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European Journal of Experimental Biology, 2012, 2 (1):75-82

ISSN: 2248 –9215
CODEN (USA): EJEBAU

Phenol tolerance of *Pleurotus florida* under varying conditions of nitrogen sufficiency

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ABSTRACT

A preliminary study of phenol tolerance of Pleurotus florida under varying nitrogen levels used Mandel and Weber's Modified Agar Glucose medium. Urea, Ammonium sulphate, Peptone: variable nitrogen components. Phenol concentrations: 200, 400, 800 mg/L; nitrogen variations: sufficient(N), deficient(N/2), deprived(N/10), excess(N×10). Tolerance studied by computation of total growth area: rate of fungal tip extension, every alternate day, for 22 days. In N, growth area higher for 200 and 400 mg/L phenol (32.48, 31.38 sq.cm) than 800 mg/L (12.55 sq.cm). In N/2, higher for 400 and 800 mg/L (14.92, 22.21 sq.cm) than 200 mg/L (0.29 sq.cm) In N/10 and N×10, high for 800 mg/L (32.07, 42.2 sq.cm) compared to 200 and 400 mg/L (12.97, 13.04 sq.cm; 11.71, 14.54 sq.cm). In N, rate of increase positive for entire growth cycle upto 400 mg/L. In 800 mg/L, stopped by day twelve. In N/2, growth stopped between day six and eight in 200 and 400 mg/L; upto day fourteen in 800 mg/L. Pattern repeated for 200 and 400 mg/L in N/10 and Nx10. In N/10 800 mg/L, growth positive throughout. In N×10 800 mg/L, stopped by day twelve. Growth pattern similar for 200 and 400 mg/L in all media, indicated by correlation of 0.871. In 800 mg/L, similar growth patterns between N/2 and N/10; N/2 and N×10; N/10 and N×10; indicated by correlation of 0.75, 0.8 and 0.99. Optimum nitrogen (N) offered better tolerance and growth for P.florida in 200 and 400 mg/L phenol, while N/2, N/10, Nx10 provided the same tolerance in 800 mg/L.

Keywords: *Pleurotus florida*, Mycoremediation, Phenol tolerance, Nitrogen sufficiency, Nitrogen limitation.

INTRODUCTION

Environmental pollution is a global problem; due to enhanced industrial activities and rising standard of living, our environment is now polluted by industrial wastes of various types [23].

Contaminated sites often contain a mixture of environmentally persistent compounds. The persistent nature of many pollutants contributes to the potential risks and difficulty of remediation. Bioremediation is a promising and potentially cost effective strategy to remediate these sites [18]. Biological treatment technologies for the remediation of soils and groundwater contaminated with organo pollutants are widely used for their environmentally friendly impact combined with low cost compared to other treatment alternatives [21,27].

Bioremediation is defined as the process whereby organic wastes are biologically degraded under controlled conditions to an innocuous state, or to levels below concentration limits established by regulatory authorities. It uses microorganisms like bacteria, fungi, green plants or their enzymes to return the natural environment altered by contaminants to its original condition. Bioremediation technologies are classified as *in situ* or *ex situ*. In *in-situ*

bioremediation, the contaminated material is treated at site while in *ex-situ* bioremediation, the contaminated material is removed to be treated elsewhere [18].

Fungi play a significant role in human life, besides their utilization in industry, agriculture, medicine, food industry, textiles and bio remediation [20]. Mycoremediation [24] is a form of bioremediation**,** the process of using fungi to return an environment (usually soil) contaminated by pollutants to a less contaminated state. Fungi are well suited for uptake and removal of metals and other pollutants from waste water and soil, because they often exhibit marked tolerance toward metals and adverse conditions like low pH, and accomplish remediation by processes such as insoluble metal oxalate formation, biosorption or chelation onto melanin-like polymers [22].

Pleurotus florida is commonly known as the *oyster mushroom*, is a common edible mushroom, highly nutritious and commonly grown in tropical West Africa and Southern part of Asia [1]. It is a saprotroph that acts as a primary decomposer on wood and other plant matter, due to the presence of ligno-cellulolytic enzyme systems. It belongs to the group of fungi, the Basidiomycetes, known as *white rot fungi*, of which there are an estimated 1,400 species from all major groups of higher basidiomycetes and xylariaceous ascomycetes [19]. The ability of white rot basidiomycetes to degrade a variety of aromatic compounds, such as lignin and aromatic pollutants is due to their ability to produce ligninolytic enzymes like lignin peroxidase, manganese peroxidase and laccase [15]. Research on fungal bleaching of Kraft pulp mill effluents demonstrated the ability of *P. chrysosporium* Burds., to degrade chlorinated organics found in the effluent [13]. Evidence for differences in their ability to degrade xenobiotics was given for the mineralization of DDT and several polynuclear aromatics [6].

