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TOLERABLE ANALYSIS OF THE FUNGI OF THE PERI-URBAN AGRICULTURAL AREA

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Abstract

The present study was conducted with the aim to scrutinize the tolerance and biosorptive capability of two strains of *Aspergillus niger* towards the prevailing heavy metals in the soil. The research focused on the fungal strains of the contaminated soils of Hudiara drain, Lahore and Chahkayra Chack, Faisalabad. The degree of tolerance of fungi was judged in correspondence with the radial growth (cm) in the presence of heavy metal containing media followed by its comparison with the control, which contain no heavy metals. Tolerance was measured in terms of minimum inhibitory concentration and *Aspergillus niger* showed minimum growth at 6mg/ml against the tested heavy metal $Cr(NO_3)_3$ and exhibited radial growth 3-4.5 cm. *Aspergillus niger* strains of Faisalabad and Lahore showed maximum biosorption at 8mM and 10mM. Data obtained from the experimentation has vast futuristic applicability in terms of biosorption, bioremediation and genetic characterization in future purposes.

Introduction

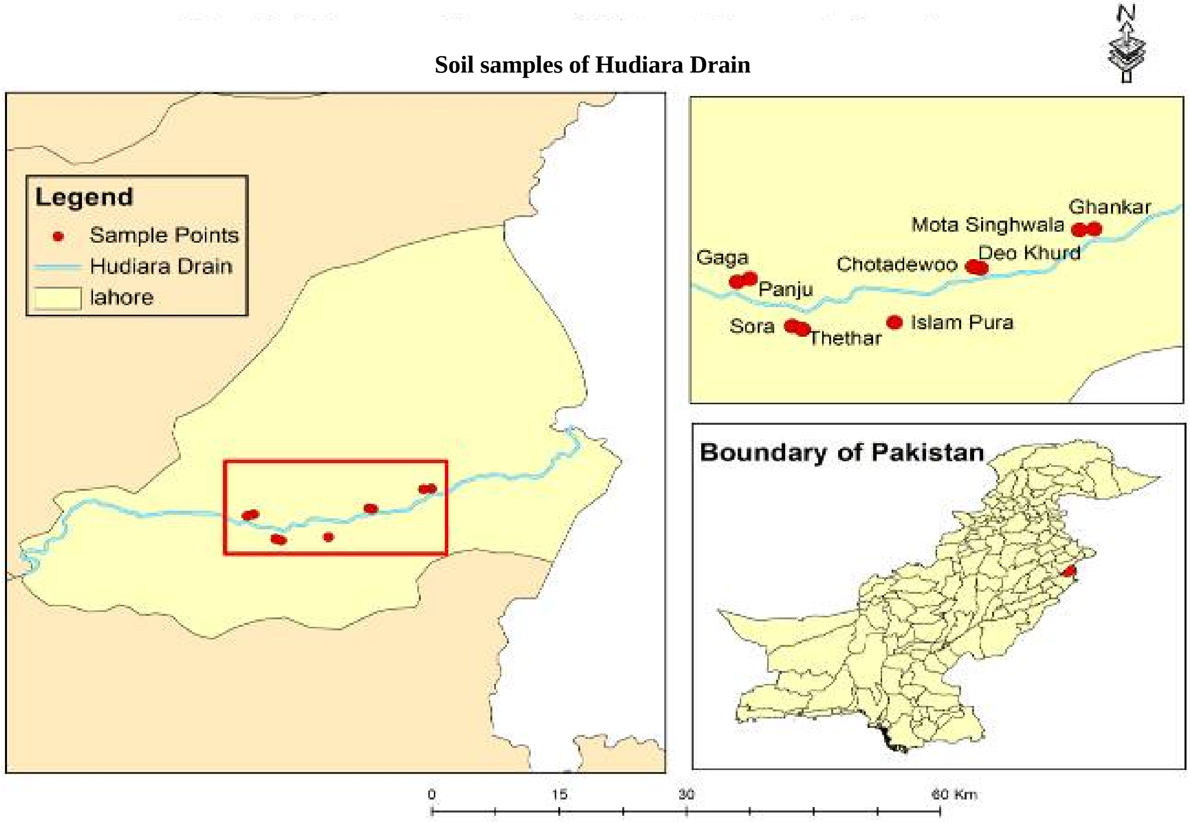
Soil pollution is a buildup of toxic chemical compound, salt, pathogens (disease-causing organisms), or radioactive materials that can affect plants as well as animals life (Singer & Munn, 1999). Discharge of industrial effluents, sewage and sewage sludge has largely contributed to the heavy metal contamination of agricultural land. These effluents contain some plant nutrients, organic matter and varying level of heavy metals (McGrath *et al.,*1988; Hayat *et al*., 2002; Korboute *et al*., 2002). Microorganisms like bacteria, fungi, algae and yeast are known to tolerate and accumulate heavy metals. Bioadsorption mechanism involved in the process may include ion exchange, co-ordination, complexation, chelation, adsorption, micro-precipitation, diffusion through cell walls and membranes which differ depending on the species used the origin and processing of the biomass and solution chemistry (Guibal *et al*., 1992). Therefore it is important to explore microbes from such ecological niches for use in metal biosorption since accustomed technologies such as ion exchange, chemical precipitation, reverse osmosis or evaporative recovery are often insufficient and very expensive especially for metals at low concentration (Spriniti *et al*., 1995; Zhou, 1999). The potential use of microbes in the treatment of heavy metal-contaminated wastewaters and in the recovery of metals in mining wastes or in metallurgical effluent is of special importance (Loren, 1979; Gadd, 1996). In recent years, the biosorption process has been studied extensively using microbial biomass as a biosorbent for heavy metal removal. Both living and dead biomass may be utilized in biosorptive processes as it often exhibits marked tolerance towards heavy metals and other adverse conditions such as low pH (Gad, 1990; Kapoor & Viraraghavan, 1997). Fungi are known to tolerate and detoxify metals by several mechanisms including valence transformation, extra and intracellular precipitation and active uptake (Ashida, 1965; Gad, 1993). The high surface to volume ratio of microorganisms and their ability to detoxify metals are among the reasons that they are considered as potential alternative to synthetic resins for remediation of dilute solution of metals and solid wastes (Kapoor *et al.,* 1999; Magyarosy *et al*., 2002). Considering the mechanisms of metal resistance in fungi, it is expected that screening of metal tolerant fungi may provide strains with improved metal accumulation. Only limited studies have been conducted in Pakistan to systematically screen filamentous fungi from metal polluted sites for their diversity, metal tolerance and their biosorption potential (Bai & Abraham, 2003). Therefore in present study filamentous fungi were studied from a polluted natural environment to assess their metal tolerance and metal removal potential from aqueous solution.

