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18S RIBOSOMAL RNA SEQUENCING AND PHYLOGENETIC ANALYSIS OF POTENTIAL CELLULASE PRODUCER *TRICHODERMA SP.*TSP ISOLATED FROM WOOD COMPOST

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Abstract: Fungi produce certain inducible enzymes like cellulases depending upon the cellulosic substrate on which they act. The cellulolytic fungi include *Trichoderma reesei*, *Trichoderma viride*, *Penicillium pinophilum*, *Phanerochaete chrysosporium*, *Fusarium solani*, *Talaromyces emersonii*, *Trichoderma koningii*, *Fusarium oxysporum*, *Aspegillus niger* and *Rhizopus oryzae* are well known . The demand for cellulosic bioethanol is increasing globally and thus the demand for cellulases which have a great potential in the production of cellulosic bioethanol. Cellulases have most promising application in the bioconversion of renewable lignocellulosic biomass or agro waste into fermentable sugars like glucose which can be fermented to ethanol by suitable yeast. Other major applications of cellulases (E.C.3.2.1.4) include use in textile and detergent industry. Diverse environmental samples like wood compost and soil samples were screened for isolation of hypercellulase producers. A fungal isolate showing significant cellulase activity was isolated. This was preliminarily identified as *Trichoderma sp*. based on colony morphology and microscopic observations. The isolate was identified upto species level by 18S r RNA sequencing and phylogenetic analysis using the Bioinformatics tool, Basic Local Alignment search tool (BLAST N) for nucleotide matching and phylogenetic tree was constructed using MEGA 6.0.6 software (Molecular Evolution Genetic Analysis). 18S r RNA gene sequencing and Phylogenetic analysis of potential cellulolytic fungal *sp* **TSP** was identified to be ***Trichoderma longibrachiatum*.** The isolate was further used for cellulase production using lignocellulosic substrate rice straw and also for synthesis of silver nanoparticles.

Keywords: *18 S r RNA sequencing, Phylogenetic analysis, BLAST N, Cellulase, Silver Nanoparticles.*

I. INTRODUCTION

Many microorganisms belonging to the groups of bacteria and fungi are cellulase producers [1]. The genera *Aspergillus and Trichoderma* are known to be efficient producers of Cellulase [2,3]. In the present day, most commercial cellulases, are produced by *Aspergillus* and *Trichoderma species* [4,5]. Cellulases have diverse applications in animal feed, food [6], textile, [7,8,9]. medical/ pharmaceutical industry, waste management, genetic engineering, protoplast production, and pollution treatment [10,11]. In addition to this, the concerns about the scarcity, shortage of fossil fuels and air pollution caused by incomplete combustion of fossil fuel like petrol, have also led to importance of production of cellulosic bioethanol from renewable lignocellulosic materials used as substrates [12,13]. Various lignocellulosic substrates like rice straw, wheat straw, wheat bran, sorghum straw etc., which can be used as renewable sources of cellulose for bioethanol production are being explored. Cellulose is the most abundant organic polymer and renewable carbon resource on earth. It makes most of the plant cell wall material and associated with other polymers like hemicellulose, lignin and pectin. Chemically cellulose is a polymer of D-glucose structural units [14]. Large amount of cellulose formed annually is degraded by bacteria and fungi to provide themselves with carbon and energy source and for recycling carbon back into the ecosystem through carbon cycle. Bioethanol production from cellulose which is a component of various lignocellulosic feedstocks could be a real time solution for the problem of depleting fossil fuels and attractive alternative for disposal of these residues. They are a promising energy source for several reasons. The main reason is that bioenergy contributes greatly to sustainable development [15,16,17] can reduce greenhouse gas emissions. Cellulosic bioethanol can be produced by degradation of cellulose to its monomeric units of glucose which can be fermented to yield ethanol. The degradation is brought about by synergistic action of cellulases which are extracellular enzymes produced by fungi [18,19,20]. The fungi also are capable of producing various chemicals which are of economic importance and also contribute to the contemporary field of Nanobiotechnology. Nanotechnology is an emerging and promising field in the area of interdisciplinary Biosciences [21]. Nanotechnology mostly deals with the synthesis of different types of nanoparticles by various chemical and physical processes. It is significant not only for the myriad of applications it has but also the way it is synthesized economically, in a green way [22]. In the present scenario there is a need to develop eco-friendly and cost effective process for synthesis of nanoparticles and hence for a 'greener' chemistry and synthesis [23]. Nanotechnology applications are conveniently suitable for molecules of biological origin, because of their unique properties. The biological molecules undergo highly controlled assembly for making them suitable for the metal nanoparticle synthesis which was found to be reliable and eco friendly. Biosynthesis of nanoparticles is gearing up for its huge potential applications [24,25]. Silver nanoparticles have tremendous applications in medical and industrial processes. The need for environmental non-toxic synthetic protocols for nanoparticles synthesis leads to the developing interest in biological approaches which are free from the use of toxic chemicals as byproducts. Thus, there is an increasing demand for "Green nanotechnology". Many biological approaches for both extracellular and intracellular nanoparticles synthesis have been reported till date using

