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# IMPACT OF ZNO NANORODS ON RNA AND PROTEIN OF *P.INDICA* TO ENHANCE ITS GROWTH

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## INTRODUCTION

*P.indica* is a symbiotic fungus which belongs to the order Sebacinales in the Basidiomycota. The fungus was initially isolated from the soil of roots of woody shrubs of northwestern part of India.[1] It has multifunctional activities as it interacts with many plant species to induces plant growth and biomass production as it mobilises and transfer all essential micronutrients, minerals like phosphorus, nitrogen from soil to plants.[2] All this makes *P. indica* a promising candidate for its use as bio-fertilizer and soil amendment.

Even though there are abundant reports for the applications of mycorrhizal fungi in the field of agriculture and horticulture, but its application in the field of biotechnology can not be subjugated to that level where they be worthy of, because they are not cultivable axenically.[3,4] *P.indica* being the tonic for the plant growth.[5] The development of technologies that would improve and maintain the overall growth of the fungus gained importance.

As Zinc is an vital material for natural world with small size (1-100nm) and high volume ratio to use as a fertilizer in plants to enhance its metabolic activities.[6] It has been stated that application of NPs like ZnO, $TiO_2$, Ag as a micronutrient fertilizers is an essential way to liberate all the necessary nutrients step by step.[7] to modify plant physiological processes to endorse the growth and development of plant. There are numeral of researchers which have reported the importance of function of zinc to boost plant growth and gross yield.[8] Zn is compulsory for production of chlorophyll, germination, fertilization and pollen function. It also plays an imperative part in biomass production.[9] ZnO nanoparticle exhibit strong adsorption capability and elevatedcatalytic efficiency. Due to their distinctive properties and versatile applications in spin electronics, transparent electronics, piezoelectric devices, UV (ultraviolet) beam emitters, and chemical sensors.[10]

As compared to Titanium dioxide, Carbon nano tubes the ZnO nanoparticles are among the most popular nanomaterials manufactured in industrial scale and are extensively studied material due to their high electron mobility, strong room-temperature luminescence, low toxicity, broad band gap, high-quality clarity and photochemical stability.[11,12] The inexpensive bioreduction of zinc oxide nanoparticles is an upsurge of research at present.[12]

The present work addresses the synthesis and characterization of ZnO nanoparticles obtained through a thermal decomposition method. The particles were then characterized, by evaluating their chemical composition through FTIR spectroscopy, their shape and size via SEM microscopy and Surface charge by Zeta Potential. *P.indica* then grow with and without ZnO nanoparticles, which leads to increase its growth by triggering the growth promoting proteins and genes.

## MATERIALS AND METHODS

### Experimental

Chemicals of analytical grade were used in all the experiments, directly without any further purification, procured from HiMedia (India). In all the conducted experiments, Millli-Q water or double distilled water (ddH2O) was used. Glassware was rinsed with Milli-Q water and air-dried before use in experiments.

### Synthesise

ZnO nanorods were prepared by a mechanical-assisted thermal decomposition process. Zinc acetate dihydrate [Zn $(CH_3COO)22H_2O$] was used as precursor for synthesis of zinc oxide nanorods. In the synthesis process, zinc acetate dihydrate (5g) was ground in a mortar and pestle for 45 min then loaded into an alumina crucible, which was then heated in a programmable

muffle furnace (at ramp rate of4 C/min) at different temperatures (275, 350, 425, and 500$^0$C) for 4 h to obtain four ZnO nanopowders with different aspect ratios. The obtained powders were then washed twice with distilled water, followed by drying in an oven at 80$^0$C for 8 hours. The nanoparticles were spherical, oblong and rod in shape. The diameter varied from 9.6-25.5 nm in size.

**Characterization**

Zinc oxide-nanorod structure and surface morphology of the samples were observed using scanning electron microscopy (SEM) (Model: JEOL-JSM-6010LA) at an accelerating voltage of 20 kV. The absorption spectrum was measured by PerkinElmer Lambda 35 UV-vis spectrometer. Band gap of the sample were calculated using Tauc's plot.