Phenol, also known as *carbolic acid*, is a highly toxic element, which also has antiseptic properties, and used as a pioneer in antiseptic surgery, besides being the active ingredient in oral analgesics, herbicides and synthetic resins. Phenol vapor is corrosive to eyes, skin and respiratory tract [5]. Repeated / prolonged skin contact can cause dermatitis and second and third-degree burns [16]. Inhalation can cause lung edema [5]. It can affect central nervous system and heart, causing seizures and coma [28]. Besides its hydrophobic effects, another mechanism for the toxicity of phenol is formation of phenoxyl radicals.

The significance of the study is that *P. florida* can be easily cultivated on various commonly found natural substrates like paddy straw [12], coconut husk [3], tea dust, saw dust and sugarcane bagasse [3]. This increases its potential as a bioremediating agent, but paradoxically, the ability to uptake phenol is a cause for concern due to its edible nature. Hence, studies concerning its rate of uptake and assimilation need to be researched in detail.

As per the Hazardous Waste Management Rules, 1989, only 5 kg of phenol is permitted by law for disposal in a year. The actual quantities being disposed off are much greater than the permissible limit. In addition, present treatment methods for phenol are highly chemical intensive and further contaminate the environment. This makes it imperative that new and non-chemical methods like mycoremediation are devised to treat these wastes prior to disposal [29].

White rot fungi can degrade a wide variety of environmental pollutants using a variety of extracellular enzymes and chemicals normally involved in lignin degradation, like pentachlorophenol, trinitrotoluene, trichloroethylene, cyanide and polyaromatic hydrocarbons [2]. Sites contaminated by recalcitrant organic compounds have often been shown to be characterized by the concomitant presence of heavy metals [4]. In such cases, the use of *white rots* may give some advantages over bacterial bioaugmentation [24].

The main objective of the study is to make a preliminary assessment of the tolerance levels of *P. florida* under various conditions of nitrogen sufficiency in the media and concentrations of phenol, to understand, in future, its potential for bioremediation of phenolic wastes in contaminated sites.

MATERIALS AND METHODS

The spawn of *P. florida* was obtained from Mushroom Laboratory, Department of Microbiology, University of Agricultural Sciences, Bangalore. It was cultured on Mandel and Weber's Modified Agar [17] glucose medium. It was maintained on agar slants at 4 to 10^{o}C in refrigerator and sub-cultured every three months. Sterilization of media was done by autoclaving at 121^{o}C for 15 minutes @ 15 psi pressure.

Inoculation was done by transferring a 1 cm disc of the pure culture onto the surface of petri plate [26]. The phenol used was 96% pure and concentrations studied were 200, 400 and 800 mg/L. For each concentration of phenol, the nitrogen content of the media was varied, in triplicate, as sufficient (N), deficient (N/2), deprived (N/10) and excess

(Nx10). The nitrogen content was varied by varying the quantities of Urea, Ammonium sulphate and Peptone in the media. In N media, 0.3 g urea, 1.4 g ammonium sulphate and 1 g peptone was used [26].

Tolerance to phenol in solid media was estimated by measuring the rate of extension of fungal mycelial tip in several directions, on the surface of the media [26], every alternate day, for a period of 22 days. A control, in triplicate, was maintained without phenol, for each nitrogen variation of the media.

The statistical analysis consisted of computation of mean values for all trials, graphical representations of growth patterns under various conditions of nitrogen sufficiency. Correlation of mean growth area between nitrogen variations of media was also computed.