microorganisms including bacteria and fungi. Biosynthesis of silver nanoparticles using fungi, including *Fusarium acuminatum* [26], *Fusarium oxysporum* [27], *Aspergillus niger* [28], *Aspergillus clavatus* [29], *Penicillium fellutanum* [30], *Fusarium solani* [31], *Alternaria alternata* [32] etc., have been successfully used for the synthesis of silver nanoparticles. The fungi which have diverse applications can be identified by morphological observations, colony characters and precisely by molecular methods using 18 S r RNA sequencing using BLASTN. BLAST is an important bioinformatics tool called Basic Local Alignment search tool. *Trichoderma sp*. pose a problem in their distinction among species using basic techniques of morphological studies [33]. Therefore, for specific study and identification of Trichoderma isolate TSP, molecular methods of identification like 18 S r RNA sequencing are used. In the present study, 18S ribosomal RNA gene, ITS1, 5.8S ribosomal RNA gene, ITS2, and 28S ribosomal RNA gene sequences were used for identification of *Trichodermasp*. TSP, upto species level, which was isolated from wood compost.

II. MATERIALS AND METHODS

A. *Isolation of Microrganism*

Diverse environmental samples like wood compost, damp soil, were collected and stored in the lab at 4ºC. The samples were serially diluted by ten fold and inoculated onto sterile potato dextrose agar (PDA) plates for the isolation of fungi. After 4 days of incubation at 28ºC, morphologically different colonies were picked and sub-cultured onto PDA slants to obtain pure cultures. The pure cultures thus obtained were maintained on PDA agar slants at 4ºC for subsequent use. Cellulase producing fungi were selected using phosphoric-acid-swollen cellulose (PASC) containing Mandel's agar medium. Mandel's Medium Composition (g/l): Urea: 0.3, $CaCl_2.2H_2O$: 0.4, KH_2PO_4: 2, $MgSO_4.7H_2O$: 0.3, NH_4SO_4:1.4, Peptone: 1, Tween-80: 0.2, $FeSO_4.7H_2O$: 0.005, $MnSO_4.7H_2O$: 0.016, $ZnSO_4.7H_2O$: 0.014, $CoCl_2.6H_2O$: 0.2, PSAC: 10, Agar: 17.5, Triton-X 100: 1 ml [34,35]. PASC was prepared by soaking cellulose powder (Hi-media) in 1% Phosphoric Acid overnight. The Mandel's media plates were inoculated with pure cultures and incubated at 28ºC for 4 days. After incubation, plates with growth were flooded with Gram's Iodine and left for 5 min., at 35ºC [36,37]. The zones of hydrolysis were observed and measured to identify cellulase producing fungi in comparison with a standard organism *Trichoderma reesei* NCIM 992 (Fig.1). The isolate which showed highest zone of hydrolysis was identified by morphological observations and designated TSP [38]. It was further subjected to molecular identification.

