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Bioremoval of toxic substances in synthetic wastewater using *Trichoderma pubescens* (NPK2), isolated from mangrove soil

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1. Introduction

Wastewater poses a serious environmental threat due to the presence of polluting chemicals in excess (Zaidi, 2007). Industrial effluents pose a potential threat to the environment of many countries. The major issue is disposal and degradation of the industrial effluents that contain toxic metals. The chemicals of the effluent are carcinogenic and mutagenic and their discharge affect the life cycle of flora and fauna. A combination of the toxic compounds varies, depending on the source of the industry. Majority of effluents have been dumped into the coastal environment without proper pretreatments and/or removal of toxic chemicals from the effluent (Gomathi et al., 2012).

When the pollutants get accumulated, they damage the biological species of terrestrial and marine environments (Travieso et al., 2002). The metal accumulations also induce environmental changes through their conversion and productions of the toxic chemicals, which may induce cancers in human and animals (Saravanakumar and Kathiresan, 2015). The wastewater contains excessive levels of rich organic carbon, phosphorus and nitrogen, as compared to raw domestic sewage (Hang et al., 1975; Brewers of Europe, 2002; Rao et al., 2007; Simate et al., 2011). The waste waters are directly discharged by many industries into water bodies or to nearby environments without undergoing any pre-treatments (Shao et al., 2008) in less developed countries (Yang and Li, 2002; Simate et al., 2011). Wastewater treatment is essential for reuse or disposal of the wastewater to ensure environmental protection. Chemical treatment of the wastewater is expensive, toxic, corrosive, and can also pollute the environment (Lee and Lui, 2000; Abu-Orf et al., 2001; Chang et al., 2001, 2002). In this regard, biological treatment of wastewater is sustainable, non-hazardous and eco-friendly, and hence it has been the main focus of many researchers (Urbain et al., 1993; Higgins and Novak, 1997; Jorand et al., 1998; Houghton et al., 2001; Sobeck and Higgins, 2002). In the biological treatment of wastewater, microorganisms including filamentous fungi are found to be useful under controlled operating conditions (Molla et al., 2001; Fakhru'l-Razi et al., 2002; Guest and Smith, 2002; Alam et al., 2003a,b; Mannan et al., 2005; Fleury, 2007). The use of filamentous fungi may provide some advantages over bacterial adsorbent in the case higher biomass and enzyme production (Annibale et al., 2006). Many fungi, such as *Mucor rouxii*, *Trichoderma*, *Aspergillus*, *Paecilomyces lilacinus* and the arbuscular mycorrhizal fungi, are known to have potential for bioremediation in metal-contaminated soils (Saravanakumar and Kathiresan, 2015).

The wastewater that is rich in nutrients is considered to be a suitable medium for the cultivation of heterotrophic organisms like fungi (Saravanakumar and Kathiresan, 2014). In biotechnology, the submerged cultivation method has been widely employed for the cultivation of fungi in the past, where the broth is inoculated with either fungal spores or mycelium. The fungal biomass is largely used for treating wastewater and the removal of organic carbon from wastewater (Shannon and Stevenson, 1975; Hang et al., 1975). Nevertheless, in the past decades, submerged cultivation of fungal biomass received less attention, even though the use of filamentous fungi for wastewater treatment being identified as an interesting area with benefits such as development of a biorefinary concept and easy harvest (Sankaran et al., 2010).

Fungi are predominantly preferred for treating waste water for their usage in large-scale treatment process. The fungi such as *Trichoderma, Aspergillus and Paecilomyces* are known to have potential for bioremediation process (Lebeau et al., 2008; Tsekova et al., 2010; Sharma and Adholeya, 2011). The fungi produce a group of extracellular enzymes that facilitate the biodegradation of recalcitrant compounds such as phenolic compounds, dyes, and polyaromatic hydrocarbons (PAH), among others, through nonspecific oxidation reactions. The fungi yield valuable byproducts such as amylase, chitin, and lactic acids during the biological wastewater treatment. In order to avoid health or environmental problems, *Trichoderma* strains are preferable (Chanda et al., 2016), but their marine counterparts are only little studied for wastewater treatment, (Saravanakumar and Kathiresan, 2015). Hence, the present work assessed the potential of *Trichoderma*, isolated from mangrove soil, for biomass production and nutrient reduction in synthetic wastewater, under submerged fungal cultivation.