RESULTS AND DISCUSSION

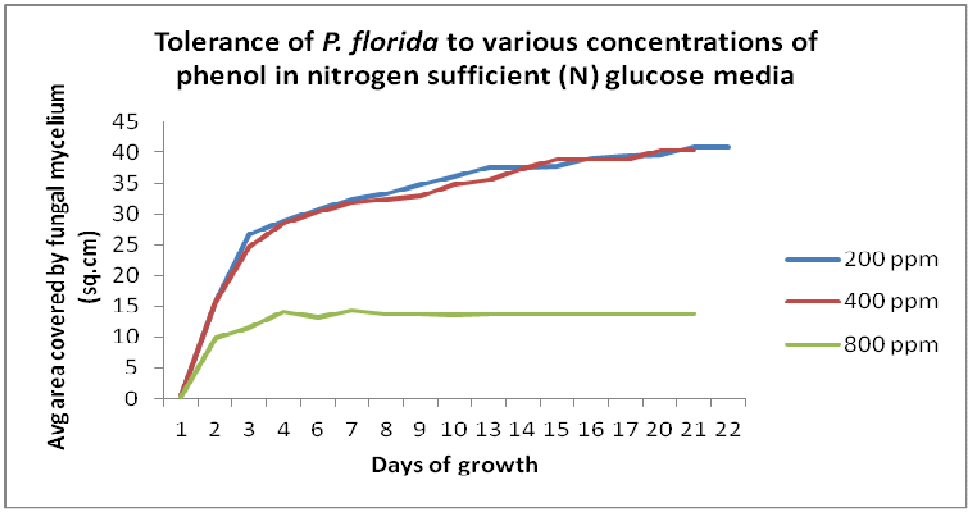
When nitrogen was sufficient in the media (N), *P. florida* showed growth area of 32.48 sq.cm for 200 mg/L phenol. This was reduced to 31.38 sq.cm when phenol was increased to 400 mg/L, and further to 12.55 sq.cm in 800 mg/L phenol [Table 1].

Table 1: **Average growth of *P. florida* under various conditions of nitrogen**

Average growth of P. florida under various conditions of nitrogen in Mandel's glucose media			
Average area covered by fungal mycelium (sq.cm)			
	200 mg/L	**400 mg/L**	**800 mg/L**
N	32.48	31.3875	12.5225
N/2	0.2966	14.92	22.218
N/10	12.979	13.04	32.074
N x 10	11.716	14.5406	42.209

When nitrogen was reduced to half (N/2), it had growth area of 0.29 sq.cm in 200 mg/L. This increased to 14.92 sq.cm in 400 mg/L and 22.21 sq.cm in 800 mg/L phenol [Table 1]. In one-tenth nitrogen media (N/10), 200 and 400 mg/L showed growth area of 12.97 and 13.04 sq.cm; however, when phenol was increased to 800 mg/L, growth area increased to 32.07 sq.cm [Table 1]. In media with ten times nitrogen (N×10), 200 and 400 mg/L had growth area of 11.71 and 14.54 sq.cm, while in 800 mg/L it had area of 42.2 sq.cm [Table 1]. This clearly indicated a much greater tolerance of 800 mg/L phenol in deficient, deprived and excess nitrogen conditions of media. Ligninolytic activities in white rot fungi are commonly known to occur under carbon-nitrogen limiting conditions [24]. The ligninolytic systems of several wood-rotting basidiomycetes, including *P. chrysosporium* are triggered by nutrient nitrogen depletion [14]. Given the poor nitrogen content of woody tissues that serve as the natural substrates for white rot fungi, it is not surprising for low nitrogen levels to trigger lignin degradation in the same way as in *P. chrysosporium* [9]. In addition, tolerance to high concentrations of a contaminant by an organism is one of the important advantages for bioremediation of highly contaminated sites [18].

