Studies on the enhanced antagonism of *Trichoderma harzianum* biofilms for water treatment using slow sand filters.

Sheherzad Akhtar

A thesis submitted to Quaid I Azam University in partial fulfillment of the reqirement for the degree of Doctor of Philosophy in Microbiology.

Sheherzad Akhtar

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بسم الله الرحمن الرحيم

Dedicated with love to my parents for teaching me, that intelligence brings out the beauty in a good heart, and to Dr Hameed for showing me that a good heart brings out the beauty in intelligence.

Declaration:

The material contained within this thesis is my original work and has not been previously submitted to any other university.

Sheherzad Akthar

This thesis submitted by Sheherzad Akhtar, is accepted in its present form to the department of Biological Sciences, Quaid-i-Azam University Islamabad, as satisfying the thesis requirements for the degree of Doctor of Philosophy (Ph. D)

Internal Examiner

Prof. Dr. Abdul Hameed

External Examiners

Dr. Allah Nawaz

Dr. Jehangir Arshad Khan

Chairperson

Prof. Dr. Safia Ahmed

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This thesis is as much yours as it is mine.

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ABSTRACT

Bacteria and fungi in the natural environment grow aggregated with each other, with solid surfaces, and at gas-liquid interfaces. There is a growing appreciation that, although clearly worthwhile, studies of standard planktonic cultures provide us with a biased view of microbial life (Parsek *et.al* 2004)

Filamentous fungi are naturally adapted to growth on surfaces and in these conditions they show a particular physiological behavior which is different to that in submerged culture; thus, they can be considered as biofilm forming organisms according to this concept (Akao *et.al.*, 2002; Biesebeke *et.al.*, 2002)

Uncertain ramifications of fungi in potable water have led to a limited number of investigations which show that fungi are present in a significant proportion of tap water samples; however, species abundance and diversity are extremely variable (Hinzelin *et.al* 1985, Nagy *et.al* 1982, Neimi *et.al* 1982, Rozenzweig *et.al* 1986)

Fungal biofilm kinetic models need to be developed yet since they have only recently been realized as true biofilms instead of simple cell immobilization systems (Keshavarz *et.al.*, 1990; Pakula and Freeman, 1996).

In the present study, a biocontrol fungi which was previously considered to be completely terrestrial, was grown and incorporated into biofilms on a laboratory scale sand filtration assembly. Although *Trichoderma* sp. have been found in numerous water distribution systems, processing plants and municipal supply lines, this is one of the first studies in seeing its role in chemical and microbial removal in submerged conditions and therefore its ability to "purify" water.

In the present research *Trichoderma harzianum* was isolated from soil in Punjab, this strain was a known bioantagonist. Its antagonistic capabilities were studied in the lab against *Fusarium oxysporum., Aspergillus niger., Salmoella typhi, Escherichia coli, Pseudomanas aeroginosa, Pseudomonas putida and Enterobacterer aerogens.* It was shown to be highly effective with an efficiency rate of upto 96% removal of pathogens.

This strain was then over a period of two months adapted to grow in water with minimal media, it adapted and remained viable upto two years of this study. Spore density was reduced by 60 %, there was almost complete reduction of

pigmentation when grown submerged in pure tap water, but green pigmentation did occur when grown in solid state on sand columns under water.

Growth rate was slowed by upto 40 %, mycelia were much weaker and thinner. The fungus incorporated well onto cellulose discs and grew with bacteria, still allowing bacterial film to form with it, although this film had 18% less bacteria. That may be due to competition and lack of space more than as a result of *Trichoderma* antagonism.

Biofim development was studied by carbohydrate production, fungal biofilms produced upto 0.6 0.55 mg/ml and in washings of planktonic cells upto 0.4 mg/ml in 10 days. Overall there was 30% greater reduction of fungal counts and 6% reduction in bacterial counts in the biofilm due to the presence of *Trichoderma* . Chemically *Trichoderma* incorporated biofilms were better for the removal of metals such as chromium (7%) and iron (40%), salts such as nitrates (5%), Chlorides (1%) and sulfates upto 12 %.

Trichoderma was also seen to be more successful in Colour (4%), Turbidity (3%), Total dissolved solids (2%) without any significant alteration in pH.

This study concluded that bioantagonist microorganisms are capable of adapting and adjusting to extreme variations in environment. *Trichoderma harzianum* does not loose completely its biocontrol ability even at high water potentials. It incorporated itself within the living system of a biofilm, there it utilized space and nutrients to its best advantage in a highly competitive manner and exerted its dominance over other organisms.

This ability can be extremely useful if manipulated and taken advantage of in wastewater, drinking water and recreational water purification.

Further studies are required to test the systems in field scale and industrial levels, it is expected that presently the results are indicative of a biofilm radius of only 3 inches, this if taken at a greater and deeper level would show even better results of the potential of *Trichoderma* in exerting its biocontrol in aquatic microbial communities.

INTRODUCTION:

1. INTRODUCTION

By 2025, over 60 percent of the world's population will continue to live in countries with significant imbalances between water requirements and supplies, largely in Asia, Africa and Latin America. Today, over one billion people lack access to safe drinking water and 2.4 billion lack access to improved sanitation. (Lawson L.R, *et.al*; 2005)

The proportion of the population with access to safe drinking water is an indicator expressed as the percentage of people using improved drinking water sources or delivered points. Improved drinking water technologists are more likely to provide safe drinking water than those characterized as unimproved.

To reach the Millennium development goals drinking water and sanitation target presents a huge challenge. Achieving the target requires the building of the drinking water infrastructure to provide services to an additional 1.1 billion people by 2015.(WHO/Unicef report 2006)

1.1 Fungal bioactive compounds

In 1940 only 20, in 1950 around 400, in 1960 approximately 1000 and in 1970 already 2500 antibiotics were known. From that time the total number of known bioactive microbial metabolites has doubled in every ten years. In 1980 about 5000, in 1990 10000 and in 2000 already almost 20000 antibiotic compounds were known. By the end of 2002 over 22000 bioactive secondary metabolites (including antibiotics) were published in the scientific and patent literature. An important part of natural products, the antibiotics, are a group of small molecular secondary metabolites of microorganisms, which exhibits some kinds of biological activity, and these compounds, these bioactive secondary microbial metabolites exhibit either antimicrobial (antibacterial, antifungal, antiprotozoal), antitumor and/or antiviral activities. The practical importance of antibiotics and other secondary metabolites is tremendous. They are widely used in the human

therapy, veterinary, agriculture, scientific research and in countless other areas. (Berdy 2005)

The total number of bioactive fungal product is approximately 8600, representing 38% of all microbial products. The most important, inherent characteristics of the bioactive microbial metabolites are their microbial origin, that is to say their specific microbial producers; their interaction with the environment, namely their various biological activities and last but not least their unique chemical structures. In the beginning of the antibiotic era the fungal (antibiotics such as penicillin, griseofulvin) and bacterial (antibiotics such as gramicidin) species were in the foreground of the interest, but after the discovery of streptomycin and later chloramphenicol, tetracyclines and macrolides the attention turned to the *Streptomyces* species.From the early nineties the number of bioactive compounds isolated from various filamentous and other microscopic and higher fungal species had continuously increased up to more than 50% by the turn of the millennium.

Among fungal species, the various microscopic (filamentous) fungi,(ascomycetes, fungi imperfecti, *etc.*) are the most frequent producers with about 6400 produced compounds. From the most common ascomycetes, namely from *Aspergillus*, *Penicillium* and *Fusarium* species 950, 900 and 350 compounds have been isolated, respectively. Besides them several other filamentous and endophytic species (*Trichoderma* , *Phoma*, *Alternaria*, *Acremonium* and *Stachybotrys*), are also good producers, each produces several hundreds of bioactive compounds. From higher fungal species - basidiomycetes or mushrooms - exemplified by *Ganoderma*, *Lactarius* or *Aureobasidium* species, altogether about 2000 active compounds have been derived. From yeasts, only 140 and from Myxomycetes (slime moulds) species 60 bioactive metabolites have been isolated.

The chemically relatively simple fungal compounds, over the antibiotic activities frequently exhibit diverse biological effects, mainly phytotoxic and pharmacological activities. We should not forget, the great practical and historical importance of beta-lactams (penicillins, cephalosporins), the

cyclosporin, and various statins (mevinolin, compactin, lovastatin, pravastatin, atrovastatin), which are all fungus derived compounds.

Some 60% of the presently known bioactive microbial metabolites, about 14000 compounds, exhibit antimicrobial (antibacterial, antifungal, antiprotozoal), approximately 5000 compounds have antitumor and about 1500 compounds exhibits antiviral activity. Additionally to antibiotic activities, some 11500 microbial metabolites, half of the all compounds, possess some kind of additional (or exclusive) "other"-non-antibiotic-bioactivities. Among them there are about 6000 metabolites without any recognized antimicrobial activity exhibiting exclusively some kinds of "other" biological activities. They are the so called "other bioactive" metabolites. 40 to 45% of the all fungal products have some kinds of antimicrobial, frequently antifungal, activities. New compounds may be prepared by chemical synthesis or may be isolated from the nature. (Table 17) (Berdy 2005)

The newly emerged combinatorial chemistry, besides the capacity to produce new compounds itself, in all means has less chances to produce useful leads for new drugs. The reason of the higher efficiency of natural products research is perhaps the fact that the natural products, in contrast to these random collections of chemicals, are somewhat prescreened by nature and they are already evolved for specific biological interactions. Furthermore, in contrast to the synthetic compounds, the products of microbes have many similarities with metabolites of other living systems.

Natural product resources, including the microbial world, are mainly unexplored both in its dimension and in the respect of geographic, ecological and environmental points of view. There surely exist, besides the presumed numbers of microorganisms, millions of microbes in the environment that are presently untouchable for science. Fungi are the second largest group of eukaryotes next to the insects and exceed not only the bacteria and actinomycetes.

Unique ecosystems as it is supposed, may result in unique organisms with unique metabolic pathways. The isolation of microbes from diverse ecosystems especially from under-represented sites (extreme circumstances, sea, *etc.*) may result in promising, new, until now unexplored producers.
Since ancient times, fungi have had a major role in food, feed and beverages. The fungal antibiotics penicillin, cephalosporin, griseofulvin, fusidic acid are significant clinically. Besides antibiotics, new therapeutic compounds with novel pharmacological activities Cyclosporin A, the most widely used drug to prevent rejection of human organ transplants; lovastatin, a cholesterol lowering drug; and ergot alkaloid, an antimigraine agent are good examples of compounds isolated from fungi. Commercial production of riboflavin by fungi may replace its synthetic counterpart in the future. Production of the anticancer drug taxol by endogenous fungi *Taxomyces andreanae* is also plausible. (Sur and Gosh, 2004)

1.2 *Trichoderma* occurrence in Natural waters and biofilms

The different stages of fungal development usually differ in the critical values of the environmental parameters. A decreased water activity was shown to promote sporulation of t.harzianum which could be used in liquid culture to produce conidiospores with increased desiccation tolerance usefull for biocontrol purposes (Harman *et.al* 1991) (jin *et.al* 1996). Conidial germination of *Trichoderma* spp was found to be particularly sensitive towards increased osmotic pressure by Jackson *et.al* (1991) whereas Harman *et.al* (1991) did not detect any such effect with T. Harzianum.
Water potential has also been reported to influence aroma formation by T.viride, particularly that of 2-heptanone (Gervais 1990) (Gervais *et.al* 1988) (Gervais and Sarrette, 1990).
A tetramic acid derivative harzianic acid was isolated from a water sample from the Hiroshima prefecture in Japan (Sawa *et.al* 1994). Trichoharzin has also been isolated from a culture of a strain found on the fresh water sponge Micale cecilia

(Kobayashi *et.al* 1993) Cultivation of the stran in both fresh and salty water was possible.

Methods to determine visually the action proportion of active vs inaction *Trichoderma* , or even *Trichoderma* vs total fungal biomass in an environmental sample, given the correct assortment of probes is possible (Harman and Kubicek 1998) comparable practical results have been achieved with diverse bacterial communities from the rumen (Schofield *et.al* 1997), activated sludge (Wagner *et.al* 1993) and environmental water samples (Lim *et.al* 1996).

Trichoderma spp. have been widely used as antagonistic fungal agents against several pests as well as plant growth enhancers. Faster metabolic rates, anti-microbial metabolites, and physiological conformation are key factors which chiefly contribute to antagonism of these fungi. Mycoparasitism, spatial and nutrient competition, antibiosis by enzymes and secondary metabolites, and induction of plant defence system are typical biocontrol actions of these fungi. On the other hand, *Trichoderma* spp. have also been used in a wide range of commercial enzyme productions, namely, cellulases, hemicellulases, proteases, and β-1,3-glucanase. (Verma 2007)

In lakes and rivers used as water supplies, mesophilic fungi and actinomycetes were common, whereas thermophilic fungi and actinomycetes were present only in low concentrations. Fungi and actinomycetes were more abundant in eutrophic and mesotrophic lakes than in oligotrophic lakes. River water contained more thermophilic actinomycetes and fungi and mesophilic actinomycetes than did lake water (Neimi *et.al*, 1982)

An investigation of drinking water showed that actinomycetes and fungi, including low concentrations of thermophilic strains, were present in raw water supplies and that they could pass through both sand filtration and disinfection and therefore can occur in drinking water.(Neimi *et.al* 1982)

A total of 70 strains of filamentous fungi were isolated from three different sources (wastewater, sewage sludge and leachate) of IWK's (Indah Water Konsortium) sewage treatment plant, Malaysia. The isolated strains were purified by conventional techniques and identified by microscopic examination. The

strains isolated belonged to the genera of *Penicillium, Aspergillus, Trichoderma* , *Spicaria* and *Hyaloflorae* The distribution of observed isolated fungi were 41% in sewage sludge followed by 39% in wastewater and 20% in leachate. (Fakhru'l-Razi *et.al* 2002)

Feasibility of production of antagonistic *Trichoderma* sp. conidial spores using wastewater sludge as a raw material employing different suspended solids concentration (10–50 g/l) was investigated in shake flasks by verma *et.al* (2005)

Studies by Gillings *et.al* (2006) showed that a biofilm in a municipal water treatment plant was composed primarily of a bacterium belonging to the genus *Acidocella*, a filamentous fungus (*Trichoderma asperellum*), and the ascomycetous yeasts.

Slime inhabiting fungi (Cooke 1961), primarily Hyphomycetes, are a diverse group of morphologically, physiologically and ecologically (Cooke 1970). The ecological roles of geofungi, especially in aquatic systems and the magnitude of those roles are not well understood. Cooke (1970) has adequately demonstrated the occurrence of geofungi in polluted waters, and while we do not fully understand the significance of this observation it seems probable that the fungi do play an important role in some waters, eg in removing excess organics in streams pollted by sewage effluents (Cooke 1961). Hynes (1970) and

Geofungi were isolated from rocks in a sewage effluent polluted stream. Samples were collected biweekly for 4 months. Amongst the most prominent genera were penicilium, *Trichoderma* and phoma. A total of 689 isolates of 14 common genera were obtained. (Noell, J. 1973)

1.3 Fungal Biofilms – lack of research

Biofilms have been used for a long time in water treatment facilities where they were called slime, mats or sludge,but not other practical used was seen until recently. This is because most of the available information is on bacterial and, in recent years, on yeast biofilms. Filamentous fungi are naturally adapted to growth on surfaces and in these conditions they show a particular physiological

behavior which it is different to that in submerged culture; thus, they can be considered as Biofilm forming organisms according to our former concept. Differential physiological behavior of most attached fungi corresponds principally to a higher production and secretion of enzymes and also to a morphological differentiation which is absent in submerged cultures (Akao *et.al* 2002; Biesebeke *et.al* 2002). The advantages of this form of growth have been industrially exploited by two culture systems: Slow sand filters and cell immobilization although there is a lack of knowledge on the molecular basis of growth on surfaces.

Technology of cell immobilization was highly developed during the last two decades based on the operative advantages in the productive process instead of physiological issues. Natural adsorption on solid supports is an immobilization technique that it has been used with filamentous fungi thus neglecting its study as a way of biofilm formation. Actually, once spores are adsorbed to the support they grow attached to it thus forming a film. The term biofilm fermentation (BF) is prefered instead of cell immobilization because the microbe is an active and differential entity. BF has been applied for the production of enzymes, amino acids, organic acids, alcohol, aromas and in bioconversion processes (Anderson, 1983; Groboillot *et.al* 1994; Norton and Vuillemard, 1994) as well as in bioremediation an effluent biotreatment (Burton, 2001; Doggett, 2000; King and Shoda, 1999; Kasinath *et.al* 2003; Rodgers *et.al* 2003; Van Driessel and Christov, 2001).

Fungal biofilm kinetic models need to be developed yet since they have only recently been realized as true biofilms instead of simple cell immobilization systems. Perhaps, the easy way to treat mathematically fungal biofilms is to adapt fungal pellet growth kinetics knowledge. An advantage of biofilms is that according to the type and form of the support almost all type of submerged bioreactors can be used, including the simple stirred tank, gaslift, packed bed columns, rotating contactors and horizontal blade-stirrer bioreactor (Keshavarz *et.al* 1990; Pakula and Freeman, 1996).

Although bacterial biofilms and their role in disease have been investigated in detail over a number of years, much less is known about fungal biofilms (Dogulas L.J, 2002, Dogulas L.J, 2003. Ramage *et.al* 2003 Ramage *et.al* 2003).

Biofilms are complex and dynamic microenvironments, encompassing processes such as metabolism, growth, and product formation, and finally detachment, erosion, or —sloughing" of the biofilm from the surface. The rate of biofilm formation and its release into a distribution system can be affected by many factors including surface characteristics, availability of nutrients, and flow veloci-ties. Biofilms appear to grow until the surface layers begin to slough off into the water (Geldreich and Rice 1987). The pieces of biofilm released into the water may continue to provide protection for the organisms until they can colonize a new section of the distribution system.

Bacteria, viruses, fungi, protozoa, and other invertebrates have been isolated from drinking water biofilms (USEPA 1992). The fact that such organisms are present within distribution system biofilms shows that, although water treatment is intended to remove all pathogenic (disease-causing) bacteria, treatment does not produce a sterile water. In fact, some otherwise harmless organisms (opportunistic pathogens) may survive the treatment process and cause disease in individuals with low immunity or compromised immune systems.

Uncertain ramifications of fungi in potable water have led to a limited number of investigations which show that fungi are present in a significant proportion of tap water samples; however, species abundance and diversity are extremely variable (Hinzelin *et.al* 1985, Nagy *et.al* 1982, Neimi *et.al* 1982, Rozenzweig *et.al* 1986)
The presence of fungi in drinking water and within biofilms of distribution systems has received limited attention. This is due, in part, to the fact that causal relationships between fungal occurrence and water quality remain uncertain. Nonetheless, waterborne fungi are likely associated with taste and odor problems, contamination in food and beverage preparation, and a variety of health-related effects (bays *et.al* 1970, Geldrich 1995, Metzer *et.al* 1976, Seeliger 1975)

This variation is often attributed to factors such as raw water source, water temperature patterns, treatment conditions, and maintenance of distribution systems (Geldrich 1995). The nature and extent of fungi within distribution biofilms remain more obscure; yet few reports demonstrated conclusively that fungi are an integral biofilm component (Nagy 1985) Although it is assumed that contamination is attributable to spore deposition within the biofilm matrix, there is limited evidence yet to support this premise.

1.4 The schmutzdecke biofilm

For over a century microbiologists have studied liquid cultures of bacterial and fungal colonies. In fact, a common criterion for choosing a microorganism to study has been its ability to grow in a suspended, homogeneous culture format, thereby simplifying examination of microbial physiology and genetics. Although these studies have been tremendously informative, they neglect the observation that many bacteria and fungi in the natural environment grow aggregated with each other, with solid surfaces, and at gas-liquid interfaces. There is a growing appreciation that, although clearly worthwhile, studies of standard planktonic cultures provide us with a biased view of microbial life (Parsek *et.al* 2004)

Biofilms are important in environmental, industrial, and clinical contexts (Costerton *et.al* 1999 Davey *et.al* 2000, Stoodly *et.al* 2002) The study of biofilm communities benefits from the efforts of investigators from many different disciplines, including environmental and clinical biologists, surface chemists, engineers, and mathematical modelers, who bring their unique questions, perspectives, and technologies to bear on this phenomenon.

One of the most recent forms of Biofilms being studied today is in the microbial layer produced for filtration and purification of drinking water known as the "schmutzdecke biofilm" A primary concern of any water treatment system is the removal and inactivation of pathogenic microorganisms.

This Biofilm is developed manually over sand filters used mostly in developing countries. The dynamics and working of this layer has yet to be characterized and formulated and very little data is available on its working.

Objectives of this thesis:

The objectives of this research are to contribute to the understanding and description of the complex and fundamental interactions in biological control. The focus of this work is to ehance the capabilities of SSF by incorporating already known fungal antagonists into the Biofilm structure so as to aid the disinfection capacity of the filter.

To achieve this purpose the specific objectives were:

- To develop a lab-scale filteration unit to simulate and control the relevant processes including chemical removal, development of schmutzdecke, microbial dynamics, headloss development and fungal-bacterial interactions.
- To identify the most important parameters in water purification in ssf
- To study the differences in fungal growth paterns and pathogenicity in varied growth conditions
- To define and quanity the contribution of a fungal enhanced schmutzdecke.

REVIEW OF LITERATURE:

2. REVIEW OF LITERATURE

2.1 Influence of water on growth of *Trichoderma* bioantagonism

Trichoderma strains have been isolated from habitats of varying moisture, temperature and nutrient status (Danielson and Davey, 1973; Papavizas 1985; Roiger *et.al.*, 1991). The ecological adaptability shown by members of these genera makes them ideal candidates for biological control applications in a variety of habitats, but the sensitivity of different isolates to abiotic environmental factors must be considered.

Santamarina (2006) reports the in vitro antagonism of an isolate of *Trichoderma harzianum* under a range of water (0.85–0.995 aw) and temperature (15–25 °C) regimes when associated with two phytopathogenic fungi: *Rhizoctonia solani* and *Verticillium dahliae*. The effects of ecophysiologic factors on the individual and dual growth of these fungal species were investigated and the index of dominance (ID) of each fungus was analysed according to its interaction. Growth rate of all the fungi tested increased as water availability in the medium increased and had the fastest growth rate at highest aw levels (0.995). In addition, *T. harzianum* inhibited the development of *R. solani* and *V. dahliae* upon contact (ID of 4) at both temperatures when aw was between 0.95 and 0.995. The results obtained show that the T. *harzianum* strain was antagonistic in vitro to *R. solani* and *V. dahliae*, and may be considered a potential biocontrol agent.

Partially purified *Trichoderma reesei* RUT-C30 acetyl esterase preparation was found to catalyze acyl transfer reactions in organic solvents, mixtures of organic solvents with water and even in water. This enzymatically catalyzed transacetylation in water, which is applied to transformation of saccharides for the first time, opens a new area in chemoenzymatic synthesis. Its major advantages are simplicity, highly regioselective esterification of polar compounds, high yields, low enzyme consumption and elimination of the need to use toxic organic solvents (Kremnicky *et.al*, 2004)

In water solution left at room temperature, the physiochemical properties of chitinolytic enzymes have been found to generally stable, as when the enzymes are dried or frozen. The occurance of N glycosylation may be responsible for the high stability of the enzyme. Acidic pH optimum from 4.0 to 5.5 and temperature optimum from 40 to 60C is reported, the enzyme activity does not require co factors is not greatly affected by metal ions, most enzymes are heat resistant. (de La Cruz *et.al*, 1993, Di Pietro *et.al.*, 1993; Draborg *et.al*, 1996; Inbar and Chet, 1995; Haran *et.al*, 1995; Harman *et.al*, 1993b, Lorito *et.al* 1994a, 1995, 1996b, Margolles-Clark *et.al*,1996a,b;Ridout *et.al*, 1988; Ulhoa and Perberdy, 1991a, 1992)

Trichoderma harzianum has the capacity to degrade water insoluble glucan polymers. The 15kDa alpha 1,3 glucanase (also called mutanase) has been used in various applications in such as the for caries prevention (Kelstrup *et.al*, 1978), and has shown good results in the water rich environment of the mouth.

Since CBH11 is an inverting enzyme, the hydrolysis is postulated to proceed through a single displacement reaction involving a general acid to donate a proton and a base to assist the nucleophilic attack of water (Koshland 1953) the active sites of inverting glycosidases are usually constructed such that a water molecule can be accommodated between the base and the anomeric carbon. The nucleophilic water thus approaches the anomeric carbon from the direction opposite the proton donor (McCarter and Withers, 1994).

The natural distribution of *Trichoderma* is greatly influenced by habitat temperature and the species groups vary in their temperature optima and tolerances (Domsch *et.al* 1980). Isolates within a species group may also vary in their antagonistic activity at different temperatures (Kohl and Schlosser, 1989; Tronsmo and Dennis, 1978) The temperature tolerance of the biocontrol isolate to that of the pathogen could be critical to the success of a number of applications.

Conidia of *Trichoderma* species require exogenous nutrients in order to germinate (Danielson and Davey, 1973b). *Trichoderma* conidia must take up water and swell considerably before the germ tubes emerge, and swelling will not

occur without a given period of exposure to sufficient nutrients (Danielson and Davey, 1973c; Hawker, 1966; Martin and Nicolas, 1970; Steiner and Lockwood, 1969).

Other than temperature, moisture is considered the environmental parameter most influencing the natural distribution of the various *Trichoderma* spp in soil (Danielson and Davey, 1973). It is also considered a factor limiting the ability of introducing antagonists to colonize the phyllosphere and thus to compete with and control foliar pathogens (Elad and Kirshner, 1992); Hannusch and Boland, 1996). Environmental moisture affects growing fungi physiologically, eg through the increased energy costs of osmoregulation under dry conditions or decreased oxygen availability in wet habitats. Furthermore, moisture affects nutrient availability, through solute transport and the presence of water films. To a certain degree *Trichoderma* can adapt to unfavorable habitat moisture conditions by cytoplasmic translocation. Cell wall synthesis is prioritized over cytoplasm synthesis and the cytoplasm is relocated to the growing tips as the hyphae extend; in this manner the mycelium can extend over areas oF low osmotic potential or nutrient content towards more suitable areas (Paustian and Schnurer, 1987a,b). Although hyphal growth of *T. harzianum* in soil thus may be relatively insensitive to low soil matric potential (Knudsen and Bin, 1990), initiation of growth from dried mycelia biomass may require addition of an osmoregulant to the formulation (Knudsen *et.al*, 1991).

Conidial germination is moisture sensitive, and *Trichoderma* isolates colonize habitats in the phyllosphere more effectively under moist conditions (Elad and Kirshner, 1993; Gullino, 1992; Knudsen and Bin, 1990; Kohl *et.al*, 1995a,b). Changes of as little as 5% relative humidity may significantly affect the ability of T.viride to control certain pathogens, best control is at 100% relative humidity (Hannusch and Boland, 1996a,b). In an experiment by Elad *et.al* (1993) it was seen that in a green house application relative humidity affected efficacy of the biological control agent more than temperature.

Despite the environmental limitations imposed by temperature and moisture, appropriately timed field applications have been shown to control disease in the

phyllosphere very well. During periods in which humidity and temperature are unsuitable for the antagonists, limited use of chemical fungicides has proven to be an effective strategy for increasing the consistency of biological disease control in the phylliosphere (Elad *et.al*, 1993; Gullino, 1992; Harman *et.al*, 1996; Tronsmo, 1991) as well as in soil (Chet 1987).

Propagule types affects the dependency on exogenous nutrients (Lockwood, 1981) both hyphae and chlaydospores of *Trichoderma* are less sensitive than conidia to soil fungistasis (Beagle-Ristaino and Papavizas, 1985; Hsu and Lockwood 1971). Propagule age also affects the nutrient requirements. Danielson and Davey (1973b) found that *Trichoderma* spores become more sensitive to a lack of nutrients as the become older.

2.2 Biological mechanisms involved in Sand Filtration

Biological filtration (or slow filtration) occurs when raw water circulates through a porous sand bed. During the process, the impurities contact the surface of the filter medium particles, and are retained. Chemical and biological degradation processes then take place, which reduce the settled matter to simpler forms. These are taken in solution or remain as inert material until they are withdrawn or cleansing takes place.