2. Material and methods

2.1. Sample collection

Soil samples were collected from rhizosphere of *Rhizophora*

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annamalayana Kathir., at a depth of 5–10 cm by using a corer in the mangrove forest at Pichavaram (N.11^{0}25′26.7″, E. 79^0 47′37.7″), located in Southeast coast of India. The samples were kept in sterile plastic bags at 4 °C until used for further processing.

2.2. Isolation of trichoderma

The samples were serially diluted in to 10^{-2} to 10^{-5} (Askew and Laing, 1993) and the diluents were placed on sterilized *Trichoderma* Selective Medium (TSM), using 50% seawater by spread plate technique, then the plates were incubated at 28 °C for 7 days. The colonies were isolated, sub-cultured and maintained in TSM. The composition of TSM as follows expressed in g/L (Magnesium Sulphate ($MgSO_4$ - 0.2, Dipotassium Hydrogen Phosphate (K_2HPO_4) - 0.9, Potassium Chloride (KCl) - 0.15, Ammonium Nitrate (NH_4NO_3) - 0.15, Glucose - 3.0, Chloromycetin ($C_{11}H_{12}Cl_2N_2O_5$) - 0.25, p-dimethyl amino benzene diazo sodium sulfonate - 0.3, Pentachloro nitro benzene ($C_6C_{l5}NO_2$) - 0.2, Rose Bengal - 0.15, Agar - 20, Distilled water - 1000 ml). All the chemicals were purchased from Sigma – Aldrich, India with 99.99% of purity.

2.3. Identification of fungal strains

The isolated colonies were identified morphologically by staining with lacto-phenol cotton blue (Hi media) under light and electron microscopes (see Fig. 1). Five strains were found predominant and confirmed as *Trichoderma* species based on the morphological key characters (Rifai, 1969). Five predominant strains - NPK1, NPK2, NPK3, NPK4 and NPK5, only NPK2 strain was selected for its rapid growth and biomass production in Potato dextrose broth. The ITS sequence of NPK2 were submitted in GenBank and the accession number is MG193751.

2.4. Maintenance of culture

A loopful of inoculum of *T.pubescens* (NPK2) was transferred to Potato Dextrose Agar (PDA) slants and maintained as pure culture for laboratory studies. Then the cultures were incubated in Petri dishes containing sterilized PDA medium at room temperature (26 ± 2 °C). After complete sporulation, conidia from the medium were harvested by washing them thoroughly with sterilized water containing Tween-20 (0.2%) for immediate use. Harvested conidia were air-dried under aseptic conditions and stored in a small air tight screw cap vials (10 cm with 2.5 cm diameter) kept at 4 °C. Colony forming units (CFUs/ml) were estimated by plating techniques. Suspension of spores was made using distilled water with Tween-20 (0.2%) and filtered through a double layered muslin cloth. Spore count was made using a double rolled Neubauer's haemocytometer under phase contrast microscope (40x). From the stock solution, further dilutions were made to obtain the required concentrations for further studies.

Table 1
Composition of syntheticwastewater (SWW) (Salvado et al., 2001).

S.No	Nutrients	Different concentrations of SWW (g.L^{-1})						
		1X	5X	10X	15X	20X	25X	30X
1.	Glucose	0.50	2.50	5.00	7.50	10.00	12.50	15.00
2.	Ammonium chloride	0.04	0.20	0.40	0.60	0.80	1.00	1.20
3.	Sodium nitrite	0.01	0.05	0.10	0.15	0.20	0.25	0.30
4.	Urea	0.01	0.05	0.10	0.15	0.20	0.25	0.30
5.	Potassium dihydrogen phosphate	0.01	0.05	0.10	0.15	0.20	0.25	0.30
6.	Potassium chloride	0.05	0.25	0.50	0.75	1.00	1.25	1.50
7.	Ferric chloride	0.03	0.15	0.30	0.45	0.60	0.75	0.90
8.	Copper chloride	0.002	0.01	0.02	0.03	0.04	0.05	0.06
9.	Zinc sulphate	0.005	0.025	0.05	0.075	0.1	0.125	0.150
10.	Lead nitrate	0.002	0.01	0.02	0.03	0.04	0.05	0.06
11.	Potassium dichromate	0.001	0.005	0.01	0.015	0.020	0.025	0.030
12.	Manganese chloride	0.005	0.025	0.05	0.075	0.1	0.125	0.150