Further the charge on ZnO is determined by Nano Particle Size & Zeta Potential Analyser by AIML ltd. Zeta Potential analysis is a technique for determining the surface charge of nanoparticles in solution (colloids).Zeta Potential is an important tool for understanding the state of the nanoparticle surface and predicting the long term stability of the nanoparticle.

**Fungal Strain and Culture Conditions**

Jaggery medium was found to be the best among different synthetic media to grow the axenically grown fungus *P. indica*.[13] Circular solidified disks (approximately 4 mm in diameter) consisting of actively grown hypha and chlamydospores of *P. indica* were placed on solidified jaggery medium (pH 6.8–7.0, 28 ± 2 ◦C in dark) as well as in broth. After 7 days, the Petri plates were found to be completely filled up with the fungal biomass.

FTIR and Zeta potential analyses were carried on *P. indica* before and after treatment with ZnO-nanorods. The *P. indica* culture without ZnO-nanorods treatment was taken as control.

**Zeta potential measurement**

Zeta potential of the particle is measured by determining the rate at which the particle moves in a known electric field. This method is commonly known as microelectrophoresis. Surface charge properties of AVB were carried out by Zeta potential measurement following the protocol of Cowan et al.4 AVB (4 g/L) was suspended in potassium phosphate (10 mM/L) buffer, adjusted to pH 2.0 to 8.0 and zeta potential was measured by Zetasizer (Malvern Zetasizer).

**FTIR analysis**

Infrared spectra of pristine and mercury adsorbed AVB were recorded on a Nicolet-Magma 750 FTIR spectrophotometer in the region 4000 to 400 cm S41 to predict the molecular interactions between ZnO and the adsorbing sites. The samples were pressed into spectroscopic quality KBr pellet with a sample/KBr ratio about 1/100. Data were collected from multiple scanning with resolution 2 cm-1.

The chemical fixation of *P. indica* was done in order to stabilize and preserve its chemical structure. *P. indica* disks were washed with 0.1 M sodium phosphate (pH 7.4) buffer for 30 min at room temperature, then put the sample in fixative, i.e., 2.5% glutaraldehyde for overnight. In order to remove the glutaraldehyde deposits, the suspension was sequentially washed with 0.1 M sodium phosphate buffer solution (pH-7.4) and distilled water, followed by centrifugation for further isolation. Sample was dehydrated with ascending series from 50 to 100% ethanol (EtOH), in 10% increments for 20 min each and finally kept for drying. The elemental analysis of the sample was carried with FTIR and Zeta Potential to confirm the presence to zinc oxide nanorods in treated sample.

**Measurement of cell growth and sugar consumption rate**

The growth of *P. indica* was expressed in terms of dry cell weight (DCW) per liter of culture broth, which was determined by filtering a known volume of culture broth through Whatman no.1 filter paper, drying to a constant weight in vacuum oven at 60±1°C for about 48 h. After complete drying, weight was determined. The dry weights of samples were taken by using mettler balance. Sugar consumption rate (y (g/g)) was calculated as grams of biomass produced per gram of substrate (carbon source) consumed.

**Spore count by Using Hemocytometer**

Spores were harvested from *P. indica* cultured on agar plate by flooding the culture with 5 ml of 0.05% (v/v) 'Tween 80' solution. The spores were carefully scraped off from the hyphae using sterile glass spreader. Spores were collected in 15 ml centrifuge tube and centrifuged for 5 min at 800 rpm to remove left over hypha fragments. Supernatant was discarded and pelleted spores were counted using hemocytometer.

**Confocal Microscopy**

*P. indica* culture with and without ZnO-nanorods were observed under a confocal laser scanning microscope LSM-780 (Carl-Zeiss, Inc., Jena, Germany). For culture staining, wheat germ agglutinin Alexa Fluor- 488 (WGA-AF488, Molecular Probes, Eugene, OR, United States) was used. Ethanol/chloroform/trichloroacetic acid in the ratio 1/4/0.15% v/v/w were used for the fixation of fungal biomass. The culture was washed three times with distilled H2O, boiled in 10% KOH for 1 min, washed with phosphate buffered saline (PBS). Afterward, biomass was stained with PBS solution containing 0.2% Silwet L-77 and 50µg/mL WGA- AF488. Vacuum infiltration of treated and control biomass in staining solution was done three times under 50 mm Hg vacuum. The cultures were transferred to PBS followed by removal of staining solution. The cultures were analyzed

under confocal microscope, fungal hyphae was strained with WGA-AFA 488.

