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Full Length Research Paper

Role of microbes in nitrogen and metal hyperaccumulation by taxilaion *Eichhornia crassipes*

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Water hyacinth (*Eichhornia crassipes*) is abundantly found in waste water and in soil (landfills) of Taxila, Pakistan. It is a well known hyper-accumulator, bioindicator and phytoremediator of nitrogen and metals. In the present study, three different experiments were performed and tried to lay down a foundation for further research in the field of environmental studies. Firstly, we assessed the metallic concentrations of Taxilian water hyacinth; secondly we isolated some selected microbes from its roots and; thirdly, these microbes were identified by biochemical tests and were purified. Our results verify the fact that besides nitrogen fixation, presence of metal binding proteins and humic acids in water hyacinth, these microbes play a key role. From the literature, we also tried to show the relationship of these microbes with absorption, adsorption, phytoextraction and phytoremoval of nitrogen and metals from waste water and contaminated soil with water hyacinth. It is also evident that water hyacinth may be used as a tool in bio mining of metals.

Key words: Water hyacinth, *Aspergillus niger*, *Azotobactor*, *Thiobacillus thiooxidans, Thiobacillus ferrooxidans,* environmental pollution.

INTRODUCTION

Taxila is an important archaeological site in Pakistan containing the ruins of the Gandharan city of Takshashila (also Takkasila or Taxila). It remained as a center of one of the oldest civilization of the world that is, Gandhara. Taxila is situated at the western region of the Islamabad Capital Territory- Rawalpindi and on the border of the Punjab and North West Frontier Province (NWFP) about 30 km west-north-west of Islamabad, just off the Grand Trunk Road. Taxila is situated at (latitude: 33° 46′ 45″ N 33.779167° longitude: 72° 53′ 15″ E 72.8875°). The water and soil pollution is increasing in Taxila due to industrialization and urbanization. The pollutants of serious concern due to pollutant's carcinogenic and mutagenic nature include lead, chromium, mercury, uranium, selenium, zinc, arsenic, cadmium, gold, silver, copper, nickel. These toxic materials may be derived from mining operations, refining ores, and sludge disposal, fly ash from incinerators, processing of radioactive materials, metal plating, and manufacture of electrical equipments, paints, alloys, batteries, pesticides or preservatives. Taxila's soil belongs to the Missa soil series (typic ustochrept) (Ali and Higgins, 1967). Aquatic plants like water hyacinth can act as phytoremediator of metals and organic waste from the Missa soil series like loamy and calcareous soil of Taxila Pakistan (Chavan et al., 2008; Lone et al., 2008, Chaney, et al., 2008). Floating water hyacinth plants are capable of assimilating large quantities of trace elements and heavy metals, some of which are essential for plant's growth. Water hyacinth in English, Jacinthe d'eav in French and *Eichhornia crassipes* in biology, it belongs to the family Pontederiaceae (Hill and Cilliers, 1999). Various studies (Zhu et al., 1999; Olivares-Rievmont et al., 2007; El-Gendy, 2008; Mishra et al., 2008) reported the phytoremediation of metals by water hyacinth. The results showed that water hyacinth is a promising candidate for the phytoremediation of wastewater polluted with As, Cd, Cr, Cu, Se, Pb, Hg, As, Zn and Ni). If the Cr (VI) is supplied to water hyacinth through culture media, it can be reduced to Cr (III) by lateral roots (Lytle, et al., 1998). Water hyacinth can remove nitrogen as contained in roots attached particulate matter very effectively.

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Nitrogen removal was 60 - 80% from waste water (Billore et al., 1998; Cozar et al., 2007; Fox, et al., 2008). Poudel and Simon (2008) found 44% total nitrogen removal. It contains large number of metal binding proteins like methionine (Matai and Bagchi, 1980; El-Enany and Mazen, 1996).

Water hyacinth grown in waste water and soil of landfills may contain some microbes and they may play some role in phytoremediation of metals and nitrogen.

Aspergillus niger, Azotobacter, Thiobacillus thiooxidans (*Acidithiobacillus thiooxidans*) and *T. ferrooxidans* (*A. ferrooxidans*) are playing vital role in bioremediation of metals and nitrogenous compounds (Simmands, 1979; Pelczar Jr. et al., 1986; Sugio et al., 1988; Wong et al., 1998; Frattini et al., 2000; Ather and Ahmed, 2002; Villar and Garcia, 2002; Allegretti et al., 2006; Mulligan and Galvez-Cloutier, 2003; Ahalya et al., 2003; Mulligan and Kamali, 2003; Spanelova et al., 2003; Mujeeb-ur-Rahman and Gul, 2003; Srivastava and Thakur, 2006; Zhigang et al., 2006; Ali et al., 2008; Kiese et al., 2008; Pathak et al., 2009).

