



## Screening, biosorption and identification of indigenous fungal strains of iron mining area, Odisha, India

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### Abstract

Heavy metal pollution of soil, water bodies and air is one of the alarming issues in all over the world. The present investigation mainly deals with the isolation, identification and screening of the metal sorption capacity of indigenous fungal strains isolated from iron mining area, Barbil, Odisha, India. Major fungal genera observed were *Aspergillus* sp., *Rhizopus* sp., *Fusarium* sp., *Penicillium* sp., *Trichoderma* sp., etc. Iron tolerance studies were carried out on Potato dextrose agar and Czapekdox agar using disc diffusion method with  $\text{FeCl}_3$  concentrations ranging from 200 to 1000 mg/l. Out of 14 isolates, 6 showed maximum tolerance at 1000 mg/l. The percentage of iron removal was maximum i.e. 96.62% by *Aspergillus japonicus* strain VIT-SB1 at 36 hrs at pH 6 and ambient temperature without any pre-treatment of fungal biomass. Hence, the isolated fungi exhibits great tolerance to iron and can be used successfully for bioremediation purpose.

**Keywords:** *Aspergillus japonicus*; Biosorption; Fungus; Heavy metal contamination; Iron; Metal tolerance

### Introduction

Environmental pollution with metals, semi-metals and organic contaminants is a serious problem of worldwide and contamination with heavy metals is one of the most dangerous pollutants (Xiezhi et al. 2005). Heavy metals showed unfavourable consequences for flora, fauna and cause to groundwater toxicity through leaching. Additionally it causes to decrease the performance and product quality in agriculture and is dangerous for living organisms as well as public health (Khosravi et al. 2009). Moreover existence of heavy metals in the soil caused the environmental stresses that can lead to reduction of plant growth (Mohsenzade et al. 2012). Heavy metals are emitted into the environment by sewage and waste materials from various resources such as metal plating, paint industry, metallurgy, released oil ingredients in the soil, combustion of fossil fuels, mining, ores washing, pesticides, coloured material, batteries, natural erosion of rocks and so on (Vadkertiova and Slavikova, 2006). There are various processes to reduce concentrations of heavy metals in the environment. By the way, it is prominent that the microorganisms utilize these

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contaminants as a source of nutrients and energy and switch them into soluble substances that process is known as bioremediation (Panda et al. 2008). In this method, there is the chance of removing of one or more impurity from environment, with a low cost, and residual products have not toxic effects on the ecosystem of contaminated sites. The using of microorganism such as algae, fungi, bacteria, and yeasts that can absorb the heavy metals, have been considered by some prior researchers for bioremediation of heavy metal polluted media (Pradhan et al. 2007). Microorganisms are nature's original recyclers, converting toxic organic compounds to harmless products, often carbon dioxide and water. Presently Researchers are exploring the bioremediation methods by exploiting microbial and associated biota inside the biological community, to degrade, remove and/or accumulate the pollutants (Gadd, 1990; Paknikar et al. 1993; Khan & Khoo, 2000). Communications between fungi and heavy metals were expatriated in many ways. Because of their resistance and detoxification to heavy metals, fungi are able to leach, absorb and transform heavy metals (Barea & Jeffries, 1995). Biological mechanism implicated in fungal survival in metal polluted aqueous solution includes extracellular precipitation, crystallization, transformation of



metals, biosorption of cell wall, decreased transport or impermeability, efflux, intracellular compartmentation and sequestration (Gadd, 1993). Heavy metals go through cells by channels and transporters, which normally assist the uptake of vital transition metal micronutrients like Fe, Mn and Zn, anions including sulphate and phosphate as well as sugars (glucose) and sugar derivatives (glycerol) (Tamás *et al.* 2005; Wysocki and Tamás, 2010). Even if heavy metals are lethal to fungal population and detrimental to its processes, a number of special fungi have been applied to the the remediation of soils polluted by heavy metals as well as treatment of industrial wastes. The use of fungal biomass and activity to evaluating the pollution situation and ecological risk of heavy metals in environment media is also important (Chen *et al.* 2002). Filamentous fungi can also show high levels of metals and metalloids resistance, being this resistance associated to the capacity to accumulate these components (Durán *et al.* 1999; Cánovas *et al.* 2003). The high incidence of heavy metal resistance detected in this work indicates the potential of fungi as bioremediation agents. Therefore the present study was undertaken to know the fungal diversity in the metal polluted soils, and to find their tolerance level at high