**Analysis of protein content in fungal biomass by Bradford method**

A sample of treated and non treated fungal biomass was treated with liquid nitrogen using precooled mortar and pestle. Proteins were extracted by homogenizing about 300 mg fresh weight of biomass in cold 0.05 M Tris buffer. A small quantity (e.g., 0.05 g) of the antioxidant polyvinyl polypyrollidone (PVPP) was added to each sample during the homogenization procedure. Homogenates were transferred to cold centrifuge tubes and centrifuged at 10,000 g for 30 min. at 4 °C. After centrifugation, 0.1 mL of each sample supernatant was transferred to assay tubes and then 3 mL of the Bradford reagent (containing the dye) was added to each tube. Finally the absorbance was recorded at 595 nm for total protein estimation using bovine serum albumin as standard (Bonjoch and Tamayo, 2001).

**Protein Analysis by Ms/Ms**

A method to directly identify proteins contained in mixtures by mass spectrometry (MS/MS) is studied. In this method, the mixture of proteins is digested with a proteolytic enzyme to produce a large collection of peptides. The complex peptide mixture is then separated on-line with a tandem mass spectrometer, acquiring large numbers of tandem mass spectra. The tandem mass spectra are then used to search a protein database to identify the proteins present. Results from standard protein mixtures show that proteins present in simple mixtures can be readily identified with a 30-fold difference in molar quantity, that the identifications are reproducible, and that proteins within the mixture can be identified at low femtomole levels.

**RNA Extraction and Quantitative Real-time PCR**

For total RNA extraction fungal materials were powdered under liquid nitrogen using a mortar and pestle. For all experiments, total RNA was extracted from fungal materials using TRIzol (Invitrogen), and aliquots were used for cDNA synthesis with the qScript cDNA synthesis kit (Quanta Biosciences). Forty nanograms of genomic DNA or cDNA were used as template for quantitative real-time PCR (qPCR) analysis, using the SYBR Green JumpStart Taq ReadyMix (Sigma–Aldrich) and the 7500 FAST Real-Time PCR System under standard conditions (Applied Biosystems).

**Statistical Analysis**

Each experiment was carried out in triplicate and final results were expressed as mean±standard error (SE).

## RESULTS AND DISCUSSION

Fig. 1 showed the SEM of ZnO-nanorods (prepared at different temperatures i.e.275, 350, 425, and $500^0$C). The nanoparticles were spherical, oblong and rod in shape. The ZnO-nanorods were of an average diameter 50 nm and length of 500 nm.

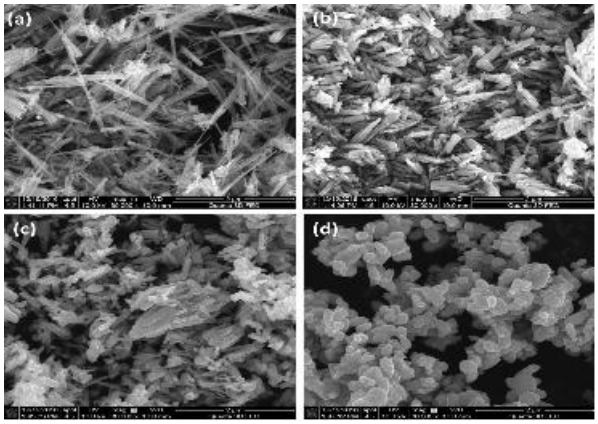



**Fig. 1: Morphology under SEM A. ZnO at 275°C B. ZnO at 325°C C. ZnO at 450℃ D. ZnO 500°C.**

Zeta potential is one of important propertied which could play a major role in effectiveness of nanoparticle. zeta potential is the measure of surface charge of nanoparticles prepared. Surface charge of particle in turn indicates how stable is your particle in the suspension system (higher the surface charge higher is repulsion between particles). Hence it is mandatory to measure zeta potential of your prepared particles. Table 1 indicates the zeta potential of ZnO prepared at different temperatures, which is varied from 8.45 to 16.50 mv. Zeta can be used to predict the long-term stability of particles. For example, particles with zeta potentials larger than ±10 mV have excellent stability, where particles with zeta values between -10 mV and +10 mV, will experience rapid agglomeration unless they are sterically protected.