2.5. Preparation of synthetic wastewater medium (SWW)

Synthetic wastewater medium (SWW) was prepared with different concentrations of constituent salts, as given in Table 1 (Salvado et al., 2001). After autoclaving, the SWW medium was allowed to cool. One milliliter of aqueous conidial inoculum with 10^{-12} spores mL^{-1} of *Trichoderma pubescens* (NPK2) was inoculated and incubated at 26 °C for a period of 28 days under shaking conditions (Saravanakumar and Kathiresan, 2014). The experimental medium was sampled aseptically starting from day 1–28 for chemical analysis.

2.6. Estimation of fungal biomass

Experiments were performed as batch reactors in 500 ml Erlenmeyer glass flasks on a horizontal orbital shaker (RIS-24 Plus, Orbital Shaking Incubator, REMI, India) at 150 rpm at 27 °C. Eight flasks were used for the experiment (Control (distilled water alone as served as a control), 1X, 5X, 10X, 15X, 20X, 25X, 30X) and all the experiments were carried out in triplicates to avoid the errors. Each reactor contained 250 mL of SWW and 50 ml of the culture filtrates were collected on 7, 14, 21 and 28th days of the experiment for their fungal biomass followed by filtration using Whatman No.1 filter paper and weighed for its fresh weight followed by dry weight. Further, the filter papers were dried in an oven at 60 °C until constant weight is achieved.

2.7. Chemical analysis of fungal biomass

The culture filtrate of different concentrations of SWW was analysed for total nitrogen (TN), ammonia nitrogen (NH_4^+-N), phosphate phosphorus (PO_4^3−P) and chemical oxygen demand (COD) following the standard methods (ISO, 1997; 1984; 2004; 1989).

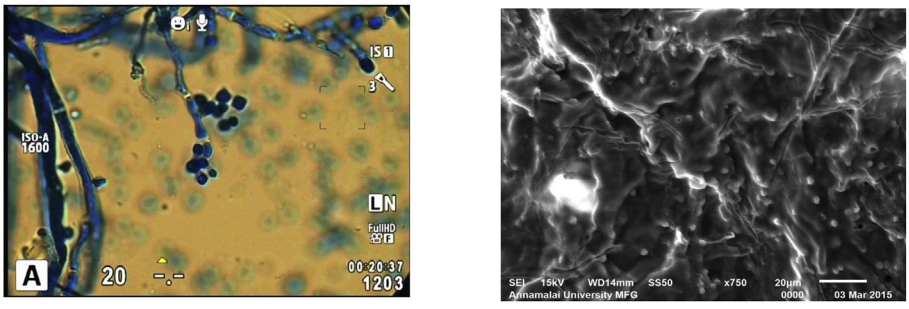

Fig. 1. Morphological views of *Trichodermapubescens*NPK2 under light microscope and Scanning Electron Microscope.

Organically bound nitrogen is oxidized to nitrate during alkaline persulphate digestion. The nitrate content of the sample is determined after reduction to nitrite. The procedure described here can be used for the simultaneous oxidation of nitrogen and phosphorous compounds, and for the determination of TN.

Blank and Standard: Measure out 50 ml of water into an oxidation bottle. Similarly transfer 50 ml of working standard solution to another bottle. Add 5.0 ml of oxidizing reagent to each of the bottles and autoclave for 30 min. Cool the autoclave to room temperature before opening. Take out the flask from the autoclave, swirl to dissolve any precipitate and open carefully to release any over pressure. Cool and transfer 5.0 ml of the digested solutions to a 50 ml volumetric flask and make upto 50 ml and add 50 ml buffer (4.3.2) and proceed as in 4.5. Follow the steps as described in 4.6 and find out the mean absorbance of standard and blanks.