The processes taking place in a slow filter complement each other and act together, simultaneously, to improve the physical, chemical and bacteriological characteristics of the treated water.

The raw water that enters the unit remains on the filter medium from three to twelve hours, depending on the filtration rate. During this period of time, the heaviest suspended particles settle and the lightest particles can agglutinate, thus facilitating their later removal. Algae grow during the daytime under the influence of sunlight, which absorb carbon dioxide, nitrates, phosphates, and other nutrients in the water to form cellular material and oxygen. This oxygen dissolves in water and reacts chemically with the organic impurities, making the impurities easier to assimilate for the microorganisms.

A layer mainly of organic origin is formed on the surface of the filter medium. This is known as the schmutzdecke or "biofilm". The water has to pass through this layer before reaching the filter medium. The schmutzdecke is mainly made up of algae and many other forms of life, such as plankton, diatoms, protozoa, rotifera, and bacteria. The intensive action of these microorganisms traps, digests and degrades the organic matter in the water. The dead algae and the live bacteria in the raw water are also consumed in this process. While nitrogenous compounds degrade, nitrogen oxygenates. Some color is removed and a considerable amount of suspended inert particles are removed by sifting.

Once the water passes through the schmutzdecke, it enters the filter bed and is forced to pass through it in a process that normally takes several hours. A physical sifting process takes place here, which is part of the total purification process. One of the most important properties of the filter layer is that of adherence, which is a phenomenon resulting from electric forces, chemical actions, and attraction of masses. To appreciate the magnitude and importance of this phenomenon, we need to visualize that one cubic meter of sand with the usual characteristics for slow filters has a grain area of almost 15 000 m2. The water passing through the sand grains with a laminar flow (which constantly changes direction), facilitates the action of the centrifugal forces on the particles and the adherence of these particles to the surface of the sand grains.

An active settling process takes place in the pores or empty spaces of the filter medium (which constitute approximately 40% of the volume). This phenomenon is considerably enhanced by the action of electrostatic forces and mass attraction.

As a result of the phenomena mentioned above, the surface of the sand grains is covered with a layer, which is similar in composition to the schmutzdecke, with low algae and particle content, but with a high content of microorganisms, bacteria, bacteriophages, rotifera, and protozoa, all of which feed and absorb the impurities and residues of the others. This biological coating is very active down to a depth of 0.40 m in the filter medium. Different forms of life predominate at

different depths. There is more biological activity near the surface of the filter layer, where conditions are optimal and food abounds.

Food consists mainly of particles of organic origin, carried by water. The organic coating maintains the suspended particles in the water until the organic matter is degraded and is assimilated by the cellular material, which in turn is assimilated by other organisms and converted into inorganic matter, such as water, carbon dioxide, nitrates, phosphates and salts that are subsequently carried away by the water.

The quantity of food diminishes at the end of the filter layer, originating another type of bacteria, which use the dissolved oxygen and solute nutrients found in water.

As a consequence of those processes, the raw water that enters the slow filter with suspended solids in a colloidal state and a wide variety of microorganisms and complex salts comes out virtually free of such impurities and with a low inorganic salt content. In the biological filtration process, not only have the harmful or dangerous organisms been eliminated, but also the nutrients in solution, which could facilitate subsequent bacteriological growth.

The effluent obtained usually has a low dissolved oxygen content and high carbon dioxide content, but both these characteristics can be improved with a later aeration process.

Since the performance of the slow filter depends mainly on the biological process, efficiency is low while the biological layer is still developing. It improves as filtration progresses. This process is known as the "ripening of the filter". (Husiman and Wood 1974)

Biological action occurs in slow sand filter beds. Because of the low hydraulic loading and smaller sand size found in slow sand filters, most of the solid particles are removed within the top 0.5 - 2 cm of sand, as opposed to rapid filters where the penetration is much deeper. With time, this area develops a biological film called the schmutzdecke as well as a zone of biological activity within the sand bed. The various purification processes in both the schmutzdecke and deeper biological zones, are interdependent and are best described

together. One example of such interdependence is where trapped impurities on and within the filter bed are broken down and rendered innocuous, with by-products of one process fuelling another deeper down.

The two principal actions that contribute to the overall purification effect are chemical and microbiological oxidation, but other biological processes involving various forms of animal and vegetable life may play a significant part. .

It is important to note that the schmutzdecke film is not the only area of biological activity in a slow sand filter. Often the term schmutzdecke appears to be used sometimes to denote the general zone of biological activity that occurs within the sand bed. However, this zone is distinct. Due to its double function, which includes mechanical filtration, the depth of the schmutzdecke can be said to correlate to the penetration zone of solid particles, that is, the top 0.5 - 2 cm of a slow sand filter bed. At this depth range, the schmutzdecke merges with the deeper biological layer, and particle-free raw water flows into this zone after passing through the schmutzdecke. This deeper zone is therefore not largely a mechanical filtration zone but rather a continuation of the area of biological action.

The schmutzdecke should remain undisturbed. This is so that the biological population in the top centimeters of sand are not disturbed or stressed, while not allowing any film that has built up to be broken, which would reduce the film's straining effect while pushing solids further into the sand. A device is usually used to disperse the energy of water as it enters a filter. This can take the form of a baffle plate, or in the case of intermittent sand filters, a diffuser plate which sits close to the water surface.

2..2.1 Adherence mechanism

Nowadays, biofilm processes used mainly for waste water treatment are also being considered for metabolite and enzyme production. Although fungal biofilms are less known than bacterial biofilms, they can be used for enzyme production as it has been recently showed (Villena *et.al.*, 2001). Both SSF and Biofilm

fermentation (BF) depend on surface adhesion and a new fermentation category was recently established. Surface adhesion fermentation (SAF) was proposed by Gutierrez-Correa (2003) as this new category.

Until adherence occurs, there can be no removal. The fraction of particles that adhere in relation to the number of collisions is, by definition, coefficient α. Research studies suggest that the development of the biofilm provides the sand grains with an absorbent surface that favors adherence. Another assumption is that the extracellular enzymes coagulate the particles, thus making adherence possible. It is unknown in what situations the value of α increases or declines.

When the filter begins to function, and before the biological film is developed, coliform removal is close to zero, therefore $\alpha = 0$ (Bryck *et.al* 1987). After the biofilm has developed, the removal rate is in the order of 2 to 4 logarithms, coefficient α being close to 1.0. This indicates the importance of the biofilm in the efficiency of the slow filter. The microorganisms can die or be ingested by predators, before they reach an absorbent surface. Therefore, removal can be due to death or predatory action, as well as to adherence. However, after adherence, predatory action and death will inevitably occur.

The filter is considered "ripe" when the biofilm has reached its maximum development for the existing conditions. The maximum development limit of the biofilm has not yet been defined. More research is needed to obtain this important information.

However, research carried out by Bellamy *et.al* (1985), Bryck (1987) and Barrett (1989) have shown that the maximum development limit of the biological layer is related to the nutrient content of the raw water.

Slow filters that treat water with a low nutrient content can be expected to present a removal of faecal coliforms in the order of 2 log after the ripening of the biological film has been produced (Bellamy *et.al*, 1985). On the other hand, it is to be expected that removals in water rich in nutrients are obtained in the order of 3 log (Bellamy, 1985); in other cases removal efficiencies of up to 4 log are found. (Barrett, 1989)

2.2.2 Depth Filtration Theory

Particle removal and retention within depth filters involves Van der Waals forces where two surfaces have attractive forces, in this case between the particle and the media surface. Van der Waals forces are short-ranged, and only become effective when the two surfaces are in close proximity. For particle-media surfaces to come close enough together for these forces to become effective, transport mechanisms must be present. These mechanisms are represented by three different processes, which include interception, inertia and sedimentation, and diffusion. These processes are attributed with most particle removal. As a particle is transported through a filter, if the streamline is within one half or less of the diameter of the particle from the media surface, the particle will be intercepted. Second, as streamlines curve around the media, particles can deviate from the streamline and continue towards the media due to inertia forces. Particles may also deviate from streamlines due to gravitational forces and settle onto the media surface. In both cases, particle will be retained at the media surface. Lastly, particles may deviate from streamlines due to Brownian motion and diffuse to the media surface. (Crittenden, J.C., *et.al* 2005)

2.3 Functional dynamics

Effective pathogen removal and success rates of filters are dependent on many factors, it is a unison of these factors which provides the biofilm layer with the right conditions to perform.

2.3.1 Effect of filter media design

Swertfeger *et.al* (1999) evaluated the effect of filter media design on cyst and oocyst removal. Designs included monomedium (sand with a depth of 750 mm), fine dual-media (anthracite and sand with depths of 900 mm and 300 mm, respectively) and deep dual-media (anthracite and sand with depths of 1500 mm

and 300 mm, respectively). The feedwater to the pilot systems was taken from the effluent of a sedimentation unit in a full-scale water
treatment plant and was in optimal coagulation condition. The authors found no statistical difference in the filtration performance for the different media. Removal of Giardia was 4.4 logs or better, with greater removal efficiency Removal processes 25 in the summer than in the winter. Removal of Cryptosporidium was similar in summer and winter, and averaged 2.7 logs or more.

Payment *et.al* (2000) reported water-quality monitoring results for a fullscale conventional water treatment plant using dual-media filters, with coagulation provided by alum and activated silica. Prechlorination was applied at 1 mg/l. The results confirmed that a properly operated conventional treatment plant provided a substantial barrier to microbial pathogens. Giardia cysts were detected in only 1 of 32 filtered water samples, with a mean removal of 3.6 logs after filtration (including removal by coagulation and sedimentation).

Removal of Cryptosporidium oocysts was lower than for Giardia. Oocysts were detected in 7 of 32 filtered water samples, with a mean removal of 2 logs. Clostridium perfringens was detected in 9 of 33 filtered water samples, with average removal of 4.4 logs. No human enteric virus was detected in 32 filtered water samples, with average removal of 3.1 logs (assuming that the concentration of humic enteric virus in filtered water was equal to the detection limit). Somatic coliphage were detected in 24 of 32 filtered water samples, with average removal of 3.5 logs

Removal of microbes:

In a review by Ellis (1985), virus removal ranging from about 1 to 5 logs was reported for bench and full-scale slow sand filters. Various studies have reported the effective removal of bacteria and protozoa by slow sand filtration in pilot and full-scale systems.

In a pilot-scale study, a new filter removed 0.82 logs of total coliform bacteria and more than 1.7 logs of Giardia (Bellamy *et.al* 1985). Once a microbiological population was established within the sand bed (after two weeks), the removal of

total coliforms increased to 4 logs and no Giardia was detected in the filtered water. The calculated cyst reduction was more than 2.6 logs, depending on influent cyst concentration. Similar results were found in another pilot study, where the removal of total coliform bacteria, heterotrophic bacteria and turbidity increased with the biological activity of the schmutzdecke (Bellamy, Hendricks & Logsdon, 1985).

In a full-scale study of a slow sand filter in Empire, Colorado (USA), Giardia cysts were detected in almost half of the influent samples, but not in the effluent (Seelaus, Hendricks & Janonis, 1986). In a full-scale study for three slow sand filtration plants in Idaho (USA), no samples positive for Giardia were found in the filtered water from two of the three treatment plants (Tanner & Ongerth, 1990). For the one positive sample found in one plant, 1-log removal of Giardia was achieved.

In the same study, removal of total coliforms and faecal coliforms varied from 84.35 to 99.5% (0.81–2.30 logs) and from 48.1 to 70.0% (0.29–0.52 logs), respectively. Removal of heterotrophic bacteria (as measured by HPC) varied from 65.8 to 91.0% (0.47–1.05 logs). These differences in removal efficiency were influenced by raw water quality, filtration rate, media size and depth. Removal of Cryptosporidium by slow sand filtration is often more difficult than removal of Giardia. In a full-scale study in British Columbia, Fogel *et.al.* (1993) reported that the average removal of Giardia was 93% (1.16 logs) but was only 48% (0.28 logs) for Cryptosporidium.

2.3.2 Effect of sand size on bacteriological quality.

Guidelines for sand size are not enforced. Results from some studies have shown that there is scope for the relaxation of typical values that have been used as benchmarks of slow sand filter design. One such study (Muhammad *et.al*, 1996) done on coarser sand with a constant uniformity coefficient of 2, found that the treatment efficiency (for removal of bacteria, turbidity and color) of slow sand filters was not very sensitive to sand sizes up to 0.45mm, although a slight

increase in treatment efficiency was observed with decreasing sand size. While it is clear that finer sand produces better quality water, filters with sand sizes larger than 0.2mm (up to 0.45mm) produce satisfactory quality water with the added advantage of a longer filter run. Hence, from the standpoint of removal efficiency the argument for using very fine sand is not strong (Muhammad *et.al*, 1996). In the same way, the ideal range for the uniformity coefficient seems to vary, for example, Ellis (1987) recommends a uniformity coefficient in the range of 1.7 – 3, while one of less than 2.7 is preferable. In practice, it seems that sand that is both finer and coarser than the recommended range still provides acceptable results in terms of filtration in continually operated systems (Barrett, 1989)

2.3.3 Effect of sand depth on bacteriological quality

Bellamy *et.al*, (1985) suggested that sand height could be reduced to 0.48m with no change in bacteriological removal efficiency. However, Muhammad, *et.al* (1996) concluded that most bacteriological purification occurs within the top 400mm of a sand bed.

They found that bacteriological treatment was not highly sensitive to sand bed depth suggesting that a continually operated slow sand filter bed could be reduced even further to 0.40m and still produce a satisfactory bacteriological quality of water. Other research confirms that the majority of biological processes occur in the top 0.4m of the sand bed (ASCE, 1991). However, while this is generally true, bacteriological treatment efficiency does become more sensitive to depth with larger sand sizes because the total surface area within the filter is reduced in a sand bed with larger grains, as well as higher flow rates potentially increasing breakthrough.

Interestingly, Ferdausi and Bolkland (2000) found adequate faecal coliform removals to below 10 per 100ml in pond filters, which only had sand bed depths of around 30cm.

2.3.4 The effect of temperature

The temperature of the water must not be allowed to fall too low for satisfactory biochemical oxidation of organic matter to take place by the organisms in the biological layer. The efficiency of slow sand filtration may also be seriously reduced by low temperatures, owing to the influence of temperature both on the speed at which chemical reactions take place and on the rate of metabolism of bacteria and other microorganisms.

Below 6°C the oxidation of ammonia practically comes to a standstill. When air temperatures drop below 2°C for any considerable period it is necessary either to cover the filters to reduce heat losses or to provide for subsequent chlorination as a precaution against incomplete purification in the filtration plant.

At low temperatures, the activity of bacteria-consuming protozoa and nematodes drops sharply, and at the same time the metabolism of the intestinal bacteria themselves slows down, increasing the chance of survival of those that are carried through the bed. The factor by which the numbers of *E. coli* are reduced, which is normally in the range 100-1000, may fall as low as 2 at temperatures of 2°C or less, and chlorination is then essential if the quality of the delivered water is to be maintained.

Research done on continually operated slow sand filters shows that the majority of biological processes occur in the top 0.4m of the sand bed (ASCE, 1991). This is confirmed by results that show that bacteriological purification occurs mostly in the top 40 cm of the sand bed below the schmutzdecke. Biological activity does, however, occur deeper within the filter bed. Below a depth of 30-40 cm (depending on the filtration rate) bacterial activity is small, but biochemical reactions take place converting organic materials such as amino acids into ammonia, nitrites, and nitrates (nitrification). These amino acids are liberated by the bacterial life cycle in the upper sand layer (Huisman and Wood, 1974). This has been confirmed by test data where oxidation of nitrogenous organic compounds at depths shallower than 40cm was found to be incomplete (Muhammad *et.al*, 1996).

For intermittently operated sand filters, the depth of biological processes is also dependent on how much water is standing on top of the sand during pause times. A shallower water depth means that more oxygen is able to diffuse to the biological layer, and as a result the biologically active zone can grow deeper within the sand. A study done by Buzunis (1995) found that with 12.5cm of standing water, the biological zone was only 10 cm deep.

For the survival of the microorganisms within the biological zone, the sand must be kept wet. The sand filter bed is kept wet by sand filter design, where the outlet level is made above the level of the sand. This always ensures that the filter bed does not dry out.

For the survival of the microorganisms within the biological zone, there needs to be a supply of food in the raw water. Seeding the filter with biologically productive raw water ensures more efficient biological filtration (Palmateer *et.al*, 1999).

There also needs to be a supply of oxygen for the microbes to survive. Oxygen is used in the metabolism of biodegradable components and the inactivation and consumption of pathogens. If it falls to zero during filtration anaerobic decomposition occurs, with consequent production of hydrogen sulphide, ammonia, and other taste- and odour-producing substances together with dissolved iron and manganese, which make the treated water unsuitable for washing clothes and other purposes. Thus the average oxygen content of the filtered water should not be allowed to fall below 3 mg/l if anaerobic conditions are to be avoided throughout the whole area of the filter bed (Huisman and Wood, 1974). This requirement may call for aeration of the raw water to increase its oxygen content or pre-treatment to lower its oxygen demand.

Finding a way to allow enough oxygen transfer to sustain the biological layer has been essential in the design of the filter. An in-depth study was carried out in 1995 (Buzunis, 1995) and a mathematical model to describe the diffusion of oxygen transfer into the filter bio-layer was developed and supported by experimental data.

For satisfactory biochemical oxidation of organic matter by the organisms in the biological layer, sufficient time must also be allowed to maintain a long enough

contact time with the sand bed. Adequate time is ensured by keeping the filtration rate down. One way of doing this is to reduce the pressure head (hydraulic loading) of the water on top of the sand, and another is to increase the depth of sand since discharge is related to both head loss and the length of the sand column).

2.4 Water purification through sand columns

Wastewater effluent treatment by soil infiltration and percolation has long been used as a simple, low-cost means of wastewater management throughout the world (Mancl and Peeples, 1991; Tchobanoglous and Burton, 1991; Wotton, 2002). When properly designed, constructed, and operated, soil infiltration systems can provide an effective means of wastewater treatment and disposal with regard to organics, nutrients, and bacteria (Anderson *et.al.*, 1994; Duncan *et.al.*, 1994; Hagedorn *et.al.*, 1981; Sundblad and Wittgren, 1996; Stevik, 1998). However, unless properly designed and operated, infiltration systems may pose a bacterial pollution risk to ground water and freshwater recipients. The majority of the studies of infiltration systems are based on the removal of fecal microorganisms from human waste (Bouma *et.al.*, 1972; Hagedorn *et.al.*, 1981; Farooq and Al-Yousef, 1993; Stevik *et.al.*, 1999a, 1999b; Harrison *et.al.*, 2000; Ausland *et.al.*, 2002). In addition, studies have shown that low-cost sand infiltration systems are promising as a way of treating fish farm effluents (Vigneswaran *et.al.*, 1991; Kristiansen and Cripps, 1996; Palacios and Timmons, 2001) and as an efficient method for removal of fish pathogenic bacteria from fish farm wastewater (Bomo *et.al.*, 2003). Bacterial removal mechanisms in infiltration systems are a combination of physical, chemical, and biological factors. The studies of Bomo *et.al.* (2003) indicated that biological wastewater filters are dynamic systems and that biological factors (i.e., predation by the indigenous protozoa) can be of major importance in these systems. According to Acea and Alexander (1988) and Acea *et.al.* (1988), protozoa are the main predators of bacteria. Increasing the knowledge about the dynamics of biological sand filters

is highly important as this will aid in optimizing infiltration systems for improved and stable bacterial removal efficiencies.

2.5 Relative roles of schmutzdecke and sand in the efficiency of the filter

Bacteria produce a slimy substance consisting of exocellular polymers as well as living and dead cells - this substance is known as zoogeal (Brock and Madigan, 1991). Within the schmutzdecke and this zoogeal film, bacteria derived initially from the raw water multiply selectively, the deposited organic matter being used as food. The bacteria oxidize part of the food to provide the energy they need for their metabolism (dissimilation), and they convert part of it into cell material for their growth (assimilation). Thus dead organic substances are converted into living matter. The dissimilation products are carried away by the water, to be used again at greater depth by other organisms.

Most of the existing documentation on slow filtration gives the schmutzdecke all the credit for the microbiological efficiency of the filter.

Hazen (1913) reported concentrations in the order of 106 bacteria/gram of layer in the filter surface and an exponential decrease with depth, to values of 105 bacteria/gram 2 cm down. Collins *et.al* (1989) informed that they had found 109 bacteria/gram of dry layer in the filter surface, decreasing from 107 to 106 bacteria/gram at a depth of 30 to 45 cm.

Bellamy *et.al*, in a study carried out in 1985 with a hydraulic rate of 0.12 m/h, indicate a removal of total coliform bacteria of three logarithmic levels when the layer was ripe. However, after the surface of the filter was scraped, the removal was of two logarithms, indicating that the schmutzdecke was not responsible for the whole efficiency of the filter, not even for the greater part of it.

2.6 Schmutzdecke and the active zone

The purification mechanisms described here originate mainly from Huisman and Wood (1974) While the exact nature and dominance of the various processes is still not completely clear, a review of the literature suggests that there are four dominant processes which contribute to the purification of the raw water:

Hostile Environment - Conditions found within a slow sand filter are generally unsuitable for the multiplication of intestinal bacteria. Normally used to a body temperature of 37°C, they do not thrive at temperatures below 3O°C.

Competition for food - Food is required by the bacteriological population for metabolism. Oxidization processes during metabolism consume organic matter in the raw water, including dead pathogens. The filter bed does not usually contain enough organic matter of animal origin to meet their nutritional needs. Within the upper layers there is competition for food from other microbes while at lower depths suitable food becomes even scarcer so that they starve, particularly at higher temperatures when their metabolic rate increases.

Predation - Many types of predatory organisms (such as protozoa and lower metazoa) abound in the upper part of the bed and feed on other cells.

Excretion of poisons - while there is little quantitative data available, it is known that the microorganisms in a slow sand filter produce various substances that act as chemical or biological poisons to intestinal bacteria.

The combined effects of this selectively hostile environment results in the death and inactivation of many pathogens (disease causing organisms). The overall result is a substantial reduction in the number of indicator bacteria such as *E. coli,* and an even greater proportional decrease in pathogens themselves. This effect becomes greater as the flora and fauna of the filter develop in the presence of adequate food, oxygen, and suitable temperatures.

The bacterial population is limited by the amount of organic material supplied by the inflowing raw water; the growth (assimilation) is therefore accompanied by an equivalent dying off. This in its turn liberates organic matter, which becomes available to bacteria at lower depths. In this way the whole of the degradable

organic matter present in the raw water is gradually broken down and converted into water, carbon dioxide, and relatively innocuous inorganic salts such as sulphates, nitrates, and phosphates (mineralization) to be discharged in the filter effluent. The bacterial activity described is most pronounced in the upper part of the filter bed and gradually decreases with depth as food becomes scarcer.

2.7 Structure of the schmutzdecke

Eighmy *et.al.* (1993) give details of microorganisms involved in degradation mechanisms and degradation pathways of aromatics, proteins, carbohydrates (aliphatics), fulvic, and humic acids. Identification of particular physiological groups of microorganisms in the schmutzdecke has generally been limited in the past to groups possessing distinct morphological characteristics such as iron bacteria, methanogenic archaea bacteria, or algae. On the other hand, isolation of microorganisms from the schmutzdecke allows further characterisation of their specific metabolism, which may be of particular interest when micro pollutants are considered. However, it should be noted that the isolation of a particular organism does not indicate that it was active in the schmutzdecke or the organism may not be culturable under standard laboratory media. Therefore it may be more meaningful to monitor the in situ activity of different groups by measuring the consumption / production of different types of metabolism, such as carbon assimilation. Hence, for simplification, and consistent broadly with the SSF literature, the microbiological community is divided into three broad categories: algae, bacteria and protozoa.

2.8 Biological populations in sand filters

The biological layer is made up of a variety of microorganisms. These include algae, bacteria, protozoa and small invertebrates. The types of microorganisms and the relative number of each species are specifically adapted to the

characteristics of the influent water source and the environment of the filter (Buzunis, 1995).

Different types of bacteria are normally found at various depths below the filter surface, the true water bacteria predominating at deeper levels. This indicates a subdivision of the filter bed into zones, in each of which specific bacteria abound, each producing well-defined effects.

Fungi are common constituents of water distribution biofilms. That fungal deposition is attributed largely to spores and not hyphal growth is significant considering that concerns such as mycotoxin production or taste and odors imply that vegetative growth occurs in situ.

2.9 Microbial community of the biofilm

The schmutzdecke supports a well developed community of micro- and macro-organisms, all of which play a role in water treatment (Wotton 2002). Knowledge of population and biomass distributions in a biofilter is important in evaluating the efficiency of BOM (Benthic organic matter) removal, as well as controlling headloss, filtration rate, and filter run times. Particulate organic carbon content of the schmutzdecke has been reported to be ~5 mg/cm (Ellis and Aydin 1995). While, nitrogen content of the schmutzdecke biopolymers is ~0.4 mg/g (Ellis and Aydin 1995; Muhammad and Hooke 2003). Similarly, Rinck-Pfeifer (2000) reported biomass densities of ~7 mg/g for a series of column experiments conducted to evaluate clogging mechanisms in porous media.

However, these reported microbial biomasses in the schmutzdecke are complicated by the range of different microbiological methods, used to quantify biomass, throughout the different studies. The different approaches used to measure biomass concentrations and the inconsistent units and sampling intervals adopted confound inter-study comparisons of biomass development and behavior during biofiltration. Most reports are of single measurements of the net biomass production at the end of a filter run, prior to cleaning, and these emphasize the significant variability apparent in schmutzdecke and filter bed

biomass accumulation in operational biofiltration. The collection of representative samples of schmutzdecke and sand material during filter operation is difficult in practice, and the lack of a simple routine method for measuring microbial biomass are probable reasons for the limited amount of field-scale investigation of the biological mechanisms of biofiltration. Detailed analyses of biomass growth in the schmutzdecke and within the filter bed during operation would improve understanding of the complex and fundamental interactions between the biological and physico-chemical processes operating in biofilter systems. (Campos *et.al.*, 2002b).

2.10 Fungal interactions and growth

The concept of a biofilm presumes either a population or a community of microorganisms living attached to a surface. Biofilms can be developed on either biotic or abiotic surfaces from a single species or as a community derived from several species (O'Toole, 2000; Fenchel, 2002). This way of growth is the prevailing lifestyle of microorganisms including bacteria, yeast, filamentous fungi and even micro-animals (Armstrong *et.al.*, 2001; Gilbert and Lappin- Scott, 2000; Morris *et.al.*, 1997; Watnick and Kolter, 2000). However, there is a strong tendency in microbiology and in the scientific literature to consider within the above concept of biofilms to those developed by bacteria in complex natural communities or those developed by bacteria and yeast in natural or artificial environments of medical relevance that are responsive of persistent infections (O'Toole *et.al.*, 2000. Rickard *et.al.*, 2003; Sauer, 2003; Stickler, 1999; Wimpenny, 2000).

It should be noted that adhesion and subsequent differential gene expression to generate phenotypes distinct from those of free living organisms are two unifying processesof the biofilm concept (Ghigo, 2003; O'Toole *et.al.*, 2000). Also, biofilms have been used for a long time in water treatment facilities where they were called slime, mats or sludge, but not other practical used was seen until

recently. This has brought that most of the available information is on bacterial and, in recent years, on yeast biofilms.

Filamentous fungi are naturally adapted to growth on surfaces and in these conditions they show a particular physiological behavior which it is different to that in submerged culture; thus, they can be considered as biofilm forming organisms according to the former concept. Differential physiological behavior of most attached fungi corresponds principally to a higher production and secretion of enzymes and also to a morphological differentiation which is absent in submerged cultures (Akao *et.al.*, 2002; Biesebeke *et.al.*, 2002). The advantages of this form of growth have been industrially exploited by two culture systems: Solid state fermentation and cell immobilization although there is a lack of knowledge on the molecular basis of growth on surfaces.. Natural adsorption on solid supports is an immobilization technique that it has been used with filamentous fungi thus neglecting its study as a way of biofilm formation. Actually, once spores are adsorbed to the support they grow attached to it thus forming a film. The term biofilm fermentation (BF) is preferred instead of cell immobilization because the microbe is an active and differential entity. BF has been applied for the production of enzymes, amino acids, organic acids, alcohol, aromas and in bioconversion processes (Anderson, 1983; Groboillot *et.al.*, 1994; Norton and Vuillemard, 1994) as well as in bioremediation an effluent biotreatment (Burton, 2001; Doggett, 2000; King and Shoda, 1999; Kasinath *et.al.*, 2003; Rodgers *et.al.*, 2003; Van Driessel and Christov, 2001).