In present study firstly our aim is to see the metallic concentrations of Taxilian water hyacinth by ICP-AES (inductively coupled plasma atomic emission spectrometry) with an ultrasonic nebulizer. As it shows maximum detection limits with less chance of error (Liao and Chang, 2004). Secondly to isolate, identify selected microbes may present in roots of water hyacinth and their contribution in phyto-removal of nitrogen and metals by hyacinth.

MATERIALS AND METHODS

Sample collection and metal analysis

The samples of soil and water were collected from roots of water hyacinth grown wildly in soil and water of Banni Mohallah, Ulistan colony, Railway crossing area and Wah Cantt area of Tehsil Taxila, district Rawalpindi of Pakistan. The soil samples were laced in polyethylene bags and the water samples were collected in sterile glass bottles with screw caps and were labeled properly (Table 1).

All samples were taken to the laboratory and were stored at 4°C until microorganism's isolation. Water hyacinth plant samples were collected washed, dried. Dry weight, moisture, ash and crude fibre contents were calculated by the method described by Okoye et al. (2002). Dry plant samples were digested in HNO_3 and $HClO_4$ as reported by Rayon et al. (2001). The digested samples were analyzed by Thermo Jarrell Ash Inductively coupled plasma atomic spectrometer. The detector was charged using following conditions: RF power was 1150 W, flush pump rate was 1000 rpm, CID (charge injection device) integration time (low) was 15 s, CID integration time (high) was 5 s, argon flow rates were 0.51/min (auxiliary) and 30 psi (nebulizer), spray chamber was cyclone spray and nebulizer was Burgenar trace. The results were expressed in µg/g of dry plant.

Samples preparation for microbial study

Rhizosphere microorganisms were isolated from each sample by serial dilution and spread plate method. One-gram soil was dispersed in 10 ml of sterile distilled water and thoroughly shaken as described by Sharpley (1960). One ml of above dilution was again transferred to 9 ml of sterile distilled water to form 10^{-2} dilution. Similarly, 10^{-3}, 10^{-4}, 10^{-5} dilutions were made from soil samples. Water samples were also diluted in the same manner. An aliquot of 0.1 ml from each dilution was taken with micropipette (0.01 - 0.1 ml range) and plated on agar and in liquid media specific for each microorganism to be isolated.

Samples were collected in screw cap glass bottles, which were cleaned sequentially with detergent, tap water, 24 h soaked in 1% HNO_3 and several times with sterile distilled water, and then were dried at 100ºC for one hour, cooled to room temperature, capped and labeled. Samples were immediately stored at 4°C until micro-organisms' isolation. Analytical grade (A.R) chemicals were used in the preparation of reagents and standards (Mahmood et al., 1997).

Media preparation and growth conditions

Various two liter media were prepared for different microorganisms. For media preparation majority of chemicals and media compounds were obtained from: BDH Laboratory Chemical Division (Poole, Dorset, England), DIFCO Laboratories (Detroit Michigan, USA) and Merck (Darmstadt, Germany).

The following media were prepared for the growth of the following microbes by already documented procedures.

(1) *T. ferrooxidans* media with ferrous sulfate (pH was 1.4).
(2) *Thiobacillus* media (pH was 2.8).
(3) *Thiobacillus* media I (for *Acidophilic thiobacillus*) (pH was 4.2).
(4) Sabouraud dextrose media for *A. niger* (pH was 5.6) (Tabassum, 2003; Verweij et al., 2007)
(5) Burk's media for *Azotobacter* (pH was 7-7.5) (Kausar 2004; Revillas et al., 2005)

Isolation and purification

Five types of media were used for isolation of microorganisms. All these media were divided into two groups. One group was consisted of solid agar while other contained broth media (without agar). Samples of water and soil were diluted and inoculated in both types of media of five groups. Some were placed in shaker (water bath with fixed temperature) while others were placed in incubator at specified temperature. Temperature was fixed at 30°C but for Burk's media it was 26°C and Sabouraud dextrose medium was incubated at 40°C. Initial pH of media was already noted by standard methods (Ryan et al., 2001). Specified conditions are given in results. After one-week colonies appeared on these media and broth became turbid. These colonies were picked and streaked on fresh solid media and broth. Colonies were inoculated in new broth for purification. This repeated process of inoculation and growth was used for the purification of these microbes. Isolated strains were enriched and purified by standard procedures (Jiao et al., 2005).

Identification and characterization of microbes

All purified microbes were physically and biochemically characterized by certified methods. Gram staining was performed as reported by Pandey (1994), and colony morphology was studied for characteristics like colony color, colony surface, colony elevation, colony surface margin, colony surface texture and shape (Smibert and Krieg, 1994; Krieg and Holt, 1984; Shakoori et al., 2003).