**Table 1: Zeta Potential of ZnO at different temperatures.**

| Zeta Potential Analyzer | Charge |
|---|---|
| Zeta potential of ZnO at 275°C | 8.45 |
| Zeta potential of ZnO at 300°C | 12.50 |
| Zeta potential of ZnO at 425°C | 16.50 |
| Zeta potential of ZnO at 500°C | 14.51 |

***Piriformospora indica* grown on Jaggery medium fortified with nanomaterials**

Fresh culture of *P. indica* was treated with ZnO-nanorods prepared at different temperature i.e. 275, 325, 450 ,500°C. The untreated culture was taken as control. The study was conducted in triplicates. The cultures were incubated at 27◦C for 8 days. After that the samples were filtered by using Whatman filter paper, and followed by drying of filtered biomass at 70◦C in hot air oven for 24 h. The dry weight of the samples was taken by using the metal balances (Balance AE240 Metler). The fungal biomass observed to be varies from 15.09- 15.87 (g/l) in case of treated sample. The best growth, i.e., 15.87 g/l was observed when the fungus was incubated with ZnO-nanorods prepared at 500°C. The fungus sporulated more faster and maximum spore density and sugar consumption rate was obtained whene *P.indica*

cultivated with ZnO-nanorods prepared at 500°C (Figure 2 and Table 2).

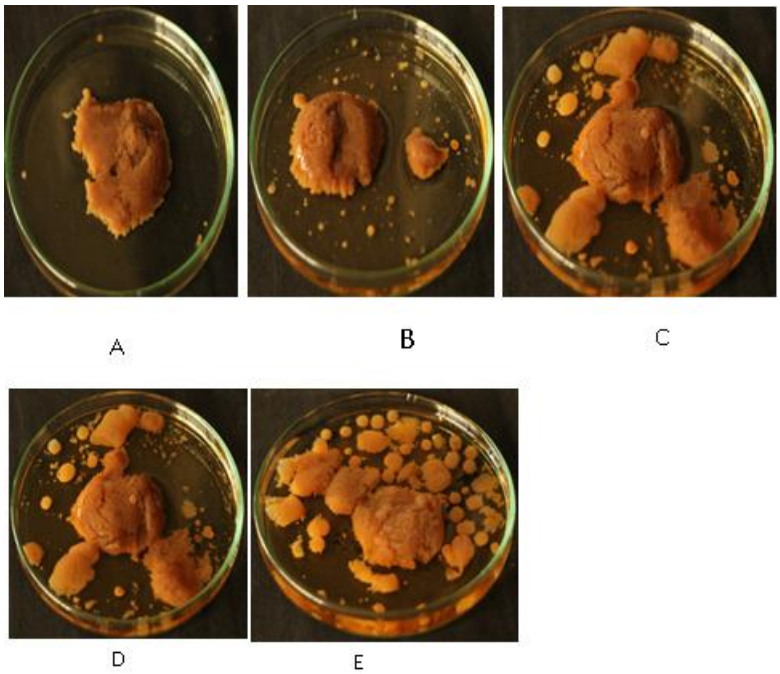



**Fig.2 *Piriformospora indica* grown on Jaggery medium fortified with nanomaterials A. Control B. ZnO at 275°C C. ZnO at 325°C D. ZnO at 450℃ E. ZnO at 500° C**

**Table 2: Effect of ZnO synthesized on different temperature on growth and sporulation of *P. indica.***

| Media | Dry Biomass weight (g/l) | Spore yield (spores/ml) | Substrate consumption rate y(g/g) | Day of start of Sporulation |
|---|---|---|---|---|
| **Control** | 14.16± 0.01 | (5.04 ± 0.01) x $10^9$ | 0.70 | 4 |
| **275°C** | 15.09±0.01 | (6.19 ± 0.005) x $10^9$ | 0.75 | 3 |
| **350°C** | 15.20±0.01 | (6.26 ± 0.01) x $10^9$ | 0.716 | 3 |
| **425°C** | 15.28±0.006 | (6.44± 0.006) x $10^9$ | 0.76 | 3 |
| **500°C** | 15.87±0.01 | (6.97 ±0.006) x $10^9$ | 0.79 | 3 |