concentration of certain heavy metals. The objective of this study was to identify and isolate fungal strains with particular tolerance toward iron. This was conducted with the specific aims of establishing the relative toxicity of heavy metals to the fungal strains, the effect of metals and their concentration on the growth of fungal strains and identifies the strains which demonstrate greater tolerance to heavy metals. The importance of the study is to develop soil health resulting from the heavy metal tolerant fungi and to analyze the tolerance of fungi against heavy metal pollutants and will be used as a biotechnological tool for bioremediation of environment. Besides it, the present study will provide a baseline and trained researchers to carry out applied research on bioremediation of soil through fungi for the betterment of environment.

## Material and methods

### Study area and samples collection

Samples were collected from the iron mining areas of Barbil, Keonjhar district of Odisha (Fig. 1). The soil of rhizosphere zone was scraped (1-10 cm depth) into a sterilized plastic bag using sterilized spatula. Similarly the tailings and overburden samples were collected individually. From each site

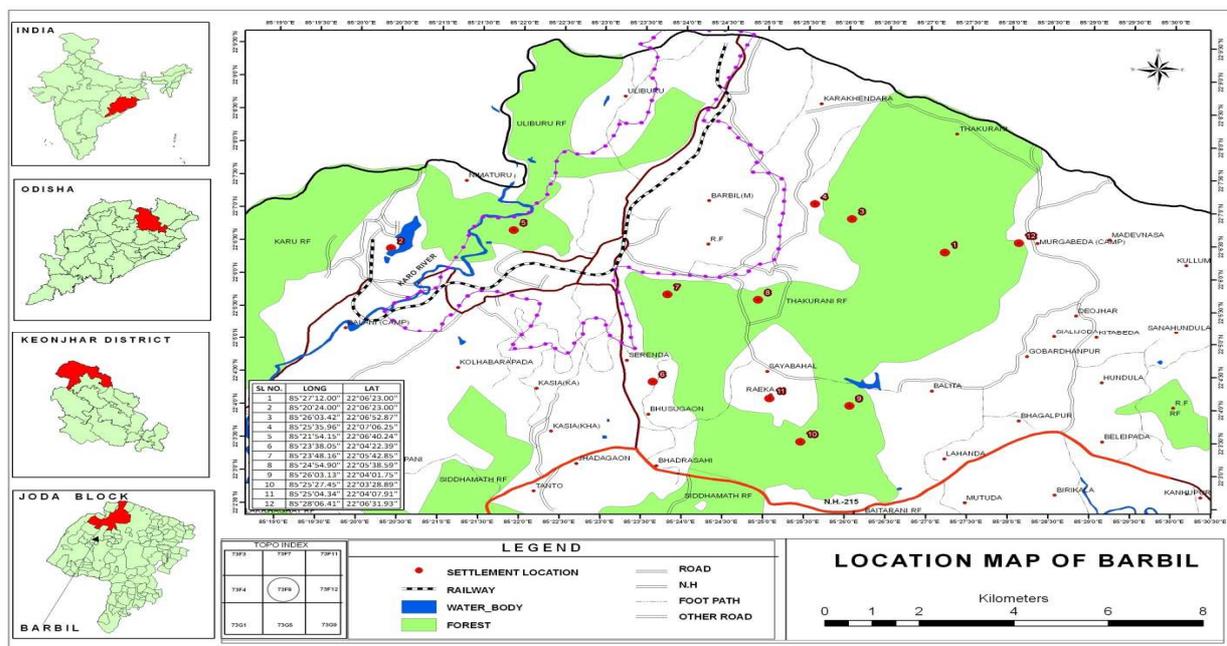


Fig. 1: Location map of sampling Sites



samples were collected from five different spots in a field and mixed together to form a composite sample. All the samples were stored at 4 °C until used for microbiological analysis.