Gram's stain has long been used to separate bacteria into two groups; the gram-negative and positive types. An already reported procedure was used for this experiment (Pandey, 1994). A smear

Table 1. Sampling scheme for microbial isolation, identification and purification

S/no.	Sample nature	Location of sample	Symbol/Abbreviation
1.	Soil	Sample of soil collected from roots of water hyacinth grown at Gulistan colony, Taxila Rawalpindi	A
2.	Soil	Sample of soil collected from roots of Water hyacinth grown at Banni Mohallah, Taxila, Rawalpindi	B
3.	Soil	Sample of soil collected from bottom of a pond. In pond water hyacinth plants were floating at Railway crossing area of Taxila city, Rawalpindi	C
4.	Soil	Sample of soil collected from roots of water hyacinth grown at Wah Cantt. Taxila, Rawalpindi.	D
5.	Water	Sample of water collected from bottom of a pond. In pond water hyacinth plants were floating at Railway crossing area of Taxila city, Rawalpindi	E
6.	Water	Sample of water collected from submerged roots of water hyacinth grown at Banni Mohallah, Taxila, Rawalpindi	F

was made on a slide. It was dried, passed through a flame two or three times and was flooded with gram's stain. It was washed, blotted dry and examined under a microscope.

Gram stained slides were prepared and observed under a microscope (Leitz, model Dialux22) at the 1000× magnification. This microscope was fitted with a camera (model Wild MPS12). Some photographs were taken and shown in the results.

A sterilized needle was used to pick a part of *A. niger* colony from the Petri dish containing the fungus and stained with aniline blue. The slide was prepared and observed under the microscope.

For biochemical characterization, motility, catalase, oxidase, hydrogen sulfide (H_2S), nitrate reduction and gas production tests were performed (Krieg and Holt, 1984; Shakoori et al., 2003). All chemicals and substances used were of high purity.

For hydrogen sulfide test the test organisms were inoculated on triple-sugar iron (TST) solid and broth media. These were incubated at 37°C for 96 h. blackening of the colonies was observed (Kausar, 2004). This method is applicable as yet (Shah, 2006). Whatman filter papers were soaked in 1% solutions of $CdSO_4.8H_2O$ and Pb $(CH_3COO)_2$. These were dried in oven. These filter papers were suspended over liquid broth and were placed over solid media for one day. These filter papers changed color. Those who were soaked in cadmium sulfate solution became yellow while Lead acetate soaked became black due to formation of sulfides. These sulfides were formed due to reaction of H_2S with cadmium and lead.

For mobility test semi-solid media was inoculated with *T. thiooxidans* and *T. ferrooxidans* bacteria with the help of a straight needle about 0.5 inches deep in the tube containing media. These tubes were incubated at 30°C and pattern of bacterial growth was checked after three days. Same procedure was adopted for *Azotobacter* at 26°C (Kausar, 2004).

For Catalase production test three ml of hydrogen peroxide (3% v/v) was taken in test tubes and mixed with 24 h old inocula with the help of glass rod. Immediate bubbling in test tubes indicated the production of catalase enzyme (Kausar, 2004).

In oxidase production test only a few organisms produce oxidase enzyme, which has the ability to utilize oxygen directly due to presence of copper or iron as the part of their molecules. Inoculated agar tubes were incubated and the growth area was covered with oxidase reagent for two minutes. Excessive reagent was poured off and the colonies were watched for change in color. Change in colony color to red then gradually black was indication of positive reaction (Kausar, 2004).

For nitrate reduction test 5 ml peptone water was taken in test tubes and autoclaved. These tubes were inoculated with isolates and incubated at 37°C for 24 h. Nitrate broth (5.0 ml) containing 0.1% (weight/volume) KNO_3 was incubated with test organisms by adding incubated peptone water and then kept at 37°C for 48 h. After incubation, 1.0 ml each of sulfanilic acetic acid and alpha napthylamine was added, thoroughly shaken and development of color was noted. Red and purple color indicated the presence of nitrite (positive reaction). Absence of nitrite (negative reaction) was further confirmed by zinc dust.

For sulfanilamide solution 5 g of AR grade was dissolved in a mixture of 50 ml concentrated hydrochloric acid and 300 ml deionized water. This solution was stable for several months.

For alpha napthylamine solution AR grade alpha napthylamine was dissolved in deionized water. It was slightly soluble in water alkaline in nature and was stored in dark glass bottle. It was stable for one month (Burlarge et al., 1998; Kausar, 2004).

RESULTS AND DISCUSSION

In our experiment, the main purpose was to quantify metallic concentrations of Taxilian water hyacinth by ICP-AES and to identify microbes from the roots of hyacinth, growing wildly in soil and floating in wastewater. So isolation, identification and purification of three bacteria and one fungus were carried out. All the samples were applied to five different media for isolation purification and identification. Results of these experiments are summarized in tables based on the type of media.