Materials and Methods

Study area and sample collection: Eight random soil samples were collected from eight different agricultural fields of Hudiara drain, Lahore and Chahkayra Chack, Faisalabad (Fig. 1). These samples constituted the upper layer not exceeding the depth of 5 cm, later mixed together to form a composite sample. Digging tools and spatula were used to gather adequate amount of soil followed by storing the soil in plastic bags and coding them as well.

Physio-chemical analysis of soil samples: Soil samples were characterized for its pH, electrical conductivity (EC) (McLean, 1982), and organic matter contents (OM). For heavy metals analysis each soil sample (1g) was taken in the conical flask (50ml), along with 10ml of HNO_3:$HClO_4$ (1:2) solution (50ml) and heated for half an hour. Solutions were filtered through Whatman 1 filter paper and volume was made to 50ml by adding distilled water. Soil samples were digested in triplicates and analyzed for Pb, Cd, Cr, Ni. The blank was prepared for quality assurance of samples, therefore it only contained 10ml of HNO_3:$HClO_4$ (1:2) solution and heated for half an hour and volume was made 50ml by adding distilled water. For the determination of heavy metals the atomic absorption spectrophotometer was powered on and warmed up for 30 minutes. After the heating of cathode lamp, the air acetylene flame was ignited and instrument was calibrated or standardized with different working standards (Nelson & Sommers, 1996).