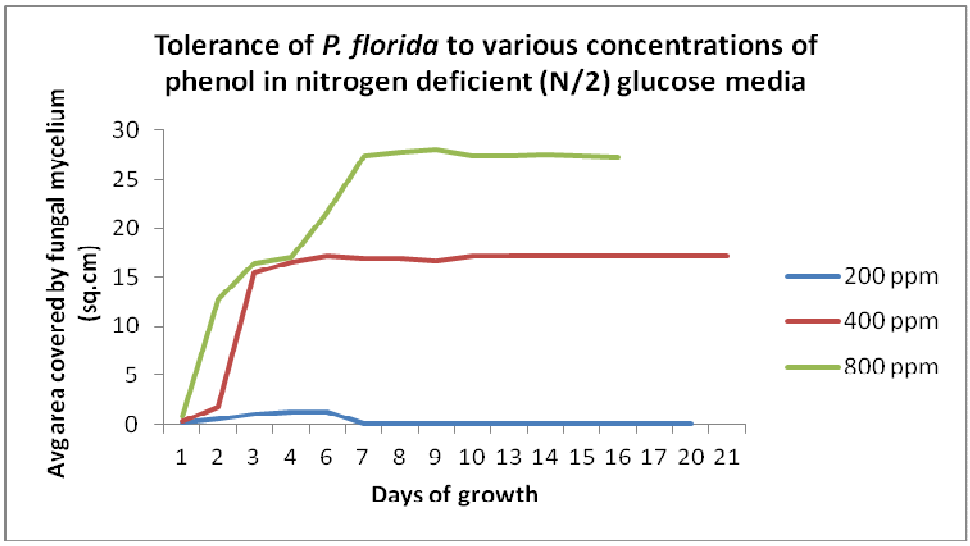
Figure 1: Tolerance of *P. florida* to varying concentrations of phenol in N media



In N media [Figure 1], rate of increase remained positive throughout the growth cycle, though slowed down, upto 400 mg/L phenol concentration. However, when concentration of phenol was increased to 800 mg/L, increase of mycelial tip completely stopped after day twelve. Tolerance and degradation of phenolic compounds in white rot fungi usually occurs as a secondary metabolic event, after the initial vegetative growth phase [13].

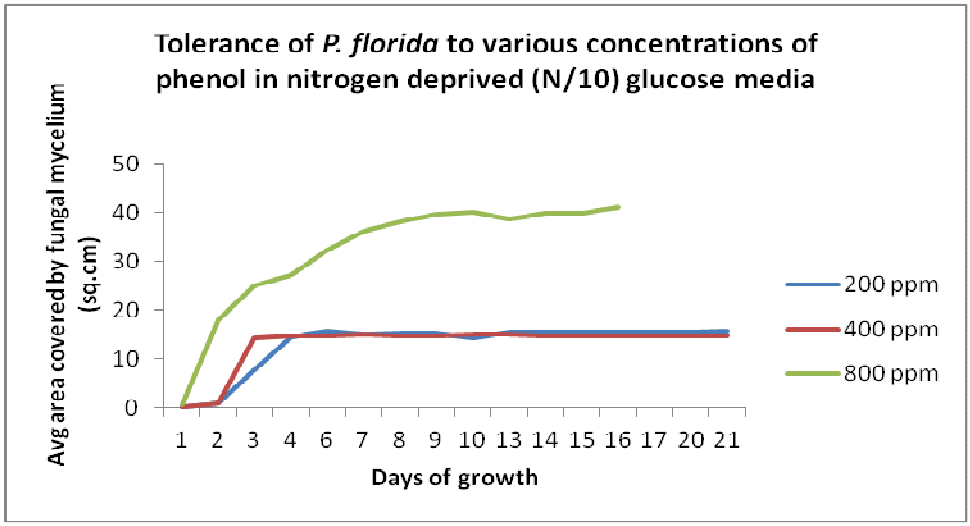
In N/2 media [Figure 2], tip extension stopped between day six and eight for 200 and 400 mg/L phenol, but continued upto day fourteen for 800 mg/L phenol.

Figure 2: Tolerance of *P. florida* to varying concentrations of phenol in N/2 media



This pattern was repeated in N/10 [Figure 3], except that, for 800 mg/L phenol, tip extension continued to be positive, throughout the growth cycle, though much slowed down.

Figure 3: Tolerance of *P. florida* to varying concentrations of phenol in N/10 media



In N×10 media [Figure 4], 200 and 400 mg/L again exhibited similar patterns of tip extension, but for 800 mg/L phenol, growth stopped after day twelve. This showed that growth pattern was similar for 200 and 400 mg/L in all media, indicated by high correlation value of 0.871 [Table 2].