The first study is based on analysis of metal hyper-accumulator and its different parameters. Fresh weight, dry weight, moisture content, ash content and crude fibre content for five samples of water hyacinth were assessed. The results were 59.2 ± 14.63 g fresh weight, 4.8 ± 1.16 g dry weight, 3.6 ± 1.0 g of ash, 90.8 ± 0.40% moisture of fresh weight. Dry plants contain 77 ± 2% ash and 1.4 ± 0.48% crude fibre. Elemental concentrations of boron and metals in water hyacinth measured by ICP-AES were 100.66 ± 3.68 B, 5979 ± 2.44 Al, 56 ± 0.81 Cu, 1218 ± 1.63 Fe, 8444 ± 3.26 Mg, 898 ± 0.81 Mn, 216±1.63 Mo,

Table 2. *T. ferrooxidans* media with ferrous sulfate and experimental conditions.

Treatments/Tests	Sample A		Sample B		Sample C	
	Broth	Solid	Broth	Solid	Broth	Solid
T. thiooxidans	+ve	+ve	+ve	+ve	+ve	+ve
T. ferrooxidans	+ve	+ve	+ve	+ve	+ve	+ve
Date of experiment	**1st to 30th November 2008**					
Temperature of oven (℃)	30	30	30	30	30	30
Temperature of shaker (℃)	30	-	30	-	30	-
Optimum growth pH	1.4	1.4	1.4	1.4	1.4	1.4
Colony morphology	Colorless for *T. thiooxidans* and yellow for *T. ferrooxidans*					
Shape	Oval for *T. thiooxidans* and rod shape for *T. ferrooxidans*					
Mobility	Polar flagella *T. thiooxidans*, perititrious flagella *T. ferrooxidans*					
Spore formation	-ve	-ve	-ve	-ve	-ve	-ve
Volume of media (ml)	100	100	100	100	100	100
H_2S Test	+ve	+ve	+ve	+ve	+ve	+ve

10 ± 0.81 Ni, 158 ± 0.81 Ti and 285 ± 0.82 Zn respectively in µg/g of dry plant.

The results of fresh weight, dry weight, moisture content, ash content and fibre content for five samples of water hyacinth were similar with the previous studies (Little and Henson, 1968; Okoye et al., 2002). All these values are showing very minor variations and were calculated by the formulas described by Okoye et al., (2002). El-Gendy (2008), studied modeling of heavy metals removal from municipal landfill leachate by using living biomass of water hyacinth. They observed the increase in concentration of total heavy metals of water hyacinth. Our results are showing its ability as hyper-accumulator and high metal content in soil or water of its habitat. It also shows that the level of metallic pollution in industrial and domestic waste water and soil (landfill or solid trash disposal site).Analytical data also shows that it contains suitable amount of Ti which can be used in the preparation of photocatalyst. This amount of Ti like other metals can be extracted/leached down by desorption with HCl by using its dry mass. Metal hyper-accumulation depends upon the impact of various factors, such as microclimate, hydrology, soil physics, soil chemistry, and soil biology (Liao and Chang, 2004). Our study provides quantitative information about metals absorbed by water hyacinth from soil of Taxila and lays the foundations for more detailed research work. The most important properties related to the thermal conversion of biomass are various but important are moisture content, ash content and volatile matter content. The moisture content depends on water's weight. It can affect the value of bio-mass. Volatile organic matter can be obtained by heating at 400 - 500℃.

Results of *Thiobacillus ferrooxidans* media with ferrous sulfate

Tables 2 and 3 show that the *T. thiooxidans* were present in all six types of samples while *T. ferrooxidans* were present in five samples but absent in one sample that is sample number (D) (Figures 1 and 2).

Results of *Thiobacillus* medium

Tables 4 and 5 show that the *T thiooxidans* were present in all six type of samples while *T. ferrooxidans* were present in four samples but absent in two samples i.e. sample number (A and D).

Results of *Thiobacillus* media I (for *Acidophilic thiobacillus*)

Tables 6 and 7 show that the *T. thiooxidans* were present in all six type of samples while *T. ferrooxidans* were present in four samples but absent in two samples i.e. sample number (A and D).

T. thiooxidans are obligate autotrophs. *T. thiobacillus* grow best at pH values of 2 - 5 and produce sulphuric acid and can decrease pH up to zero (Pelczar Jr. et al., 1986). Pathak et al. (2009), found that *T. thiooxidans* are much more effective in removing heavy metals from digested sewage sludge. Villar and Garcia (2002) studied two patterns of solubilization of metal ions resulting from bioleaching of sewage sludge by sulfur-oxidizing *Thiobacillus sp*. These patterns were established as a function of pH. Chromium and copper ions required a pH of 2 - 3 to initiate their solubilization, where as nickel and zinc ions had their solubilization initiated at pH 6 - 6.5. Chromium (VI) reduction by *A. ferrooxidans* (*T. ferrooxidans*), was higher than that observed for *A. thiooxidans* (*T. thiooxidans*). However, at pH close to 7, Chromium (VI) reduction by *T. thiooxidans* was as high as that by *A. thiooxidans* and much higher than that observed for *A. ferrooxidans* at the same pH. The capacity of these to reduce chromium (VI) was associated

Table 3. *T. ferrooxidans* media with ferrous sulfate and experimental conditions.