2.11 Related Bacteria of the biofilm and ripening time

Bacteria are involved in most process related to water treatment by biofiltration. Despite this, relatively little is know about the species of bacteria which are commonly found in biofilters, or the role of particular species in filter performance. The major works characterising the composition of the bacterial community of schmutzdecke were completed several decades previously (Calaway *et.al.* 1952; Brink, 1967). In these studies, the dominant bacteria were

identified (using standard culture based techniques) as *Zooglea ramigera, Bacillus cereus, Flavobacterium aquatile, Alcaligenes faecalis, Nocardia* and Streptomyces.

Eighmy *et.al.* (1993) conducted a literature search to summarise the known bacterial species diversity in filtration systems, but summary results were given for GAC media only. Interestingly, only 3-4 isolates were found to dominate the community in each of the samples. In sand-based filtration, Eighmy *et.al.* (1993) tentatively identified *Pseudomonas, Achromobacter (now Alcaligenes), Chromobacterium violaceum, and Agrobacterium radiobacter*. Again, none of these isolates are involved in important functions such as nitrification which are known to occur in SSFs. Muhammad and Hooke (2003) investigated the biomass of the schmutzdecke, reporting the bacterial counts down to 120 mm of a SSF bed. The highest bacterial levels (4×10 cfu/cm) were found in the top 30 mm and steadily declined to 1×10 cfu/cm at 90 - 120 mm. Other authors report micro-organism densities of ~ 1×10 cfu/cm (Ellis and Aydin 1995), ~2 ×10 cfu/g (Rinck-Pfeifer 2000) ~1×10 cfu/g (Muhammad and Hooke 2003).

A biofilm takes some time to develop naturally. This is usually around 2 - 3 weeks for a fully mature film to develop. Palmateer, *et.al* (1998) measured the development of a biofilm and found that at 21 degrees C it took 16 days for the biological film to develop to 85-90% cover. They noted that having a raw water that is more biologically productive will mean that the biofilm will develop more quickly and that the filter will operate more efficiently.

The composition of the layer is complex and variable, but can generally be considered as a gelatinous biofilm containing a consortium of bacteria, fungi, algae, protozoa, rotifers, and a range of aquatic insect larvae and their products. The activity of the microbial community in the schmutzdecke is directly responsible for much of the treatment function; although it should be stressed that the underlying sand or other bed material (e.g. GAC) is also responsible for removal of various fractions from the water.

A key role of biofiltration is removal of major nutrients, carbon, nitrogen, sulphur, and phosphorus, from the source water. This removal (assimilative uptake)

occurs through active growth of organisms in the biofilter; particularly the schmutzdecke layer itself. Although assimilation of major nutrients is important, the importance of this process in SSF, in relation to other SSF processes, is often taken for granted. However, it is generally considered that the water purification process of biofiltration begins in the supernatant layer. Microorganisms, such as algae, absorb carbon dioxide, nitrogen, phosphorous, and other nutrients from the water to form new cellular material. The dissolved oxygen in the water reacts with organic carbon rendering it more assimilable to bacteria and other microorganisms. Dead algae in the supernatant water and bacteria are consumed within the schmutzdecke and inorganic salts are released.

Filter ripening is important area when considering the role of the schmutzdecke in Slow sand filter performance. A filter is traditionally considered ripened to maturity when coliform removals reach optimum levels, as opposed to the removal of other contaminants such as benthic organic matter. The ripening stage consists of complex and poorly known phenomena including both physical and biological processes like the synthesis of polymeric compounds by microorganisms or the establishment of some protozoa responsible of bacterivory. Currently, the theoretical time of ripening varies, from a few hours to several days. For the restarting of a SSF, there are no direct measurements of the fixed biomass in order to estimate its density, and only indirect measurements such as turbidity of the outlet water are used. Actually, classical enumeration of fixed culturable bacteria would be too lengthy a process, requiring several days before results. Delahaye *et.al.* (2005) discuss a simple and inexpensive method that could represent a useful tool for investigation of SSF biofilms. That is they measured the number of micro-algae in successive washings as a direct, simple, and quickly measurable indicator of the fixed total biomass and of the degree of filter ripeness.

2.12 Schmutzdecke biomass

This schmutzdecke layer, up to a depth of ~40 mm (Ellis and Aydin 1995), comprises both deposited and synthesised material, characterised as a gelatinous mat in which micro-organisms thrive and where major contaminant removals occur (Sharp *et.al.* 1999). According to Barrett *et.al.* (1991) the schmutzdecke can vary widely in character ranging from gelatinous zooglea to carbonaceous detritus. The size (biomass) of the schmutzdecke layer is integrally linked with both the filtration performance and clogging of SSF (Muhammad and Hooke, 2003). The dominant role of total biomass in clogging (head loss) has been identified (Campos *et.al.* 2002b). A detailed understanding of the interactions between biomass formation and clogging would enable the development of mechanistic models of SSF systems which may improve the operational management of SSF through the prediction of head loss rate, and the frequency of sand cleaning and renewal. Total biomass may be divided into various subgroups based on function (activity) or operational (extraction process used) basis. For example, biomass may be considered in terms of: total biomass; active biomass; active cell biomass; living cell biomass (Muhammad and Hooke, 2003). The measure of each component can provide different levels of information relating to the function of biofilters. Due to methodological constraints, biomass measures of any kind are rarely apportioned across the different classes of organisms which comprise the overall microbial community. There are numerous methods to measure biomass; each has inherent limitations. The chloroform fumigation extraction method has been employed (Campos *et.al.* 2002a), but the method can have large variation particularly when analysing from small sample volumes (e.g. < 10 g).

2.13 Biomass Activity

Measuring activity (= active biomass) of microbial communities provides important insights into the functioning of biofilters. For example, Urfer and Huck

(2001) investigated the link between total biomass and activity on removal of biodegradable organic matter from water (using composite filters). Whilst total biomass deep down the filters was low, the activity per unit biomass was very high. A number of direct measurements of activity are also possible. Active biomass can be measured through the respiration by C-glucose (Servais *et.al.* 1991) or by the assay of high-turnover of biomolecules such as phospholipids or ATP (e.g. Unger and Collins 2006; Magic-Knezev *et.al.* 2004; Findlay *et.al.* 1989). ATP appears to be useful on materials such as GAC where many carbon-based extraction protocols may experience interference form the media itself.

The measure of extracellular enzymes can be used to measure activity related to metabolism of specific compounds. In particular, new methodologies based on flurogenic assays based on MUF (4-methylumbelliferone; alt MUB) - labelled substrates have been developed. These assays are sensitive enough to measure the activities of a number of key enzymes including β-glucosidase and phosphatase (Hendel *et.al.*, 2001).

MATERIALS & METHODS:

3 MATERIALS & METHODS

The bioantagonist, *Trichoderma harzianum* was obtained for the culture collection center of the Punjab University Lahore. The strain was isolated from infected crop land near Lahore. The pathogens against which this strain was tested were *Aspergillus sp and Fusarium sp.* These and the bacterial pathogens *Escherechia coli, Pseudomonas aeroginosa, Pseudomonas putida* and *Enterobacter aerogens* were isolated from tap water samples from Islamabad, the source of which was simli dam. These strains were identified using Biomerieux API identification kits.

3.1 Growth optimization for *Trichoderma harzianum*

3.1.1 Media:

Strains were grown on SDA, PDA, and MEA media to see which gave best growth patterns for maximum metabolic growth.

POTATO DEXTROSE AGAR OXOID Code: CM0139

Formula	gm/litre
Potato extract	4.0
Glucose	20.0
Agar	15.0
pH	5.6 ± 0.2

39 g was suspended in 1 liter of distilled water, and brought to the boil to dissolve completely. The media was then sterilized by autoclaving at 121°C for 15 minutes. Before poruring the media was mixed well. Plates were poured according to ASTM methods. Plates were inoculated with an 8mm disc from the growing edge of *Trichoderma harzianum* colony for 7 days at 21°C.

MALT EXTRACT AGAR *OXOID Code: CM0059*

Formula	gm/litre
Malt extract	30.0
Mycological peptone	5.0
Agar	15.0
pH	5.4 ± 0.2

50 g was suspended in 1 liter of distilled water, and brought to the boil to dissolve completely. The media was then sterilized by autoclaving at 115°C for 15 minutes. Before pouring the media was mixed well. Plates were poured according to ASTM methods. Plates were inoculated with an 8mm disc from the growing edge of *Trichoderma harzianum* colony for 7 days at 21°C.

SABARAUD DEXTROSE AGAR *DIFCO*

Formula	*gm/litre*
Enzymatic Digest of Casein	10.0 g
Dextrose	40.0 g
Agar	15.0 g
pH	5.6 ± 0.2

65 g was suspended in 1 liter of distilled water, and brought to the boil to dissolve completely. The media was then sterilized by autoclaving at 121°C for 15 minutes. Before pouring the media was mixed well. Plates were poured according to ASTM methods. Plates were inoculated with an 8mm disc from the growing edge of *Trichoderma harzianum* colony for 7 days at 21°C

3.1.2 pH and temperature

In order to check the most suitable growth conditions that produced best inhibitory metabolites, *Trichoderma* was grown on shake flask using Oxoid Potato dextrose broth at pH 5, 5.5, 6, 6.5, 7 and 7.5 at temperatures, 27°C, 30°C and 35°C for 7 days at 100 rpm. At the end of the experimental procedure mycellial biomass was filtered out, and the filtrate was kept for further metabolite inhibitory assays for Biocontrol potential.

3.2 Inhibition assays to check for Biocontrol potential of *Trichoderma harzianum*

Trichoderma sp are bioantagonists producing suppressive metabolites of a volatile and non volatile nature, which adversely affect the growth of different fungi (Bruce *et.al* 1984, Corey *et.al*, 1994, Dennis and Webster 1971, Hovarth *et.al*, 1995, Moss *et.al* 1975, Mimpuni *et.al* 1998) In order to check our strains inhibition potential bioassays were performed.

Growth rates were recorded daily by measuring colony diameter according to Lilly and Barnett (1951). Using a slide ruler, colony diameter was measured from growing tip to tip and recorded in mm.

3.2.1 Fungal inhibition

Dual culture metabolites :

An assay method for *Trichoderma harzianum* dual culture described by Perez *et.al* 2002 was used. An 8-mm disk of a pure pathogen (of *Aspergillus* and *Fusarium)* culture was seeded on one side of a Petri dish containing PDA and cultured for two days at 27° C. The growth of the pathogen was then checked, and the plate was inoculated with another 8-mm disk of a pure culture of T. harzianum), placed on the site opposite the pathogen. The plates were cultured for another 5 days at 27° C. The growth diameter of the pathogen was measured and compared to control plates of pathogen growth. Controls were run on plates inoculated with pathogen and mock inoculated with an 8-mm disk of PDA.

Volatile metabolites:

Volatile inhibitory metabolites were tested in an assemblage described by Dennis and Webster (1971b). The centers of half Petri dishes containing PDA were seeded with an 8-mm disk of pure cultures of Pathogen on one side and T. harzianum isolate on the other. One half-plate containing a recently-seeded T. harzianum isolate and another containing Pathogen seeded two days earlier were placed face-to-face preventing any physical contact between fungi, and the growth of pathogen was measured at this time. The half-plates were sealed with parafilm tape to isolate the interior atmosphere and to prevent the loss of any volatiles that formed. The plates were incubated at 27°C for another 5 days and the growth of the pathogen was measured and compared to controls developed in the absence of the bioantagonist (mock inoculation with an 8-mm disk of PDA).

Diffusible metabolites:

The effect of non-volatile metabolites from *Trichoderma* species against *C. paradoxa* was by the method described by Lundberg and Unestan (1980) and Dennis and Webster (1971a). The centers of PDA plates covered with a cellophane paper membrane were inoculated with an 8-mm disk of T. harzianum. After incubation for 72 h at 27°C, the membrane with the grown *Trichoderma* isolate was removed, and the center of the plate was inoculated with an 8-mm disk of a pure pathogen culture. The plates were further incubated at 25°C for 5 days, at which time the pathogen growth was measured. Controls were run with mock inoculated plates on the cellophane membrane and inoculated wit pathogen.

3.2.2 Bacterial inhibition

Kirby bauer using media overlay and fungal discs:

The evaluation of inhibitory activity of *Trichoderma* cultures against bacteria was performed according to the modified method of Spelhaug and Harlander (1989).

The mycelial discks (7 mm dia) of fungi were transferred to potato dextrose water agar at 25 % concentration. After three days incubation at 27 C, 7 ml of semi solid tap water agar inoculated with strains *of Salmoella typhi, Escherichia coli, Pseudomanas aeroginosa, pseudomonas putida* and *enterobacterer aerogens.* All which were isolated from local water samples and identified using biomerieux API 20E, was carefully poured on the plate surface as an overlay.

The Petri dishs were allowed to stand for 8 hours at 4C, for diffusion of metabolites from *Trichoderma* cultures to the tapwater overlay, then incubated at 37C for 24 hours.

Kirby bauer is a method in which antibacterial activity is determined by measuring the radius of the clear inhibition zone around each mycelial disc. (Bauer A,W 1966)

Kirby bauer using crude extract :

Plates of the above bacteria spread as lawns were also tested against crude extract which was poured into wells and clear zones of inhibition were measured.

3.3 Development of water tolerant strains and incorporation of *Trichoderma harzianum* into sand columns

3.3.1 Growth of strains in water agar:

Fungal strains were grown on Potato dextrose water agar, in a step down procedure, using first 100% strength of agar (normal formulation of PDA), The conidial suspension was recovered by flooding the surface of the agar plates with 5 ml phosphate buffered saline (Oxoid) containing 0.025 % tween 20 and rocking gently these were then added to 75%, similarly to 50%, 25 % and then 1%. Each plate was given one week growth time.

Finally the harvested conidia were inoculated onto sand-agar plates, containing a 3 mm layer of sand covered with a 1% strength agar layer.

Suppression potential of water grown strain by direct counts of low density spores and bacterial suppression:

The antifungal ability of the water grown strains were checked. *Trichoderma* was grown in 50 ml flask containing a mixture of pathogen spores. A combined spore solution was diluted so as to give no more than 3-4 spores some fields of of neubaur heamocytometer view and no spores in others (25-50% of fields examined) In this case, spores are few enough to be counted directly. The spore suspension after vortex was placed in a watch glass and counted directly. The quantity of spores in the solution with *Trichoderma* was compared to a similar control flask and by direct observation, counts (suppression) were observed.

Bacterial Counts:

Bacterial counts were taken by Kirby bauer method as described in section 3.2.2. Counts were taken from *Trichoderma* grown on semi solid water agar, mycellial discs of 7 mm dia were transferred to PDA Plates of 25 % concentration. Three days after incubation at 27C, 7 ml of semi solid tap water agar inoculated with cultures of Salmoella typhi, Escherichia coli, Pseudomanas aeroginosa, pseudomonas putida and enterobacterer aerogens All which were isolated from local water samples and identified using biomerieux API 20E, was carefully poured on the plate surface as an overlay.
The Petri dishs were allowed to stand for 8 hours at 4C, for diffusion of metabolites from *Trichoderma* cultures to the tapwater overlay, then incubated at 37C for 24 hours. Antibacterial activity was determined by measuring the radius of the clear inhibition zone around each mycelial disc.

Kirby bauer using crude extract :

Plates of the above bacteria spread as lawns were also tested against crude extract of 7 day shake flask cultures of *Trichoderma* water tolerant strain grown

on PDB which was poured into wells and clear zones of inhibition were measured.

3.4 Construction of the column filter:

An assembly was constructed to hold a sand column made of glass, which drained into a collecting tube below. Water from this collecting tube was pumped into an overhead holding tank and back into the columns through an outlets from the head of the pump, thus creating a recirculating flow system, levels of contaminants in the water were maintained by making additions into the tank.
The following parameters were standardized for the filter:
The water layer above the filter bed provides the head to push water through the filter bed. It is convenient as a water storage zone and provides an effective temperature buffer to stabilize the filter and protect the biological activity occurring in the top layers of filter bed. A minimum depth of .5 m up to 1.5 m is most commonly used. In this experimental set a depth of 3 inches was maintained.

i. Height of column:

Columns were prepared at the glass factory, dept of chemistry at quaid I azam university. They were made from glass tubing, and had a working length of 0.5 m.

ii. Temperature

All experiments were done at room temperature, between November to January for a three month period. Temperature variations were between 18 to 25C. These were in the thermic and hyperthermic soil temperature ranges (USDA)

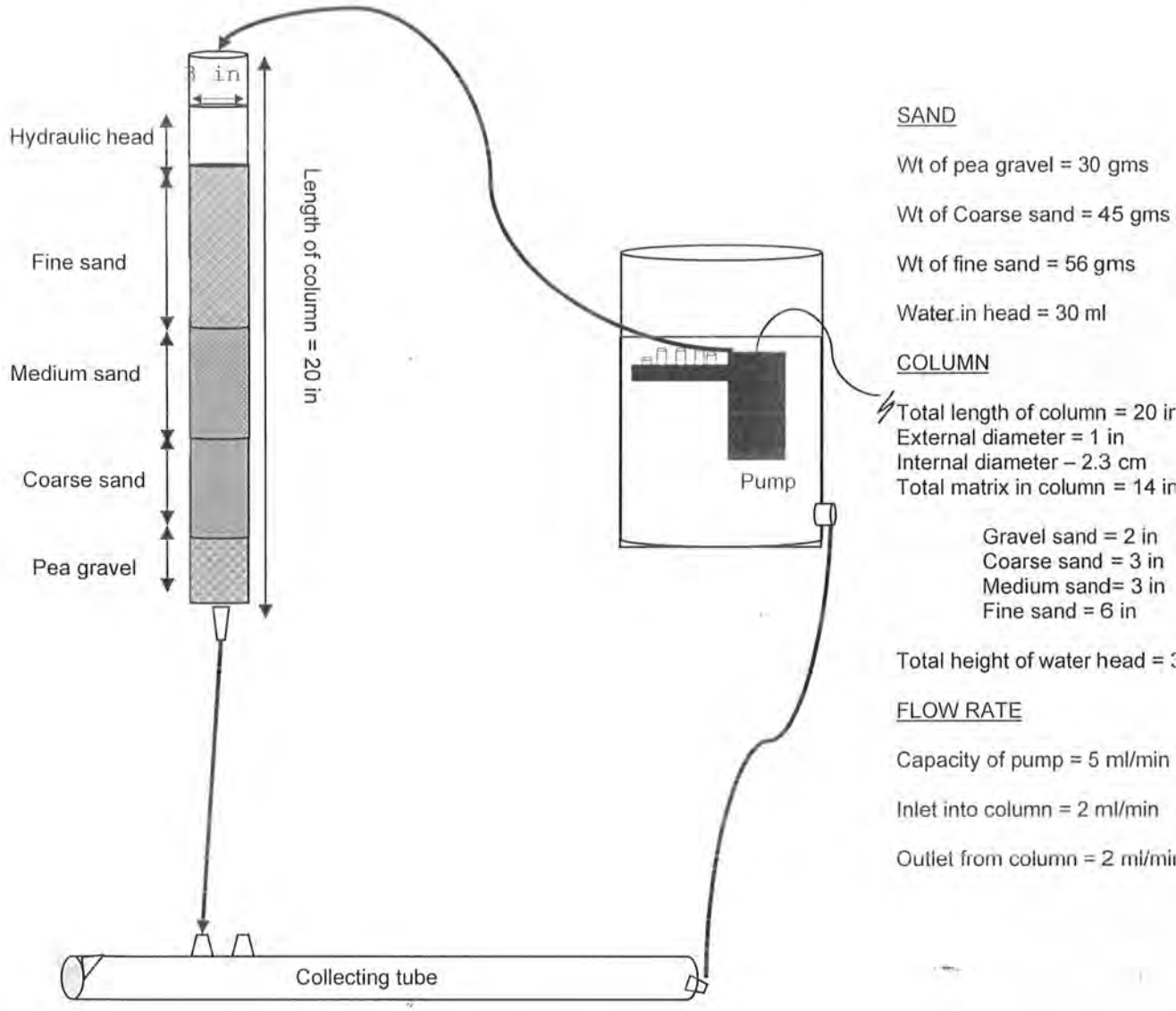


Fig 01 : sand column assembly for Biofilm production

iii. Turbidity

Turbidity levels of the feed water were kept between 20 to 40 FAU by using autoclaved supernatant of soil suspension.

iv. Feed Water quality

Feed water quality was prepared using 100 ml of tap water, and 2 gms of unsorted soil. The water was agitated in a mechanical stirrer at low speed for 10 minutes at 30C. Turbidy, Colour, Conductivity and total dissolved solids were measured.

1 ml replicates were plated onto nutrient agar and potato dextrose agar for bacterial and fungal counts. Fungal spores were also counted using a heamocytometer. Feed water was tested for total hardness, iron, sulphates, nitrates, calcium and magnesium salts.

Mathematical characteristics of the sand column were calculated, using general mathematical formulae:

i. **Total (Contact) surface area** $SA = 2(\pi r^2) + (2 \pi r)^* h$

(the surface area is the sum of all the areas of all the shapes that cover the surface of the object).

ii. **Surface area of the Biofilm** πr^2

(the surface area of the biofilm is the sum of all the areas on which biofilm is growing).

iii. Kinetic energy $E_K = ½$ M(gms)Volume(l)

(The kinetic energy of an object is the energy which it possesses due to its motion.)

iv. **Flow rate of the pump** FR = Volume (l)/time(s)

(Flow rate is the volume of fluid which passes through the pump per unit time)

v. **Flow rate of the filter** FR = Volume (l)/time(s)

(Flow rate is the volume of fluid which passes through the filter per unit time)

vi. **Volume of the sand column** Volume = πr^2 x h

(Volume of the sand column is is how much three-dimensional space the sand occupies within the column)

vii. **Sand texture :** Wentworth scale (Wentworth, 1922)

Coarse Sand 0.5-1 mm

Medium Sand 0.25 - 0.5 mm

fine sand 0.125-0.25

(Sand texture is the relative proportions of sand, silt, and clay particles in a mass of soil.)

viii. **Bulk Density** $\rho = MS/Vt$

M_S = Mass of oven dryed sand

V_t = Volume of sand

(Bulk density is a measure of the weight of the soil per unit volume)

ix. **Porosity (water saturation method)** $\phi \% = Vf /vt$

V_f = Volume of void space

V_t = Volume of total material

(Porosity or void fraction is a measure of the void spaces in a material, and is a fraction of the volume of voids over the total volume, between 0–1, or as a percentage between 0–100%)

3.5 Development of Biofilm and microbial dynamics: establishment of colony diameter.

Biofilm colonies were developed on the surface of the sand layer using cellulose fiber held in position over a fine wire mesh. This provided a solid support for enabling removal of the entire colony for study, and provided a carbon booster support for mycellial growth of the fungi.

Three Separate columns were set up for these removable discs so as not to disturb the discs placed on the sand columns being used for water filtration tests. The cellulose fiber discs were marked into 6 partitions before running the experiment. Each partition represented a 5 day growth period for the film

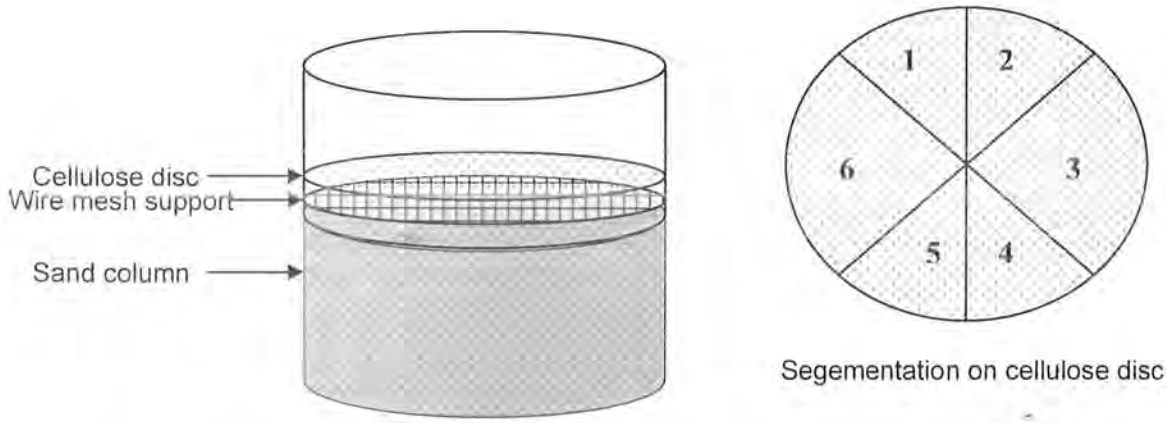


Fig 02 . Cross section of filter showing cellulose support for biofilm development

3.5.1 Bacterial counts

Bacteria feed into the system came from normal tap water, no additional strains were added. Plate counts were done in accordance to ASTM test methods using standard plate count agar. Counts were taken daily of pre and post filter samples, both for the biofilms with *Trichoderma* incorporated in them, and for those without (control films)

3.5.2 Fungal counts

2 ml of fungal spore suspension each was added to the head space water of the column. Fungal counts were done in accordance to ASTM test methods using potato dextrose agar. Counts were taken daily of pre and post filter samples, both for the control and *Trichoderma* enhanced films

3.5.3 Biofilm growth measurement by determination of total carbohydrate

Growth of the Biofilm was measured by performing an anthrone reagent method for total carbohydrate The method used is based on those of Fales (1951) and Schlegel (1956) but was modified by Hewitt (1958)

Anthrone regent was prepared by , standard glucose solution by preparing 10 mg of glucose in 100 ml distilled water, and pipetting out 0.1 to 1.0 ml dilutions. 2.5 N HCl and Sodium carbonate was used.

100 mg of cut cellulose discs from placed above the column were taken and ground with a pestle and mortar with 20 ml ethanol. This was then centrifuged to get a clear supernatant. The residue was washed again with ethanol and centrifuged. . 1 ml of extract was added to 4 ml of anthrone reagent and placed in a boiling water bath for 10 minutes to allow development of colour. The tube was capped to prevent loss of evaporation. When the tube cooled it was measured for optical density at 620 nm against a blank.

Carbohydrate present in 100 mg of disc = mg of glucose/vol of test solution x 100

3.6 Growth ability of Trichoderma; estimation in submerged conditions

3.6.1 Katz mean tip extension rate

Katz (Katz et al 1972) studied the growth kinetics of Aspergillus nidulans on three different media, each with a distinct specific growth rate. From these observations they proposed a number of general relationships that are conveyed in equation (1), elucidated by Steele & Trinci (steele & Trinci 1975):

Eq 1 : $E = \mu_{max} G$ (1)

where E is the mean tip extension rate,
μ_{max} is the maximum specific growth rate,
and G is the hyphal growth unit. (G is defined as the average length of a hypha supporting a growing tip) according to equation (2):

Eq 2: $G = L/Nt$ (2)

where Lt is total mycelial length,
and Nt is number of tips.

The hyphal growth unit is approximately equal to the width of the peripheral growth zone (more accurately, the volume of the hyphae within that zone), which is a ring-shaped peripheral area of the mycelium that contributes to radial expansion of the colony (Pirt S.J 1976) (Trinci 1971). Hyphal tips growing outside this zone will only fill space within the colony. G is an indicator of branching density; Katz also postulated that a new branch is initiated when the capacity for a hypha to extend increases above E , thereby regulating $G \approx 1$ unit.
Trichoderma strains that were optimized for water, were grown in confrontational assays against aspergillus and compared to plate grown *Trichoderma* of the same strain. The mean tip extension rates of both were calculated to see the effect of being submerged had on Biocontrol.

Growth measurement by time lapse photography

A method was devised to visualize the growing tips at the corners of growing colonies. Petri plates were marked into 1 x 1 cm divisions with permanent marker and then inoculated with Trichoderma. These plates were then fixed under microscopes with camera attachments. And growth was photographed after every five minutes until the tip had grown by 1 cm.

3.7 Improvement of Water quality.

To check for chemical removal efficiency, water quality standards and guidelines as detailed by House and Reed (1997) were followed. According to these guidelines, which encompass amongst others, WHO guidelines, water testing has been categorized into four groups.