Soil samples of Hudiara Drain



Soil samples of Chahkayra Chack

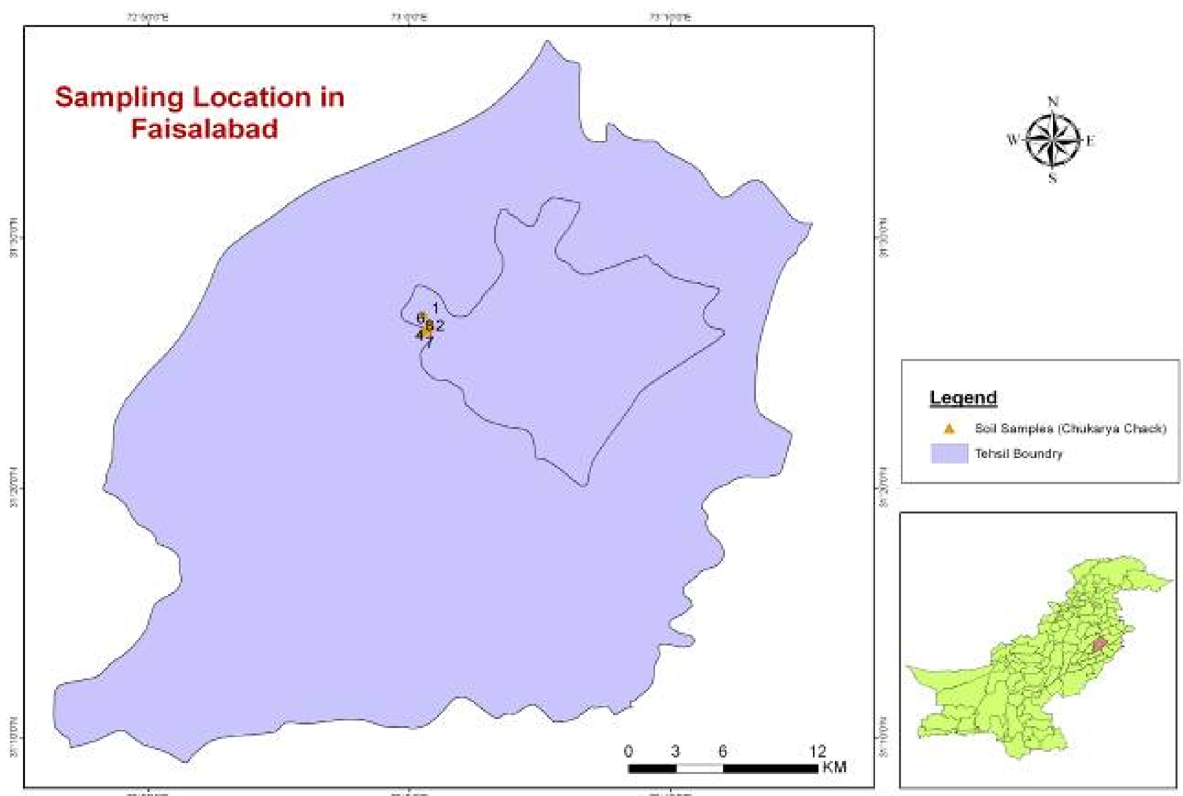


Fig. 1. Soil samples collection from the per-urban agricultural fields of hudiara drain, Lahore and Chahkayra Chack, Faisalabad.

Fungal isolation from soil: Fungal cultures were isolated from the contaminated peri-urban agricultural areas of Lahore and Faisalabad. Isolation of fungal strains was done on potato dextrose agar media under sterilized condition. The soil samples were processed with isolation procedure using the soil dilution plate method (Waksman, 1922). Inoculations were done aseptically. After incubation distinct colonies were counted and identified. In identifying fungi, microscopic (Domsch *et al.,* 1980; Barnett & Hunter, 1999) and macroscopic examinations including staining for morphological characteristics were carried out (hyphae, mycelium, sporocarp, septa). Fungi were preserved on Potato Dextrose Agar slants.

Fungal tolerance to heavy metals and Minimum Inhibitory Concentration (MIC): Among different fungal strains only *Aspergillus niger* cultures of Lahore and Faisalabad were tested for tolerance against different concentrations of heavy metal $Cr(NO_3)_3$. Potato Dextrose Agar (PDA) media was used for heavy metal resistance experiment. Different concentrations (0.0, 0.05, 2, 4, 6, 8 and 10mg/ml) of heavy metal Cr $(NO_3)_3$ were used for the selection of metalophilic fungi. Tolerance to heavy metals was determined as the MIC against the test fungi. PDA medium was prepared and amended with various amounts of heavy metal to achieve the desired concentration ranging from 0.05 to 10 mg/ml. Potato Dextrose Agar media pH (5.6) was maintained by 3M NaOH. After media was autoclaved and cooled. An inoculum of test fungi was spotted in center of metal and control plates (plates without metal). The plates were incubated at 29 ± 1°C for 2-5 days to observe the growth of fungi on the spotted area. Fungal colony morphology and radial growth diameter were measured, for the latter fungal culture was monitored from the point of inoculation or centre of the colony for radial diameter. Tolerant fungi were studied by the comparison among fungi grown in the presence of metals and in the absence of metals as control (Zafar *et al*., 2006).