Sample analysis: Measure out 50 ml of the sample aliquot and add 5.0 ml oxidizing reagent. Follow the digestion procedures as described in 5.6. Dilute 5.0 ml of the digested solution to 50 ml with water, add 50 ml buffer and pressure as described above. The balance 50 ml of the solution left in the oxidation flask is used for the determination of total phosphate, Ammonia present in water reacts with sodium hypochlorite and phenol in alkaline condition to produce indophenol blue. Sodium nitroprusside is used as a catalyst and color intensifier. Transfer 50 ml water into a conical flask and add 2 ml of phenol solution. Mix well. Add 2 ml of sodium nitroprusside solution followed by 5 ml of oxidizing solution. Mix thoroughly. Cover the flask with polythene sheet and wait for 1 h. Measure the extinction (O.D) of the reacted sample against ammonia free distilled water reagent blank at 640 nm using 1 cm or 10 cm cell.

Phosphate in Synthetic Waste Water is allowed to react with ammonium molybdate in acid medium, forming a phosphomolybdate complex, which is reduced by ascorbic acid, in presence of antimony ions (to accelerate the reaction), to a blue coloured complex containing 1:1 atomic ratio of phosphorous to antimony. The absorption of the complex is measured at 880 nm using a 50 mm cell. To avoid interference of silicate, the pH is kept below 1. Organically bound phosphorous is completely decomposed to phosphate by a strong oxidizing agent (alkaline persulphate). Inorganic forms of phosphorous in lower oxidation state are also oxidized to phosphate. The pH of the reaction is initially maintained at 9.7, so that after oxidation the pH is between 4 and 5. These conditions are obtained by a boric acid-sodium hydroxide system. The orthophosphate formed is determined by the method outlined in section 1.

2.8. Statistics

In all the experiments, each concentration of SWW was carried out in triplicates. Mean values and standard deviations were calculated. Data were subjected to analysis of variance (ANOVA) followed by Tukey's multiple comparison test. Differences were considered significant at $P < 0.05$, where Mean is the average, SD denotes as standard deviation was carried out by using ORIGIN Pro 8 as a Statistical tool and SE as standard error.

3. Results

Biomass production: Data on dry biomass of *T.pubescens* NPK2 strain at different concentrations of synthetic wastewater for different incubation days are shown in Fig. 2 and Table 2. The biomass significantly varied between days of treatment and also concentrations of wastewater. It was significantly high between the incubation days of 7 and 28 in a range from 63% in high concentration (30x) to 383% in low concentration (1x) of wastewater. The biomass production was higher in low concentration than that in high concentration of wastewater.

Nutrient reduction: The filtrate of *Trichoderma* cultured in different concentrations of wastewater was drawn for different days of culture,

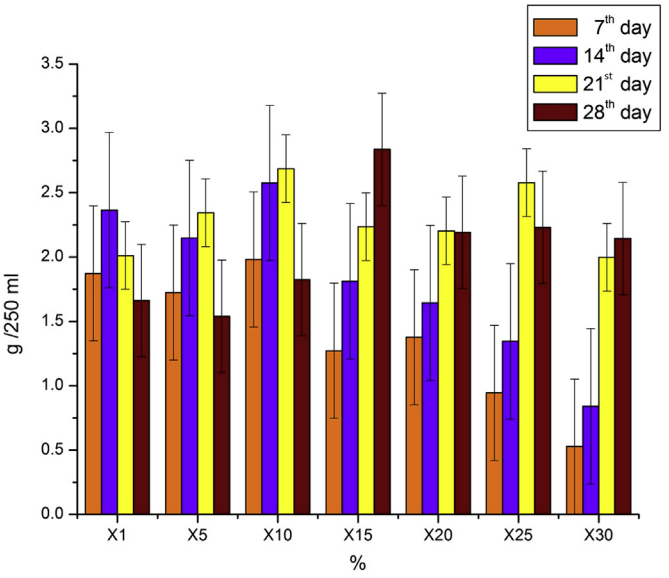

Fig. 2. Biomass production of *Trichoderma pubescens* in different concentrations of wastewater for various days of incubation.

and analysed for pH, ammonium nitrogen (NH_4^+-N), phosphate-phosphorus (PO_4^{3-}-P), total nitrogen (TN) and chemical oxygen demand (COD). The result is shown in Figs. 3–7 and Table 2.

Change of pH *in wastewater:* The pH reduced with increasing concentration of wastewater and days of treatment. The pH reduction ranged from 1.23% to 2.8% in low (1x) and high (30x) concentrations of wastewater between 7 and 28 days of fungal treatment of wastewater.