Figure 4: Tolerance of *P. florida* to varying concentrations of phenol in Nx10 media

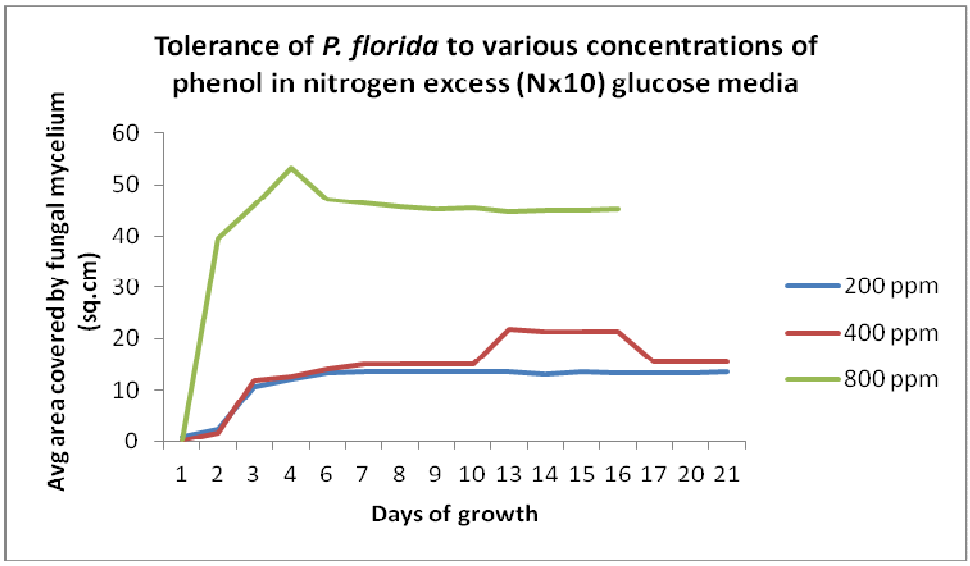
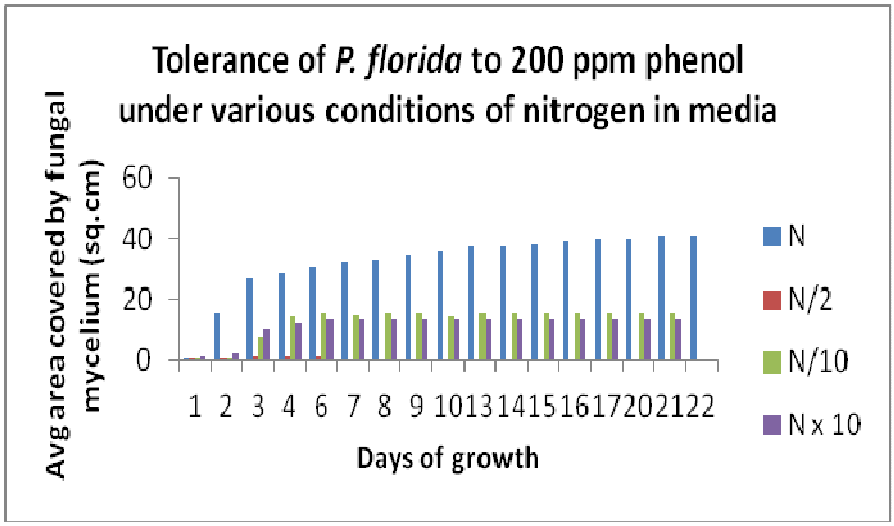


Table 2: Correlation between growth patterns of *P. florida* under various conditions of nitrogen and phenol concentrations

Correlation between growth patterns of P. florida under various conditions of nitrogen and phenol concentrations in Mandel's glucose media								
Increase of Phenol Concentration			Variations in Nitrogen Content of Media					
B/w 200 & 400 mg/L	B/w 400 & 800 mg/L	B/w 200 & 800 mg/L	B/w N & N/2	B/w N & N/10	B/w N & N x 10	B/w N/2 & N/10	B/w N/2 & N x 10	B/w N/10 & N x 10
0.871	-0.776	-0.473	-0.786	-0.998	-0.999	**0.757**	**0.807**	**0.996**

Figure 5: Tolerance of *P. florida* to 200 mg/L phenol under varying nitrogen levels



When phenol concentration was increased to 800 mg/L, there was similarity of growth pattern between N and N×10, and also between N/2 and N/10 media, with a better rate of increase in depleted media [Figure 1,2,3,4]. This showed that nitrogen depletion of media enhanced its capacity to tolerate higher phenol concentration. There was high correlation between N/2 and N/10 media (0.75), between N/2 and N×10 (0.8) and between N/10 and N×10 (0.99) [Table 2].