Metal biosorption assay: Biomass of the test fungi (*Aspergillus niger*) was grown on PDA medium with pH adjusted to 5. The fungi were cultured in filamentous form under aerobic conditions for 3 days in shake flask (125 rpm) at 29°C. The biomass was harvested by filtration through a sieve of a 60 µm size and thoroughly washed with distilled deionized water to remove residual growth medium. Briefly, 1-5g of biomass of the above fungi was added to 100ml metal solution containing 0.0, 0.05, 2, 4, 6, 8 and 10 mM of $Cr(NO_3)_3$ in double distilled water, at an initial pH of 5.0 in a 250 ml conical flask (Kapoor & Viraraghavan, 1995; Yan & Viraraghavan, 2003). The flasks were agitated on a rotatory shaker for 4 h at 25^0C followed by filtering the solution to separate the biomass. The supernatant and control (metal solution without biomass) were digested in 65% HNO_3.The digested solution was evaporated till to dryness and solids were redissolved in 0.1M HCl (Yan & Viraraghavan, 2003). The heavy metal concentration was analyzed by atomic absorption spectrophotometer Perkin Elmer (A Analyst 700). Biosorption capacity i.e. the amount of metal ions (mg) biosorbed /g of biomass was calculated by the following equation:

$$Q = (Ci - Cf / m) \quad V$$

where Q =mg of metal ion bioadsorbed per gm of biomass, Ci=initial metal ion concentration mg/l, Cf = final metal ion concentration mg/l, m=mass of biomass in the reaction mixture gm, V=volume of the reaction mixture (l).

Results and Discussion

According to the strategy conceived from the present investigation, soil was considered the most endangered component of the environment in the proximity of tanneries and industries in Pakistan. These considerations formed the basis of Lahore and Faisalabad contaminated soils metal analysis towards assessing the pollution status and detecting the potential sources. The data in Table 1 provides the summary of Pb, Cd, Cr, Ni, pH, EC and OM in the peri urban areas of Lahore and Faisalabad due to application of sewage sludge, industries and tanneries wastewater for many years, a large portion of soil has been contaminated with heavy metals such as Pb, Cd, Cr and Ni (Anonymous., 1999; Khan *et al.,* 2012). Among all the peri-urban areas of Punjab and Lahore had the higher concentrations of Cr with values 90.6 mg/kg. In Faisalabad the minimum and maximum values of Pb, Cd, Cr and Ni were higher (Bowen, 1979). Similar results have also been reported, i.e. the continuous use of polluted effluents for crop production could result in accumulation of trace elements in concentration that may be come phytotoxic (Ghafoor *et al.,* 1995; Lacatusu, 1998; Younas *et al.,* 1998; Ahmad *et al.,* 2011).

The pH range of the soil of Lahore and Faisalabad varied from 7.4-7.8 and 8.1-9.4. As opposed to the mean pH of Lahore, the mean pH of Faisalabad was higher. In present study, pH of collected soil samples of peri-urban areas ranged from 7.6 to 8.8 indicating that soil pH was falling in the alkaline range. This is due to the fact that arid and semi arid environment (like in Pakistan) have soil rich in bases and contain soluble carbonates and bicarbonates which produce OH^- making soil pH alkaline.

Long-time exposure of soil fungi to heavy metals can lead to physiological adaptation or considerable modification of their microbial populations, reducing their activity and their number and such changes may be associated with increased metal sorption capacity (Doelman *et al.,* 1994). Lahore and Faisalabad possessed high fungal diversity. *Aspergillus* spp was isolated from both the sampling sites. In present investigation the richest class was Ascomycota as compared to Deutromycota. Azaz, 2003 obtained results from the soil dilution plate method and said that a bulk of fresh soil equivalent to 1g of oven-dried soil. An acceptable number of micro fungi in 1g of fertile land soil are around 400,000 (Fig. 2).