Levels of COD in wastewater: The levels of COD in wastewater were found to be reduced with days of experiment and also concentration of wastewater. The COD reduction ranged from 50% in low concentration (x1) to 25% in high concentration (30x) of wastewater between 7 and 28 days of experiment. Thus fungal treatment was found efficient particularly in low concentration of wastewater.

Determination of ammonium-nitrogen (NH_4^+-N): The ammonium-nitrogen were found to increased with concentration of wastewater and reduced with days of fungal treatment. The reduction of NH_4^+—N was maximum (88%) with high concentration (x30) and minimum (53%) with low concentration (1x) of wastewater. Thus the fungal treatment was found efficient to remove NH_4^+—N especially in higher concentration of wastewater (Fig. 8a and b).

Phosphate- Phosphorus levels (PO_4^{3-}-P): The levels were did not significantly vary between days, but between concentrations of wastewater. The reduction of PO_4^{3-}-P was maximum (77%) in low concentration (x1) and minimum (18%) in high concentration (30x) of wastewater. Thus the fungal treatment was found useful for removal of PO_4^{3-}-P particularly in low concentration of wastewater.

Total nitrogen levels (TN): The total nitrogen levels were significantly between days of experiment and also concentrations of wastewater. The reduction of TN was higher (67%) in high concentration (x30) than that (56%) in low concentration (x1) of wastewater. Thus the fungal treatment was found potent for removal of TN especially in high concentration of wastewater.

4. Discussion

Fungi play an important role as trickling filters in aerobic biological wastewater treatment systems. The present study recorded the fungal biomass production by *T.pubescens* NPK2 in a range of 2.2–3.9 g/L within 28 days of incubation for the COD value of 30–66 mg/L. However, Hultberg and Bodin (2017) have recorded the biomass

Table 2
Percent increase or decrease between 7 and 28 days of fungal treatment in different concentrations of wastewater.

Concentration of waste water	Biomass (%)	pH	COD (%)	NH_4^+-N(%)	PO_4^{3-} −P (%)	Total Nitrogen (%)
1X	+383	−1.23	−50.00	−53.00	−77.00	−56.00
5X	+272	−1.68	−41.00	−55.00	−44.00	−58.00
10X	+221	−1.89	−36.00	−55.00	−36.00	−59.00
15X	+199	−1.91	−35.00	−57.00	−28.00	−62.00
20X	+136	−2.13	−33.00	−62.00	−26.00	−60.00
25X	+71	−2.79	−27.00	−82.00	−17.00	−60.00
30X	+63	−2.80	−25.00	−88.00	−18.00	−67.00

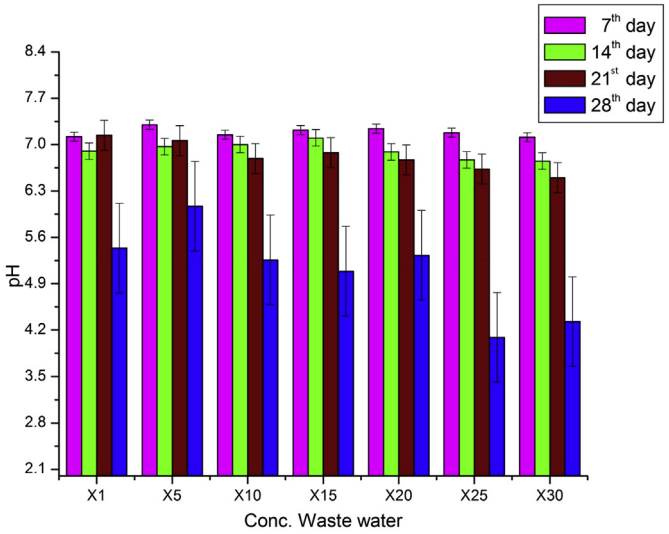

Fig. 3. Changes in pH in different concentrations of wastewater for various days of fugal treatment with *Trichoderma pubescens*.