B. 18 S R RNA Sequencing and Phylogenetic Analysis:

The fungus, which produced zone of hydrolysis of maximum size indicated hyper cellulase production and was subjected to genotypic identification by analyzing genomic DNA using the Bioinformatics tool, Basic Local Alignment search tool (BLAST N). This tool was used for nucleotide matching and constructing phylogenetic tree using MEGA 6.0.6 software (Molecular Evolution Genetic Analysis). Total genomic DNA for PCR amplification of 18 S r DNA was extracted from the isolate TSP following the method described in the literature [39]. The 18 S r DNA was amplified by PCR with universal primers used for PCR and bidirectionally sequenced. The primers used were Internal Transcribed Spacers (ITS) 1 and 4. ITS1 Forward primer (5' – TCC GTA GGT GAA CCT GCG G - 3') ITS4 Reverse primer (5' – TCC TCC GCT TAT TGA TAT GC - 3') [40]. Fungal ITS sequences generally provide greater taxonomic resolution than sequences generated from coding regions [41]. Identification was done by matching the isolate 18 S r RNA sequence with NCBI database and the phylogenic tree was constructed using MEGA 6.0.6 software [42] by performing boot strap analysis [43].
In this study Sequence of *Trichoderma* isolate TSP was compared to other r DNA sequences using BLASTN algorithm at the website http://www.ncbi.nlm.nih.gov. The nucleotide sequence of *Trichoderma isolate TSP*. was submitted at the NCBI Genbank [44] so as to obtain Accession number.

C. *Production of Cellulase by Trichoderma Isolate TSP under Submerged Fermentation (SmF)*

Inoculum : *Trichoderma* isolate TSP was maintained as stock culture on PDA slants. They were cultured at 28^0C for 5 days and stored at 4^0C for regular sub culturing. 50 ml of Inoculum medium was prepared in 150 ml flask.

Inoculation media composition (g/l): Corn steep liquor [CSL]: 26.8; KH_2PO_4: 2; $CaCl_2$: 0.3; MgSO4.7H2O: 0.3, $FeSO_4.7H_2O$: 0.005; $ZnSO_4.7H_2O$: 0.0015; $MnSO_4.H_2O$: 0.0014; $CoCl_2$: 0.002; Cellulose: 20g; $(NH_4)_2SO_4$: 1.4g; Urea: 0.3g; Peptone: 0.1g; Tween-80: 0.3%. To 50 ml of the sterile inoculation medium, four or five loopfuls of culture from PDA slant were inoculated. The medium was then incubated in an orbital shaker at 28^0C with shaking at 120 rpm for inoculum development before using for SmF**.**

Submerged Fermentation: For carrying out submerged fermentation using lignocellulosic substrate rice straw, 100 ml of sterile fermentation medium was prepared in a 250 ml flask. The fermentation medium was supplemented with delignified rice straw at 5 % level (w/v), which is a carbon source for the organism. Fermentation medium for cellulase production was prepared with composition (g/l): KH_2PO_4: 1; $CaCl_2$: 0.3; Urea: 0.3; $MgSO_4$: 0.3; $(NH_4)_2SO_4$: 1.4; Peptone: 5; $FeSO_4.7H_2O$: 0.005; $MnSO_4.H_2O$: 0.006; $ZnSO_4.7H_2O$: 0.0014; $CoCl_2$: 0.002; Tween-80: 1; Na_2HPO_4:1; Yeast extract: 1; Rice straw powder: 50; PASC: 10; pH-5. 10% inoculum from inoculation medium was added to the fermentation medium and incubated at 28°C, 150 rpm. The fermentation experiment was done in duplicates. Sampling was done from 3rd day up to 6th day of incubation to perform enzyme assay (Filter Paper Assay).

Determination of enzyme activity: Fermented samples were withdrawn from 3rd day upto 6 days, for determination of enzyme activity. Cellulase activity (FPA) was analysed on filter paper, according to Ghose [45]. One unit of enzyme corresponds to the amount of enzyme necessary to form 1 µmol of glucose per ml per minute. The reducing sugars were measured by DNS method according toMiller [46].