Treatments/Tests	Sample D		Sample E		Sample D	
	Broth	**Solid**	**Broth**	**Solid**	**Broth**	**Solid**
T. thiooxidans	+ve	+ve	+ve	+ve	+ve	+ve
T. ferrooxidans	-ve	-ve	+ve	+ve	+ve	+ve
Date of experiment	**1st to 30th November 2008**					
Temperature of oven (℃)	30	30	30	30	30	30
Temperature of shaker (℃)	30	-	30	-	30	-
Optimum growth pH.	1.4	1.4	1.4	1.4	1.4	1.4
Colony morphology	Colorless for *T. thiooxidans* and yellow for *T. ferrooxidans*					
Shape	Oval for *T. thiooxidans* and Rod shape for *T. ferrooxidans*					
Mobility	Polar flagella for *T. Thiooxidans*; perititrious flagella for *T. ferrooxidans*					
Spore formation	-ve	-ve	-ve	-ve	-ve	-ve
Volume of media (ml)	100	100	100	100	100	100
H_2S test	+ve	+ve	+ve	+ve	+ve	+ve

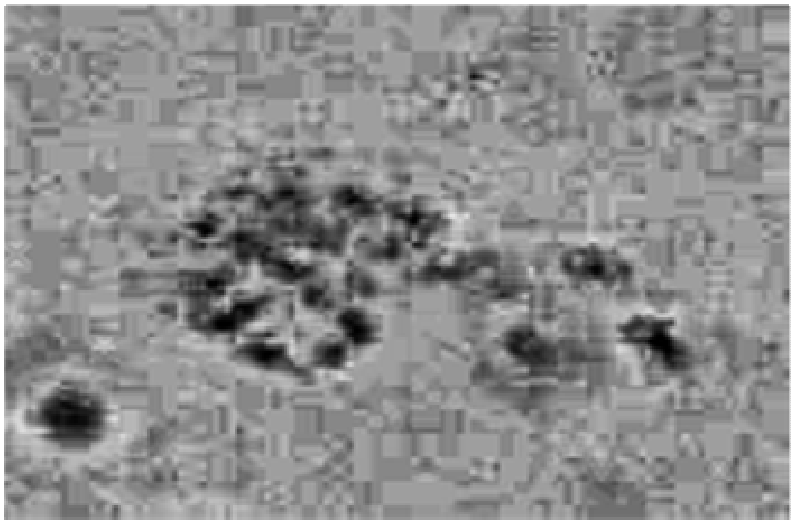

Figure 1. *T. ferrooxidans* colony (1000×).

Figure 2. *T. thiooxidans* (2 m).

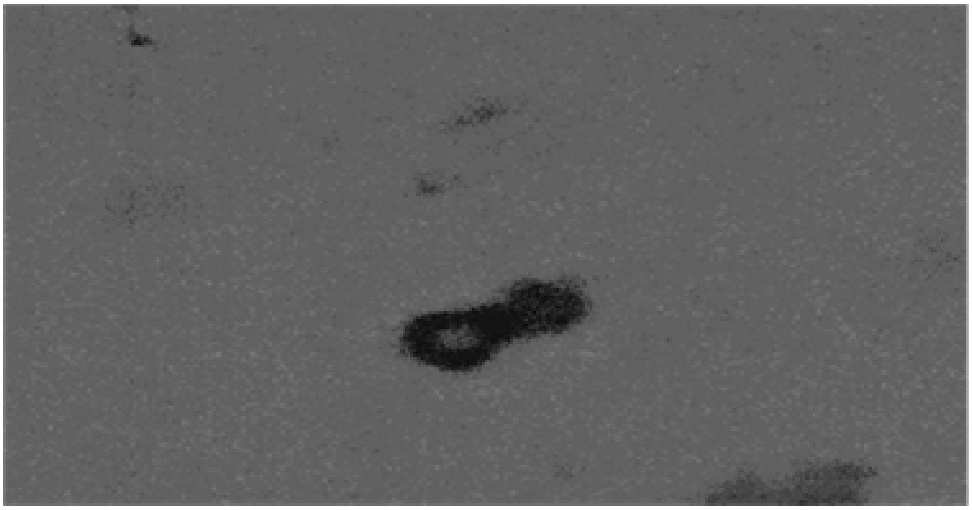

Figure 3. *Zotobacter* (1000×).

Table 4. *Thiobacillus* media and experimental conditions.