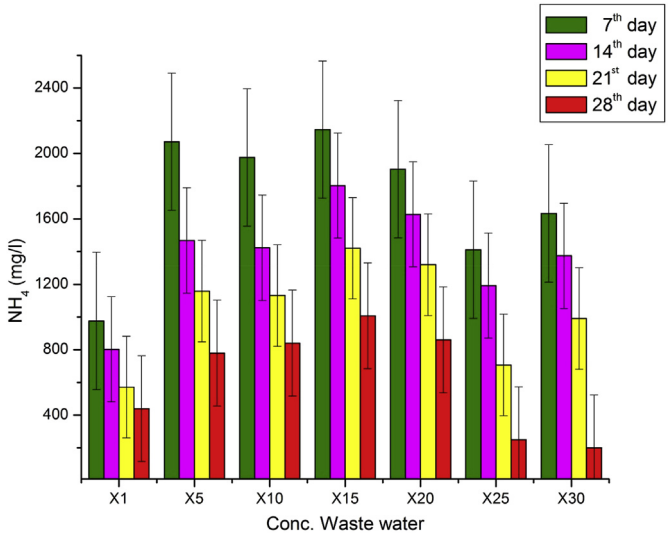

Fig. 5. Levels of Ammonium - Nitrogen in different concentrations of wastewater for various days of fungal treatment with *Trichoderma pubescens*.

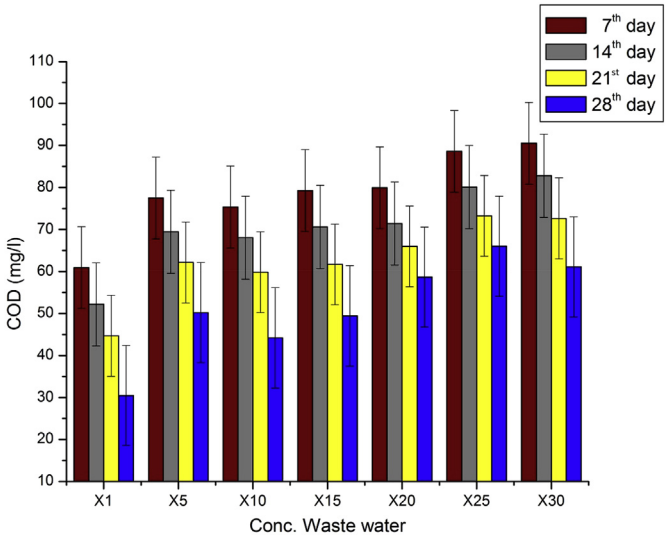

Fig. 4. Levels of COD in different concentrations of wastewater for various days of fungal treatment with *Trichoderma pubescens*.

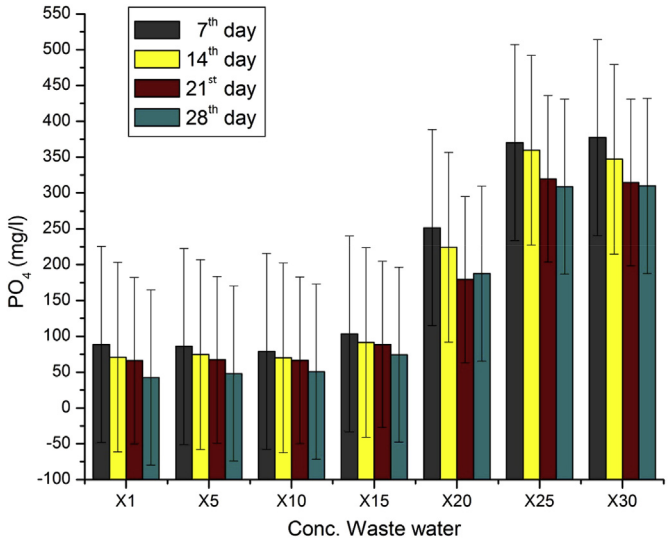

Fig. 6. Levels of Phosphate -Phosphorus in different concentrations of waste water for various days of fungal treatment with *Trichoderma pubescens*.