The fungal biomass was observed to be 16.09,15.42, 14.78,8.01 for ZnO-nanorod concentrations (prepared at 500°C) of 500,600,700,1000 ppm, respectively. The best growth, i.e., 16.09 was observed when the fungus was incubated with 500 ppm of ZnO-nanorods concentration (prepared at 500°C). The 500 ppm concentration of ZnO-nanorods is regarded as 'optimized ZnO-nanorods' for interaction with *P. indica*. In all followed studies, *P. indica* is treated with optimized ZnO-nanorods (Table 3).

**Table 3: Effect of different concentration of ZnO (500° C) on growth and sporulation of *P. indica.***

| ZnO Conc. (ppm) | Dry Biomass weight (g/l) | Spore yield (spores/ml) | Substrate consumption rate y(g/g) | Day of start of Sporulation |
|---|---|---|---|---|
| Control | 14.36± 0.01 | (5.04 ± 0.01) x $10^9$ | 0.89 | 4 |
| 500 | 16.09±0.01 | (6.97) ± 0.005) x $10^9$ | 1.00 | 3 |
| 600 | 15.42±0.01 | (5.91 ± 0.01) x $10^9$ | 0.96 | 3 |
| 700 | 14.78±0.006 | (4.99± 0.006) x $10^7$ | 0.92 | 4 |
| 1000 | 8.01±0.01 | (2.07 ±0.006) x $10^5$ | 0.50 | 5 |

**Confocal Microsocpy of *P.indica* spores treated with different ZnO Nanorods**

Morphology of *P. indica* was viewed under confocal laser scanning microscopy (CLSM) using Wheat Germ Dye. Laser excitation at 488 nm resulted in emission in the visible range. In the control specimen, the hyphae (Fig. 3A) were thin walled; spore (Fig. 3A) count was low with morphological deformities and disaggregation. In ZnO-nanorods treated *P.indica*, the hyphal walls were thick and hyaline (Fig. 3E); spores (Fig. 3E) were large in size, count was more with smooth surface topology.