Treatment	Sample A		Sample B		Sample C	
	Broth	**Solid**	**Broth**	**Solid**	**Broth**	**Solid**
T. thiooxidans	+ve	+ve	+ve	+ve	+ve	+ve
T. ferrooxidans	-ve	-ve	+ve	+ve	+ve	+ve
Date of experiment	**1st to 30th November 2008**					
Temperature of oven (℃)	30	30	30	30	30	30
Temperature of shaker (℃)	30	-	30	-	30	-
Optimum growth pH.	1.4	1.4	1.4	1.4	1.4	1.4
Colony morphology	Colorless for *T. thiooxidans* and yellow for *T. ferrooxidans*					
Shape	Oval for *T. thiooxidans* and rod shape for *T .ferrooxidans*					
Mobility	Polar flagella for *T .thiooxidans*, Perititrious flagella for *T. ferrooxidans*					
Spore formation	-ve	-ve	-ve	-ve	-ve	-ve
Volume of media (ml)	100	100	100	100	100	100
H_2S Test	+ve	+ve	+ve	+ve	+ve	+ve

Table 5. *Thiobacillus* media and experimental conditions.

Treatment	Sample D		Sample E		Sample D	
	Broth	**Solid**	**Broth**	**Solid**	**Broth**	**Solid**
T. thiooxidans	+ve	+ve	+ve	+ve	+ve	+ve
T. ferrooxidans	-ve	-ve	+ve	+ve	+ve	+ve
Date of experiment	**1st November to 30th November 2008**					
Temperature of oven (℃)	30	30	30	30	30	30
Temperature of shaker (℃)	30	-	30	-	30	-
Optimum growth pH.	2.8	2.8	2.8	2.8	2.8	2.8
Colony morphology	Colorless for *T. thiooxidans* and yellow for *T. ferrooxidans*					
Shape	Oval for *T. thiooxidans* and rod shape for *T .ferrooxidans*					
Mobility	Polar flagella for *T. thiooxidans*; perititrious flagella for *T .ferrooxidans*					
Spore formation	-ve	-ve	-ve	-ve	-ve	-ve
Volume of media (ml)	100	100	100	100	100	100
H_2S Test	+ve	+ve	+ve	+ve	+ve	+ve

Table 6. *Thiobacillus* media I (for *A. thiobacillus*) and experimental conditions.

Treatments	Sample A		Sample B		Sample C	
	Broth	Solid	Broth	Solid	Broth	Solid
T. thiooxidans	+ve	+ve	+ve	+ve	+ve	+ve
T. ferrooxidans	-ve	-ve	+ve	+ve	+ve	+ve
Date of experiment	**1st to 30th November 2008**					
Temperature of oven (℃)	30	30	30	30	30	30
Temperature of shaker (℃)	30	-	30	-	30	-
Optimum growth pH.	4.2	4.2	4.2	4.2	4.2	4.2
Colony morphology	Colorless for *T. thiooxidans* and yellow for *T. ferrooxidans*					
Shape	Oval for *T. thiooxidans* and rod shape for *T. ferrooxidans*					
Mobility	Polar flagella for *T. thiooxidans*; perititrious flagella for *T. ferrooxidans*					
Spore formation	-ve	-ve	-ve	-ve	-ve	-ve
Volume of media (ml)	100	100	100	100	100	100
H_2S Test	+ve	+ve	+ve	+ve	+ve	+ve

Table 7. *Thiobacillus* media I (for *A. thiobacillus*) and experimental conditions.

Treatment	Sample D		Sample E		Sample D	
	Broth	Solid	Broth	Solid	Broth	Solid
T. thiooxidans	+ve	+ve	+ve	+ve	+ve	+ve
T. ferrooxidans	-ve	-ve	+ve	+ve	+ve	+ve
Date of experiment	**1st to 30th November 2008**					
Temperature of oven (℃)	30	30	30	30	30	30
Temperature of shaker (℃)	30	-	30	-	30	-
Optimum growth pH.	4.2	4.2	4.2	4.2	4.2	4.2
Colony morphology	Colorless for *T.thiooxidans* and yellow for *T. ferrooxidans*					
Shape	Oval for *T.thiooxidans* and Rod shape for *T. ferrooxidans*					
Mobility	Polar flagella for *T. thiooxidans*; Perititrious flagella for *T. ferrooxidans*					
Spore formation	-ve	-ve	-ve	-ve	-ve	-ve
Volume of media (ml)	100	100	100	100	100	100
H_2S test	+ve	+ve	+ve	+ve	+ve	+ve

specifically with cells, sulfur and associated sulfur compounds. All these come from *A. thiooxidans* culture (Allegretti et al., 2006).