production of 0.695 g/L for *Trichoderma harzianum* within 8 days with the COD value of 1221 mg/L. In the same fungal species, Zhang et al. (2008) have registered higher biomass production within a short period of 24 h in winery wastewater with COD values that are 2–4 times higher than the study of Hultberg and Bodin (2017). This is in accordance with an earlier study of Shannon and Stevenson (1975) who have found higher biomass production of 3–20 g/L by *Pleurotus ostreatus* in brewery wastewater that contained 10–30 times higher COD than the study of Hultberg and Bodin (2017). The higher biomass is attributed to higher content of carbon available for the fungal growth and to higher COD values (Zhang et al., 2008). However, our study could not support this observation. For instance, the biomass production by *Trichoderma* was 3.9 g/L in normal synthetic wastewater (1x), which was higher than that (2.2 g/L) in concentrated wastewater (30x) on 28 days of incubation. Fungal biomass is habitually present in larger proportions in wastewater treatment systems containing lignocellulosic material and

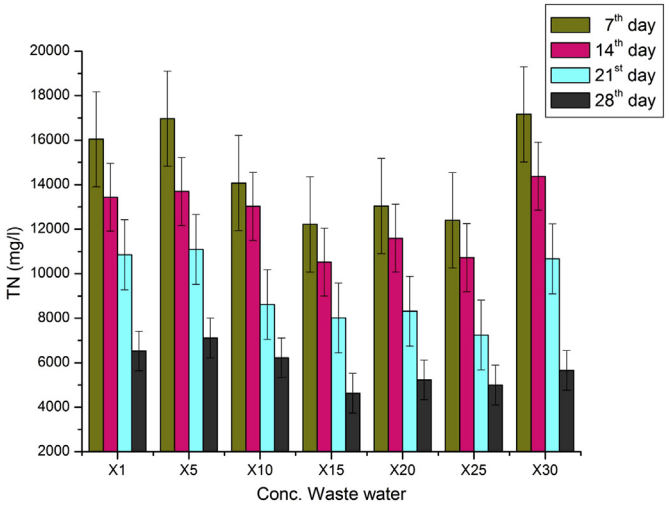

Fig. 7. Levels of Total Nitrogen in different concentrations of wastewater for various days of fungal treatment with *Trichoderma pubescens*.

lignin because they are more effective degraders of these materials than bacteria.

The main function of nutrient removal is depends on nutrient concentration and in other words, higher the nutrient concentration results in higher the rates of nutrient removal and increased biomass production (Henze et al., 2002). In the present study, the reduction of pH and ammonium-nitrogen was increased with increasing concentrations of wastewater. However, the reduction of COD, phosphate - phosphorous and total nitrogen was decreased with increasing concentration of wastewater. In other words, the fungal treatment was efficient in higher concentration of wastewater for removal of ammonium nitrogen, whereas the treatment was better in lower concentration of wastewater for removal of COD, phosphate - phosphorous and total nitrogen.

In the present study *Trichoderma pubescens* NPK2 was proved to have a considerable ability to reduce COD, similar to earlier reports. Hultberg and Bodin (2017) have recorded that *Trichoderma harzianum* reduces COD by 79–89% with potential for its utility for wastewater treatment of breweries. Similarly Zhang et al. (2008) have achieved the COD reduction of 86–91% by using *Trichoderma viride,* while Singh (2006) have reported 90% COD reduction by *Trichoderma versicolor* in anaerobially digested plant waste.

Hultberg and Bodin (2017) have observed that *Trichoderma harzianum* reduces the maximum concentration of NH_4^+ −N, due to mineralisation in the medium. The authors also demonstrate that lowering the pH levels favours the higher reductions in NH_4^+ −N in association with and this process is attributed to the transport of NH_4^+ being transported into the fungal cell as ammonia, leaving the hydrogen ion in the medium. The present study is in accordance with the findings that *Trichoderma pubescens* strains reduced the NH_4^+ −N up to 88% with high concentration (30x) of wastewater between 7 and 28 days of incubation, and this is also in line with the reduction in pH levels of the culture filtrate. The solubility of pollutants in wastewater is dependent on pH, and hence pH is playing a vital role in fungal treatment of wastewater (Singh, 2006).