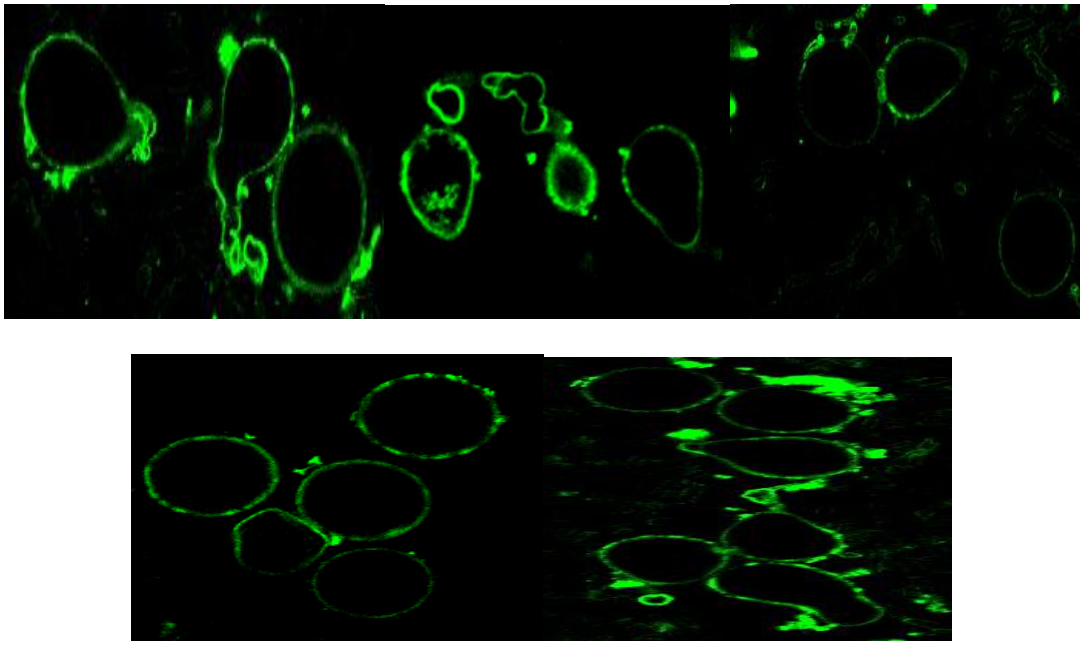


**Fig. 3: Confocal Microsocpy of *P.indica* spores trated with different ZnO Nanorods.**

**Protein Content in treated and Non treated Fungal Biomass**

Biochemical analysis of treated and non treated fungal biomass revealed that ZnO Nano rods prepared at 500°C when used in 500ppm concentration significantly increased the protein content (7.1±0.05 and 7.6±0.05 mg/g respectively) than the non-treated control (5.6±0.01 and 6.1±0.01mg/g). although, all the nanaorods significantly exalted the protein content at 9th d as compared to control (Table 4 and 5).

**Table 4: Protein Content in treated and Non treated Fungal Biomass.**

| Day | Control (mg/g) (Without ZnO) | Treated (mg/g) (With ZnO) | % increase over Control |
|---|---|---|---|
| 6th | 5.6 ±0.01 | 6.1 ± 0.03 | 8.9 |
| 9th | 6.2 ± 0.06 | 7.1 ± 0.05 | 14.5 |

**Table 5: Effect of different concentration of ZnO (500° C) protein content.**

| ZnO Conc. (ppm) | Protein content (mg/g) |
|---|---|
| Control | 6.1± 0.01 |
| 500 | 7.6 ± 0.05 |
| 600 | 6.7± 0.06 |
| 700 | 5.9± 0.01 |
| 1000 | 4.1± 0.01 |

**Zeta Potential analysis for adsorption of ZnO by fungal biomass**

The zeta potential represents the charge of sample surface. At pH 6.5 the surface charge of fungal biomass is more negatively charged which is favourable to adsorption of ZnO (Table 6). This high adsorption is believed to be associated with the formation of positively charged metal-hydroxy species,having strong affinity for the surface functional groups.At higher pH values (6.0–7.0),more functional groups are available for metal ion binding due to deprotonation, resulting in high adsorption. The adsorption of the negatively charged particles at the positively charged sites via electrostatic interaction can lead to localized neutralization and a subsequent bending of the membrane favoring in turn endocytosis for cellular uptake.

**Table 6: Zeta Potential analysis for adsorption.**

| Zeta Potential Analyzer | Charge |
|---|---|
| Zeta potential of ZnO at 500° C | +14.5 mv |
| Zeta potential of nano emebedded fungal biomass | -35.5 mv |

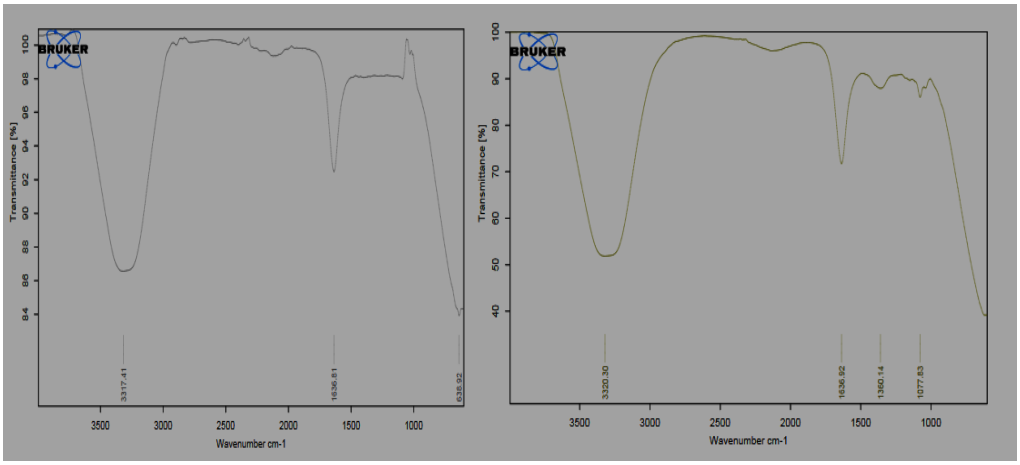
**FTIR analysis**

Functional groups present on the cell surface can be identified by Fourier transform infrared spectroscopy as each group has a unique energy absorption band.