### Physico-chemical analysis

Various physical and chemical analysis of tailing and soil samples were carried out as per the standard protocol. The electrical conductivity (EC) and pH were done using the pH and EC meter (Systronics water analyser). By rapid dichromate oxidation technique, Organic carbon (OC) content was determined (Walkey and Black, 1934); by 1(N) ammonium acetate extraction method CEC (Jackson 1973); exchangeable Ca, Na, K and Mg extracted by 1(N) ammonium acetate solution (Gupta, 2000); Water holding capacity (WHC, in percent) of the samples was calculated using the following equation (Basu *et al.* 2015):

$$WHC(\%) = \frac{W_3 - W_2}{W_2 - W_1} \times 100$$

Here,

W1 Weight of the (WHC instrument/perforated disc+ filter paper)

W2 W1plus+weight of the dry soil/tailings sample

W3 Weight of (the wet soil sample + filter paper) after 24 h.

### Heavy metal analysis of sample

Heavy metals viz., Pb, Cd, Fe, Ni and Cr of mine waste analysed using Atomic Absorption Spectrophotometer (AA-6300 SHIMADZU) after digestion with the mixture of concentrated HNO<sub>3</sub>, H<sub>2</sub>SO<sub>4</sub> and H<sub>2</sub>O<sub>2</sub> (2:6:6) for 30 minutes. All the reagents and reference standards were of analytical grade from Merks (Darmstandt, Germany). Suprapure sulphuric and nitric acids (Merks, Darmstandt, Germany) were used for sample digestion and preparation of standards.

### Preparation of media and Enumeration of fungi from iron ore samples

Two common fungal growth media were used for the initial isolation of the fungi, namely Potato Dextrose Agar (PDA) and Czapek Dox Agar medium (CDA)(Himedia Pvt. Ltd., Mumbai, India) (Mehta and Nautiyal, 2001). The fungal isolation process was carried out under sterile conditions and involved the addition of 250 mL deionised water to

100 g iron ore materials. The mixture was shaken for 24 h at 60 rpm using an orbital shaker (Genei, Bangalore, India) at room temperature. Thereafter, 1 ml of the homogenised mixture was vortexed and 100 µl of the vortexed mixture was inoculated onto pre-prepared plates of PDA and CDA agar. All the plates were incubated at 37 °C for 38-72 hours. Distinct growing mycelia of the fungi were sub-inoculated onto new plates to obtain pure cultures of the fungi. This method enhanced the purity of the isolates by encouraging growth from individual hyphae.

### MTC study

Disc diffusion method was carried out in order to determine the maximum tolerance potential among isolated fungal strains. PDA and CDA medium was prepared and amended with various concentrations (200, 400, 600, 800 and 1000 ppm) of metal FeCl<sub>3</sub>. Media was autoclaved for 20 min at 121 °C and poured into Petri plates. The plates were incubated at 28 °C for 7 days. The growth of fungi was monitored from the point of inoculation or the centre of the colony. Tolerance was measured by observing maximum tolerant concentration (MTC). Fungi that tested positive by this method were then identified using the molecular methods described next.

### Biosorption study

The selected fungal strains which showed maximum tolerance capacity towards iron were further screened out for biosorption study. 0.025 gm of selected dry fungal biomass was added to 50 ml of 200 ppm of FeCl<sub>3</sub> solution. The flasks were kept on the rotary shaker at 150 rpm for 48 hrs at room temperature. The concentration of iron after adsorption was determined by using Atomic Adsorption Spectroscopy (AA-6300 SHIMADZU). The experiment was done in duplicate and the efficiency of biosorption (E) was calculated using following equations (Mamman *et al.* 2011):

$$E = \frac{C_i - C_f}{C_i} \times 100$$

C<sub>i</sub> = initial concentration of the metallic ions (mg/L)

C<sub>f</sub> = final concentration of metallic ions (mg/L)

### Molecular identification of the isolates

The fungal isolates were characterized biochemically and morphologically following



standard microbiological techniques as explained by Gerhardt *et al.* (1994). Isolated genomic DNA was amplified using PCR consensus primers (Bangalore Genei, India), for 16S rDNA sequence analysis. The DNA sequence acquired was analyzed at National Center for Biotechnology Information server using BLAST tool and corresponding sequences downloaded ([www.ncbi.nlm.nih.gov](http://www.ncbi.nlm.nih.gov)). Phylogenetic tree was constructed using MEGA 5.