T. ferrooxidans convert metal sulfite ore into metal ions by oxidation process and convert Fe (II) - Fe (III), Copper chalcopyrite to soluble Cu (II) form (Mujeeb-ur-Rahman, and Gul, 2003). The effects of naturally-occurring organic compounds on ferrous iron oxidation by the bacterium *T. ferrooxidans* were examined by Frattini et al., (2000). Sugio et al. (1988) studied that *T. ferrooxidan* plays a crucial role in the bacterial leaching of sulfide ores. The Cobaltous Co^{2+} has been shown to be toxic metal for the growth of *T. ferrooxidan* on sulfur salts medium. They purified an enzyme or sulfur: Ferric ion oxidoreductase (SFRase) from *T. ferrooxidans,* which catalyzes the reduction by Fe^{3+} of elemental sulfur in the presence of reduced glutathione (GSH). All these studies show that these microbes are growing in roots of hyacinth. These are solublizing the insoluble metal compounds and making available to hyacinth for phytoremoval. The conversion of chromium (VI) to chromium (III), copper (I) to copper (II) and iron (II) to iron (III) in the roots of water hyacinth is due these microbes.

Previous studies indicated the possible role of microbes in nitrogen removal, metals removal, oxidation, reduction or biosorption. In present study our aim is to see the presence of these microbes in roots of water hyacinth and their contribution in phytoremoval of nitrogen and metals by hyacinth.

Results of Sabouraud dextrose medium for *A. niger*

Tables 8 and 9 show that the *A. niger* was present in all six types of samples. The presence of *A. niger* in the roots of water hyacinth shows that it has some interesting

Table 8. Sabouraud dextrose media and experimental conditions.

Treatment	Sample A		Sample B		Sample C	
	Broth	Solid	Broth	Solid	Broth	Solid
A. niger	+ve	+ve	+ve	+ve	+ve	+ve
Date of experiment	**1st to 30th November 2008**					
Temperature of oven (°C)	40	40	40	40	40	40
Temperature of shaker (°C)	40	-	40	-	40	-
Optimum pH.	4	4	4	4	4	4
Mobility	-ve	-ve	-ve	-ve	-ve	-ve
Spore formation	+ve	+ve	+ve	+ve	+ve	+ve
Volume of media (ml)	100	100	100	100	100	100
H_2S test	-ve	-ve	-ve	-ve	-ve	-ve

Shape & Colony Morphology; colony consists of compact yellow basal felt covered by a dense layer of dark-brown to black conidial heads. Conidiophores were long, smooth-walled and terminate in pale-brown colored globose vesicles.

Table 9. Sabouraud dextrose media and experimental conditions.

Treatment	Sample D		Sample E		Sample F	
	Broth	Solid	Broth	Solid	Broth	Solid
A. niger	+ve	+ve	+ve	+ve	+ve	+ve
Date of experiment	**1st to 30th November 2008**					
Temperature of oven (°C)	40	40	40	40	40	40
Temperature of shaker (°C)	40	-	40	-	40	-
Optimum pH.	4	4	4	4	4	4
Mobility	-ve	-ve	-ve	-ve	-ve	-ve
Spore formation	+ve	+ve	+ve	+ve	+ve	+ve
Volume of media (ml)	100	100	100	100	100	100
H_2S Test	-ve	-ve	-ve	-ve	-ve	-ve
Gram staining	-ve	-ve	-ve	-ve	-ve	-ve

Shape and colony morphology; colony consists of compact yellow basal felt covered by a dense layer of dark-brown to black conidial heads. Conidiophores were long, smooth-walled and terminate in pale-brown colored globose vesicles.

role in hyper accumulation of metals. *A. niger* is a fungus and one of the most common species of the genus *Aspergillus*. It causes black mould on certain types of fruit and vegetables, and is a common contaminant of food. Wong et al., (1998) reported the adsorption of metals and reduction of Cr (IV) to Cr (III by *A. niger* at pH 7 (30°C). Leaching of copper from biomining tailings is feasible by using *Aspergillus niger* (Mulligan and Galvez-Cloutier, 2003). Metal biosorption on *Aspergillus sp*. was well narrated by Ahalya et al., (2003). Transmission electron microscopy (TEM), scanning electron microscopy (SEM), and Energy-dispersive X-ray spectroscopy (EDX), indicated an accumulation of chromium in the fungal mycelium and maximum adsorption of Cr (IV) was 75% at 30°C (Srivastava and Thakur, 2006). Ali et al. (2008), studied production and partial purification of cellulase complex by *A. niger* which was grown on water hyacinth blend. Various other studies narrated the biosorption of metals by *A. niger* (Mulligan and Galvez-Cloutier, 2003; Mulligan and Kamali, 2003; Spanelova et al., 2003). All these studies indicate that *A. niger* is also important partner in metal phytoremoval by hyacinth.