FTIR spectrum of the treted and non treated biomass (Figure 4A) shows the presence of amine, carboxyl, and hydroxyl groups. The strong band in the region of 3500–3300 cm-1 is characteristics of N–H and O–H stretching vibrations and those for alkyl chains around 2920–2850

cm-1. A distinct peak at 1745.5 cm-1 can be attributed to the C=O stretching band from the carboxyl or ester groups, and that at 1712.3 cm-1 to the C=O of the carboxylic groups of amino acids. The amide I band is primarily a C=O stretching mode and is centered at 1645.7 cm-1 while the amide II band is a combination of N–H bending and C–N stretching, being centered near 1550.0 cm-1. Peak positions at 1541.0 cm-1, and 1418.5 cm-1 can be attributed to the COO- of the carboxylate group present in the biomass. The more complex amide III band is located near 1330.0 cm-1. The strong band in the region of 1100–1000 cm-1 is due to C–O bond, which is the characteristic peak for polysaccharides.

FTIR spectrum of the ZnO adsorbed biomass (Figure 4B) shows many changes (appearance or disappearance) of band over the fungal biomass. Transmittance at wave no 3431.1 cm-1 is shifted to 3411.8 cm-1 after adsorption. This change might be responsible for chemical interaction of ZnO with N-H and O-H groups present on the biomass. The disappearance of the carboxyl stretching band at 1745.5 cm-1 and 1712.3 cm-1 is also noted in the mercury adsorbed biomass. These changes, which are typical for the interaction of the carboxyl group with metal ions, suggest that the chemical interactions take place between ZnO and carboxyl groups on the biomass. Similarity in peaks (in control and treated samples) shows that the fungal biomass absorbed ZnO which leads to increase in fungal biomass Conspicuous changes i.e appearance or disappearance of band (peak 3 and 4) in the FTIR spectrum are observed on the ZnO adsorbed biomass, which demonstrate that the amine, carboxyl and hydroxyl groups are involved in chemical interactions involving ionic as well as covalent bonding with ZnO




**A. Control B. Treated**

**Fig. 4: FTIR Results.**

**Real Time PCR**

In order to investigate the impact of ZnO Nanorods on the fungal genes, the cellular proteome of *P. indica* was generated in absence (control) and presence of ZnO. Based on the proteomic results obtained, RT-PCR was done for the selected genes.Four fungal growth regulators genes i.e hexose transporter (Hex T5), urease (Ure A), Glutamate synthase (Glut N) and glutamine synthatase (Glut S) were selected.Hex T5 was upregulated by 12 folds, Ure A was upregulated by 10 folds, Glut N was upregulated by 13 folds, Glut S was upregulated by 17 folds. This observation suggests that ZnO boosts up carbon and nitrogen assimilation in *P.indica* (Fig. 5).

