTCGCCTACATGAAGCTGGAATCGCTAGTAATCGCGG
ATCAGCATGCCGCGGTGAATACGTTCCCGGGCCTTGT
ACACACCGCCCGTCACACCACTCGGTT

Phylogenetic Analysis

The genomic sequence of the *Bacillus subtilis* TS22 were obtained and deposited to the NCBI and accession number obtained JQ727436. The phylogenetic analysis showed the 98% similarity with *Bacillus subtilis* as shown in Figure 2.

Chitinase Assay

The chitinase enzyme of *Bacillus subtilis*TS22 showed 127 U/ml of chitinolytic activity.

Molecular weight of protein

The molecular of purified protein was found 50KDa by using the 12% sodium deodyle sulphate poly acryl amide gel electrophoresis

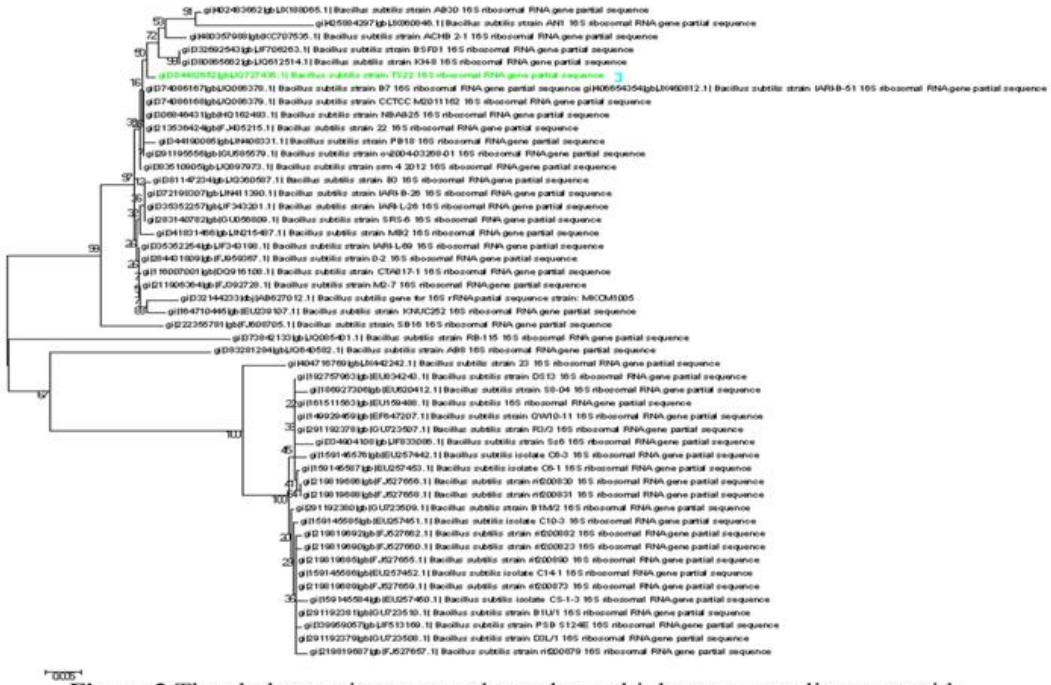

Figure 2.The phylogenetic tree was drawn by multiple sequence alignment with neighbour joining method. The sequences were submitted to Treetop, online tree construction tool, A.N.B Institue, Russia and strain TS22 showed 97% similarities with that of *Bacillus subtilis.*

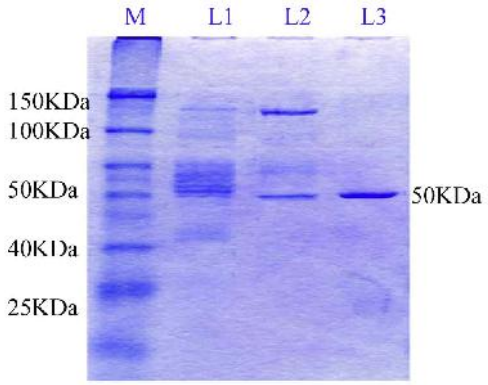

Figure 3. Protein bands of chitinase in 12% SDS-PAGE Where M-is marker protein, L1- cultrual filterate, L2-ammonium sulphate precipitate and L3-DEAE-purified protein.