Table 1. Concentration of heavy metals in soils (mg/kg) of peri-urban areas of Lahore and Faisalabad.

	Pb	Cd	Cr	Ni	pH	OM	EC
Lahore							
Min	51.3	1.1	44.9	42.2	7.4	0.6	0.0
Max	106.1	4.0	135.8	82.0	7.8	1.3	0.8
Mean	68.4	2.6	90.6	55.8	7.6	1.0	0.5
Median	63.9	2.5	90.2	52.8	7.6	1.1	0.6
S.D	±16.2	±0.9	±39.8	±13.3	±0.1	±0.2	±0.2
Kurtosis	5.57	-0.08	-2.56822	1.014	-1.80	**0.17**	3.920
Skewness	2.13	-0.11	0.005	1.097	-0.29	-0.82	-1.776
Faisalabad							
Min	54.0	2.3	46.9	31.6	8.1	0.83	1.0
Max	87.9	3.4	122.4	66.9	9.4	1.29	4.0
Mean	63.9	2.7	76.9	46.9	8.8	1.04	1.9
Median	60.1	2.6	71.1	45.0	9.0	1.04	1.6
S.D	±12.3	±0.4	±28.0	±13.7	±0.5	±0.17	±0.9
Kurtosis	0.917	-1.673	-0.464	-1.664	-1.698	-1.155	4.667
Skewness	1.2904	0.4859	0.9577	0.2770	-0.4721	0.2158	1.995
****C. Limit Values**							
a Bowen (1979) b Prior to global contamination	35 (12^b)	0.35	70	50			
c Ewers, 1991	100	3	100	50			

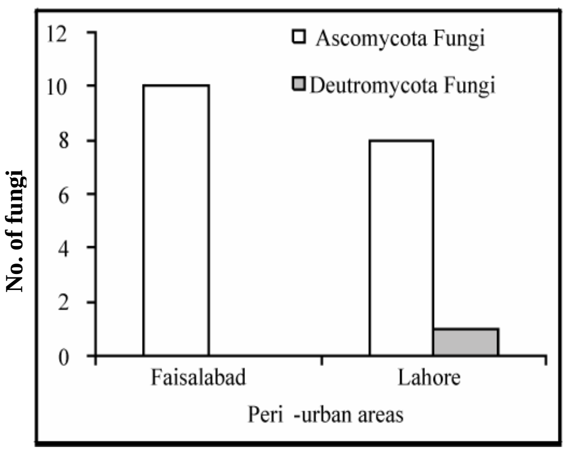


Fig. 2. Number of fungi of peri-urban agricultural fields of Lahore and Faisalabad.

In this particular experimentation few types of fungi were isolated because peri-urban areas are contaminated of heavy metals and sources are automobiles exhaust, sewage water, industrial and ternaries waste and it can be argued that the peri urban fields of present investigation are poor in quality and highest level of contamination. Major differences among the species in terms of numbers of spores and tolerance to metals suggest that fungi follow different strategies to establish symbiosis and probably reveal *Aspergillus* spp were preferentially found in soil samples with the differences in functioning (Johnson *et al.,* 1992; Allen *et al.,* 1995; Bever *et al.,* 1996).

Exposure of soil fungi to heavy metals can lead to physiological adaptation or the selection of mutants (Gadd, 1993), and such changes may be associated with increased metal sorption capacity. Soil fungus belonging to the genera *Aspergillus* able to grow in the presence of heavy metal (Cr) was isolated. The purpose of the investigation was to obtain filamentous fungus from polluted habitats for their possible exploitation in metal tolerance biosorption studies. So *Aspergillus niger* was selected from the both sites to determine the metal tolerance and biosorption analysis for the Cr since it was present in higher concentration in peri-urban soils of Lahore and Faisalabad.