Thus, when the phenol concentrations were low to moderate in the media (200 and 400 mg/L), nitrogen sufficient condition offered better tolerance capacity and growth to *P. florida* [Figure 5,6 and Table 1], but for high phenol

concentration of media (800 mg/L), nitrogen depleted, deprived and excess conditions provided the same benefit [Figure 7 and Table 1].

Figure 6: Tolerance of *P. florida* to 400 mg/L phenol under varying nitrogen levels

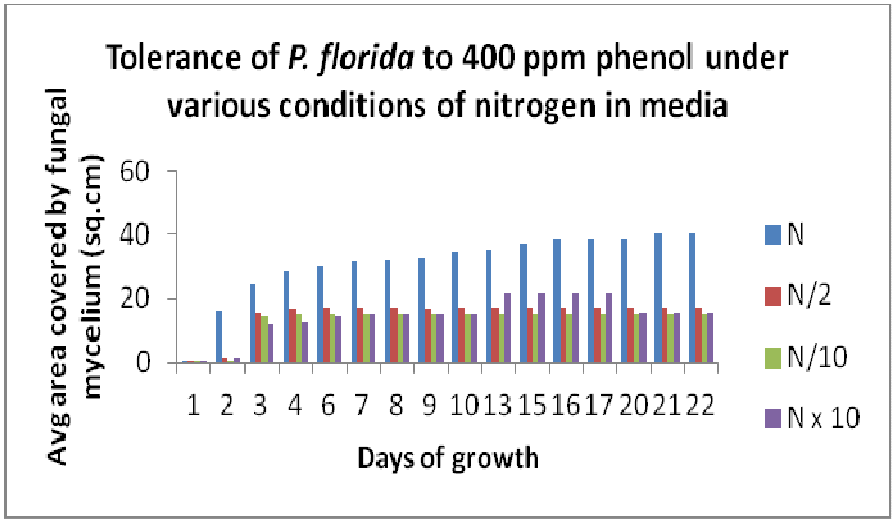


Figure 7: Tolerance of *P. florida* to 800 mg/L phenol under varying nitrogen levels

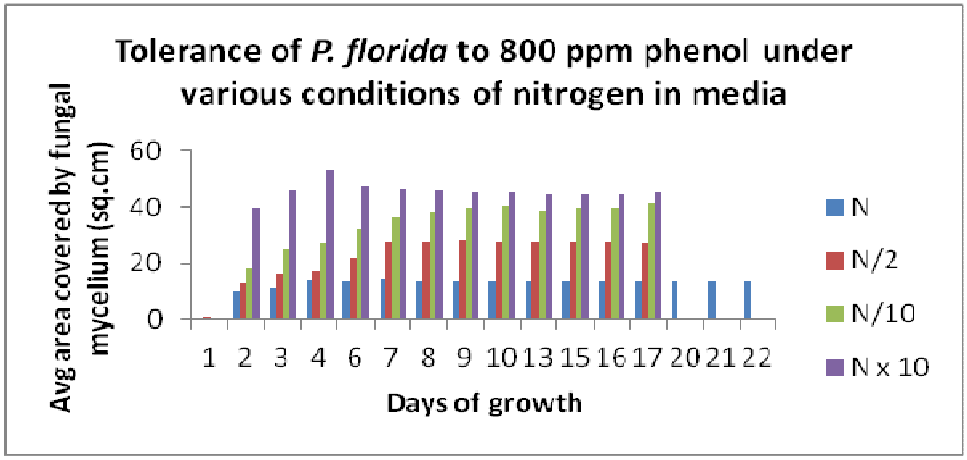


Figure 8: Analysis of variance for growth of *P. florida* in different phenol concentrations