Antifungal activity of *Bacillus subtilis* TS22

The *Bacillus subtilis* TS22 showed antifungal activity against the fungal pathogens such as *Phytophthora parasitica* with zone of inhibition measured 13mm, *Alternaria solani* showed 14mm a zone of inhibition and *Pythium aphanidermatum* showed 15mm zone of inhibition respectively. The *Bacillus subtilis* TS22 showed the maximum zone of inhibition against *Alternaria solani* which is more than 15mm. The *Bacillus subtilis* TS22 effectively maintained the antifungal activity for a long time against *Phytophthora parasitica* and *Pythium aphanidermatum.*

DISCUSSION

The bacterial isolate *Bacillus subtilis* TS22 bearing genomic molecular weight 870bp exhibited the chitinolytic by showing a clear zone in chitin media (Wang *et al*., 2002; Pelletier and Sygusch, 1990). It indicated the bacteria produced the chitinase enzyme and degrade the chitin in this media, for utilizing the carbon element (Yoon *et al*., 2000; Wang *et al*., 2006). Chitin is a virtuous source of carbon and it is identical essential for the bacterial growth (Liu *et al*., 2010; Tariq *et al*., 2012). The *Bacillus subtilis* TS22 showed the highest chitinolytic activity when compared to *Bacillus subtilis* W-118, *Bacillus* sp. DAU101 and *Bacillus licheniformis* TP1 (Watanabe *et al*., 1990). The assay of chitinase enzyme resulted 127U/ml of chitinolytic activity in colloidal chitin media when used as substrate (Liu *et al*., 2007; Yoohat and Yamabhai 2013; Imoto and Yagishita 1971). The reducing sugar level was determined relative to N-Acetyl -β- D glucosamine standards of 10-200µl/mol (Yang *et al*., 2000; Miller 1959). One unit of chitinase activity is defined as the amount of enzyme producing 1µl/mol reducing sugar per minute (Li *et al*., 2007). The molecular weight of the protein in bacterial strain *Bacillus subtilis* TS22 was found to be 50KDa when stained with 10% methanol, 7% acetic acid and 0.2% coomassie brilliant blue for 4 hours and destained with 10% methanol, 25% acetic acid solution for 12 hours (Laemmli *et al*., 1970). The antimicrobial study revealed that *Bacillus subtilis* TS22 exhibited antifungal activity against the three fungal pathogens which commonly affect in egg plant such as *Phytophthora parasitica, Alternaria solani and Pythium aphanidermatum* (Wang and Hwang 2001; Lang *et al*., 2002). The zone of inhibition indicates the antifungal activity and the highest zone indicates the strong activity (Chandrasekaran *et al*., 2012). The bacterial isolate *Bacillus subtilis* TS22 showed the maximum zone of inhibition against *Alternaria solani* which is more than 15mm (Liu *et al*., 2011). But *Bacillus subtilis* TS22 was effectively maintained the antifungal activity for a long time in *Phytophthora parasitica* and *Pythium aphanidermatum* (Ilina *et al*., 2004; Roberts and Selitrennikov1986).

Conclusion

In this present study, we isolated strain TS22 from the shrimp shell waste was identified by biochemically and gnomically found to be as *Bacillus subtilis*. After analyzed for chitinolytic activity it showed the highest chitinolytic activity when compared the previous studies on *Bacillus subtilis* W-118, *Bacillus* sp DAU101 and *Bacillus licheniformis* TP1. It shows the 127U/ml of chitinolytic activity in colloidal chitin media. The molecular weight of the protein in bacterial strain *Bacillus subtilis* TS22 was found to be 50 KDa. The *Bacillus subtilis T*S22 was effectively shows antifungal activity against, the eggplant fungal pathogens such as *Phytophthora parasitica, Pythium aphanidermatum, Alternaria solani* which is more than 15mm.

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