In tolerance analysis Minimum Inhibitory Concentration (MIC) values suggest that the resistance level depends on different metal concentrations. The occurrence of various fungi such as *Aspergillus, Rhizopus, Penicillium, Fusarium, Paecilomyces* species in soil polluted with heavy metals (Cu, Cd, Pb, As and Zn) has been reported by other workers from different parts of the world (Babich & Stotzky, 1985).The variation in the metal tolerance to different concentrations might be due to the presence of one or more tolerance strategies or resistance mechanisms exhibited by fungus *Aspergillus niger.* Both selected strains of *Aspergillus niger* showed minimum inhibitory concentration at 6mg/ml. In present study *Aspergillus niger* was able to tolerate heavy metals 0-10mg/ml. Similar study was reported by Price *et al*., 2001 and showed that *Aspergillus niger* is better to grow and tolerance to heavy metals. According to the outcomes of the analysis *Aspergillus niger* turned out to be more tolerant at lower concentrations. The behavior of Lahore isolate was similar to Faisalabad and MIC was observed at 6mM. At 10 mM the isolate did not showed any growth or tolerance. Minimum Inhibitory Concentration (MIC) of Faisalabad isolate is listed in Table 1. The tolerance of *Aspergillus niger* to different concentrations of metal was observed in order of 0.0>0.05>2>4>6>8>10 mg/l. Table 2 represents that isolate of Faisalabad showed MIC at 6mM while at 6 and 8 mM the MIC was similar and at 10 mM the isolate did not show any tolerance or growth.

Table 2. Minimum inhibitory concentration (MIC) of *Aspergillus niger* isolates against Cr $(NO_3)_3$.

Cr $(NO_3)_3$	*Aspergillus niger* (Radial growth Cm)	
	Faisalabad	Lahore
0.0	9	9
0.05	9	9
2	9	9
4	9	9
6	3	4
8	3	3
10	0	0

Biosorption of the toxic metals is based on ionic species associated with the cell surface or extra cellular polysaccharide, protein and chitins (Volesky, 1990; Ali *et al.,* 2012). Biosorption factor and other factors such as contact time, biomass dosage, temperature and pH are known to influence biosorption of metals (Kapoor & Viraraghavan, 1997; Zhou, 1999; Bai & Abraham, 2003; Yan & Viraraghavan, 2003). The objective of this examination was to explore *Aspergillus niger* (isolates of Faisalabad and Lahore) ability as a effective biosorptive weapon for Cr. As a result maximum biosorption for Cr was detected at 8mM concentration. Biosorption was influenced by the initial metal concentration. *Aspergillus niger* biosorption at different concentration of Cr from 0 to 10mM (Fig. 3). It was noted that biosorption of Cr starts at 0.05mM and increased at 2mM, 4mM, 6mM and 8mM but at 10mM biosorption decreased. Maximum biosorption was observed at 8mM however for the Lahore isolate it came out to be at 10mM. *Aspergillus niger* biosorption at different concentration of Cr from 0 to 10mM.

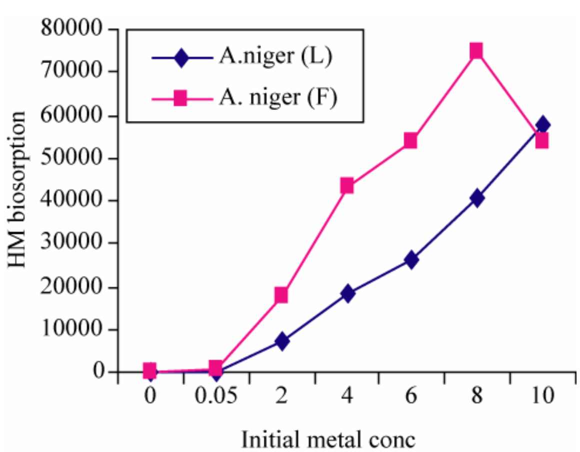


Fig. 3. Biosorption of Cr by *Aspergillus niger*.

From the perspective of environmental biotechnology the data generated is compelling enough, suggesting that such tolerance and co tolerance could be acquired in natural environments. Such simple bioremediation strategies could enhance the detoxification of polluted areas due to resistant microbes which are present in soils under adverse conditions.

Conclusion

Preliminary findings attained imply the employment of other fungi for metal removal from contaminated material. The present investigations gives no evidence for a direct relationship between level of metal resistance and biosorption capacity in *Aspergillus* isolates. Further investigations are needed to optimize the conditions for metal removal from multimetal aqueous solutions and diluted wastewater for large scale operation.

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