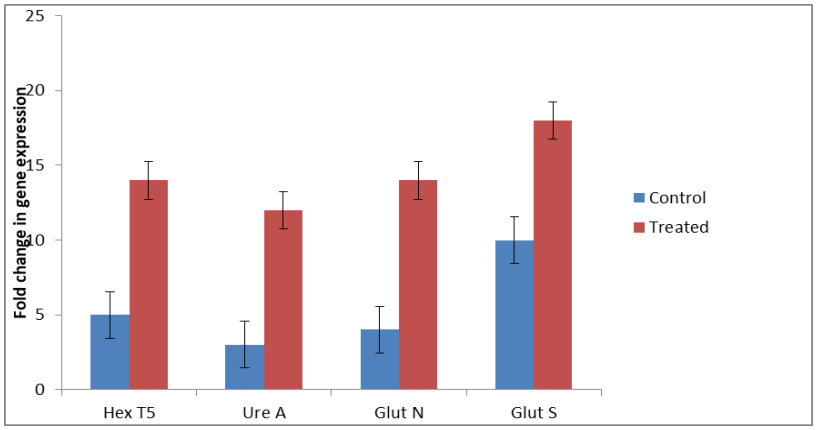



**Fig. 5: Real Time PCR Analysis of fungal biomass.**

**Protein Analysis by Ms/Ms**

As it is proved that ZnO binds with *P.indica* and after binding it boosts up carbon and nitrogen assimilation in *P.indica* by upregulated the growth regulator genes. Now, we have to check the changes at protein level in control and treated fungal biomass for this we go for protein analysis by Ms/Ms interaction. Some of the important proteins that are responsible for energy consumption, Maturation and play important role in various pathways are identified which include Eno1, Glut S, Arginase, Ure D, Ypt 1 and Rtm1. In order to investigate the impact of ZnO on the fungal biomass, the cellular proteome of *P. indica* was generated in absence (control) and presence (treated) of ZnO by MS to detect upregulation of proteins. Important proteins include Eno1, Glut S, Arginase, Ure D, Ypt 1 and Rtm1. Eno 1 was upregulated by 4.3 folds, Glut S was upregulated by 3.4 folds, Arginase was upregulated by 5.3 fold, Ure D was upregulated by 3.1 fold, Ypt 1 was upregulated by 2.9 folds, Rtm 1 was upregulated by 5.4 folds (Fig. 6). The observation suggest that ZnO helps in upregulation of those proteins that play important role in metabolism, maintain pH and regulation of pathways. Due to which there is increase in fungal biomass and spore count.

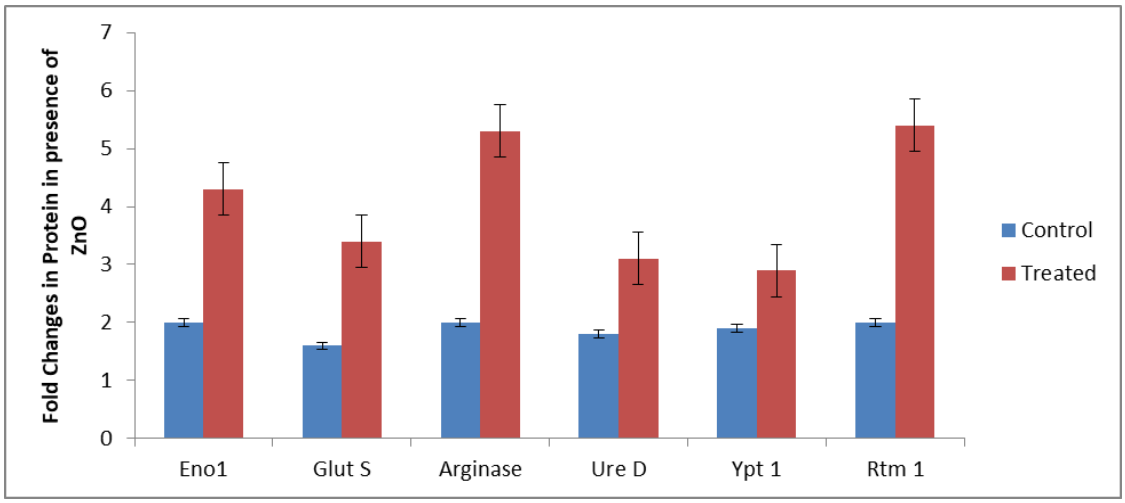



**Fig. 6: Proteome of *P. indica* in presence and absence of ZnO.**

## CONCLUSION

*P.indica* when grown with ZnO (synthesised at 500°C) gives maximum fungal biomass and spore count**.** The ZnO enhances fungal biomass and spore count only when its concentration is 500ppm. If we increase its concentration then it shows negative impact on fungal growth**.** There is increase in protein content in presence of ZnO (synthesised at 500°C) (500 ppm)**.** ZnO binds with *P.indica* and after binding it upregulates the growth regulator genes and proteins which plays very important role in maturation of fungus.

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