### Statistical analysis

The experiments were set up with three replicates. Results were processed and analyzed using SPSS 16 statistical analysis package for Windows®. Data is represented as mean  $\pm$  standard error of the mean (SEM) unless otherwise expressed. A p-value of  $<0.05$  was considered significant. Two-way analysis of variance was performed (ANOVA) on

the pairs of variables likely to exhibit correlation (Zar, 1999).

## Results and Discussion

### Physico-chemical analysis of IOT

The physicochemical and heavy metal concentration of the collected samples from and around iron mining area of Odisha have indicated a strong influence of mining activities over the soil quality and associated flora and fauna. The physical chemical and heavy metal analysis of the tailings sample was presented in table 1 and 2. The tailing samples were acidic in nature. The heavy metal was in order Fe > Zn > Pb > Ni > Cr > Cd and most of them are more than the permissible limits. The elevated concentrations of iron in the sample are most likely due to long-term continuous mining operations.

**Table 1: Showing the various physico-chemical characteristics of tailing samples (n =3, Mean $\pm$ SE)**

Sample	pH	EC( $\mu$ S/cm)	CEC	Na	K	Ca	Mg	WHC (%)
IOT	5.4 $\pm$ 0.02	188 $\pm$ 0.04	11.51 $\pm$ 0.05	3.98 $\pm$ 0.01	4.62 $\pm$ 0.03	1.83 $\pm$ 0.01	1.41 $\pm$ 0.01	27.5 $\pm$ 0.01

**Table 2: Metal contents (ppm) in control and various treatments (n = 5, Mean $\pm$ SE)**

Treatment	Heavy metal (mg/kg)					
	Fe	Cr	Cd	Pb	Ni	Zn
IOT	478 $\pm$ 0.010	3.09 $\pm$ 0.011	0.256 $\pm$ 0.005	15.27 $\pm$ 0.017	14.83 $\pm$ 0.001	21.55 $\pm$ 0.014

### Fungal Diversity

Prolonged exposure of fungi to elevated heavy metals contents has developed resistance in them (Gadd, 1993). In the present study, a total of 14 fungal strains were isolated from the iron tailing samples. As described by Barnett and Hunter (1999), the isolates were characterized and identified by fine tuning of their morphological characteristics. The isolated fungal isolates belonged to genera *Aspergillus* sp., *Rhizopus* sp., *Fusarium* sp., *Penicillium* sp., *Trichoderma* sp. were the most frequently encountered. The *Aspergillus* sp. appeared to be the most commonly occurring in the contaminated sample as also reported elsewhere (Zafar *et al.* 2007).

### MTC study

The MTC values, the highest concentration of metals that show visible growth, suggest that all the fungal isolates tested were qualified for MTC.

However, the tolerance level of each isolate was independent against individual metal ions. The maximum tolerable concentration (MTC) of all the iron ore tailings resistant isolates were determined by disc diffusion method in PDA and CDA medium with FeCl<sub>3</sub> concentrations ranging from 200 to 1000 mg/l. Out of 14 isolates, 6 showed maximum tolerance at 1000 mg/ml (Table 3) and these were subjected to further screening. However, the MTC values observed in this study are very much similar to those reported by Ahmad *et al.* (2005) and Sunani *et al.* (2015). These results showed that a variety of fungal isolates responded differently to different concentrations of iron. Physiological and morphological differences were seen between fungal genera, species and strains, and therefore, their response was not same to the concentrations of the metal ions (Al-Garni *et al.* 2009). The metal tolerant fungi can successfully be used for bioremediation as it is an efficient strategy due to its low cost high efficiency and eco-friendly nature.



