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Morphological characterization of isolates novel strain of *Trichoderma* which accumulate the heavy metal (Cobalt)

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Abstract
Heavy metal pollution of soil currently becomes a key environmental problem throughout the whole world. Conventional methods for the removal of heavy metals are not economically and environmental friendly because it has produced massive quantity of toxic chemical compounds. Recently, biological treatments, especially filamentous fungi have gained an increasing attention for heavy metal accumulation due to their upright performances, low cost and huge quantities. The filamentous fungi have a great potential to produce large amount of biomasses which are widely used for metal adsorption capacities ofcobalt. Production of biomass has offered great potential for adopting metal-recovery system. The main aim of this review paper is to discuss the available information of heavy metals accumulation for the utilization of filamentous fungi biomass and scrutinize the practical of exploiting them for heavy metal remediation.

Keywords: Morphological characterization, isolates novel strain, heavy metal (Cobalt)

Introduction
Trichoderma spp. are free living filamentous fungi that are common found in soil and root ecosystem, belongs to the genus *hypocrea.* Hypocera species are the telomorph of some strain of trichoderma.There are five species belong to genus trichoderma i.e, *Trichoderma harzianum, trichoderma viride, trichoderma pseudokoningii, trichoderma longibrachiatum* and *trichoderma koningi.* This fungi are commonly known as biocontrol agent against several soil born phytopathogens (Benitez *et al.* 2004). It's also play an important role in ecology by taking part in decomposition of natural residue, biodegradation of man -made chemicals and bioaccumulation of various metals from industrial scrap and waste water (Ezzi 2005, Anand 2006) [7]. Recent studies have shown that the strains isolated from contaminated areas have significant potential to tolerate such toxic condition (Gadd 1990).

Cobalt is naturally occurring element that does have beneficial applications. For instance, cobalt is an essential component of vitamin B12. Cobalt has been added to pigments to produce a distinct blue color. Lithium ion batteries contain cobalt. In the medical field, cobalt-60 is used in radiotherapy and for sterilizing medical equipment. Regardless of these perceived advantages, cobalt is not without its problems. Cobalt can accumulate to toxic levels in the liver, kidney, pancreas, and heart, as well as the skeleton and skeletal muscle. Cobalt has been found to produce tumors in animals and is likely a human carcinogen as well. (Edward, 2015) [6].

So present study main aim to improve strain of *Trichoderma* strain by protoplast fusion between *trichoderma harzanium* and *trichoderma viride* for enhance secondary metabolite and extracellular enzymes used for heavy metal accumulation.

Materials and Methods
Sample Collection: Fungi collected from plant pathology department, Junagadh Agricultural Unniversity, Junagadh. The fungi were then sub-cultured and preserved on fresh Potato Dextrose Agar (PDA) medium for further studies. All cultures were maintained on PDA slants at 5°C.

Selection of cobalt -tolerant isolates of *trichoderma* spp
In-vitro tolerance of *trichoderma* spp. to different concentration of cobalt was determined by the poisoned food technique (Dhingra *et al.*, 1995).

The cobalt stock solutions were added to molten PDA to get required concentration (40,60,100,150 and 200 mg/ml) and resulting media were poured into petri plates after gentle shaking.

Cultural and morphological observations

The characteristics of *Trichoderma* spp. like colony appearance and sporulation pattern were examined from cultures grown on media: potato dextrose agar (PDA), at 28°C for 4 days. The Petri dishes (9 cm) each containing 25 mL of media were used for each isolate. For observing colony characteristics and growth rate, inoculation were taken from the actively growing margin of 4 days culture, grown on PDA. Radial growth were measured at 24 h intervals until colony covers the whole Petri dish. The microscopic examination and measurements of conidiophores and conidia were made from slide preparations stained with lacto phenol-cotton blue.