1. Core tests;
2. Secondary tests;
3. Treatability tests;
4. Assessments for industrial pollution.

Core testing and secondary testing was done for these filters because they provide Key information to determine treatment requirements (turbidity, pH, bacteriological analysis), they are simple to perform and indicate acceptability which can highlight other potential pollution problems, and hence the need for further tests. (Table A)

Table A. Test Parameters to study the efficiency of Filters with incorporated *Trichoderma* biofilms

	PARAMETER	METHOD	UNIT	Detection range	
5.1				Min	Max
CORE TESTS	Turbidity	EN ISO 7027	FAU	1	100
	Conductivity	Electrometric	µS/cm	14	1500
	pH	USEPA 150.1	-	1	14
	TDS	USEPA 160.1	mg/L	10	1000
	Colour	APHA 2120	TCU	0.2	100
	Bacteriology	USEPA 9223	CFU/ml	<1	> 5700
5.2 SECONDARY TESTS	Nitrates	APHA 4500	mg/L	0.10	25
	Iron	APHA 3500	mg/L	0.005	5
	Chromium	APHA 3500	mg/L	0.01	3
	Sulfates	Photometric	mg/L	25	300
	Chlorides		mg/L		

TEST METHODS

Testing of core parameters for turbidity, conductivity, pH, TDS and Colour was done using a Nova 60 Photometer (Merck). Samples were transferred into 50 mm cuvettes and selecting method code on the LCD.

Bacteriological counts were taken using Merck Plate count agar and colonies were read on an electric colony counter.

Secondary parameters of Nitrates, Iron, Chromium and Sulfates were also tested automatically on the Nova 60 Photometer. Samples were prepared in accordance to USEPA certified test method kits and measured by selecting method code on the LCD.

Standard Solutions for secondary tests:

Standard solutions were prepared for testing Iron and salts. 1 ml of each was then added to the columns and removal rates recorded.

Standard Iron Solution:

Dissolve 0.7022 gram of ferrous ammonium sulfate crystals [FeSO4 (NH4)2SO4.6H2O] in about 50mL of distilled water and 20mL of concentrated sulfuric acid [H2SO4]. Warm and add potassium permanganate [KMnO4] until a slight pink color persists. Dilute to 1 liter with distilled water.

1mL = 0.1mg Fe

Standard Nitrate Solution:

Dissolve 2.4455 gram of pure potassium nitrate [KNO3] in 1 liter of distilled water. Evaporate 50mL of this solution to dryness on the water bath. Moisten the residue with 2mL of phenoldisulfonic acid rubbing it well in the residue to insure intimate contact. Dilute to 500mL with distilled water.

1mL = 0.15 mg of NO3

Standard Chloride Solution:

Dissolve 164.7 gram of pure sodium chloride [NaCl] in 1 liter of distilled water.

1mL = 100mg of Cl-

Standard Chromate Solution:

Dissolve 2.511 gram of Di-potassium chromate in 1 liter of distilled water.

1mL = 1.5mg of Cro4-

Standard Sulfate Solution:

Dissolve 140.4 gram in 1 liter of distilled water.

1mL = 100mg of SO4-

3.8 Statistical analysis

The suppression potential of *Trichoderma* strains against fungal pathogens *Aspergillus* sp. and *Fusarium* sp (table 1) ; selection of best media for growing *Trichoderma* (Table 2) and optimization of pH and temperature (Table 4) were statistically analyzed by two factor analysis of variance tables and p-values were calculated.

Two sampled T-test was performed by the following formula

$$T^2 = \frac{n_1 n_2}{n_1 + n_2}(\overline{\mathbf{x}}_1 - \overline{\mathbf{x}}_2)'\mathbf{S}_{\text{pooled}}{}^{-1}(\overline{\mathbf{x}}_1 - \overline{\mathbf{x}}_2).$$

Statistical analysis was done by calculating the standard deviation for culture assays (table 4),Reduction potential of *Trichoderma* (table 5) Fungal spore counts and bacterial counts in headwater and outlet water (table 6,7,8) and reduction in contamination levels of total dissolved solids, pH, conductivity, turbidity, and colour by the following formula.

$$\sigma = \sqrt{\frac{1}{N}\sum_{i=1}^{N}(x_i - \overline{x})^2}$$

σ = standard deviation

x_i = each value of dataset

x bar = the arithmetic mean of the data

N = the total number of data points

$\Sigma (x_i - \text{mean})^2$ = The sum of $(x_i - \text{mean})^2$ for all data points

Percentage reductions were also calculated by the formula :

100 - (Value after filtration/value before filtration x 100)

RESULTS & DISCUSSION:

4. RESULTS AND DISCUSSION

4.1 Selection and identification of strains

Three Strains of *Trichoderma harzianum Trichoderma koningii* and *Trichoderma Virens* where obtained from the Culture Collection Center at Punjab University, Lahore. These strains had been isolated from infected cropland near Lahore. The climatic conditions there are tropical. Most cropland is of wheat, barley and vegetables. These strains were tested for their biocontrol potential against crop pathogens, *Fusarium oxysporum,* and *Aspergillus niger* which were isolated from tap water whose source was Simli Dam in Islamabad. It was seen that *Trichoderma harzianum* had the best suppressive potential against these strains as compared to *Trichoderma koningii* and *Trichoderma virens* (Fig 1, Table 1) This strain was therefore carried forward in further experiments.

Fungal strains were identified according to microscopic morphological characteristics after refreshing these strains at Quaid I Azam University.

For *Trichoderma harzianum* septate hyaline hyphae, conidiospores, phialides and conidia were observed. Phialides were hyaline, flask shaped and inflated at the base, attached to the conidiophores at right angles, the phialides were arranged in clusters, conidia were an average of 3 micrometers in dia and ellipsoidal in shape, they were mostly smooth walled and grouped in sticky heads at the tips of the phialides, the colour of the conidia was green to greenish yellow. This was in confirmation with identification of *Trichoderma harzianum* species. (de Hoog *et.al* 2000, Larone 1995)

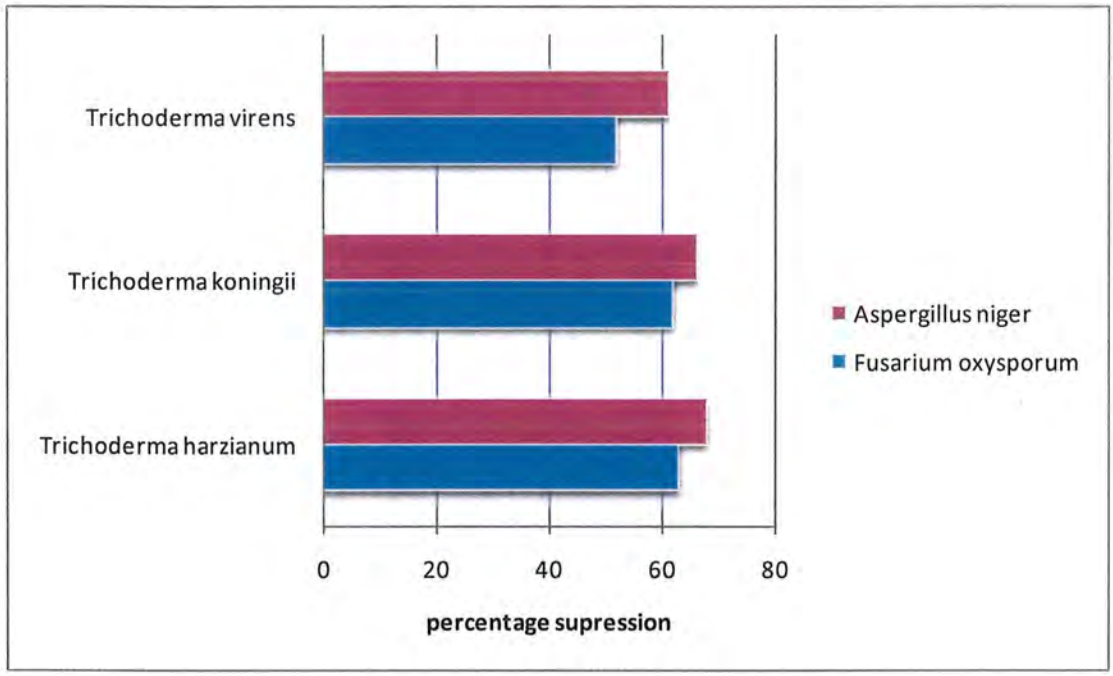


Fig 1: Suppression potentials of *Trichoderma* strains against soil pathogens

Fusarium oxysporum had hyaline and septate hyphae with shorter conidiophores. Macroconidia ere abundant and sickle shaped, thin walled they were three to five septate. Microconidia were also abundant, mostly non septate, ellipsoidal to cylindrical and slightly curved. This identification was in accordance to Sutton (1998) and Nelson (1983)

Strain of Aspergillus niger was typical. Hyphae were septate and hyline. Conidial heads radiate. Conidiophores were long, smooth, hyaline, becoming darker at the apex and terminating in a globose vesicle. Metulae and phialides covered the entire vesicle. Conidia were brown to black, very rough and globose. This confirmed to identifications for Aspergillus niger by Sutton (1998) and de Hoog (2000)

Bacterial pathogens *Salmonella typhi, Escherichia coli, Pseudomonas aeroginosa, Pseudomonas putida and Enterobacteri aerogens* were identified using an API-20E bioMerieux .test kit, These bacteria were isolated from the same source of water as were the *Fusarium* and *Aspergillus* sp.

4.2 Optimization of *Trichoderma harzianum* growth:

Growth optimization for the strain was done for media, pH and temperature. Growth patterns were checked on Potato Dextrose Agar (PDA), Sabaurad Dextrose Agar (SDA) and Malt Extract Agar (MEA) (Merck). *Trichoderma harzianum* was seen to grow best on PDA with an average growth of 3.3 mm, As compared to 3.2 mm on MEA and 3.1 mm on SDA which represented a 6.5% better growth rate.

PDA was also seen to be suitable for the growth of *Fusarium* by 5 % having average growth at 1.9 as compared to 1.6 mm for SDA and 1.8 mm for MEA and *Aspergillus* by 10% as compared to 2.0 mm for other media and was therefore selected as the growth media for the sake of uniformity. (Fig 2. table 2)

Statistical analysis of the data comparing all three media gave p-value of 0.0016, which was less than confidence interval level 0.01 which shows that the values are highly significant at confidence limits of 99% . Although there was not much

difference in growth on each media, PDA was chosen as the better of the three. PDA showed better growth patterns other than size also in terms of woolliness of the colony, and colour; which was initially white and as condia formed became blue green to yellow green, which later formed concentric rings.

PDA has been documented by St Germain (1996) and Sutton (1998) as producing more readily visible growth rings than Sabouraud dextrose agar.

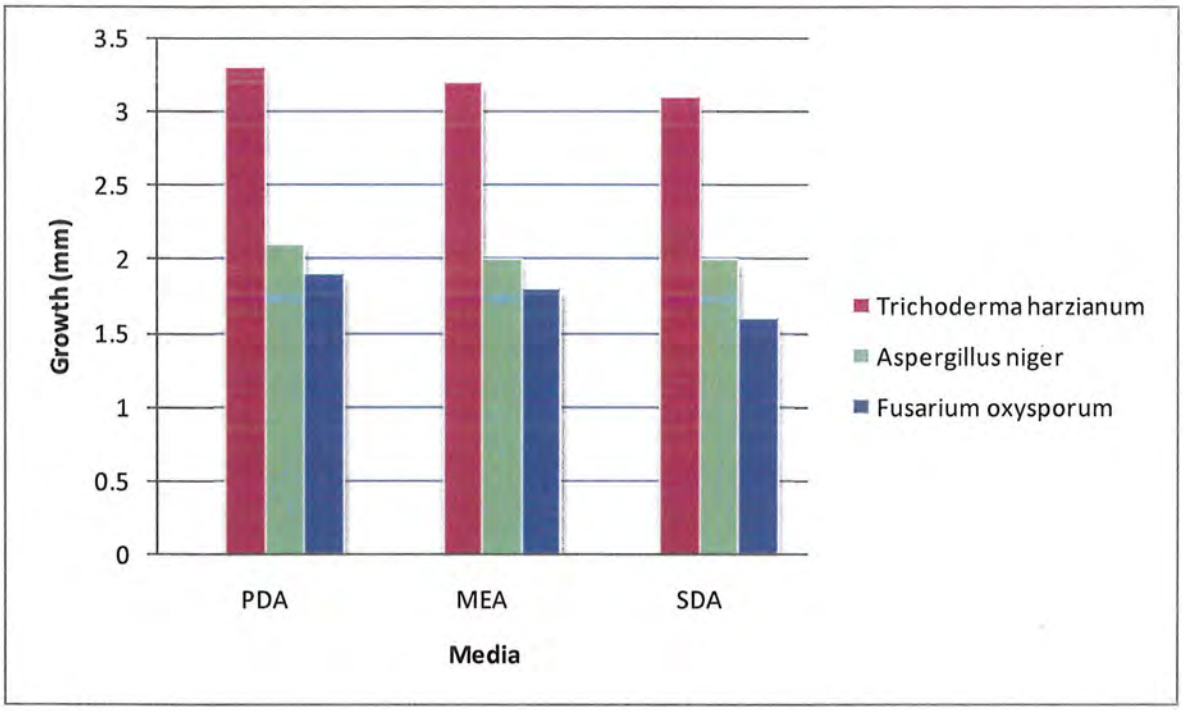


Fig 2: Selection of best media for growth of fungal strains

4.2.1 Production of Suppressive metabolite:

Optimal pH and temperature for the production of best suppressive metabolites was checked for *Trichoderma harzianum,* by growing the strain in shake flask fermentation at various ranges the metabolites produced were then tested by filtering out the biomass and spreading the filtrate over PDA plates and pathogen discs grown on them.

Statistical analysis of data using Analysis of Variance tables gave a calculated value of $p < 0.0035$ for pH and $p < 0.0230$ for Temperature (Table 3)

Overall maturation was complete within five days with best growth temperature at 30C. Best pH for production of suppressive metabolites against soil pathogens *Aspergillus oxysporum* and *Fusarium niger.* was 6.5 (Fig 3a,3b, Table 3)

4.3 Culture assays for Biocontrol Potential :

Three types of culture assays were done between *Trichoderma harzianum* and the two soil pathogens. In Dual culture assays, the strains were inoculated on the same plate and suppressed growth rate was measured as compared to controls.

For dual culture assays *Fusarium* was suppressed by 66%, Standard deviation was 1.4 for normal growth (the control) and 0.5 for suppressed growth and *Aspergillus* by 61% Standard deviation was 1.4 for normal growth (the control) and 0.7 for suppressed growth.

In volatile culture assays, pathogens were inoculated on the base plate of a petri plate and *Trichoderma harzianum* inoculated inside the lid, the effect of volatiles produced by *Trichoderma harzianum* was checked. *Fusarium* was suppressed by 14 % Standard deviation was 1.3 for normal growth (the control) and 1.2 for suppressed growth and *Aspergillus* by 12% Standard deviation was 0.6 for normal growth (the control) and 0.5 for suppressed growth.

For checking the effect of diffusible metabolites, first *Trichoderma harzianum* was grown on cellophane paper membrane, which was then peeled off to allow the pathogen to grow on the plate beneath it., diffusible metabolites suppressed

Fusarium by 54% Standard deviation was 0.9 for normal growth (the control) and 0.4 for suppressed growth and *Aspergillus* also by 54% Standard deviation was 0.9 for normal growth (the control) and 0.4 for suppressed growth (Fig 4, Table 4).
In dual cultures *Trichoderma harzianum* isolates have been shown to have marked significant inhibitory effect on the growth of pathogens compared with their controls. (Hajieghrari *et.al* 2008) (Shalini 2003, Rini 2007)
Hajieghrari *et.al* (2008) also found best suppressive pH for *Trichoderma harzianum harzianum* to be 7 against *Fusarium* species. with best temperature to be 25C.

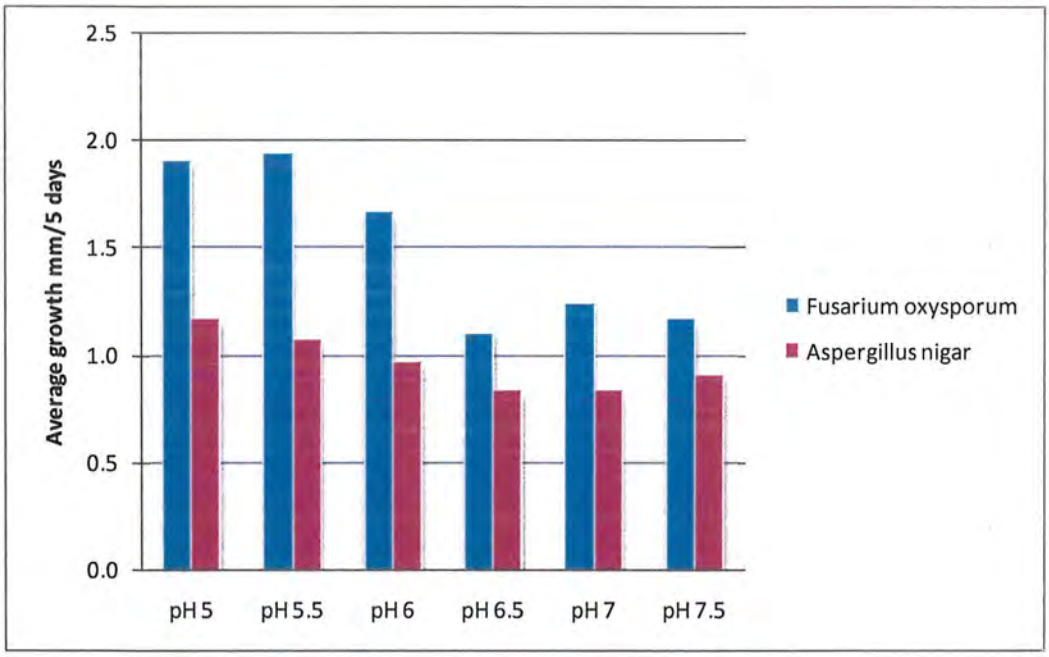


Fig 3a: Optimization of pH for the production of best suppressive *Trichoderma harzianum* metabolites against fungal pathogens

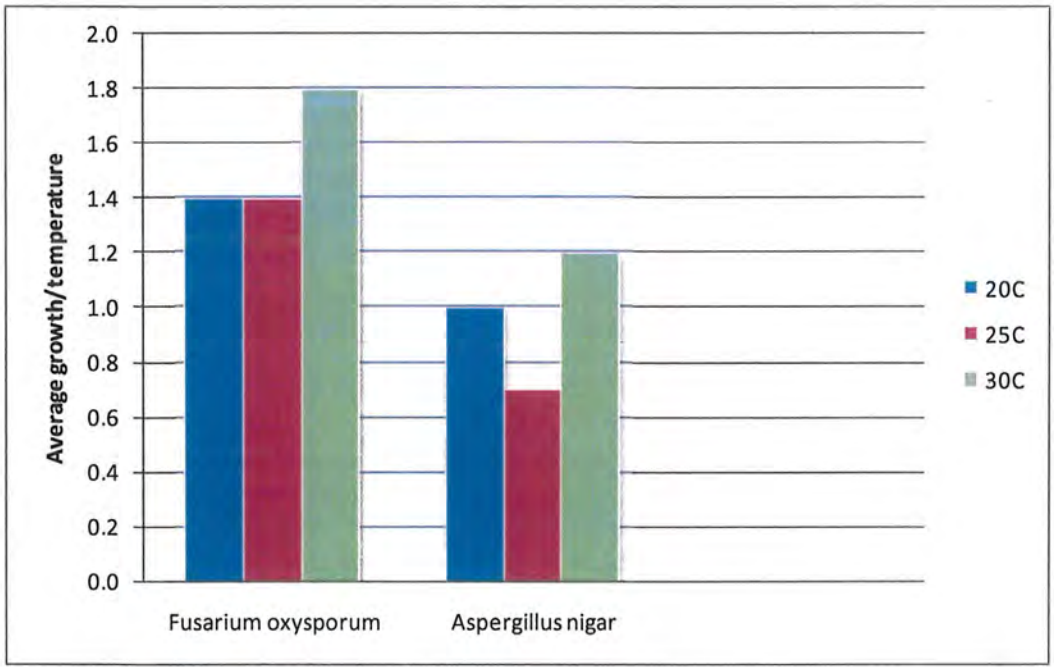


Fig 3b: Optimization of temperature for the production of best suppressive *Trichoderma harzianum* metabolites against fungal pathogens

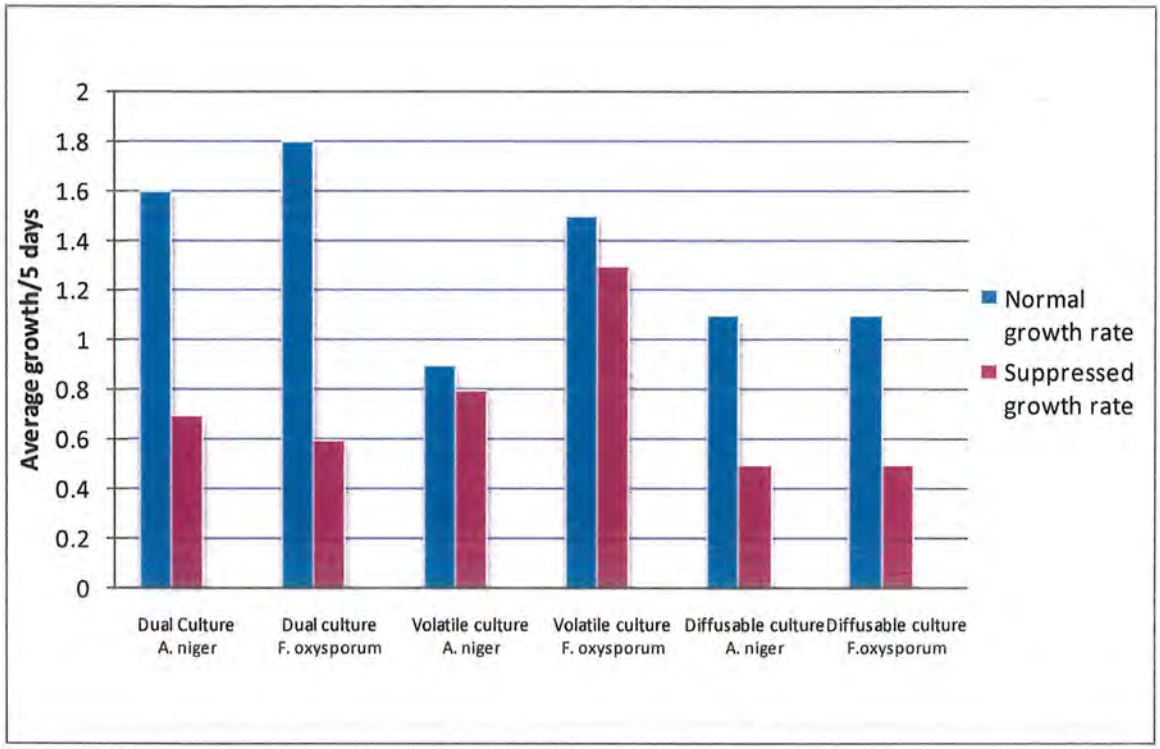


Fig 4: Culture assays to check for the best form of suppressive metabolites produced by *Trichoderma harzianum*

In studies by Eziashi *et.al* 2006, The inhibition varied from 2.0% to 64% in volatile, 0.0% to 74% in non-volatile and 0.0% to 81% in direct-diffusible metabolites of *Trichoderma harzianum* against Ceratocystis paradoxa.

Bacterial inhibition by *Trichoderma harzianum harzianum* extracts was checked against *Enterobacter aerogens, Escherichia coli, Salmonella typhi, Pseudomonas aeroginosa, and Pseudomonas putida*. All strains were suppressed by both crude extract in which supernatant extract from shake flask cultures was poured into wells and zones of inhibition were measured in bacterial lawns, and media overlay in which the extract was added to cooled media before pouring into plates, on which then bacteria was spread. The results of which are shown in table1a

This was due to multiple factors such as competition for space and nutrients in order to exclude the presence of the pathogen, mycoparasitism in order to use the metabolic machinery of the pathogen for its benefit, production of antibiotics that interfere with cellular functions of the pathogen; and production of hydrolytic enzymes that degrade the cell wall of the pathogen.

Biological activity of antagonist fungi and bacteria may partially be associated with production of antibiotic (Etebarian *et.al.*, 2000; Faull *et.al.*, 1994; Pusey and Wilson, 1984). The production of antibiotics; Trichodermin, ergokonin (Kumeda *et.al.*, 1994), viridin (Grove *et.al.*, 1996; Grove *et.al.*, 1965) and viridin fungin A, B and C (Harris *et.al.*, 1993) are commonly documented.

Table 1a Bacterial inhibition zones by Kirby bauer of *Trichoderma harzianum* strain

	Plate	*Enterobacter aerogens*	*Escherichia coli*	*Salmonella typhi*	*Pseudomonas aeroginosa*	*Pseudomonas putida*
Zone of inhibition by fungal disc	1	++	++	+	++	+
	2	++	+	++	++	++
	3	+	+	+	++	++
	4	+	+	+	++	+
Zone of inhibition by crude extract	1	+	++	++	+++	++
	2	+	++	++	+++	+++
	3	+	++	++	+++	+++
	4	++	++	++	+++	+++

≥ + 0.5 cm

≥ ++ 1cm

≥ +++ 1.5 cm

4.4 Development of water tolerant strains and incorporation of *Trichoderma harzianum* into sand columns

During the step down procedure for adjustment in water the reduction in spore counts was recorded for each stage, overall spore counts reduced by approximately 60% as compared to those grown on PDA, the spores and mycelia grown were almost colorless and very weak. Standard deviation of the number of spores produced in the absence of *trichoderma harzianum* is 12.57 and the standard deviation n of the number of spores produced in the presence of *trichoderma harzianum* is 4.95(Fig 5, Table 5)

Similarly direct spore counts showed a 10 % less production of pathogen spores in the headwater with *trichoderma* (Standard deviation is 12.45) as compared to spore counts in columns without the biocontrol agent (Standard deviation is 11.73 (Fig 6, Table 6). The strain retained suppression for all bacterial pathogens previously tested (Table 2a) showing that although weaker than the original this strain still had good capacity for biocontrol.

The strain of *Trichoderma harzianum* chosen was taken from infected crop land. Since it was isolated from soil, it had to be first adapted to increased water potentials before its characteristics could be studied in submerged conditions.

In studies of flood tolerance, it has been seen that obligate species do not necessarily have superior flood tolerance. To further complicate the situation, different ecotypes of a single species can respond very differently to flooding. The availability of carbon and water strongly governs the activities of specific microbial populations and functions. Major changes occur in the physiology of an organism under stress. *Trichoderma harzianum* is known to have physiological acclimation mechanisms to survive under stress, however these adaptation and acclimation strategies create physiological costs at the organism level and can alter the composition of the active microbial community, creating shifts in ecosystem level carbon, energy and nutrient flows.