**Table 3: MTC analysis of isolated fungal strains**

Sl/no	Code	200ppm	400ppm	600ppm	800ppm	1000ppm
1	T1	+	+	+	+	+
2	T2	+	+	+	+	+
3	T3	+	+	+	+	+
4	T4	+	+	+	+	+
5	T5	+	-	-	-	-
6	T6	-	-	-	-	-
7	T7	+	+	+	+	+
8	T8	+	+	-	-	-
9	T9	+	-	-	-	-
10	T10	-	-	-	-	-
11	T11	+	+	+	+	+
12	T12	-	-	-	-	-
13	T13	+	-	-	-	-
14	T14	-	-	-	-	-

**Biosorption study of selected fungal strains**

Biosorption of Iron by selected fungal strains was separately carried out at different periods and its efficiency was given in Figure 2. Fungal biosorption was largely dependent on pH, metal ion and biomass concentration and physical and chemical pre-treatment of biomass (Kapoor & Viraraghavan, 1995). The percentage of iron

removal was maximum i.e. 96.62% by T11 species. The T11 fungal strain was identified to remove maximum at 36 hrs at pH 6 and ambient temperature without any pre-treatment of fungal biomass. There by a decrease in the percentage removal was observed after 36 hrs as shown in Figure 3. This explains the adsorption - desorption behaviour of the fungal mass for the given metal.