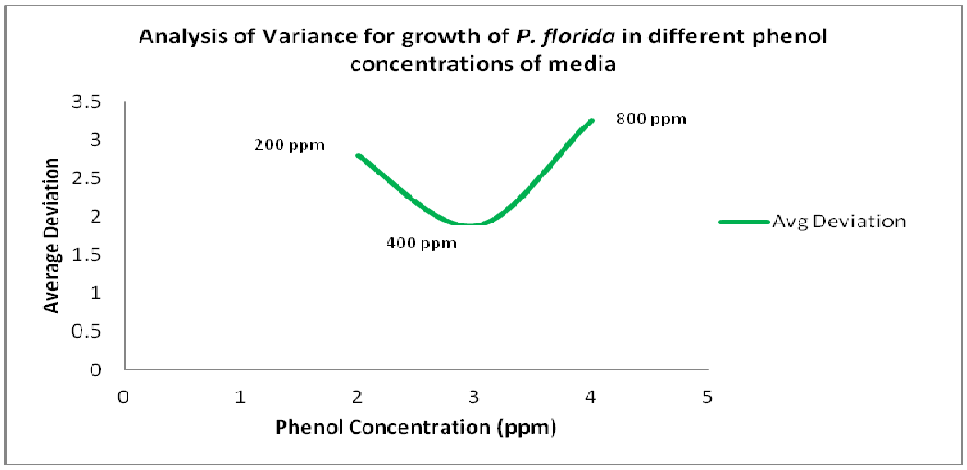
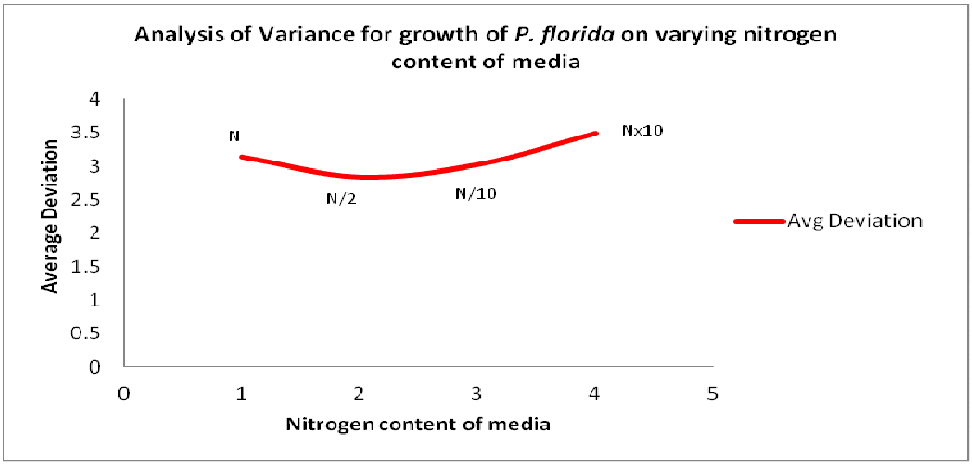


Figure 9: Analysis of variance for growth of *P. florida* in varying nitrogen content



Analysis of Variance indicated that it was least in 400 mg/L phenol concentration (1.8) and nitrogen deficient and deprived conditions (2.7 and 2.8) [Figure 8, 9].

CONCLUSION

Microorganisms as biological control agents have high potential to control specific point pollution and no effect on the environment (or) other non-target organisms [11]. White rot fungi have a methylation system that is important in the degradation of phenolic pollutants and phenolic lignin degradation products and this is mainly accomplished by the formation of both oxidative and reductive species by lignin peroxidases, manganese peroxidases, cellobiose dehydrogenase and laccases [19]. Lignin peroxidase catalyzes the oxidation of various aromatic compounds to form aryl cation radicals [10] while manganese peroxidase oxidizes Mn(II) to Mn(III), which diffuses from the enzyme and oxidizes various phenolic compounds. Species which produce lignin peroxidases generally secrete a secondary metabolite, veratryl alcohol, which is a substrate for further production of lignin peroxidase. Thus, the system is self-catalyzing in nature [16].

Laccase too catalyzes the oxidation of various phenolic compounds and aromatic amines by catalyzing the reduction of O_2 to H_2O using a range of phenolic compounds like polyphenols, aromatic amines and methoxy-substituted monophenols [25]. Membrane-associated methyl transferases and a plasma membrane redox potential also contribute to the detoxification and degradation of compounds [2]. The pH optima for these reactions are usually between 4 and 7, being more efficient in acidic pH.

It is also suggested that manganese peroxidase in combination with either laccase or lignin peroxidase may be the necessary minimum complement of oxidative enzymes for lignin degradation. The enzyme could have a function during lignolytic growth other than direct involvement in lignin cleavage. All enzymes involved in lignin cleavage produce highly reactive and toxic intermediates from which the fungal mycelium must be protected. It is suggested that one of the functions of laccase is to scavenge these compounds by promoting polymerization, before they can enter the hypha [8].

The role played by nitrogen in the manganese peroxidase enzyme system of white rot fungi have far-reaching implications for more efficient substrate colonization and utilization in enhancing their bioremediation potential [7].

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