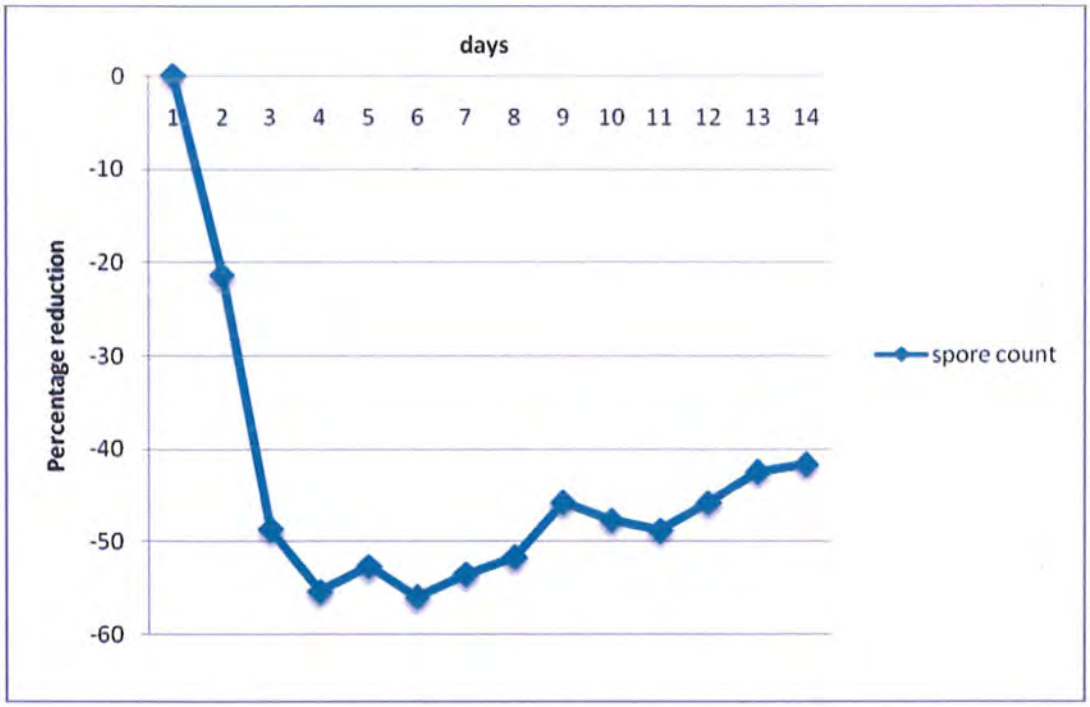


Fig 5: Average percentage reduction in spore counts in the water grown strain of *Trichoderma harzianum*

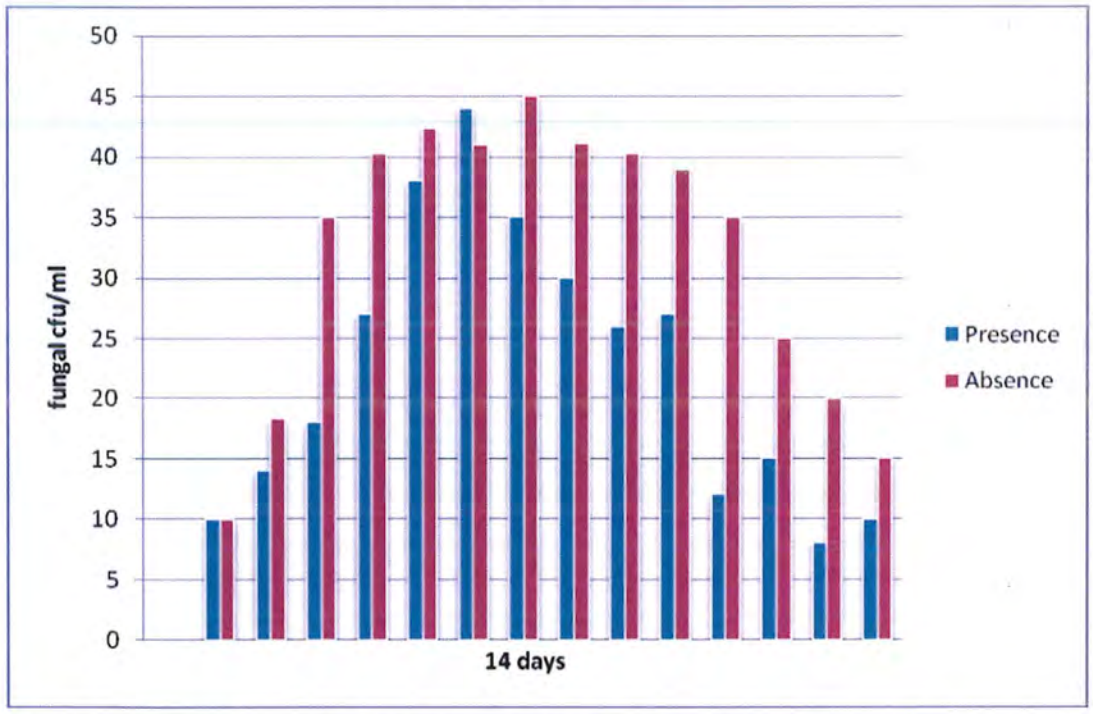


Fig 6. Reduction in fungal pathogen counts in headwater of films with *Trichoderma harzianum* in the biofilm.

In the current study, *Trichoderma harzianum* was slowly adapted to increased water levels through a step-down procedure of growth in shake flask at increased water to broth percentages until the strain was growing in water with sand particals as the only nutrient source. The plates were kept flooded. Mycellia were weaker and thinner and there was a significant loss of pigmentation. (Fig 1a, 2a)

The organism likely experienced ecosystem scale physical disturbances primarily through altered micro climate and resources. Microbes must acclimate to immediate stress by altering their resources from growth to survival pathways, because stress that is too extreme will force them to dormancy or kill them. In the case of water, because microbes are small, they have an intimate contact throughout their surface semi permeable membranes, thus cellular water potential rapidly equilibrates with that of surrounding water, similarly in the case of soil organisms when soils dry and water potentials decline, cells must accumulate solutes to reduce their internal water potential and avoid dehydration and dying (Harris 1981).As their primary osmolytes, microorganisms use simple organics with a good balance of high solubility and limited physiological effects (Csonka 1989). Bacteria typically use amino compounds such as proline, glutamine, and glycine betaine (Csonka 1989). Fungi, on the other hand, use polyols such as glycerol, erythritol, and mannitol (Witteveen and Visser 1995).

Although changes in the physiology and biochemistry of *Trichoderma harzianum* strain under water must be altered from its terrestrial ancestors that is not the scope of this study. Our research is based only on its adaptation and growth in submerged conditions and its biocontrol potential therein.

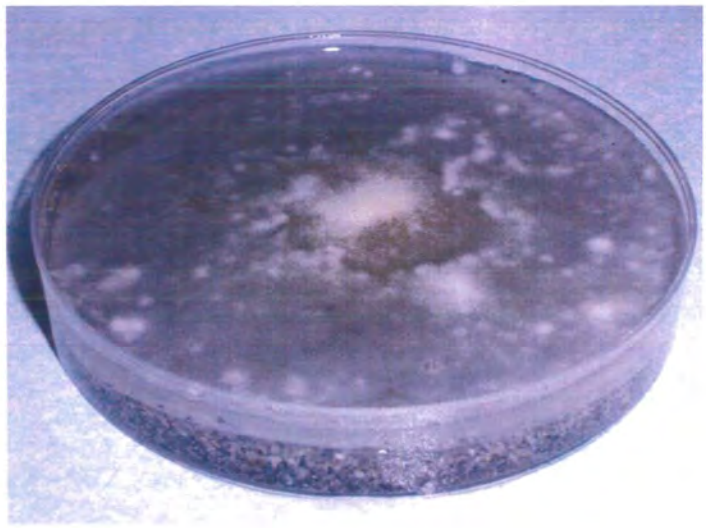


Fig 1a Growth of *Trichoderma harzianum* on water with sand underlay (4 days)

Fig 2a Growth of *Trichoderma harzianum* on water with sand underlay (18 days)

Table 2a Bacterial inhibition zones by Kirby bauer of water tolerant strain of *Trichoderma harzianum*

	Plate	*Enterobacter aerogens*	*Escherichia coli*	*Salmonella typhi*	*Pseudomonas aeroginosa*	*Pseudomonas putida*
Zone of inhibition by fungal disc	1	-	+	+	++	+
	2	-	++	+	++	++
	3	+	+	+	++	++
	4	+	+	+	++	+
Zone of inhibition by crude extract	1	+	++	+	+++	++
	2	+	++	+	+++	++
	3	+	++	+	+++	++
	4	+	++	+	+++	++

≥ + 0.5 cm

≥ ++ 1cm

≥ +++ 1.5 cm

4.5 Construction of the biosand filter

The column was constructed in accordance to specifications for biosand filters (Huisman and Wood, 1974, Schulz and Okun, 1984). The matrix used was construction material sand. Supernatant water level was maintained at 4 inches. The total height of the column was 20 inches. Temperature was not maintained in order to replicate field conditions. The temperature during the study was between 22 to 28 C. Turbidity of feed water was kept high from between 20 to 40 FAU (Formazine attenuation units). The feed water was taken from raw sources originating from Simli dam, Islamabad known to have bacterial content.

The mathematical workings of the column were determined using excel mathematical modeling software.

The Total (contact) surface area was 86.19 in^2 , whereas the surface area of the Biofilm was 2.65 in^2. The flow rate of the pump was 5 ml/min. The flow rate of the column was 2 ml/min. Sand texture of course sand was 0.5-1 mm and amount used was 45 gms, Medium sand (0.25 -0.5 mm) used was 30 gm. Fine sand (0.125 – 0.25 mm) was 56 gm. The internal Volume of sand column was 37.20 in The Bulk density was 3.52 gm/in3, Porosity 45 %, and retention time was 40 sec. (Table 3a)

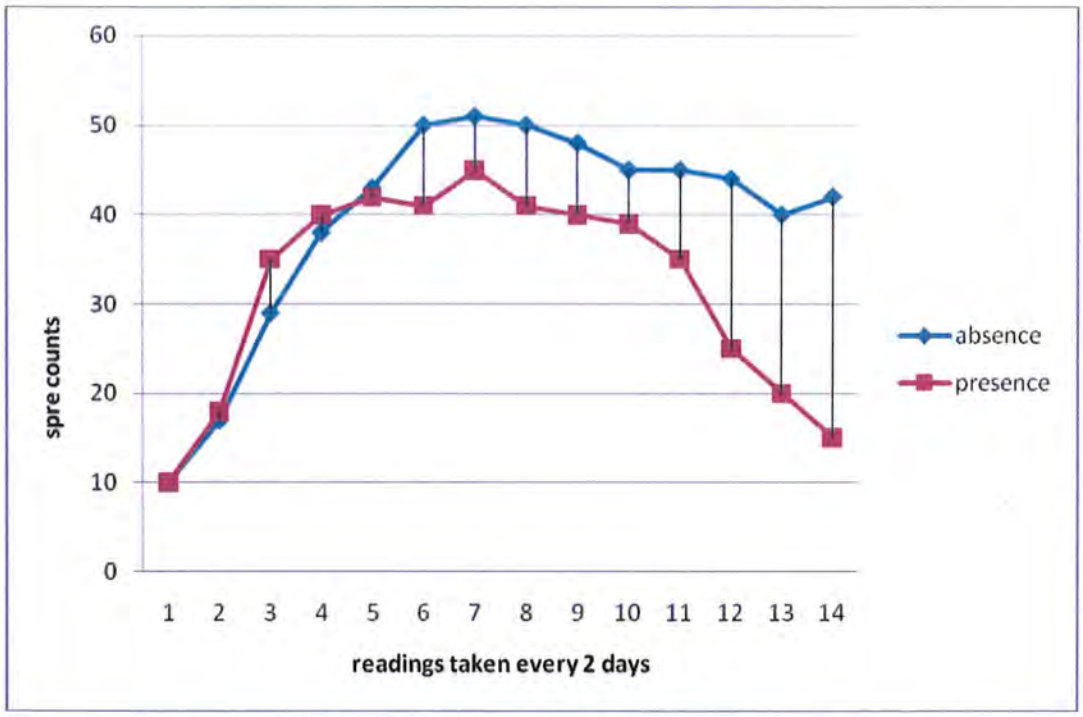
Table 3 a Mathematical modeling and hydrolysis of the column

Parameter	*Value*
Total (contact) surface area	86.19 in^2
Surface area of the Biofilm	2.65 in^2
Flow rate of the pump	5 ml/min
Flow rate of the column	2 ml/min
Sand texture	Course sand (0.5-1 mm) = 45 gms Medium sand (0.25 -0.5 mm) = 30 gm Fine sand (0.125 – 0.25 mm) = 56 gm
Internal Volume of sand column	37.20 in
Bulk density	3.52 gm/in^3
Porosity	45 %
Retention time	40 sec

4.6 Development of Biofilm and microbial dynamics: establishment of colony diameter.

Biofilm colonies were developed on the surface of the sand layer using cellulose fiber held in position over a fine wire mesh. Strain grew well on aerobic PDA plates with reduction in spore counts and color in water agar, however growth on cellulose cloth fiber over the sand matrix of the column was much better, due to increased nutrients coming from sand, influent water and accumulation of dead and growing bacterial cells and debris on the surface. This film later became the trapping and filtration zone from where the efficiency of the filter would be determined.

The diameter of the Biofilm colony produced was 2 inches. Counts were taken of fungal and bacterial colonies in the head space of the column representing the microbial environment just above the Biofilm and was then compared to control columns without *Trichoderma harzianum.* In both cases it took approximately one weeks for the Biofilm to begin formation. Initially the counts were equal, but by the end of the week once the Biofilm had established itself and *Trichoderma harzianum* got incorporated reduction rates were seen, by the end the efficiency of fungal spore removal was more than 30% better in columns with *Trichoderma harzianum,* (Fig 7, Table 7) Similarly for Bacteria there was seen a 18 % better reduction in bacterial counts of the headwater. Standard deviation in headwater counts in absence of *trichoderma* was 8.36.88, and standard deviation in headwater counts in presence of *trichoderma* was 597.09 (Fig 8, Table 8)



Standard deviation (presence) is 11.6 and Standard deviation (absence) is 11.7 (given in table 7)

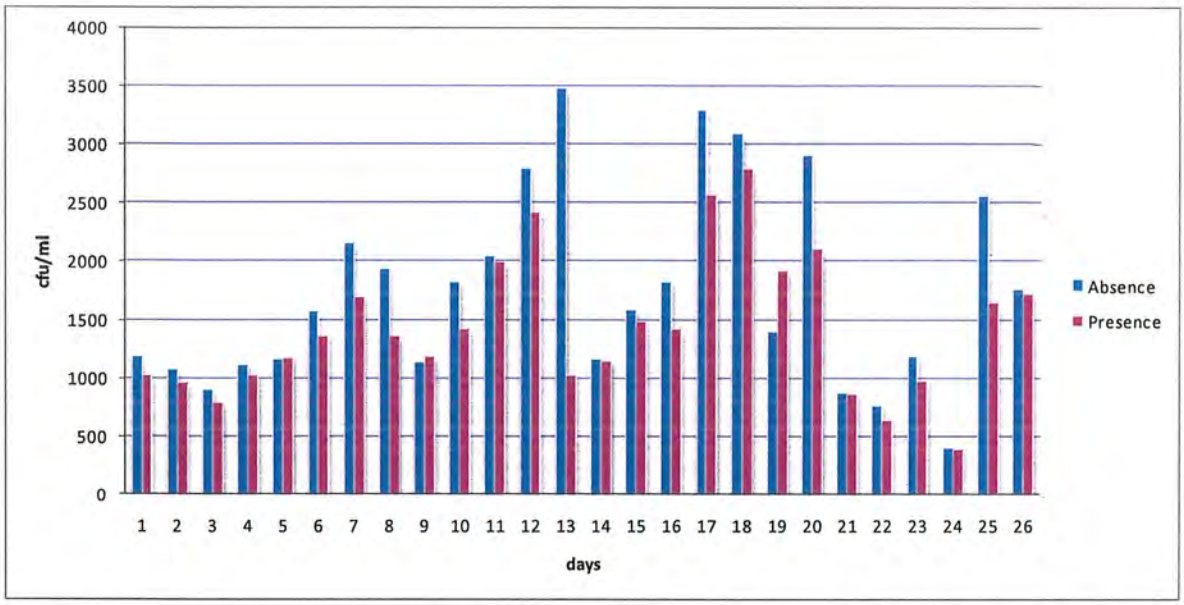
Fig 7: Suppression potential of water grown strain of *Trichoderma harzianum*: reduction in total spore counts in the presence and absence of the strain

Biofim quanitifaction was done by a method of Vilena *et.al* 2001, in which disc pieces were removed and weighed, the weight difference was about 0.003 mg by the end of the experiment period which was two months, the disc was perhaps too small for a noticeable weight difference of the biofilm, therefore a chemical method for calculation of total carbohydrate was used.

A method devised by Fales *et.al* (1951) was employed by which anthrone reagent is used to determine the total carbohydrate content, this can be translated into Biofilm growth of living and dead cells. Total Carbohydrate content increased in disc upto 0.55 mg/ml and in washings of planktonic cells upto 0.4 mg/ml. (Fig 9)

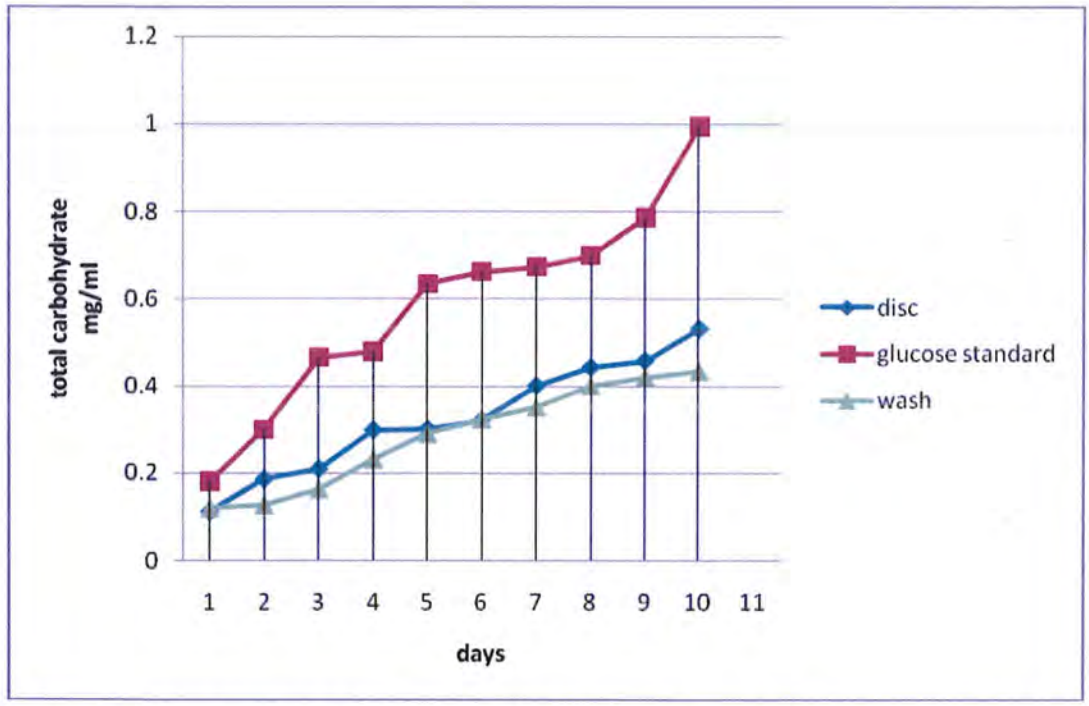
Considering that submerged free floating fungal growth is not natural, growth on and within solid substrates is fundamentally related to cell adhesion. The morphogenetic and physiological responses derived from this biological process are the basis of surface adhesion fermentation proposed as a new fermentation category by Gutierrez Correa and Villena (2003). Fungal adhesion is known to be a natural yet complex process.

The application of the above biological process is Biofilm fermentation in which there are some advantages of slow sand filtration such as high enzyme productivities. Fungi can be considered as regular biofilm forming organisms with two inherent and fundamental processes: adhesion and subsequent differential gene expression to develop new and distinct phenotypes different from those of free living conditions (Wimpenny *et.al.* 2000).



Standard deviation (presence) is 597.09 & Standard deviation (absence) is 836.83 (given in table 8)

Fig 8: Bacterial counts in the presence and absence of water grown *Trichoderma harzianum* incorporated biofilm



Standard deviation (disc) is 0.133 & Standard deviation (wash) is 0.120 (given in table 9)

Fig 9. Growth determination of biofilm by measurement of total carbohydrate

4.7 Growth potential of *Trichoderma harzianum* ; estimation in submerged conditions

To measure the extension rate, 1cm ^{2} grids were drawn on the base of Petri plates, then poured with PDA. Using time lapse photography of 5 minute intervals growth rate was measured. It took 105 minutes for a single mycelia to cover 1 cm^2 (Fig 4a)

The same technique was used for submerged strains by flooding the plate with water and measuring growth after every 5 minutes. For the water grown strain this time was 175 minutes for 1 cm^2. (Fig 5)

Results showed that growth rate slowed by 40 % in water, the causes for this are resistance that the growing hyphae has to face for traveling in a different medium, weakening of the hyphae due to environmental stress and lack of nutrients and reduction in sporulation.

Growth rate on PDA = 105 minutes /cm^2

= 6300 S / cm^2

Growth rate in submerged culture = 175 minutes /cm^2

= 10500 S/ cm^2

In 1972 Katz proposed a model to study growth of the colony from spore germination, to hyphal development and branching. Branching is thought to be an extension of the basic tip growth mechanism Katz proposed that precursors from such distant regions are transported to growing tips and that accumulation of one or more of these precursors could be the trigger for branch initiation. Specifically, branching would be initiated when the rate of supply of vesicles outpaces the rate of their incorporation at the growing tip. This vesicular basis of hyphal growth and branching was incorporated into a model by Trinci (Prosser and Trinci 1974). A key element of this model was the hyphal growth unit, defined as the ratio of total hyphal length to the total number of tips. This growth unit represents the mean length of hyphae that contribute to the extension of an

individual tip. The initiation of a new branch has been proposed to be controlled by changes in the cytoplasmic volume, so that branching occurs when a critical value of the mean hyphal growth unit is attained. In this way, the protoplasm considerably distant from the growing tip could have a contributing role in branch initiation. In a further elaboration of this model, Prosser and Trinci (1979) proposed that the concentrations of vesicles and nuclei regulate the increase in hyphal length and the occurrence of branches and septa. The model requires that the rates of supply and consumption of vesicles be proportional to the metabolic rate and that the deposition rate determine the tip growth rate. Growth of filamentous fungi is a somewhat unpredictable thing. Current means of measurement are based on colony diameter for growth on plates and biomass for growth in broth. Both these forms are arbitrary and measurement is taken of the overall colony.

The model also predicts that branching at the tip should enter into a transitional hypo branching phase following physical separation from the colony, as was observed. The model explains how branching can be independent of tip extension rate under steady-state conditions while responding dramatically to changing conditions. The model also is consistent with the results of tip isolation experiments and explains the lack of mutations resulting in longer branch intervals as well as the observed temperature and extension-rate dependence of branching in colonial mutants.

This is helpful in giving insight into changes taking place in the cytoplasm of the hyphae, these changes could be due to mutations or adaptations under stress.

Katz model was used in these experiments of this study to estimate the reduction in growth of mycelia and sporulation. This gave us an estimate of how growth was effected in submerged conditions.

This method is based on a factor known as the mean tip extension rate in which the number of new hyphal tips is measured and multiplied by the specific growth rate. (Fig 6).