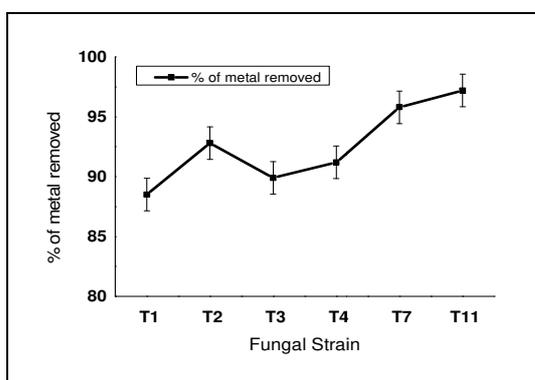


Figure 2: Iron removal efficiency by selected fungal strain

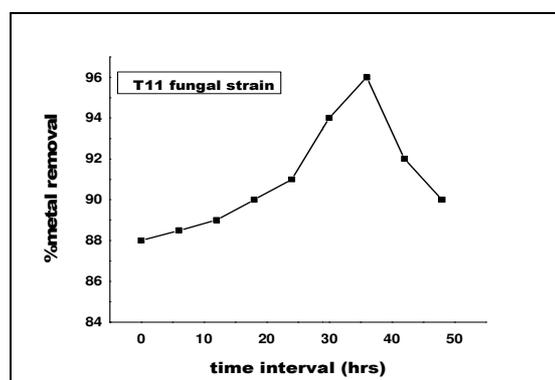


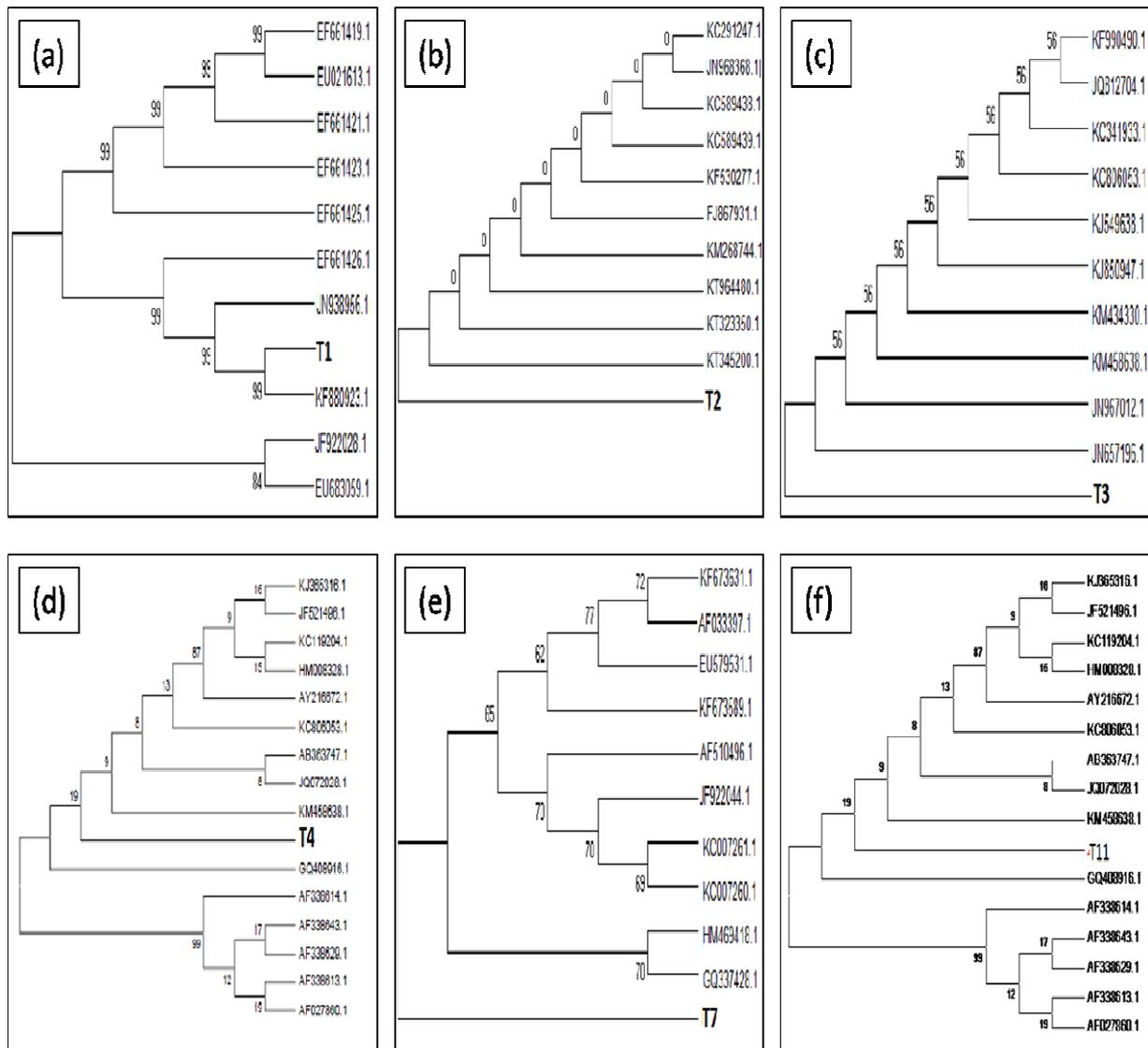
Figure 3: % of removal of iron by T11 at different time interval



**Molecular Identification of isolated Fungi**

Pure culture of all the selected fungus was performed for identification of the cultivable fungi species through phenotype identification and 28S rDNA method. Usually when the sequence of an isolated strain had more than 97% similarity with the sequence of a related strain in GenBank, they were considered as the same species (Jiang *et al.* 2015). However, this study adopted the phenotype observation of colonial morphologies and 28S

rDNA sequence alignment methods including nucleotide homology and phylogenetic analysis. The most potent strain, T11 was identified as *Aspergillus japonicus* strain VIT-SB1. The assessments seemingly are in agreement with observations recorded in the case of *A. niger* (Kapoor and Viraraghavan, 1998) and *Mucor rouxii* (Yan & Viraraghavan, 2000) and *A. fumigates* (Figure 4 a-f).



**Figure 4: Phylogenetic tree of selected fungal isolates based on 28S rDNA based molecular method (a) T1- *Aspergillus ochraceus*(b) T2-*Aspergillus flavus* (c) T3- *Aspergillus niger* (d) T4- *Aspergillus niger* strain SF-6095 (GenBank Accession Number: KM458638.1) (e) T7- *Penicillium pinophilum* (f) T11- *Aspergillus japonicus* strain VIT-SB1 (GenBank Accession Number: KC128815.1)**



## Conclusion

It is concluded from our research that iron and still dusts that have settled on the soil during the mining activities which alter the physiochemical characteristics of soil and microbial diversity. Fungal populations isolated from heavy metal-contaminated sites have the ability to resist higher concentrations of iron. Derived from the present findings, it can be concluded that *Aspergillus japonicus* strain VIT-SB1 is a potential biosorbent for the removal of iron from aqueous solution which is also an effective low-cost material. It is likewise presumed that, pH affects the adsorption capability of the metals on the biosorbent. The overall findings of our laboratory studies are encouraging and can be applied to industrial waste waters biosorption.

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