In the present study, *Trichoderma* reduced the phosphate-phosphorus to the maximum of 77% in low concentration of wastewater (1x) between 7 and 28 days of incubation. Previous workers have recorded only low level of reduction in a range of 28.3–44% (Guest and Smith, 2007; Hultberg and Bodin, 2017). This differential reduction of phosphate phosphorus can be attributed to the differences in organic nitrogen. It is also supported by the fact that fungal cellular P content has positive relationship with organic nitrogen in wastewater (Ye et al., 2015). Therefore, addition of organic matter stimulates the utilization of phosphorus (Guest and Smith, 2007). Moreover, filamentous fungi can be easily harvested due to profuse mycelium growth that has a greater potential in P recovery. The P is also accumulated in the form of intracellular polyphosphate granules in some fungal species. For example, *Mucor racemosus* accumulates polyphosphate granules over 6.7% of its dry weight.

Nutrients found in waste streams are mostly compounds of carbon, nitrogen, and phosphorus (CNP) and they are important for sustenance of various life forms. Nitrogen and phosphorus are essential components of cell's DNA, aminoacids, and chlorophyll. In eukaryotic cells, phosphorus is the “energy currency” of the cells in the form of adenosine triphosphate, or ATP (Tornroth-Horsefield and Neutze, 2008). Nitrogen and phosphorus play critical roles in plant growth and food supply. While nitrogen abundantly exists in atmosphere (78%) in a highly stable and nonreactive form N2 gas, its content is limited in soils. Therefore, in order to make it useable and increase its availability in soils, nitrogen is fixed in reactive forms such as amino-acids, nitrate, and ammonia (Sengupta et al., 2015). The nutrient value of sewage in terms of nitrogen 30 mg/L, phosphate 7.5 mg/L, and potassium 25 mg/L is provided by CPCB [1997].

T. harzianum is reported to reduce more than 50% total nitrogen for wastewater treatment, which is the legal measure set by Swedish Agency for marine and water management (HaV, 2016). This supports our results that *Trichoderma* reduced total nitrogen up to 67% in high concentration (x30) of wastewater. The present study concludes that

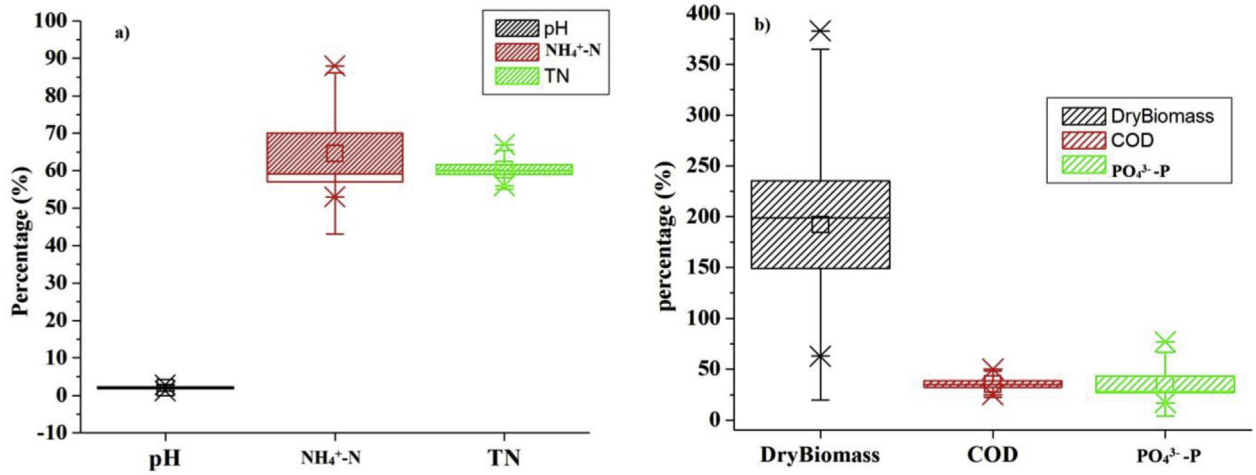

Fig. 8. a) decrease (%) over pH, ammonium-nitrogen & total nitrogen (TN), b) increase (%) of dry biomass, COD and Phosphate - Phosphorous over the initial and after 28 days of incubation (SD ± SE).

Trichoderma strain is much more efficient in the treatment of wastewater, due to its high capability for reducing not only COD, but also other nutrients (NH_4^+ −N, PO_4^{3-}-P and total nitrogen). *Trichoderma* is also advantageous as a biocontrol agent (Damle and Shukla, 2010) and it also provides cost effective method of treating the wastewater.

Acknowledgements

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bcab.2019.101100.

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