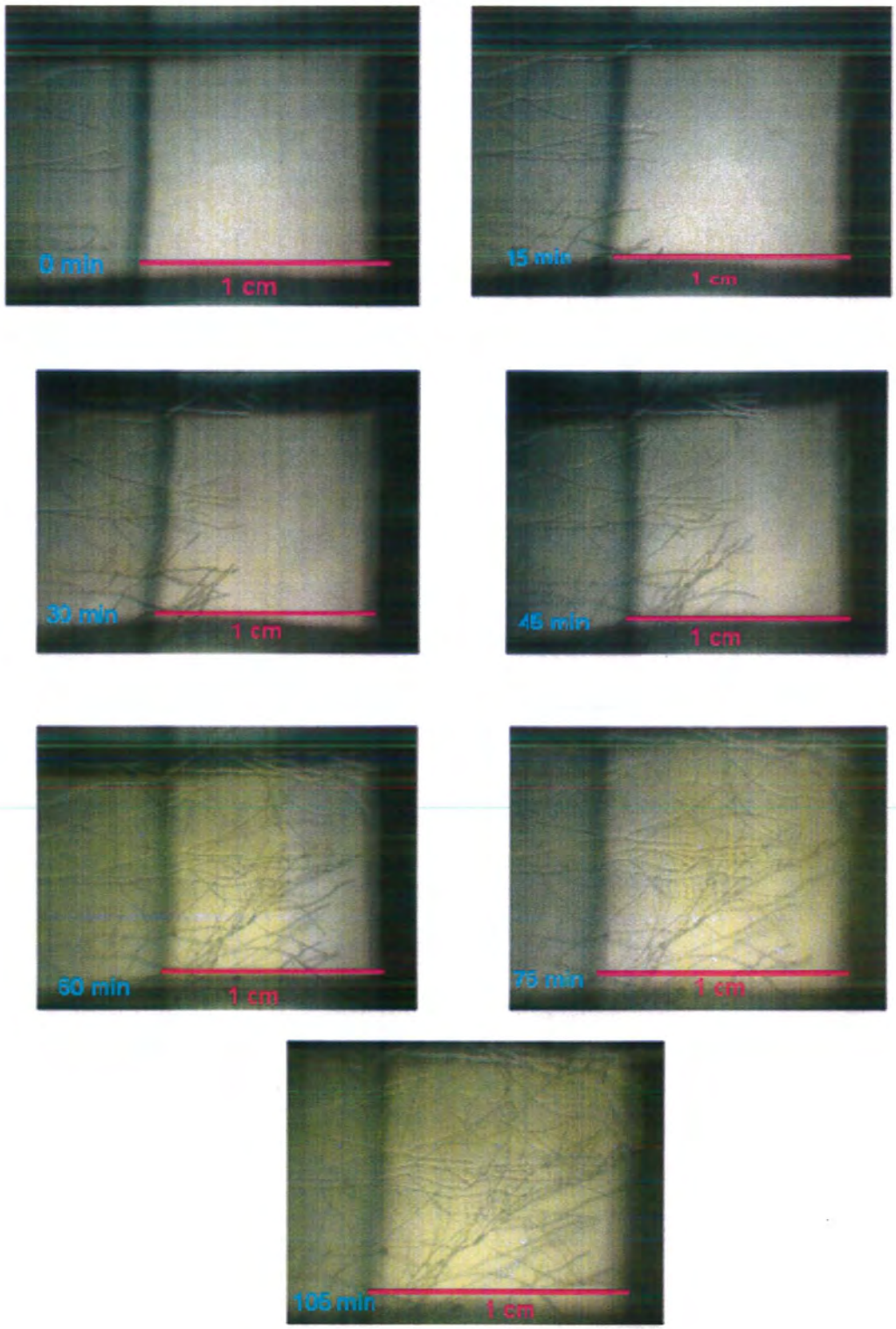


FIG 4a Growth rate of *Trichoderma harzianum* on PDA

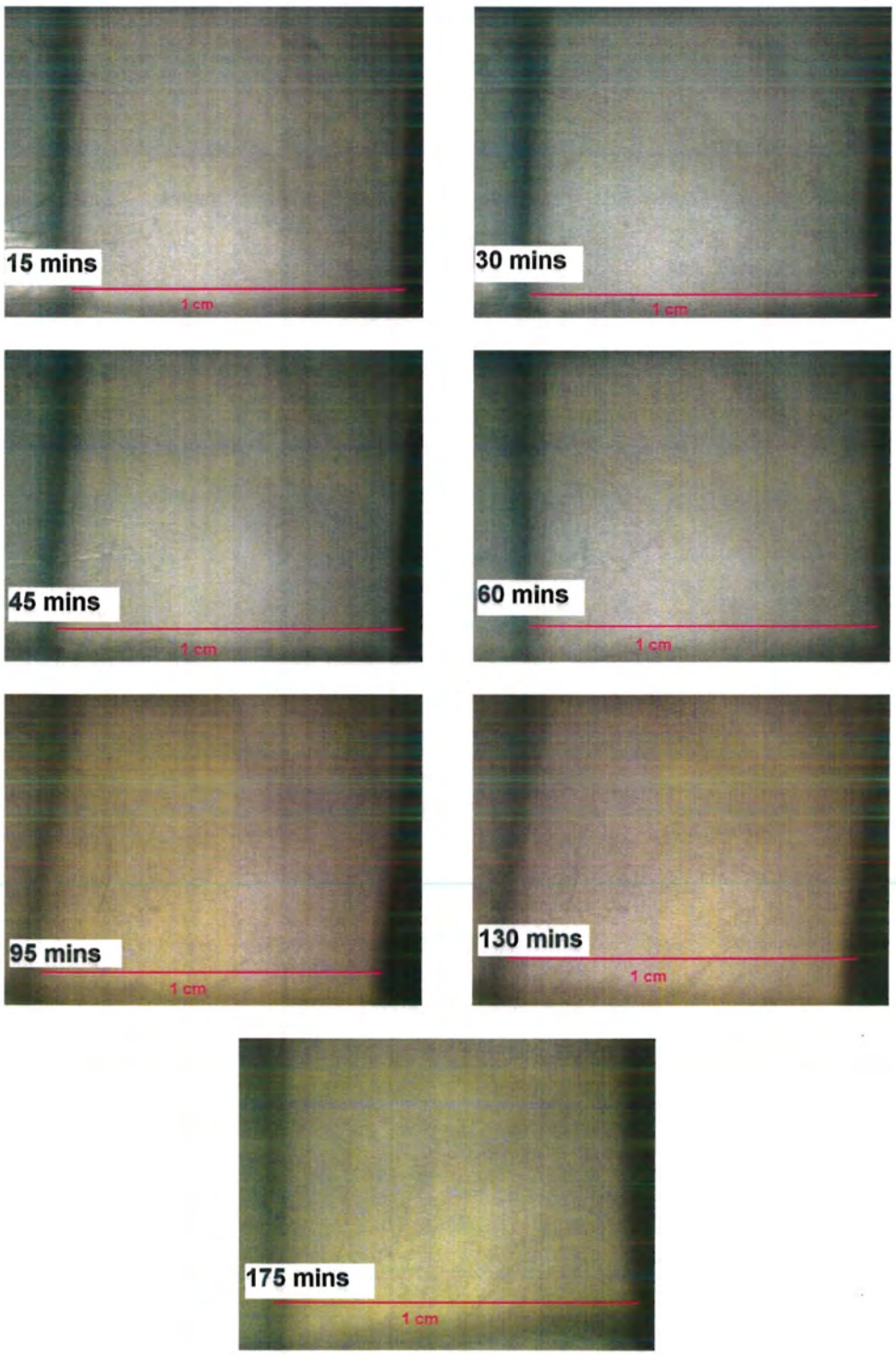


FIG 5 Growth rate of *Trichoderma harzianum* on PDA with water overlay

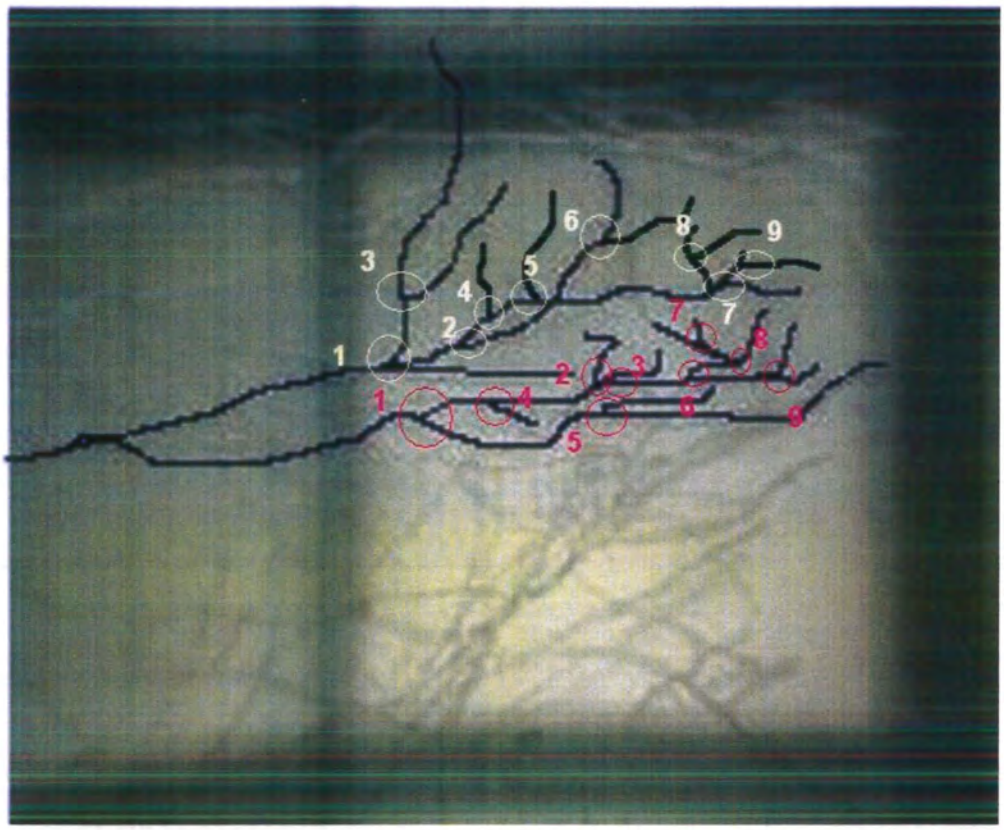


Fig 6. Count of new tips produced on mycelia

4.8 . Improvement in quality of water

Test methods:

There are 3 types of water quality features Physical (for example, colour, pH, taste, odour, temperature and turbidity) Chemical (for example, arsenic, fluoride, chloride, conductivity, dissolved oxygen, iron, manganese, nitrate, nitrite, sulphates, pesticides and heavy metals) and Microbiological and biological (for example, bacteria, protozoa, viruses, helminths and higher organisms) (House and Reed, 1997)

Using this classification *Trichoderma harzianum* incorporated into the biofilm was tested for its removal potential of physical parameters metals, chemical organics and pathogens. Testing of core parameters for turbidity, conductivity, pH, TDS and Colour was done using a Nova 60 Photometer (Merck). Samples were transferred into 50 mm cuvettes and selecting method code on the LCD for an automatic measurement. Bacteriological counts were taken using Merck Plate count agar and colonies were read on an electric colony counter.

Chemical parameters of Nitrates, Iron, Chromium and Sulfates were also tested automatically on the Nova 60 Photometer. Samples were prepared in accordance to the provided kits and measured by selecting method code on the LCD.

4.8.1 TOTAL DISSOLVED SOLIDS:

Total dissolved Solids (TDS) were reduced well by the fungal incorporated filter. In filters with *Trichoderma harzianum* the average removal of total dissolved solids was 14% whereas in filters without *Trichoderma harzianum* the removal rate was around 12%. Indicating a 15% better removal in the enhanced biosand filter. (Fig 10a, 10b Table 10a,10b) Total dissolved solids gives an idea of the soluble content in the water, it is a combined total of organic and inorganic contents and is used as an indication of aesthetic characteristics of drinking water and as a general aggregate indicator of the presence of chemical contaminants. Primary sources maybe agricultural runoff or sewerage. Standard

deviation values of outlet water in columns with *trichoderma* was 55.57, and standard deviation values of outlet water in columns without *trichoderma* was 63.68.

4.8.2 pH and CONDUCTIVITY:

Very little variation was seen in pH values over the study period. Pre and post filter samples remained within the neutral range. Samples taken from the filter enhanced with *Trichoderma harzianum* showed a pH range of 7 + - 2.19 whereas for samples from the control filter, the range varied from 7 + - 2.05, showing a slight difference. Standard deviation values of outlet water in columns with *trichoderma* was 0.156, and standard deviation values of outlet water in columns without *trichoderma* was 0.16. (Fig 11a, 11b, Table 11a,11b) pH values are important because they not only estimate acidity or alkalinity of the water, they also indicate removal efficiency of pathogens. Adsorption of pathogens is also improved by lower pH raw water. Gerba *et.al* (1988) documented that low pH favors virus adsorption whereas high pH favors virus elution. Lukasik *et.al* (1999) also found the same trend for ordinary sand columns where a pH of 5 favoured adsorption of E. coli and MS2 coliphage compared to water with a pH of 7. However, this adsorption was found to be depend on the media used. - the sand columns with metal-coated media had removal efficiencies that were best at pH 7 for both E. coli and MS2, while removing MS-2 equally well over the pH range tested. In contrast to ordinary sand, the adsorption of E. coli to modified sand was found to be lower at pH 5 than pH 7.

Because conductivity is directly associated with the dissolved ions in the water, its results showed correspondence to TDS results Conductivity in filters with *Trichoderma harzianum* was showed an average variation by 14.2 %, which was a negligible difference from filters without *Trichoderma harzianum* which showed variations in conductivity by 14.3% over the study period. Standard deviation values of outlet water in columns with *trichoderma* was 79.38, and

standard deviation values of outlet water in columns without *trichoderma* was 92.12. (Fig 12a,12b Table 12a,12b)

4.8.3 TURBIDITY AND COLOUR:

Both filters showed significant results in their ability to remove turbidity from influent water. Turbidity levels are generally reported between 60 to 90 %, depending upon the type of sand used. Good filters show removal rates by 98%. Gerba *et.al* (1988). Our control filter without *Trichoderma harzianum* showed efficiency of removal by 95%, but the filter with *Trichoderma harzianum* had an improved turbidity removal of 98 %. Standard deviation values of outlet water in columns with *trichoderma* was 0.358, and standard deviation values of outlet water in columns without *trichoderma* was 0.468. (Fig 13a,13b Table 13a, 13b)

In drinking water, the higher the turbidity level, the higher the risk that people may develop gastrointestinal diseases due to an inability to digest chemicals or fight pathogens. This is especially problematic for immune-compromised people, because contaminants like viruses or bacteria can become attached to the suspended solid. The suspended solids interfere with water disinfection with chlorine because the particles act as shields for the virus and bacteria. Similarly, suspended solids can protect bacteria from ultraviolet (UV) sterilization of water. Turbidity and colour removal efficiency decline considerably with higher filtration rates in continually-operated sand filters, although the filtrate quality remains reasonably good. (Muhammad *et.al*, 1996)

Colour removal in filters with *Trichoderma harzianum* showed 99% removal, those without the fungi showed removal rates of 95%. Standard deviation values of outlet water in columns with *trichoderma* was 0.094, and standard deviation values of outlet water in columns without *trichoderma* was 0.211. (Fig 14a, 14b Table 14a, 14b)Removal rates for standard filters are reported between 90 to 95% (Bellamy 1985)

4.8.4 MICROBIOLOGY

Both filters showed good removal rates for bacteria and fungi upto 96% in *Trichoderma harzianum* enhanced films and 92% in the filters without *Trichoderma harzianum* . Over the whole study period the improvement over the control filter was by 6% .Initially removals were similar, after about one week of filter maturation removal rates significantly improved. (Fig15a, 15b Table 15a 15b) Bacteriological removal depends upon a number of factors. Most importantly the age and development of the Biofilm on the filter. A well operated slow sand filter will remove protozoa and helminthes (IRC 1981), in percentage reductions this means 95% removal of coliforms and a 99% removal of cysts. (WEDC 1999). Many authors have documented the high efficiency rates of biosand filters.

The removal efficiency of two salts and two metals was tested in the control and the fungal enhanced filter in a secondary series of tests, all of which were done using a Merck Nova 60 Spectrophotometer. Samples were prepared according to the Kits and loaded into cuvettes, and placed in the meter for an automatic measurement. (Graph 16, Table 16)

4.8.5 CHEMISTRY

Nitrates

Nitrate removal was 5 % improved by the filter with *Trichoderma harzianum* incorporated. Showing a 20% removal in these films as compared to only 15% removal in filters without them.

Sulfates

Sulfate removal was better by 12% in filters with *Trichoderma harzianum* , having a removal of rate of 52% by the Biofilms incorporated with the fungi, over a 40 % nitrate removal in filters without them.

Chromium

Chromium removal was 7% better in the enhanced filters, having removal rate of 20% in these filters, over 13% in filters without the fungi.

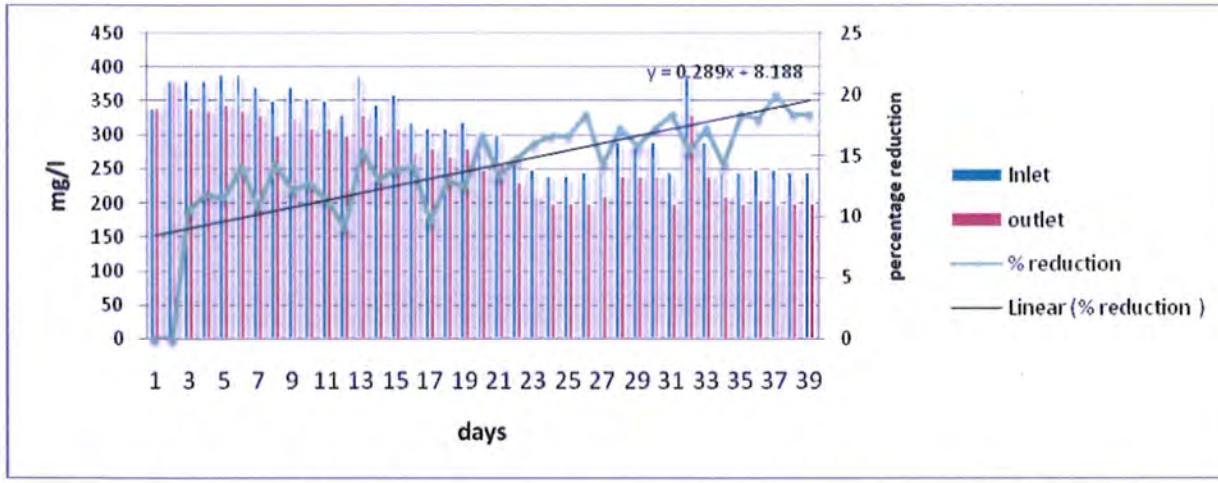
Iron

Iron reduction was 30% in filters without *Trichoderma harzianum* and 70% in filters with the fungi, showing a 40% better removal in filters with *Trichoderma harzianum*

Chlorides

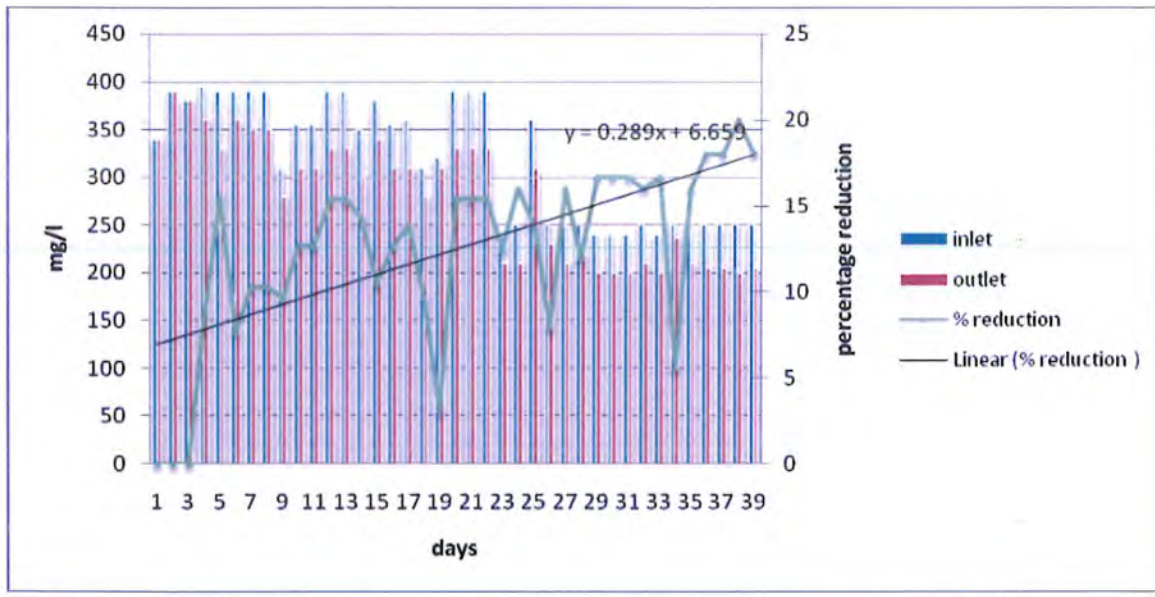
Chloride was overall 1% improved, but showed little variation with removal rate of 8 %in filters without *Trichoderma harzianum* and 9 % in filters incorporated with *Trichoderma harzianum* .

Slow sand filters are known not to completely remove all organic chemicals, dissolved inorganic substances, such as heavy metals, or trihalomethane (THM) precursors. Studies by Collins (1998) showed that if properly established the schmutzdecke can remove upto 95-99% heavy metals.



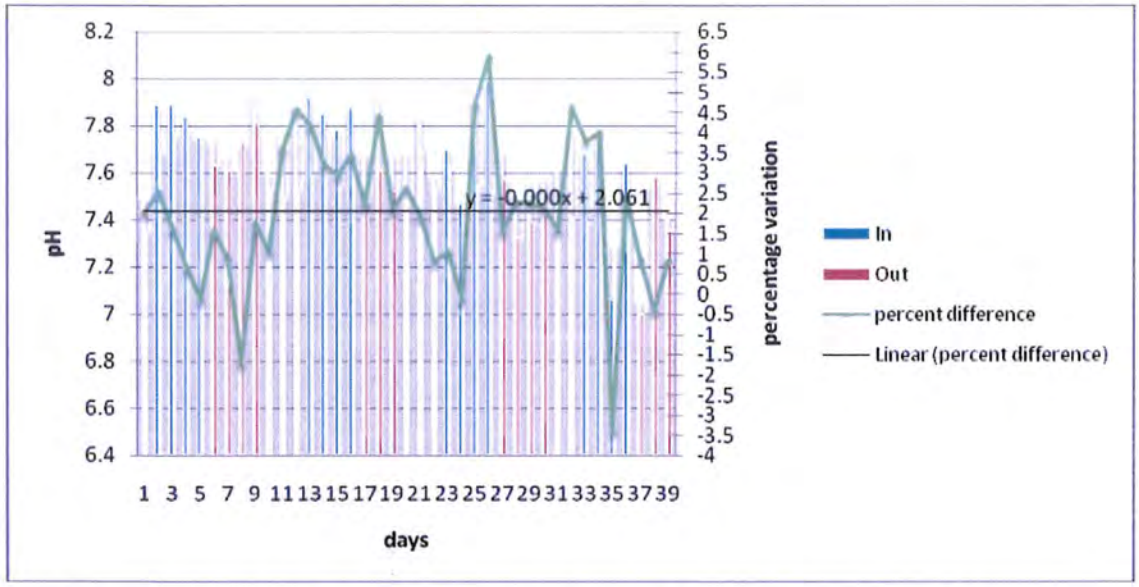
Standard deviation (inlet) is 54.23 & Standard deviation (outlet) is 55.57 (given in table10a)

Fig 10 a. Reduction in Total Dissolved Solids in filters with *Trichoderma harzianum*



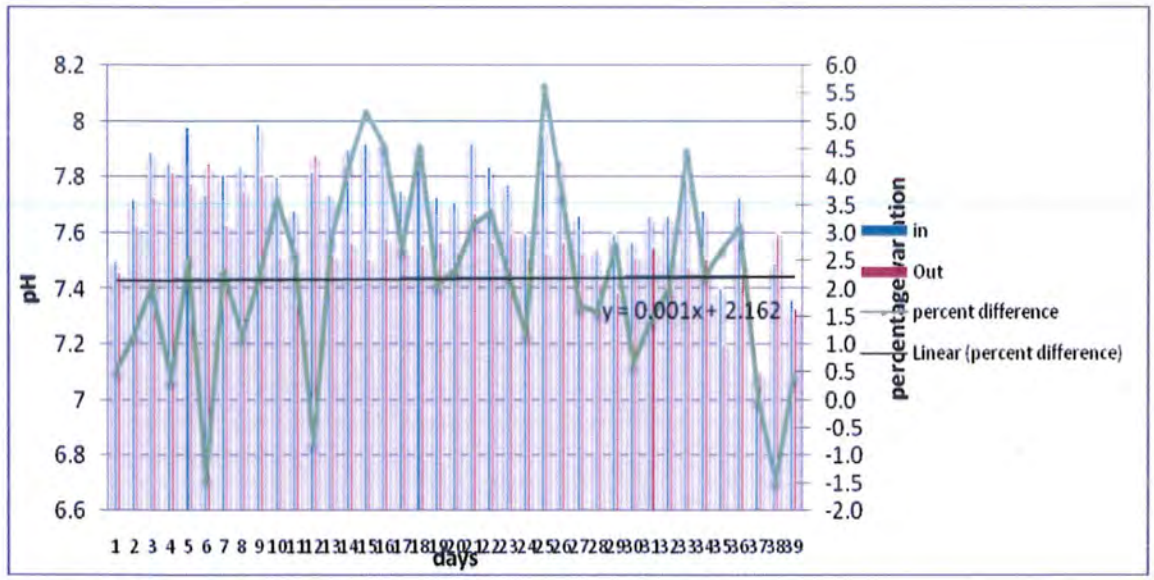
Standard deviation (inlet) is 64.07 & Standard deviation (outlet) is 63.68 (given in table10b)

Fig. 10b. Reduction in Total Dissolved Solids in filters without *Trichoderma harzianum*



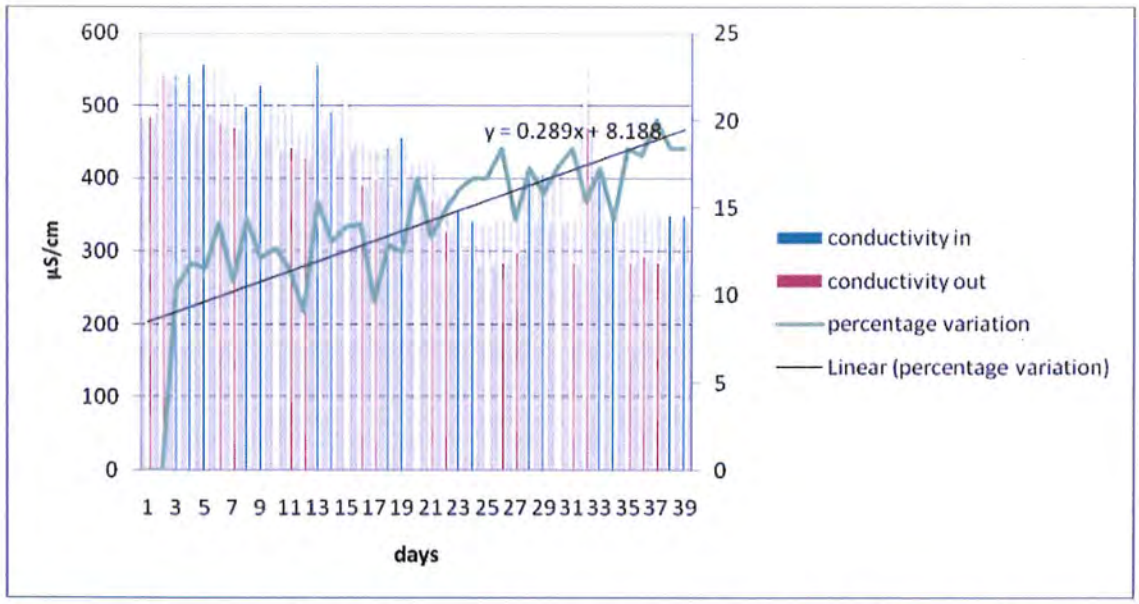
Standard deviation (inlet) is 0.21 & Standard deviation (outlet) is 0.156 (given in table11a)

Fig 11 a. Variation in pH in filters with *Trichoderma harzianum*



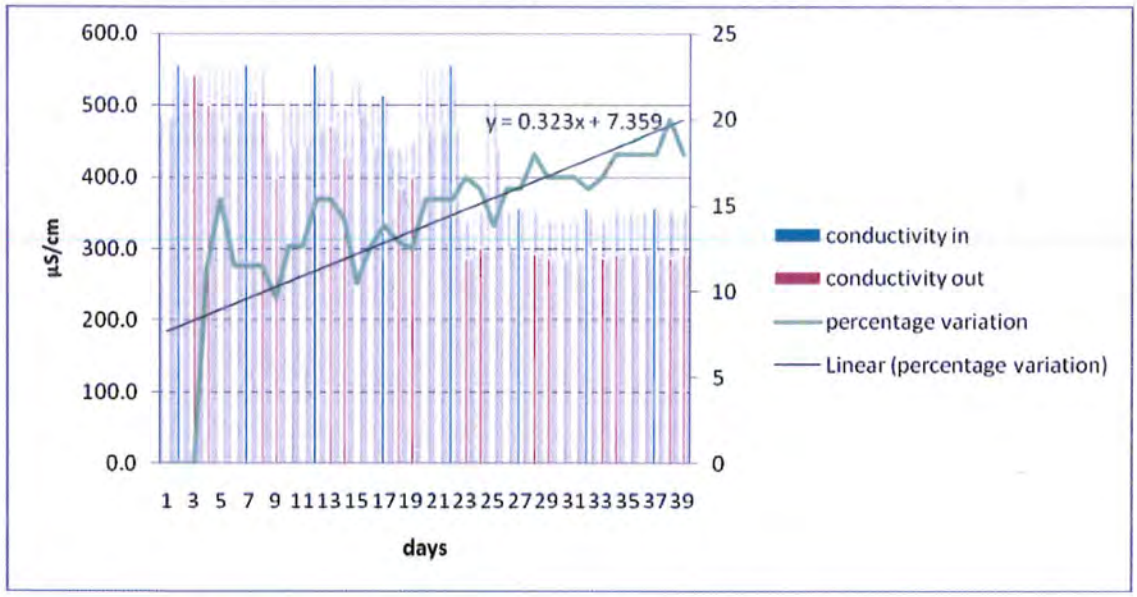
Standard deviation (inlet) is 0.19 & Standard deviation (outlet) is 0.16 (given in table 11b)

Fig 11 b. Variation in pH in filters without *Trichoderma harzianum*



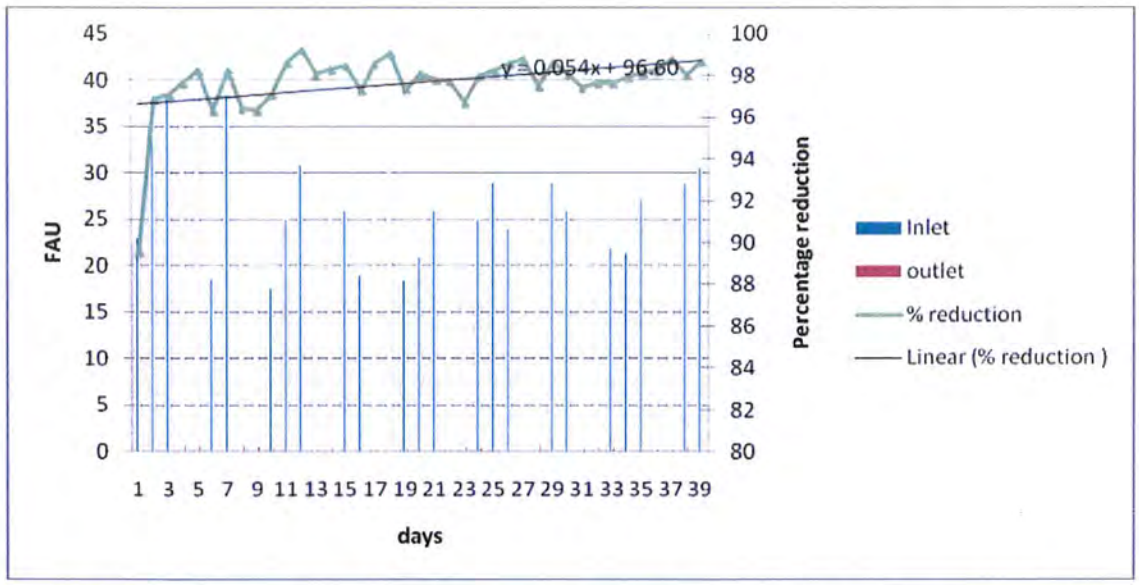
Standard deviation (inlet) is 77.47 & Standard deviation (outlet) is 79.38 (given in table 12a)