Results and Discussion

Cobalt is found in the Earth's crust only in chemically combined form, save for small deposits found in alloys of natural meteoric iron and it comprises 0.0029% of the Earth's crust. cobalt is essential in trace amount for human life. But Exposure to very high levels of cobalt can cause health effects. Cobalt ions in the presence of oxidants can cause increased levels of DNA damage in vitro. (2006, WHO). So here we done invitro experiment for heavy metal accumulation using fungal novel strain of *Trichoderma* by Protoplast fusion technique.

Protoplasts are important tools in genetic research, as well as genetic manipulation which can be successfully achieved through fusion of protoplasts in filamentous fungi that lack the capacity for sexual reproduction (Kushwaha, 2014 and Mrinalini, 1998). Ten fusant strain isolated according to tolerant capacity of cobalt from 15 fusant strains.

Studies revealed that all ten fusant strain isolates are differ in cultural characteristics with most isolates exhibiting slow growth, effuse conidiation and /or loosely arranged conidia in pustules (table 2). As observed by (samules, 2002) the color of colonies pigment varying white, pale yellowish to various green shades, or sometimes grey or brown. Conidia broadly rounded to obovoid, both ends broadly rounded or with the base narrower. Phialides were hung like banana in the conidiophore, base and apex were more narrow than middle. Chlamydospores are generally simillar in many species, although they tend to be uniformly globose or ellipsoidal, terminal and intercalary, smooth-walled, colourless, yellowish or greenish, and 6-15 um diameter in most species. (Anees *et al*., 2010; Gams and Bissett, 2002) [2, 9]. All characteristic is describe as earlier disccuse by (Bisset, 1991a and b, Samuals *et al*., 1996) [4, 5].

After 72 hours of incubation the maximum growth rate recorded at 40 ppm in Tk+Tv 4 and Tk+Tv 9 (90 mm) followed by Tk+Tv 8 (89.4 ppm)), Tk+Tv 5 (86.5 ppm) and Tk+Tv 1 (84.2 ppm). Howerver minimum growth rate was observed at 200 ppm in Tk+Tv 4 (35.1 ppm) followed by Tk+Tv 2 (48.6 ppm), Tk+Tv 3 (57.7 ppm) and Tk+Tv 10 (58.3 ppm) (Table 1).

This experiment agree with the report of Poonam *et al*., (2016) which demonstrated that, at higher concentration of cobalt the colony growth rate of fungal strain was inhibited, but at lower concentration of cobalt either inhibited or accelerated. Reduction in growth of fungi in the presence of higher concentration of heavy metal may be due to increase in length of the initial (lag) phase. It's also observed during the study on filamentous fungi (Aspergillus spp.) in case of Cr metal by (Valix *et al*., 2000).

As is shown in fig.1, with increasing concentration of cobalt from 40 to 200 mg/l the growth rate of *trichoderma* isolates decrease due to its potential to bioremediation.

The microbes and fungus reciprocation to heavy metals by several ways, including transport across the cell membrane, biosorption to the cell walls and entrapment in extracellular capsules, precipitation, complexation and oxidation-reduction reactions (Akhtar *et al*., 2013). Fungi are mainly made up of protein, contain large amounts of polymer of N-acetyl, chitin and chitosan, and deacetylated glucose-amine on their cell wall. Rather than microbes, fungi showed a great affinity for metal ions. These can accumulate metals by means of biological and physiochemical mechanisms from their external environment (Cabuk *et al*., 2004; Preetha and Viruthagiri, 2005) [4, 19].

Although Fungi being widespread in soil and have high metabolic activity. So fungi have dynamic role for removal of heavy metal (Sosak - Swiderska, 2010; Lacina *et al*., 2003) [21, 22].

Conclusion

Our study conclude that isolates of *Trichoderma* sp. have the ability to tolerant heavy metals cobalt at certain level. And based on the morphological characteristics and previous research on the capability of *Trichoderma* sp. in removing heavy metals, it is completely suggested that *Trichoderma* sp. should be considered as the agents in bioremediation process.