D. **Biosynthesis of Silver Nanoparticles:**

Production of *Trichoderma* isolate TSP Biomass: The spores of TSP were used for the production of required biomass. 100 ml of potato dextrose broth (Hi-Media) was inoculated with 10^5 spores/mL of spores in 250 ml Erlenmeyer flask. The flask was kept in a shaker incubator at 120 rpm, 28° C, for 3 days for the biomass to develop. After 3 days the fungal culture was filtered through Whatman No.1 filter paper to separate out the culture filtrate and biomass.

Synthesis of Silver Nanoparticles (AgNPs) using culture filtrate of *Trichoderma* isolate TSP: The biomass was washed thoroughly with sterile distilled water to remove residual medium components. Approximately 25 g of the biomass was aseptically transferred to a 250 mL flask having 100 ml of distilled water and incubated for 3 days in a shaker incubator at 120 rpm, 28° C. After incubation, the culture was filtered through Whatman No.1 filter paper to obtain clear fungal filtrate. 20 ml of the fungal filtrate was added with 20 ml of 0.1mM Silver Nitrate ($AgNO_3$) solution in a 100 ml flask and incubated in dark in shaker incubator at 120 rpm, 28° C. During incubation the silver nanoparticles were produced by reduction of silver ions to metallic silver with $AgNO_3$ acting as substrate for the fungal enzymes.

Characterization of biosynthesized nanoparticles:

Visual Analysis:
After incubation for 24 hours, change in the color of the culture filtrate and $AgNO_3$ mixture was observed. This mixture was further subjected to **UV-Visible spectroscopic analysis.**

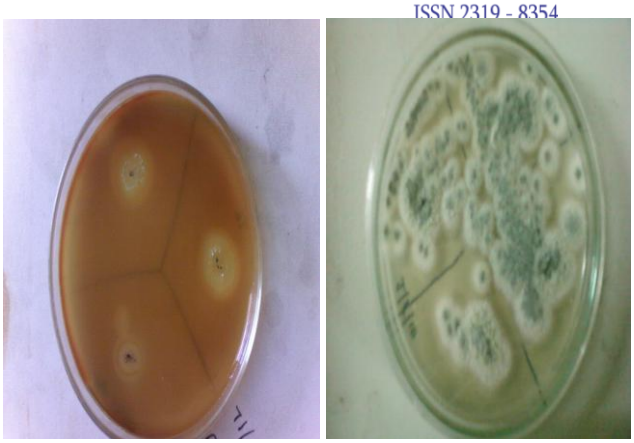
UV-Visible spectroscopic analysis:
The UV-Visible spectra of the culture filtrate and $AgNO_3$ mixture was recorded from a wavelength of 320 to 500 nm, using UV-Vis spectrophotometer. The peak value of wavelength gave the surface Plasmon resonance of silver nanoparticles.

III. RESULTS

A. Isolation of Microorganism:

Based on the size of zones of hydrolysis, one fungal strain (*Trichoderma sp*.) was selected, isolated and cultured onto fresh PDA medium plate (**Fig.1**). The fungus *Trichoderma sp*. TSP was identified based on its cultural characteristics and microscopic morphology. This strain *Trichoderma sp*.TSP was used for the production of cellulase under Submerged Fermentation (SmF).

Fig: 1 Zones of Hydrolysis on PDA medium plate and fungal isolate *Trichoderma sp*. TSP



B. BLAST N analysis:

The sequence of TSP was 1046 bp in length. The sequence was deposited at the website http://www.ncbi.nlm.nih.gov in the NCBI, Genbank with the accession Number Bank It 1858505 TSP: KT844471.