Fig 12 a. Variation in conductivity in filters with *Trichoderma harzianum*



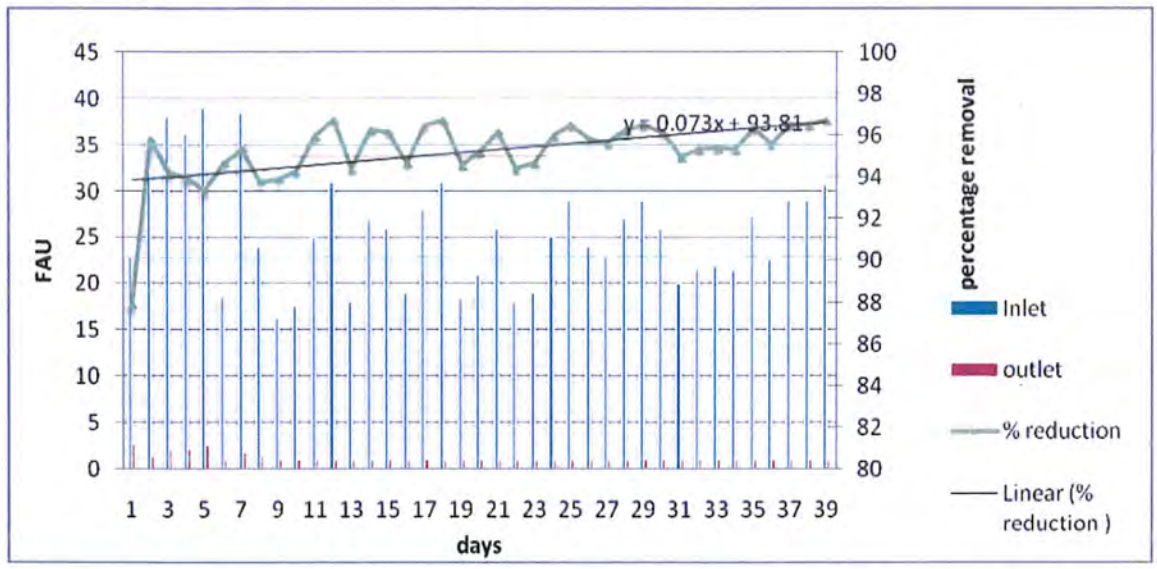
Standard deviation (inlet) is 91.53 & Standard deviation (outlet) is 92.12 (given in table 12b)

Fig 12 b. Variation in conductivity in filters without *Trichoderma harzianum*



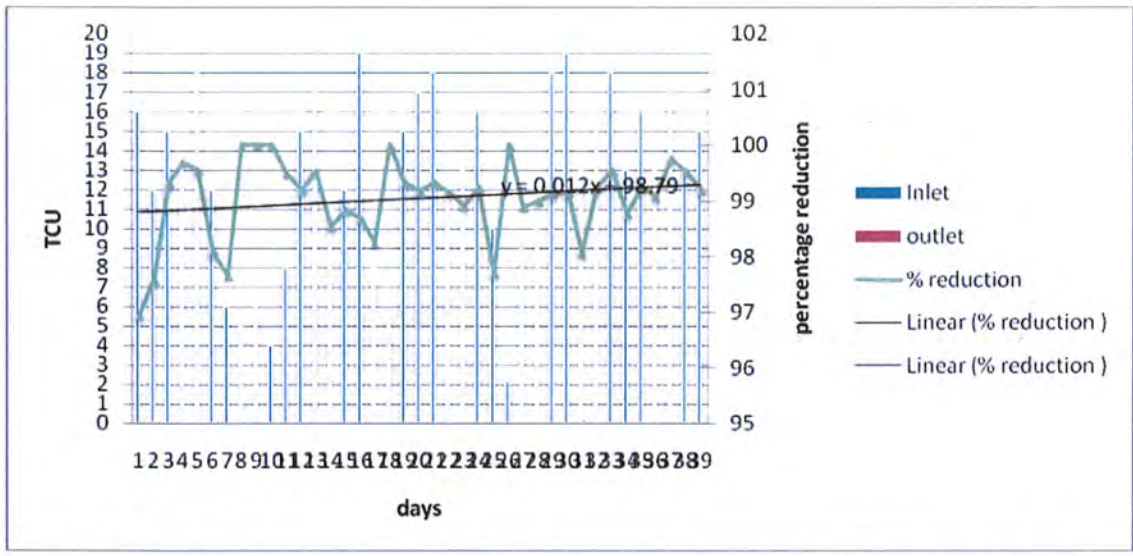
Standard deviation (inlet) is 6.155 & Standard deviation (outlet) is 0.358 (given in table 13a)

Fig 13 a. Reduction in Turbidity in filters with *Trichoderma harzianum*



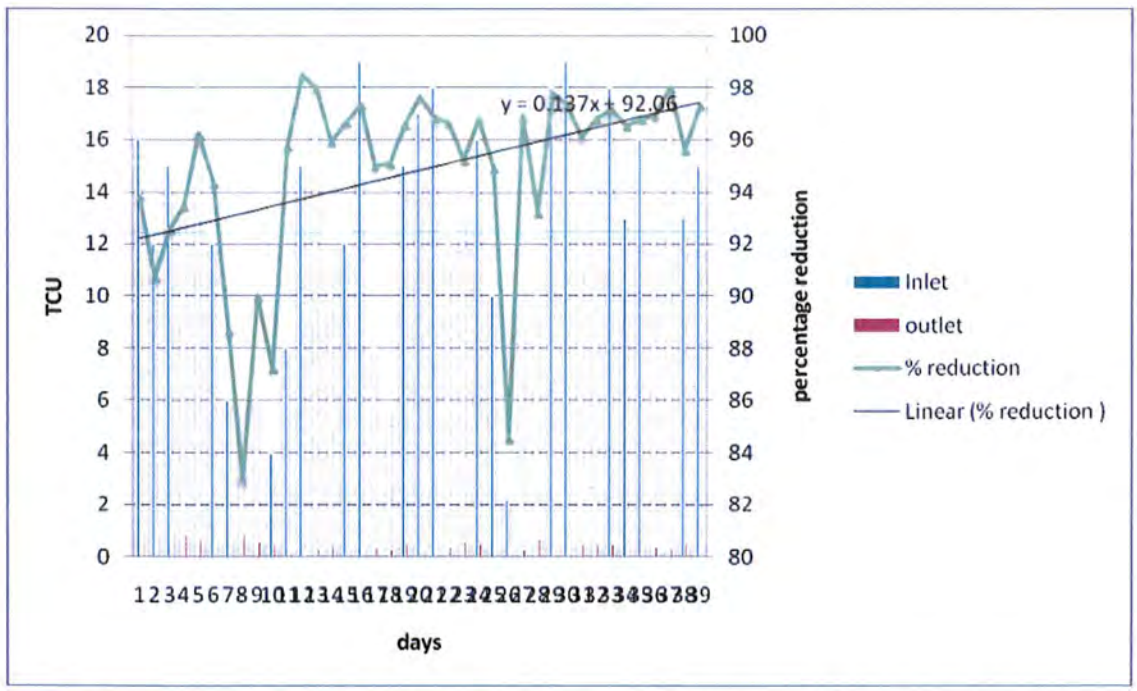
Standard deviation (inlet) is 6.155 & Standard deviation (outlet) is 0.468 (given in table 13b)

Fig 13 b. Reduction in Turbidity in filters without *Trichoderma harzianum*



Standard deviation (inlet) is 4.512 & Standard deviation (outlet) is 0.094 (given in table 14a)

Fig 14 a. Reduction in Color in filters with *Trichoderma harzianum*



Standard deviation (inlet) is 4.512 & Standard deviation (outlet) is 0.21 (given in table 14b)

Fig 14 b. Reduction in Colour in filters without *Trichoderma harzianum*

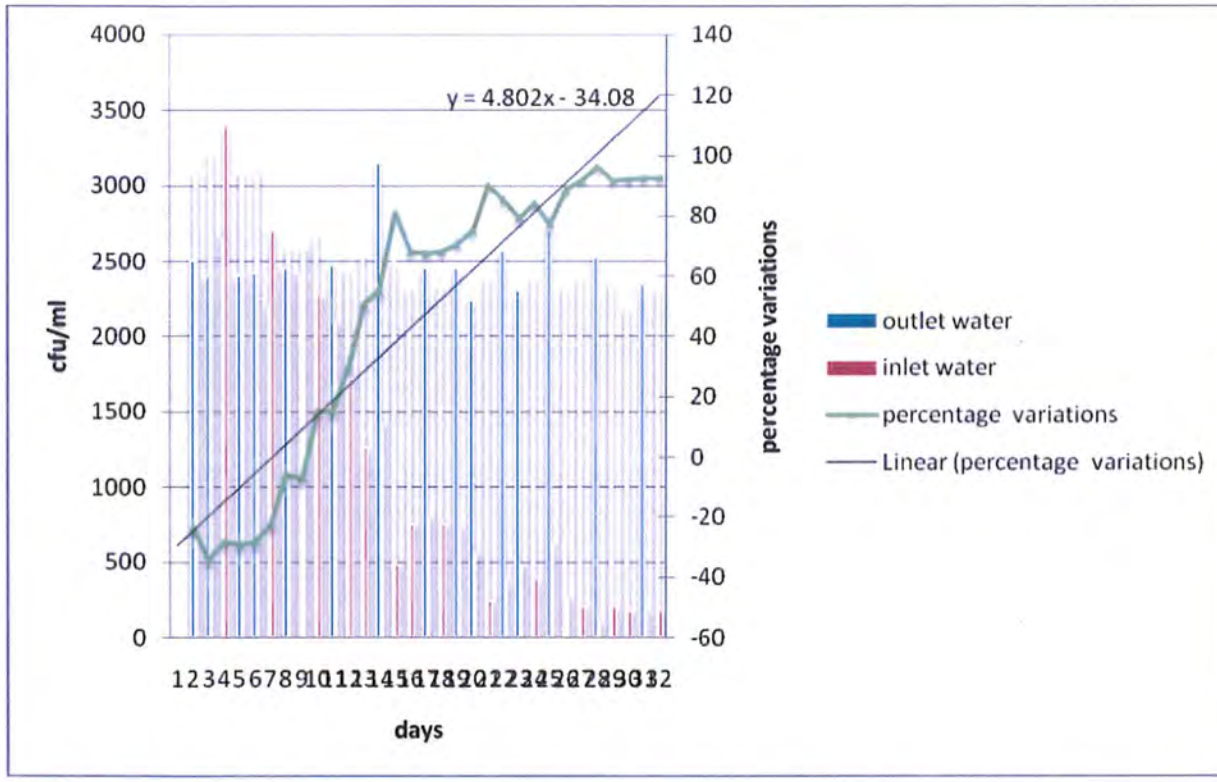


Fig 15 a. Reduction in Bacterial Counts in filters with *Trichoderma harzianum*

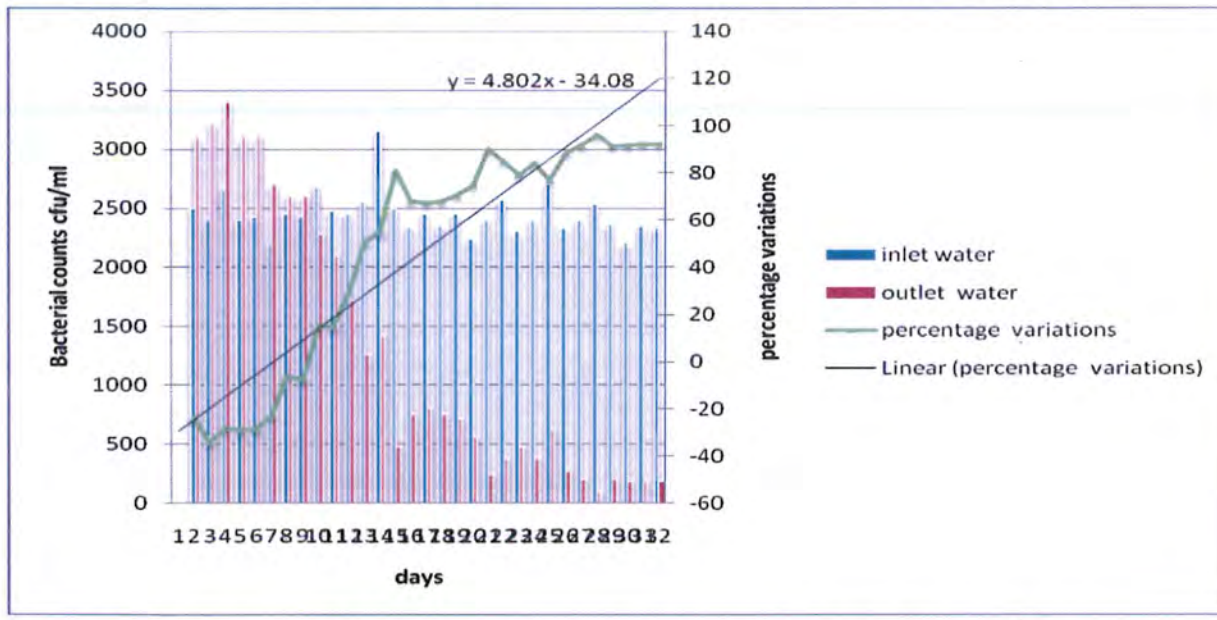


Fig 15 b. Reduction in Bacterial counts in filters without *Trichoderma harzianum*

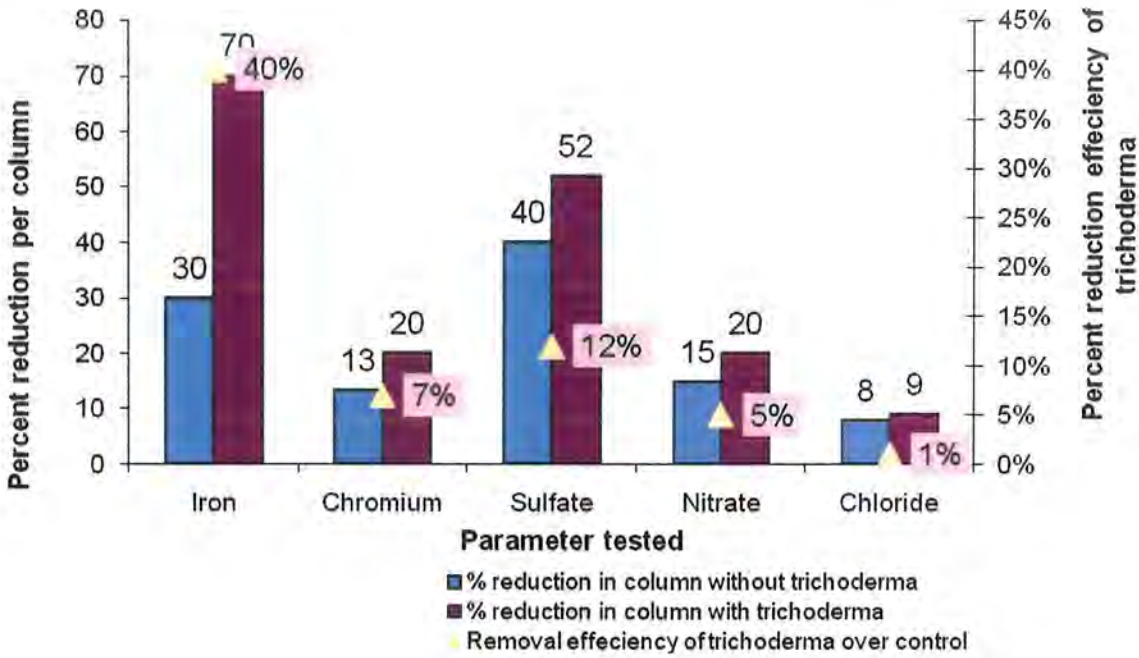


Fig 16. Efficiency of *Trichoderma harzianum* incorporated biofilm for the removal of salts and metals.

Both bacteria and fungi play critical roles in decomposition processes in many natural environments, yet only rarely have they been studied as an integrated microbial community. (Buchan et. al 2003) In the few studies that have considered the activity of salt marsh bacterial and fungal saprophytes simultaneously, interactions between fungi and bacteria have been hypothesized to be based on temporal resource partitioning (Newell et. al 1989). Yet, there has been no satisfactory explanation of the mechanisms by which microbial communities are replaced during temporal resource partitioning. A broader view that recognizes the potential for physiological and ecological interactions between co-occurring bacterial and fungal groups may be a more valuable perspective . (Mille-Lindhlom and Transvik 2003).

Culturing approaches are generally not successful for identifying ecologically dominant bacterial species in marine environments (Giovanni 2000), Positive or negative interactions between co-occurring taxa (e.g., in the acquisition of

nutrients, in the sequential attack of substrate, in deterring of predators, or in competition for space on the plant blade) may affect microbial community composition (Wohl and McArthur 2001), as might selective predation of specific taxa by higher trophic levels in the microbial food web (Newell 2001)

Buchan *et.al* 2003 examined the potential importance of microbe-microbe interactions within the *S. alterniflora* decomposer community by exploring statistical associations between taxa. Although such associations reflected similar responses to the same environmental factor (or combination of factors) or unrelated to decomposition, they may also be indicative of ecological interactions between bacteria and fungi that would warrant future study.

In contrast to that of aquatic organic matter decomposition, bacteria have not been able to monopolize decomposition processes in terrestrial ecosystems. The emergence of fungi in terrestrial ecosystems must have had a strong impact on the evolution of terrestrial bacteria. On the one hand, potential decomposition niches, e.g. lignin degradation, have been lost for bacteria, whereas on the other hand the presence of fungi has itself created new bacterial niches. Confrontation between bacteria and fungi is ongoing, and from studying contemporary interactions, we can learn about the impact that fungi presently have, and have had in the past, on the ecology and evolution of terrestrial bacteria. (Boer *et.al* 2005)

During the evolution of terrestrial microbial life, fungi have become the major decomposers of recalcitrant organic matter. Bacteria on the other hand have been able to maintain a significant role in the degradation of simple substrates. However, this is only the general picture and, there is an ongoing confrontation between fungi and bacteria for both complex and simple substrates.

Hence, it is often not clear to what extent an antifungal trait has actually evolved as a strategy of competition. Experimental manipulation of fungal density could be used as an initial approach to study the impact of fungi on the selection of bacteria with anti-fungal properties.

The fungi themselves often produce compounds with antibacterial activity, and have developed several strategies to counteract bacterial antagonism including

detoxification, removal of antibiotics by efflux, and modification of bacterial gene expression (Duffy *et.al* 2003)

It is widely assumed that fungal exudates are a major or exclusive source of nutrients for the bacteria living in their vicinity. (Nurmiaho-Lassila *et.al* 1997)

Associated bacteria may have negative, neutral or positive effects on fungal fitness. There are several indications of mutualistic relationship between fungi and their associated bacteria, the most obvious being found in the cyanolichens. The fungi benefit from the relationship by obtaining a supply of energy from the cyanobacteria. Unlike the green algal photobionts in most lichens, some cyanobacterial partners fix nitrogen and supply a portion of the yield to the fungus (Honegger, R *et.al* 1998). Nitrogen transfer may also be important in the association of organotrophic bacteria with fungi.

Obviously, uptake of bacteria by fungi is a less common event than is uptake by protozoan bacterial predators, which utilize phagocytosis as a mechanism for incorporating the bacterial cells. The fungal cell wall as a physical barrier strongly tends to prevent acquisition of bacteria (Bianciotto *et.al* 2000). The wall is not rigid at the hyphal tip (Wessels, 1994) and bacterial acquisition may occasionally occur at this location. Another scenario for bacterial acquisition by fungi could be the lysis of hyphal tips by bacteria, followed by entry into the fungal mycelium.

Besides being competitors or suppliers of exudates, living fungi may also serve directly as sources of nutrients for other microorganisms. A nutritional strategy based on targeting fungi as a substrate is well known for the so-called mycoparasitic fungi.

Several studies have addressed the mechanism and regulation of attack on fungi by *Trichoderma harzianum* spp. (Zeilinger,*et.al* 1999) (Jeffries *et.al* 1997) (Chet *et.al* 1997)

The following steps can be recognized: (1) chemotropic growth towards the host fungus, (2) coiling around the host fungus and appressorium formation, (3) secretion of cell wall degrading enzymes, (4) penetration and (5) degradation of hyphal content In a previous study, coiling around and penetration of a host fungus by a Streptomyces strain was also described (Rehm 1959) However,

with the exception of studies on penetration and killing of living fungal spores no further attention appears to have been paid to the occurrence of mycophagy in actinomycetes. Bacterial pathogens of fungi may be considered as a special group of mycophagous bacteria. The best studied case is that of brown blotch disease of the cultivated mushroom (Agaricus bisporus) by Pseudomonas tolaasii (Soler-Rivas *et.al* 2000).

Not only are there bacteria that feed on fungi, but also there are fungi that are able to lyse and consume bacteria (Barron 1988) It has been suggested that bacteria may be an important source of nitrogen during fungal degradation of resources with a high C/N ratio, e.g. wood (Tsuneda and Thorn 1994)

Fungi with the ability to lyse bacteria appear to be attracted by bacterial colonies. It has been speculated that density-dependent control of antifungal gene expression, which is apparent for several soil bacteria, could be a strategy to defend bacterial colonies against fungal attack (De Boer *et.al* 1998).

During two laboratory studies documented by Stauber *et.al* (2000), mean *E. coli* reductions were 94% and they improved over the period of filter use, reaching a maximum of 99%. Field analysis conducted on 55 household filters in the Dominican Republic averaged *E. coli* reductions of 93%. The *E. coli* reductions by the BSF in laboratory and field studies were less than those typically observed for traditional slow sand filters, although as for traditional filters microbial reductions were found to improve over the period of filter use. Similarly an evaluation carried out by Kaiser *et.al* (2002) of 577 intermittent household sand filters located in 6 countries found that on average, 93% of faecal coliforms in the raw water were removed by the filters.

In 2001, a total of 39 sets of samples were tested from intermittent bio-sand filters in Nepal by Hurd *et.al*. The results of filters that were working properly showed using presence/absence tests that 75% removed total coliforms, 83% removed E. coli and 89% removed H2S-producing bacteria. Also in 2001, a set of presence/absence tests carried out by Lee indicated that the filter technology was effective at removing total coliforms with an average removal of 99.5%. Out

of 5 tests, the filter reduced total coliforms from an average of 630 to 3 per 100 ml.

The Global Outreach Students Association (GOSA) in Guatemala carried out 3 tests in 2001. About 25 filters were built and installed during the project. The tests revealed a raw water coliform average of 1781 per 100 ml that reduced to an average of 7 coliforms per 100 ml after 14 days. The average removal rate was 99.6%.

Medair carried out two sets of bacteriological tests in 1999 and 2000 on filters installed in Machakos District, Kenya (Medair, 2000). An average removal rate of 96% was established, while in all but 11 cases turbidity was reduced to less than 5 NTU.

In Kenya and Uganda, a Samaritan's Purse project distributed 25 bio-sand filters, A report of this project produced by Snider (1999) showed removal rates on the 25 filters. The total coliform removal rate was 88.9% during the first week, improving to 98.3% by week eight. The average % reduction was 95 % for total coliforms and 94 % for E. coli.

A report was carried out on a bio-sand filter project in Brazil in 1998 (Liang, 1998). fiftyfive filters were constructed and installed in the Amazon area east of the city of Santarem from July to November 1998. After the filters had been in operation for at least two weeks, the average removal rate for the project was 99.7% for faecal coliform removal, and 98.6% for removal of *E. Coli.*

In 1996 SERVE began research on an appropriate slow sand filter for use in households (Gresham, 1998). After three months of testing with heavily polluted water, the filter was removing 98% to 99% of all contaminating organisms.

An in-depth thesis on the functioning of the household bio-sand filter was carried out in 1995 (Buzunis, 1995). Considerable data was taken over a 55 day test period using influent water averaging 1300 coliforms/100 ml taken from a river lagoon. The experimental filter, which had a water layer of 12.5 cm, was found to be effective in removing 96% of faecal coliform indicators while reducing turbidity levels to < 1 NTU.

The most important aspect of designing a filtration system is its end result. The incorporation of *Trichoderma harzianum* or any other biocontrol agent would only be of any use if it enhanced the working of that system.

Fungi have a long history of being very efficient in the removal of industrial byproducts and waste, in recycling water and in reclamation of hazardous land.

Bioreactors play an important role in industrial economics. Reaction rates should be high and reactor configuration should be easy. Under optimized parameters such as pH, temperature, substrate and media reaction rates can be increased by increasing cell mass concentration. There are two methods commonly used for increasing cell mass concentration inside the reactor, first is the use of a permeable membrane to retain the cells; and the second is the use of immobilized cell technique.

In this study cellulose fiber was used a permeable support for the growing colony. This had the advantage of providing an initial nutrient source for the biofilm. The nature of mixed culture biofilms is dependent on which species are present and what role each species fills. For instance a single species may utilize anaerobic fermentation deep within one biofilm environment but may use aerobic metabolism in another (Costerton *et.al* 1999). Multi-species biofilms are used industrially to achieve several aims including the treatment of wastewater for removal of organics and heavy metals. The presence of multiple species allows for the treatment of waste water that is diverse in composition and that fluctuates in component concentration. In industrial applications including wastewater treatment, usually two types of biofilms are employed, namely, biofilms that grow onto supports such as charcoal, resin, bone charcoal, concrete, clay brick, or sand particles, and biofilms that are formed as a result of flocs and aggregate formation.

Bioremediation is an expanding area of environmental biotechnology and may be defined as the application of biological processes to the treatment of pollution. Much bioremediation work has concentrated on organic pollutants, although the range of substances that can be transformed or detoxified by microorganisms includes both natural materials and inorganic pollutants, such as toxic heavy

metals. There is a distinct lack of appreciation of the potential roles and involvement of fungi in bioremediation, despite clear evidence of their metabolic and morphological versatility. The fundamental importance of fungi in the environment with regard to decomposition and transformation of both organic and inorganic substrates and resultant cycling of elements is of obvious relevance to the treatment of wastes, while the branching filamentous role of growth can allow efficient colonization and exploration of contaminated substrates. This together with the growing importance of fungi as model systems in eukaryotic cell and molecular biology, physiology and biochemistry provides a further rationale for this study.

Processes of natural bioremediation of lignocellulose involve a range of organisms but predominantly fungi (Hammel, 1997). Most studies in the laboratory have shown that fungal species grow well in laboratory conditions and can be readily manipulated in liquid culture to express results of our interest.

Whereas bioremediation of polluted environments is of great importance, bioremediation of water for medical reasons is also very important. Metals when present in our bodies are capable of causing serious health issues by interfering with our normal body functions. Toxic metals accumulate in vital organs and glands of the human body. They also displace vital nutritional minerals from their proper place in the body to provide biological functions e.g. lead and cadmium displace calcium in enzyme reactions disrupting the enzyme reaction to a large extent. Metals can cause genotoxicity as the affect the DNA and immunotoxicity as they are major irritants to the body. The genomic instability by these metals induces cancer (Leonard *et.al* 2004)

Contaminants of this type in drinking water are unfortunately common. They are readily absorbed and therefore difficult to remove by general filters. Biosand filters do not do a good job of removing chemical pollutants such as heavy metals, pesticides, herbicides and petroleum contaminants as completely as pathogens and organic particles. Based on slow sand filter research, the biosand filter may also remove some heavy metals (Muhammad, 1997; Collins, 1998).The incorporation of a fungal bioremediant to the filter gives the added

benefit of enhanced reduction in such contaminants. "Biocurtain" is a term that has been used to describe the process by which large amounts of biomass stop or slow contaminant movement. The biomass can then absorb hydrophobic organic molecules (National Research Council, 1993). A large biomass also can hinder the migration of a contaminant. This is perhaps the main mechanism taking place in sand filters.

Considering the low costs of sand filteration and the number of filters being used in developing countries it is certainly worthwhile to further research the prospects for improving the effectiveness of this system. In conjunction with the introduction of the technology to communities, these filters have been tested by various government, research and health institutions, as well as non governmental agencies. Analysis of 107 long term biosand filters used in Haiti by the Center for Affordable water and sanitation technology in 2005 found an average removal effectivness of 98.5%.

As this study has shown with improved methods and a better understanding it is possible to improve removal rates, with further research and studies it may be possible to make removal rates of pathogens uptill 100%, thereby saving millions of lives through simple Microbial techniques and good sense. This is both the purpose of Biotechnology and the proper use of our knowledge for the betterment of mankind.