Results of Burk medium for *Azotobacter*

Results of morphological studies of *Azotobacter:* Tables 10, 11, 12 and 13 show that the *Azotobacter* was present in all six types of samples. Its presence is confirmed by morphological and biochemical tests. The presence of *Azotobacter* in the roots of water hyacinth shows that it has some important role in hyperaccumulation of metals and nitrogen fixation. *Azotobacter* is a bacterium that lives freely in soil. It can fix nitrogen. Study of *Azotobacter sp.* for the effect of heavy metals like Cd, Cu, Ni, Zn, Pb and Cr, indicates that these metals play some

Table 10. Burk's media and experimental conditions.

Treatment	Sample A		Sample B		Sample C	
	Broth	Solid	Broth	Solid	Broth	Solid
Azotobacter	+ve	+ve	+ve	+ve	+ve	+ve
Date of experiment	**1st to 30th November 2008**					
Temperature of oven (℃)	26	26	26	26	26℃	26
Temperature of shaker (℃)	26℃	-	26℃	-	26℃	-
Optimum pH.	7-7.5	7-7.5	7-7.5	7-7.5	7-7.5	7-7.5
Spore formation	+ve	+ve	+ve	+ve	+ve	+ve
Volume of media (ml)	100	100	100	100	100	100

Table 11. Burk's media and experimental conditions.

Treatment	Sample D		Sample E		Sample F	
	Both	Solid	Both	Solid	Both	Solid
Azotobacter	+ve	+ve	+ve	+ve	+ve	+ve
Date of experiment	**1st to 30th November 2008**					
Temperature of oven (℃)	26	26	26	26	26	26
Temperature of shaker (℃)	26	-	26	-	26	-
Optimum pH.	7-7.5	7-7.5	7-7.5	7-7.5	7-7.5	7-7.5
Spore formation	+ve	+ve	+ve	+ve	+ve	+ve
Volume of media (ml)	100	100	100	100	100	100

Table 12. Results of morphological studies of *Azotobacter* based on Burk's media.

Treatment/ /Parameters	Observations
Colony color	Creamy
Colony surface form	Circular
Colony elevation	Low convex
Colony surface margin	Entire
Colony surface texture	Smooth
Gram staining	Negative
Shape	Rod shape

Table 13. Results of biochemical characteristics of isolated *Azotobacter* based on Burk's media.

Biochemical tests	Result/Inference
Catalase test	+ve
Oxidase test	+ve
Gas production	-ve
H_2S test	-ve
Nitrate reduction test	+ve

roles in nitrogen fixing (Ather and Ahmed, 2002). Simmands, (1979) studied that in wastewater's treatment in 10 days the 99% of the applied nitrate nitrogen and potassium were removed by hyacinth. This would represent a very significant relationship/role of *Azotobacter* a nitrogen fixing bacterium with hyacinth. Soil microbial

nitrification and emission of N_2O was studied in by Kiese et al., (2008).

Zhigang et al., (2006) studied chromium in growth media of *Azotobacter* and reported that Cr-Fe protein might be a new nitrogenase component. This shows possible role of *Azotobacter* in nitrogen removal, metals removal, oxidation, reduction and biosorption in hyacinth. Our results show that *T. thiooxidans, T. ferrooxidans, Azotobacter* and *A. niger* grow in the rhizosphere and on roots of water hyacinth. Lytle et al. (1998) studied reduction of Cr (IV) to Cr (III) on roots, Allegretti et al. (2006) studied same process by *T. thiooxidans* and *T. ferrooxidans* while Wong et al. (1998) studied on *Aspergillus niger.* Our study suggests the role of these microbes in reduction of chromium. Our findings about *Azotobacter* verify water hyacinth's role in nitrogen phytoremoval. We concluded that;

(1) *T. thiooxidans, T. ferrooxidans, Azotobacter* and *A. niger* are showing relationship with metals and nitrogen.
(2) In hyperaccumulation of metals and nitrogen fixation there is a possible role of these microbes.
(3) Metal sulfide ore's solubilization by *T. thiooxidans* and *T. ferrooxidans,* is also responsible for the phyto-removal of these metals by water hyacinth.
(4) Metal's biosorption by *A. niger* may also be a reason for phytoremoval of these metals by water hyacinth.
(5) Involvement of Mn, Mo, Fe, Cr and some other metals in nitrogenase enzymes of *Azotobacter* may also be responsible for phytoremoval of these metals by water hyacinth.
(6) Behind the phytoremoval, waste metals monitoring, pollution indication and nitrogen removal properties of water hyacinth these microbes play a vital role.
(7) Presence of *A. niger* shows valuable use of water hyacinth that is in biofuels, enzyme extraction or purification etc.
(8) Presence of *Azotobacter* shows valuable use of water hyacinth i.e. in biofertilizers.
(9) Presence of *T. thiooxidans* and *T. ferrooxidans* shows valuable use of water hyacinth i.e. in biomining of Zn, Cd, Ni, Fe, Cu, Sb, Sn and Cr.
(10) The growth of water hyacinth in soil of Taxila area also confirms its use in phytoremediation of soil (landfills) besides waste water.

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