1aaagttgtga ttgcatctac agacgccccg cgaggtccgc ccagagaggc tcagagcaaa
61aaataaaata gagtcgcgag ggacgccgag gagagaaaaa aaaaagattg agtgtgcttt
121cggcggcgcc atggatccgg gttggacgcg cccgggctat gaatcccgc cgaggcaaga
181 gattggtaac gttcacattg gggtatggag ttgtaaactc ggtagttttt cctagagggg
241 gctgcggagg gatcattacc gagtttacaa ctcccaaacc ccaatgtgaa cgttaccaat
301 ctgttgcctc ggcgggattc tcttgccccg ggcgcgtcgc agccccggat cccatggcgc
361 ccgccggagg accaactcca aactcttttt ttctctccgt cgcggctccc gtcgcggctc
421 tgttttattt ttgctctgag cctttctcgg cgaccctagc gggcgtctcg aaaatgaatc
481 aaaactttca acaacggatc tcttggttct ggcatcgatg aagaacgcag cgaaatgcga
541 taagtaatgt gaattgcaga attcagtgaa tcatcgaatc tttgaacgca cattgcgccc
601 gccagtattc tggcgggcat gcctgtccga gcgtcatttc aaccctcgaa ccccctccggg
661 gggtcggcgt tggggatcgg cccctcaccg ggccgccccc gaaatacagt ggcggtctcg
721 ccgcagcctc tcctgcgcag tagtttgcac actcgcaccg ggagcgcggc gcggccacag
781 ccgtaaaaca ccccaaactt ctgaaatgtt gacctcggat caggtaggaa tacccgctga
841 acttaagcat atcaaagtcg gggagagaaa aattaccgag tttacaactc caaaccccaa
901 tgtgaacgtt accatctatt gcctcggcag gaattctcta gcccgggcgc tcacaccctg
961 atccatgggg ccccgagaga gaaatctcac atttattttt ttctttcctc cggcttcgtc
1021 gcgctttatt tttatgtttt gctctc.

C. Cellulase production by *Trichoderma* isolate TSP (KT844471) under Submerged Fermentation with Rice straw:

When submerged fermentation was carried out using the lignocellulosic substrate rice straw, by the isolate Trichoderma TSP, a maximum average cellulase yield of 0.43 U/ml was recorded on 5th day of incubation.

Table: 1Enzyme activity (FPA) of crude cellulase enzyme

	Filter Paper Assay (U/ml)			
Day	**3rd day**	**4th day**	**5th day**	**6th day**
Enzyme activity	0.09	0.11	**0.43**	0.14

D. Biosynthesis of Silver Nanoparticles (AgNPs) using culture filtrate of *Trichoderma* isolate TSP :

Figure 2 shows Flask **A** of the fungal culture filtrate and before immersion in 0.1 mM $AgNO_3$ solution. The pale yellow color of the fungal cells can clearly be observed The fungal cells after immersion in 0.1 mM $AgNO_3$ solution for 24 hours is shown in Flask **B**. It can be observed that the previous pale yellow color of the reaction mixture is changed to the brownish color after 24 hours of incubation in dark. The appearance of brown color in solution containing the biomass is a clear indication of the formation of silver nanoparticles in the reaction mixture. The color of the solution is due to the excitation of surface plasmon vibrations in the silver nanoparticles.

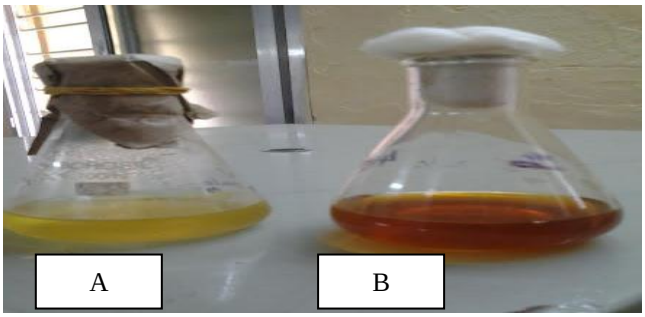


Fig: 2 Flasks A and B containing *Trichoderma sp.* TSP culture filtrate before and after exposure to Ag+ ions for 24 hours

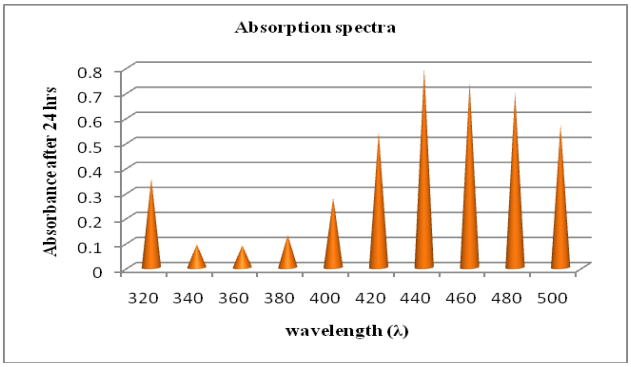
Ultraviolet-Visible (UV-Vis) Spectroscopic Analysis:

The absorption spectra recorded at various wavelengths (λ) from the ***Trichoderma* TSP** reaction flask after 24 hours are given in **Table 2, Figure 3**. The strong surface plasmon resonance centered at 440 nm is characteristic of colloidal silver and similar result was reported by Mukherjee et al. [47] where the peak was found at 410 nm. According to most reports the absorption spectrum of spherical silver nanoparticles is found maximum between 420 nm and 450 nm [48].

Table: 2 Absorption spectra recorded at various wavelengths

S.No	Wave length (λ)	Absorbance (24 hrs)
1	320	0.355
2	340	0.092
3	360	0.088
4	380	0.129
5	400	0.279
6	420	0.542
7	**440**	**0.797**
8	460	0.739
9	480	0.700
10	500	0.571
11	320	0.355

Fig: 3 Maximum Absorption Spectra shown at 440 nm.



IV. DISCUSSION:

In the present study, fungus from natural environmental sources like wood compost was isolated and identified upto genus level as *Trichoderma* and coded as TSP. For the first time a single isolate was studied for its diverse applications like, its potential for producing cellulase and mycosynthesis of silver nanoparticles. The cellulase was produced in an economical and cost effective method, utilizing lignocellulosic agro waste substrate like rice straw. Using fungal isolate *Trichoderma* sp. TSP for synthesis of nanoparticles itself is most economical, greener and safe method in nanotechnology. Molecular methods of identification were performed for identification of this potential organism TSP upto species level using standard forward and reverse primers ITS1 and ITS4. The fungal isolate was successfully identified T*richoderma longibrachiatum.* with the accession Number KT844471

Among several organisms studied *Aspergillus niger* is found to be efficient in degrading various agricultural wastes like Rice husks, Millet husks, wheat bran etc for the production of cellulases [49].

Pasha et. Al., [50] reported 0.41 U/ml of filter paper activity by *Aspergillus niger*, using crystalline cellulose as carbon source supplemented with the basal medium under submerged conditions. The isolate *Trichoderma sp.* TSP showed similar yields of cellulase using rice straw under submerged fermentation. The Filter paper activity was found to be 0.43 U/ml.

In the mycosynthesis of silver nanoparticles, enzymes are produced which reduce a salt to its metallic nanoparticles through the catalytic effect. In the current study the color change from faint yellow to brown color was observed within 24 hrs after incubation with 0.1 mM silver nitrate. However, *Nigrospora sphaerica*, isolated from the soil samples ofcultivated localities, had synthesized silver nanoparticles after 72 hrs incubation with 1 mM silver nitrate solution [51]. *Aspergillus flavus* NJP08, another soil fungus, also shown brown color after 72 hrs incubation [52]. Therefore, it is apparent that the isolated fungus *Trichoderma sp.*TSP is a potent organism showing synthesis of nanoparticles in a short time of 24 hrs than the fungi reported previous studies [51,52].

V. CONCLUSIONS:

The results underline the importance of using molecular approach which serves as a valuable tool in inferring phylogenetic relationships among species in *Trichoderma and* also provides systematic information about identification of *Trichoderma* strain upto species level. The molecular methods being highly selective and sensitive, stand important in generating a robust database about the fungal isolate from wood decompost. Due to impact of various environmental conditions on morphological and physiological characteristics, identification of fungal isolates is a difficult task. Therefore, use of molecular techniques may address this problem and proves to be more significant for the characterization of the new fungal isolates. The present investigation, with the aid of morphological observations and molecular techniques, identified *Trichoderma sp*. TSP which could able to produce both cellulase and silver nanoparticles thereby proving the diverse applications of the organism. Hence, *Trichoderma* isolate TSP identified as *Trichoderma longibrachiatum* with accession number **KT844471** in this study can be effectively exploited for the cost effective production of important enzymes like cellulase and also greener synthesis of silver nanoparticles both of which have their own applications in the field of industrial biotechnology and also medicine

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