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APPENDICES:

APPENDICE TABLES

TABLE 1 : Suppression potentials of Trichoderma strains against fungal pathogens

Suppression potential of Trichoderma harzianum against Aspergillus niger

	normal growth rate of pathogen mm	Suppressed plate 1	suppressed plate 2	suppressed plate 3	Average suppression	reduction percentage
day 1	0	0	0	0	0.00	0
day 2	0.4	0.1	0.1	0.1	0.10	75
day 3	0.9	0.3	0.1	0.2	0.20	78
day 4	1.6	0.4	0.2	0.2	0.27	83
day 5	2.5	0.6	0.3	0.3	0.40	84
day 6	3	1	0.4	0.5	0.63	79
day 7	3.4	1.1	0.5	0.8	0.73	78
				Average		**68**

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Rows	6.767143	6	1.127857	3.881726	0.011596	2.661305
Columns	9.9	3	3.3	11.35755	0.000206	3.159908
Error	5.23	18	0.290556			
Total	21.89714	27				

Suppression potential of Trichoderma koningii against Aspergillus niger

	normal growth rate of pathogen mm	Suppressed plate 1	suppressed plate 2	suppressed plate 3	Average suppression	reduction percentage
day 1	0	0	0	0	0.00	0
day 2	0.4	0.1	0.2	0.1	0.13	67
day 3	0.9	0.2	0.2	0.1	0.17	81
day 4	1.6	0.3	0.3	0.3	0.30	81
day 5	2.5	0.5	0.6	0.5	0.53	79
day 6	3	0.7	0.6	0.6	0.63	79
day 7	3.4	1.0	0.6	0.7	0.77	77
				Average		**66**

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Rows	7.098571	6	1.183095	4.352409	0.005627	2.661305
Columns	9.207143	3	3.069048	11.29051	0.000213	3.159908
Error	4.892857	18	0.271825			
Total	21.19857	27				

Table 1.cont.

Suppression potential of Trichoderma virens against Aspergillus niger

	normal growth rate of pa mm	Suppressed plate 1	suppress plate 2	ssed pl 3	Average suppression	Reduction percentage
day 1	0	0	0	0	0.00	0
day 2	0.4	0.1	0.2	0.1	0.13	67
day 3	0.9	0.2	0.2	0.3	0.23	74
day 4	1.6	0.5	0.5	0.3	0.43	73
day 5	2.5	0.8	0.6	0.5	0.63	75
day 6	3	0.9	0.9	0.8	0.87	71
day 7	3.4	1.1	1	1.2	1.10	68
				e		**61**

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Rows	9.519286	6	1.586548	7.241623	0.000474	2.661305
Columns	7.571429	3	2.52381	11.51965	0.00019	3.159908
Error	3.940476	18	0.219087			
Total	21.03119	27				

Table 2. Selection of media for optimum growth of fungi

i. Growth rate of Trichoderma harzianum in different media

	PDA (mm)	MEA (mm)	SDA (mm)
day 1	1.3	1.1	1
day 2	2.3	2.3	2.1
day 3	3.2	3.1	3
day 4	4.1	4	3.9
day 5	4.2	4.1	4
day 6	4.4	4.3	4.4
Average	[illegible]	3.2	**3.1**

Anova: Two-Factor Without Replication

SUMMARY	*Count*	*Sum*	*Average*	*Variance*
day1	3	3.4	1.133333	0.023333
day 2	3	6.7	2.233333	0.013333
day 3	3	9.3	3.1	0.01
day 4	3	12	4	0.01
day 5	3	12.3	4.1	0.01
day7	3	13.1	4.366667	[illegible]
PDA (mm)	6	[illegible]	3.25	[illegible]
MEA (mm)	6	[illegible]	3.15	[illegible]
SDA (mm)	6	18.4	3.066667	[illegible]

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Rows	[illegible]	5	4.808889	[illegible]	1.29E-13	3.325835
Columns	[illegible]	2	[illegible]	[illegible]	0.001654	4.102821
Error	[illegible]	10	[illegible]			
Total	[illegible]	17				

Table 2. cont.
ii. Growth rate of Aspergillus niger in different media

		MEA (mm)	SDA (mm)
day1		0.3	0.3
day 2		0.8	1
day 3		1.5	1.4
day 4		2.5	2.8
day 5		3.1	3
day 6		3.6	3.3
Average		2.0	**2.0**

Anova: Two-Factor

SUMMARY		*Sum*	*Average*	*Variance*
day1		1	0.333333	0.003333
day 2		2.7	0.9	0.01
day 3		4.4	1.466667	0.003333
day 4		8	2.666667	0.023333
day 5		9.1	3.033333	0.003333
day 6		10.7	3.566667	0.063333
PDA (mm)		2.05		
MEA (mm)		11.8	1.966667	
SDA (mm)		11.8	1.966667	

ANOVA

Source of Variation		*df*		*F*	*P-value*	*F crit*
Rows					E-10	3.325835
Columns					824	4.102821
Error						
Total						

Table 2. cont

iii. Growth rate of Fusarium oxysporum in different media

	PDA (mm)	MEA (mm)	SDA (mm)
day 1	0.2	0.2	0.1
day 2	1	1	0.9
day 3	1.4	1.3	1.2
day 4	2.5	2.2	2
day 5	3	2.8	2.7
day 6	3.2	3.1	2.9
Average	1.9	1.8	**1.6**

Anova: Two-Factor Without Replication

SUMMARY		*Sum*	*Average*	*Variance*
day 1	3	0.6	0.2	0.01
day 2	3	2.9	0.966667	0.093333
day 3	3	3.9	1.3	0.01
day 4	3	6.7	2.233333	0.063333
day 5	3	8.5	2.833333	0.063333
day 6	3	9.2	3.066667	0.063333
PDA (mm)	6	11.3	1.883333	1.[illegible]
MEA (mm)	6	10.7	1.783333	1.[illegible]
SDA (mm)	6	9.8	1.633333	1.[illegible]

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Rows	[illegible]03	8	7.814067	[illegible]	1.25-11	3.325835
Columns	[illegible]	2	0.005	[illegible]	0.001964	4.102821
Error	[illegible]367	10	0.007667			
Total	[illegible]	17				

Table 3 Optimization of pH and Temperature for the production of suppressive metabolites

		20C	25C	30C	Average growth/pH
Fusarium oxysporum	5	2	1.7	2	**1.9**
	5.5	1.5	2.2	2.1	**1.9**
	6	1	1.5	2.5	**1.7**
	6.5	1.7	0.5	1	**1.1**
	7	0.9	1.3	1.5	**1.2**
	7.5	1	1	1.5	**1.2**
Average growth per temperature		1.4	1.4	1.8	
Aspergillus niger	5	1.1		1.5	**1.2**
	5.5	1.1	1	1.1	**1.1**
	6	0.5	0.3	1.5	**1.0**
	6.5	1	0.5	0.9	**0.8**
	7	1	0.5	1	**0.8**
	7.5	1.2	0.3	1	**0.9**
Average growth per temperature		1.0	0.5	1.2	

Anova: Two-Factor Without Replication

SUMMARY	*Count*	*Sum*	*Average*	*Variance*
F-pH 5	3	5.7	1.9	0.0
F-pH 5.5	3	5.8	1.933333	0.1
F-pH 6	3	5	1.666667	0.3
F-pH 6.5	3	3.3	1.1	0
F-pH 7	3	3.7	1.233333	0.0
F-pH 7.5	3	3.5	1.166667	0.0
A-pH 5	3	3.5	1.166667	0
A-pH 5.5	3	3.2	1.066667	0
A-pH 6	3	2.9	0.966667	0
A-pH 6.5	3	2.5	0.833333	0
A-pH 7	3	2.5	0.833333	0
A-pH 7.5	3	2.7	0.9	0.1
20C	12	14	1.166667	0.1
25C	12	12.7	1.058333	0
30C	12	17.6	1.466667	0

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Rows	1.0	11	0.157854	1.3	0.00153	2.258518
Columns	1.0	2	0.536944	4.5	0.023026	3.443357
Error	2.6	22	0.119369			
Total	8.7	35				

Table 4: Culture assays of pathogens against Trichoderma harzianum

	metabolite assay		
Aspergillus niger	NORMAL	SUPPRESSED	PERCENTAGE SUPPRESSED
	0	0	
	0.2	0.1	
	1.4	0.4	
	2.5	0.9	
	3.2	1.8	
Average		0.6	61%
Standard Deveiation		0.7	
Fusarium oxysporum		0	
		0.1	
		0.3	
		0.5	
		0.8	
		1	
		1.2	
Average		0.6	66 %
Standard Deviation		0.5	
	metabolite assay		
	NORMAL	SUPPRESSED	
Aspergillus niger		0	
		0.4	
		0.5	
		0.6	
		1	
		1.2	
		1.6	
Average		0.8	12 %
Standard Deviation		0.7	
Fusarium oxysporum		0	
		0.13	
		0.5	
		1.1	
		1.9	
		2.7	
		3	
Average		1.3	14 %
Standard Deviation		1.2	

Table 4.cont	[illegible] metabolite assay		
	[illegible]L	SUPP[illegible]D	
Aspergillus niger		0	
	[illegible]	0.1	
	[illegible]	0.9	
	1.1	0.2	
	1.5	0.5	
	1.9	0.6	
	[illegible]	0.9	
Average	[illegible]	0.5	**54 %**
Standard Deviation		0.4	
Fusarium oxysporum		0	
	[illegible]	0.03	
	[illegible]	0.1	
	[illegible]	0.6	
	[illegible]	0.5	
	[illegible]	0.8	
	[illegible]	1.2	
Average		0.5	54%
Standard Deviation		0.4	

Table 5: Reduction in number of spores produced by *trichoderma* in submerged conditions (water tolerant strain)

	No of spores grown in PDB	No of spores grown in water	Percentage reduction
	10	10	0
	17	13	-21
	29	15	-49
	38	17	-55
	44	21	-53
	50	22	-56
	51	24	-54
	50	24	-52
	48	26	-46
	45	24	-48
	45	23	-49
	41	24	-46
	40	23	-42
	42	24	-42
Average	4		
Standard Deviation	57	1.95	

Table 6. Average spore counts in headwater in the presence and absence of water grown Trichoderma harzianum

Absence		Presence	
10		10	
17		18	
29		35	
38		40	
43		42	
50		41	
51		45	
50		41	
48		40	
45		39	
45		35	
44		25	
40		20	
42		15	
Average	39.4	Average	**31.9**
Standard Deviation	12.45		**11.73**

Table 7. Fungal counts in the biofilm headwater in the presence and absence of Trichoderma harzianum

Presence		Absence
10		10
14		18
18		35
27		40
38		42
44		41
35		45
30		41
26		40
27		39
12		35
15		25
8		20
10		15
average	**22**	32
Standard Deviation	11.60	11.76

Table 8. Bacterial counts in the biofilm headwater in the presence and absence of *Trichoderma harzianum*

Absence		Presence
1197		1026
1083		969
912		798
1123		1027
1168		1182
1576		1370
2166		1700
1938		1368
1140		1107
1824		1425
2052		2001
2800		2420
3480		1[illegible]6
1168		1154
1596		1[illegible]00
1824		1[illegible]25
3300		2[illegible]80
3100		2[illegible]00
1410		1[illegible]30
2907		2[illegible]40
880		8[illegible]1
770		6[illegible]2
1197		9[illegible]3
413		3[illegible]3
2565		1[illegible]38
1767		1[illegible]30
Average	1744	1[illegible]67
Standard Deviation	836.88	5[illegible]6.89

Table 9. Biofilm quantification by total carbohydrate production

	Carbohydrate on disc mg/ml	glucose standard mg/ml	Carbohydrate from washings mg/ml
Day 1	0.112	0.181	0.12
Day 2	0.186	0.3	0.127
Day 3	0.21	0.467	0.164
Day 4	0.299	0.481	0.232
Day 5	0.301	0.636	0.291
Day 6	0.322	0.664	0.324
Day 7	0.401	0.675	0.352
Day 8	0.444	0.701	0.401
Day 9	0.459	0.787	0.422
Day 10	0.532	0.996	0.435
St Dev	**0.133288**		**0.120359**

Anova: Two-Factor Without Replication

SUMMARY	*Count*	*Sum*	*Average*	*Variance*
Day-1	3	0.413	0.137667	0.00147
Day-2	3	0.613	0.204333	0.00773
Day-3	3	0.841	0.280333	0.026662
Day-4	3	1.012	0.337333	0.016602
Day-5	3	1.228	0.409333	0.038558
Day-6	3	1.31	0.436667	0.038761
Day-7	3	1.428	0.476	0.030364
Day-8	3	1.536	0.5153	0.0
Day-9	3	1.458	0.5	0.040363
Day-10	3	1.963	0.654333	0.089069
disc	10	3.266	0.3266	0.017766
glucose standard	10	5.588	0.5588	0.056523
wash	10	2.868	0.2868	0.013486

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Rows	0.704631	9	0.078292	14.87727	1.27E-06	2.456281
Columns	0.538456	2	0.269228	51.17277	3.78E-08	3.554557
Error	0.094799	18	0.00526			
Total	1.337886	29				

Table 10a Percentage reduction in Total Dissolved Solids in biofilm with **Trichoderma harzianum**

	Headwater	Outlet water	Percentage reduction
	340	340	0
	380	380	0
	380	340	11
	380	335	12
	390	345	12
	390	335	14
	370	330	11
	350	300	14
	370	325	12
	355	310	13
	350	310	11
	330	300	9
	350	300	15
	345	300	13
	340	290	14
	320	275	14
	310	280	10
	310	270	13
	310	270	13
	300	250	17
	300	260	13
	280	230	15
	280	240	14
	280	230	17
	280	230	17
	275	230	16
	275	240	14
	290	240	17
	270	230	16
	270	240	17
	280	250	18
	260	220	15
	280	240	17
	270	240	14
	270	230	18
	260	220	18
	260	230	20
	260	230	18
	260	230	18
Average			14
Standard Deviation	41.13	55.57	

Table 10 b. Percentage reduction in Total Dissolved Solids in biofilm **without Trichoderma harzianum**

	Headwater	Outlet water	Percentage reduction
	340	340	0
	390	390	0
	380	380	0
	395	360	9
	390	330	15
	390	360	8
	[illegible]	350	10
	[illegible]	350	10
	340	280	10
	355	310	13
	355	310	13
	390	330	15
	390	330	15
	390	330	14
	[illegible]	340	11
	[illegible]	310	13
	[illegible]	310	14
	310	310	10
	310	310	3
	[illegible]	330	15
	[illegible]	330	15
	[illegible]	330	15
	[illegible]	310	13
	[illegible]	310	16
	[illegible]	310	14
	[illegible]	290	8
	240	210	16
	240	220	12
	240	220	17
	240	220	17
	240	220	17
	250	210	16
	240	200	17
	240	226	6
	240	210	16
	240	205	18
	[illegible]	205	18
	250	200	20
	[illegible]	205	18
	Average		12
Standard Deviation	[illegible]	[illegible]	

Table 11 a Percentage reduction in pH in biofilm with Trichoderma harzianum

	Headwater	Outlet water	Percentage reduction
	7.5	7.35	2.0
	7.89	7.69	2.5
	7.887	7.76	1.6
	7.84	7.79	0.6
	7.75	7.76	0.1
	7.75	7.63	1.5
	7.69	7.61	0.9
	7.8	7.73	1.7
	7.85	7.81	1.8
	7.6	7.52	1.1
	7.79	7.5	3.6
	7.83	7.53	4.6
	7.82	7.59	4.2
	7.75	7.6	2.2
	7.79	7.55	3.0
	7.88	7.61	3.4
	7.83	7.51	4.2
	7.86	7.61	3.4
	7.76	7.52	3.1
	7.83	7.49	4.6
	7.75	7.7	1.9
	7.81	7.53	3.8
	7	7.62	1.0
	7.87	7.49	4.1
	7.91	7.54	4.7
	7.79	7.52	3.9
	7.93	7.57	4.6
	7.89	7.32	2.3
	7.85	7.29	4.2
	7.83	7.42	4.1
	7.75	7.49	4.6
	7.7	7.41	3.8
	7.69	7.39	3.8
	7.75	7.44	4.0
	7.06	7.3	4.4
	7.71	7.46	3.4
	7.68	7	4.8
	7.82	7.58	4.4
	7.56	7.25	4.3
Average			3.3
Standard Deviation		0.156	

Table 11 b. Percentage reduction in pH in biofilm without Trichoderma harzianum

	Head[illegible]	Outlet water	Percentage reduction
	7.5	7.46	0.5
	7.72	7.63	1.2
	7.80	7.73	2.0
	7.85	7.82	0.4
	7.982	7.78	2.5
	7.74	7.85	1.0
	7.81	7.63	2.3
	[illegible]	7.75	1.1
	[illegible]	7.91	2.3
	7	7.52	3.6
	7.[illegible]	7.48	2.6
	7.[illegible]	7.88	1.0
	7.[illegible]	7.52	2.8
	7.[illegible]	7.57	4.2
	[illegible]	7.51	5.2
	[illegible]	7.58	4.5
	[illegible]	7.74	2.7
	[illegible]	7.56	4.5
	7.[illegible]	7.57	2.1
	7.[illegible]	7.53	2.3
	7.[illegible]	7.57	3.2
	7.[illegible]5	7.57	3.4
	[illegible]	7.5	2.3
	[illegible]	7.51	1.2
	[illegible]	7.73	5.6
	[illegible]	7.57	3.7
	7.[illegible]	7.53	1.7
	7.[illegible]	7.42	1.6
	7.[illegible]	7.39	2.8
	[illegible]	7.52	0.7
	7.[illegible]	7.46	1.4
	7.[illegible]	7.51	2.0
	[illegible]	7.48	4.5
	[illegible]	7.51	2.2
	7.[illegible]	7.5	2.7
	7.[illegible]	7.49	3.1
	7.[illegible]	7.4	0.0
	7.[illegible]	7.6	1.0
	7.[illegible]	7.73	0.4
Average			2.4
Standard Deviation	[illegible]	0.16	

Table 12 a Percentage variation in conductivity in biofilm with Trichoderma harzianum

	Inlet water	Outlet water	Percentage reduction
	[illegible]	485.7	0.0
	542.9	542.9	0.0
	[illegible]	485.7	10.5
	[illegible]	478.6	11.8
	[illegible]	492.9	11.5
	[illegible]	478.6	14.1
	[illegible]	471.4	10.8
	[illegible]	[illegible]	11.3
	[illegible]	464.3	12.2
	[illegible]	442.9	12.7
	[illegible]	442.9	11.4
	[illegible]	[illegible]	9.1
	[illegible]	471.4	15.4
	[illegible]	[illegible]	13.0
	[illegible]	[illegible]	[illegible].9
	[illegible]	[illegible]	[illegible].1
	[illegible]	[illegible]	9.7
	[illegible]	[illegible]	12.9
	[illegible]	[illegible]	12.5
	[illegible]	[illegible]	16.7
	[illegible]	[illegible]	13.3
	[illegible]	[illegible]	[illegible].8
	[illegible]	[illegible]	[illegible].0
	[illegible]	[illegible]	[illegible].7
	[illegible]	[illegible]	[illegible].7
	[illegible]	[illegible]	[illegible].4
	[illegible]	[illegible]	[illegible].3
	[illegible]	[illegible]	[illegible].2
	[illegible]	[illegible]	[illegible].8
	[illegible]	[illegible]	[illegible].2
	[illegible]	[illegible]	[illegible].4
	[illegible]	[illegible]	[illegible].4
	[illegible]	342.9	[illegible].2
	[illegible]	[illegible]	[illegible].3
	[illegible]	[illegible]	[illegible].4
	[illegible]	[illegible]	[illegible].0
	[illegible]	[illegible]	[illegible].0
	[illegible]	[illegible]	[illegible].4
	[illegible]	[illegible]	[illegible].4
Average			**[illegible].2**
Standard Deviation		[illegible]	

Table 12 b. Percentage variation in conductivity in biofilm without Trichoderma harzianum

	...ter	Outlet water	Percentage reduction
	4...	485.7	0.0
	5...	557.1	0.0
	5...	542.9	0.0
	5...	500.0	11.4
	5...	471.4	15.4
	5...	492.9	11.5
	5...	492.9	11.5
	5...	492.9	11.5
	4...	492.0	9.7
	5...	485.9	12.7
	5...	485.9	12.7
	5...	471.4	15.4
	5...	471.4	15.4
	5...	478.6	14.3
	4...	485.7	10.5
	5...	485.9	12.7
	5...	485.9	12.9
	4...	485.7	12.9
	5...	485.0	12.5
	5...	471.4	15.4
	5...	471.4	15.4
	5...	471.4	15.4
	4...	485.7	12.7
	4...	485.0	12.0
	5...	485.9	12.9
	4...	485.0	12.0
	4...	485.0	12.0
	4...	485.9	12.0
	4...	485.7	12.7
	4...	485.7	12.7
	4...	485.7	12.7
	4...	485.0	12.0
	4...	485.7	12.7
	4...	485.9	12.0
	4...	485.9	12.0
	4...	485.9	12.0
	4...	485.0	12.0
	4...	485.7	12.0
	4...	485.9	12.0
Average			12.3
Standard Deviation			1.2

Table 13 a. Percentage reduction in turbidity in biofilm with Trichoderma harzianum

	Outlet water	Percentage reduction
23	2.39	90
36	1.11	97
38	1.11	97
36	0.85	98
39	0.68	98
19	0.68	96
30	0.68	98
24	0.85	96
17	0.6	96
18	0.51	97
25	0.34	99
23	0.23	99
49	0.34	98
32	0.45	98
26	0.4	98
19	0.51	97
33	0.4	99
31	0.28	99
21	0.51	97
29	0.4	98
34	0.57	98
20	0.4	98
19	0.62	97
33	0.51	98
33	0.51	98
31	0.74	99
31	0.28	99
32	0.68	97
31	0.4	99
39	0.51	98
27	0.51	97
29	0.51	98
31	0.51	98
23	0.45	98
31	0.51	98
21	0.4	98
29	0.34	99
30	0.57	98
33	0.4	99
Average		98
Standard Deviation	6.155	0.158

Table 13 b. Percentage reduction in turbidity in biofilm without Trichoderma harzianum

	Outlet water	Percentage reduction
[illegible]	2.8	88
[illegible]	1.5	96
[illegible]	2.2	94
[illegible]	2.2	94
39	2.6	93
19	1	95
39	1.8	95
[illegible]	1.5	94
[illegible]	1	94
[illegible]	1	94
[illegible]	1	96
[illegible]	1	97
[illegible]	1	94
[illegible]	1	96
[illegible]	1	96
[illegible]	1	95
[illegible]	1	96
[illegible]	1	97
[illegible]	1	95
[illegible]	1	95
[illegible]	1	96
[illegible]	1	94
[illegible]	1	95
[illegible]	1	96
[illegible]	1	97
[illegible]	1	96
[illegible]	1	96
[illegible]	1	96
[illegible]	1	97
[illegible]	1	96
[illegible]	1	95
[illegible]	1	95
[illegible]	1	95
[illegible]	1	95
[illegible]	1	96
[illegible]	1	96
[illegible]	1	97
[illegible]	1	97
[illegible]	1	97
Average		95
Standard Deviation	0.468	

Table 14 a **Percentage** reduction in colour in biofilm with Trichoderma harzianum

	Outlet water	Percentage **reduction**
16	0.49	97
12	0.29	98
15	0.1	99
13	0.04	100
18	0.08	100
12	0.23	98
6	0.14	98
5	0	100
6	0	100
4	0	100
8	0.04	100
15	0.12	99
17	0.08	100
11	0.16	99
12	0.14	99
19	0.25	99
8	0.14	99
6	0	100
15	0.1	99
17	0.14	99
19	0.12	99
12	0.1	99
13	0.14	99
16	0.12	99
10	0.23	98
2	0	100
9	0.1	99
10	0.1	99
14	0.16	99
12	0.16	99
13	0.35	99
16	0.12	99
18	0.08	100
13	0.16	99
16	0.12	99
13	0.12	99
17	0.14	99
10	0.06	100
15	0.12	99
Average		
Standard Deviation	4.512	0.094

Table 14 b Percentage reduction in Colour in biofilm without Trichoderma harzianum

	Hard water	Outlet water	Percentage reduction
	16	1	94
	12	1.11	91
	15	1.11	93
	13	0.85	93
	18	0.68	96
	12	0.68	94
	6	0.68	89
	5	0.85	83
	6	0.6	90
	4	0.51	87
	8	0.34	96
	15	0.23	98
	17	0.34	98
	11	0.45	96
	12	0.4	97
	10	0.31	97
	8	0.4	95
	6	0.33	95
	15	0.71	97
	17	0.4	98
	11	0.37	97
	12	0.4	97
	10	0.52	95
	15	0.71	97
	13	0.71	95
	2	0.11	85
	9	0.33	97
	10	0.73	93
	10	0.4	98
	11	0.31	97
	13	0.51	96
	10	0.71	97
	12	0.31	97
	13	0.45	97
	10	0.31	97
	11	0.4	97
	12	0.24	98
	11	0.37	96
	11	0.31	97
Average			95
Standard Deviation	4.12	0.211	

Table 15 a Percentage reduction in Bacteria in biofilm with Trichoderma harzianum

	Headwater	Outlet water	Percentage reduction
	2500	3100	-24
	2400	3220	-34.
	[illegible]	3400	-28
	2400	3100	-29
	2420	3110	-29
	2200	2700	-23
	[illegible]	2600	-6
	[illegible]	2600	-7
	[illegible]	[illegible]	15
	[illegible]	2100	15
	[illegible]	1720	30
	[illegible]	1260	51
	[illegible]	1420	55
	[illegible]	480	81
	[illegible]	750	68
	[illegible]	800	67
	[illegible]	750	68
	[illegible]	720	71
	[illegible]	560	75
	[illegible]	240	90
	[illegible]	380	85
	[illegible]	480	79
	[illegible]	380	84
	[illegible]	620	77
	[illegible]	270	88
	[illegible]	200	[illegible]
	[illegible]	100	[illegible]
	[illegible]	200	92
	[illegible]	180	92
	[illegible]	180	92
	[illegible]	180	92
Average			[illegible]

Table 15 b Percentage reduction in Bacteria in biofilm without Trichoderma harzianum

Headwater	Outlet water	Percentage reduction
2500	3100	-24
2400	3000	-25
2650	2980	-12
2400	3230	-35
2420	2800	-16
2200	2565	-17
2451	2820	-15
2420	2600	-7
2680	2600	3
2470	2300	7
2451	1500	38
2550	1030	60
3150	930	70
2500	840	66
2340	1210	48
2450	740	70
2350	650	64
2450	680	72
2240	200	9
2400	350	85
2570	670	74
2300	840	63
2400	690	71
2700	1130	58
2330	700	70
2400	650	73
2530	1200	52
2360	2300	02
2200	1100	50
2345	1150	50
2330	1160	50
Average		48

Table 16 Efficiency of Trichoderma harzianum biofilms in reducing salts and metals from influent water

	Inlet	outlet biofilm without Trichoderma harzianum	% difference	outlet biofilm with Trichoderma harzianum	% difference	Efficiency comparison
Iron	0.1	0.07	30	0.03	70	0.57
chromate	0.15	0.13	13	0.12	20	0.35
sulfate	100	60	40	16	52	0.52
nitrate	1.5	1.4	15	1.2	20	0.25
chloride	100	92	8	91	9	0.11

Table 17: Approximate number of bioactive microbial metabolites according to their producers and bioactivities

Source	Bioactive secondary microbial metabolites				
	Antibiotics		Bioactive metabolites		Total bioactive metabolites
	Total	With other activity	No of antibiotic activity	Antibiotics plus other bioactives	
Bacteria	[illegible]	780	900	[illegible]	3800
Eubacteriales	2170	570	580	1150	2750
Bacillus sp	[illegible]	235	65	[illegible]	860
Pseudomonas sp	[illegible]	185	185	370	795
Myxobacter	[illegible]	130	10	[illegible]	410
Cyanobacter	[illegible]	80	[illegible]	[illegible]	640
Actinomycetales	[illegible]	2100	[illegible]	[illegible]	10100
Streptomyces sp	(6550)	1020	[illegible]	[illegible]	7630
Rare actinos	[illegible]	590	[illegible]	[illegible]	2470
Fungi	[illegible]	[illegible]	[illegible]	[illegible]	8600
Microscopic fungi	[illegible]	[illegible]	[illegible]	[illegible]	6450
Penicillium/Aspergillus	[illegible]	[illegible]	[illegible]	[illegible]	1950
Basidiomycetes	[illegible]	[illegible]	[illegible]	[illegible]	2000
Yeasts	[illegible]	[illegible]	[illegible]	[illegible]	140
Slime moulds	[illegible]	5	[illegible]	[illegible]	60
Total Microbial	[illegible]	[illegible]	[illegible]	[illegible]	22500
Protozoa	[illegible]	[illegible]	5	[illegible]	50

Ref :

Berdy J., 2005. Bioactive microbial metabolites, a personal view. J Antibiot. 58(1):1-26