Table 1: Growth rate of *Trichoderma* fusants strain on PDA at different concentration of cobalt.

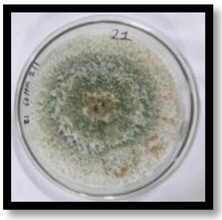
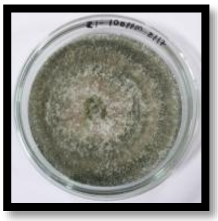
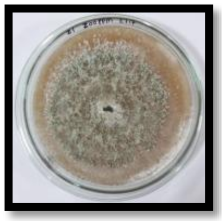
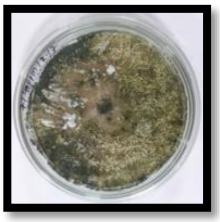
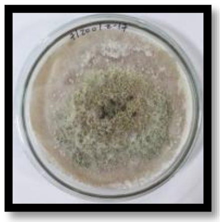
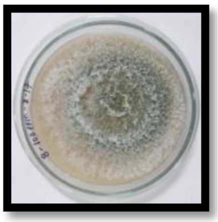
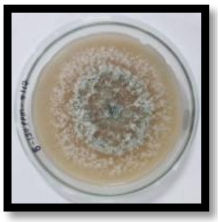
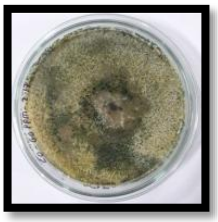
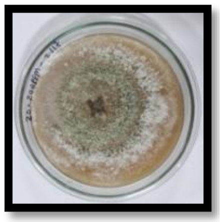
Strain	Control	Colony growth after 72 hours of incubation (mm)				
		40 ppm	60 ppm	100 ppm	150 ppm	200 ppm
Tk+Tv1	90	84.2	80.1	75.6	69.8	60.5
Tk+Tv2	90	73.6	70.9	61.2	55.2	48.6
Tk+Tv3	90	79.7	76.4	68.6	60.2	57.7
Tk+Tv4	90	90.0	87.1	80.9	76.7	35.1
Tk+Tv5	90	86.5	82.4	76.7	70.3	68.2
Tk+Tv6	90	79.5	76.6	70.1	64.8	60.7
Tk+Tv7	90	80.1	79.7	70.2	68.3	62.3
Tk+Tv8	90	89.4	86.3	79.8	76.5	70.3
Tk+Tv9	90	90.0	88.4	78.4	76.2	70.1
Tk+Tv10	90	73.4	72.9	68.5	60.7	58.3

*Mean of three replicate

Table 2: Morphological characteristic of *Trichoderma* isolates at 200 ppm concentration of Cobalt

STRAIN	Morphology	Conidiation (Effuse/flat pustule)	Pigmentation	Sporulation initiate After (hrs)
Tk+Tv1	White & green mycelium	E+F	Green	48
Tk+Tv2	Thick dense Green mycelium	E	Greenish yellow	48-72
Tk+Tv3	Very thick greenish yellow	E	Dark green	48
Tk+Tv4	White & green mycelium	E	Dark green	48
Tk+Tv5	Thick dense Green mycelium	E	Whitish green	48-72
Tk+Tv6	Thick Green & white mycelium	E+F	Whitish green	48
Tk+Tv7	Very thick greenish yellow	E	Green	48
Tk+Tv8	Thick Green & white mycelium	E	Dark green	48
Tk+Tv9	Thick dense Green mycelium	E	Green	48
Tk+Tv10	Sparse Yellowish brown	E	Dark green	48

Fig 1: Growth rate of *Trichoderma* isolates at different concentration of Cobalt

Isolates	Concentration of Cobalt at several concentration				
	40 ppm	60 ppm	100 ppm	150 ppm	200 ppm
Tk+Tv1					
Tk+Tv3					
Tk+Tv7					
Tk+Tv10					

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