International Journal of Science and Research (IJSR)
ISSN (Online): 2319-7064
Impact Factor (2012): 3.358

Using Bacterial Recombinants for Heavy Metals Uptake from Liquid Industrial Wastes Resulted from Chemical Fertilizer Industry

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Abstract: ***This study aimed to use bacterial recombinants for removing heavy metals from wastewaters in contaminated industrial sites which resulted from chemical fertilizer manufactures in Egypt, as a unique process. The significance of this study was due to heavy metals pollution which become one of the most serious environmental problems suffering society today. Ten bacterial strains belong to four genera were used in conjugation process conducted in this study, as well as, 22 transconjugants resulted from 11 mating were used in biosorption of heavy metals from factory effluents. The results appeared that some of transconjugants appeared positive hybrid efficiency in heavy metals uptake than their mid-parents, as well as, the better parent. This efficiency was exceeded one hundred percentage in relation to mid-parents, as well as, the better parent. The results indicated the higher effectiveness of bacterial recombinants than their parents in reducing the toxicity of heavy metals from factory effluents to be used as a good biosorpant agents in bioremediation experiments.***

Keywords: bacterial recombinants, bioremediation, factory effluents, heavy metals, hybrid effectiveness.

1. Introduction

Water is not only a resource, it is a life source. It is well established that water is important for life. Water is useful for several purposes including agricultural, industrial, household, recreational and environmental activities. Despite its extensive use, in most parts of the world water is a scarce resource. Ninety percent of the water on earth is seawater in the oceans, only three percent is fresh water and just over two thirds of this is frozen in glaciers and polar ice caps.

The contamination of water resources with environmentally harmful chemicals represents a problem of great concern not only in relation to the biota in the receiving environment, but also to humans. The continuing growth in industrialization and urbanization has led to the natural environment being exposed to ever increasing levels of toxic elements, such as heavy metals. Approximately 10% of the wastes produced by developed countries contain heavy metals. (**Duffus, 2002**). The contamination of the environment by heavy metals is an important environmental problem due to the toxic effects of metals, and their accumulation throughout the food chain, leads to serious ecological and health problems. Common sources of metal polluted wastes include electroplating plants, metal finishing operations, as well as, many mining, nuclear and electronics industries. All of these contribute to anomalously high concentrations of metals in the environment relative to the normal background levels **(Neytzell-De Wildes, 1991).** Metal causes genotoxicity as they 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).**

The contaminated wastewater cannot be treated by traditional methods, like active sludge, the treated water still contains higher concentrations of heavy metals that are allowed by the environment laws. Traditional technologies of treating contaminated wastewater, including precipitation, ion exchange or reverse osmosis still generate too large costs. Moreover, metals are still insufficiently removed from the equipment, especially after a long term of usage what is mostly seen in case of ion exchange and reversal osmosis (**Kita and Skoblewski, 2010)**.

Biological methods such as biosorption or bioaccumulation strategies for the removal of metals ions may provide an attractive alternative to existing technologies (**Preetha and Viruthagiri, 2005**). Microorganisms have evolved coping strategies to either transform the element to a less-harmful form or bind the metal intra- or extracellularly, thereby preventing any harmful interactions in the host cell. Microbial species, have been shown to be relatively efficient in bioaccumulation of uranium, copper, lead, and other metal ions from polluted effluents (**Gupta *et al.*, 2001).**The uptake of heavy metals, present in industrial wastes, and detoxification of metal ions by bacteria provide an additional mechanism of environmental bioremediation. Surprisingly, little information is available regarding the incidence and distribution of plasmids in contaminated subsurface environments. Such studies will provide greater knowledge on the ecology of plasmids and their contributions to the genetic adaptation of naturally occurring subsurface microbial communities (**Coombs and Barkay, 2004).**

Gene change and exchange are mechanisms that promote physiological diversity. The potential to alter metabolic functions is advantageous, because most microbes in nature live in changing environments. Hybrids arise from the mating between different strains, varieties, or species, resulted partial polyploids through some genome duplication events. Heterosis is defined as greater biomass, fertility or other traits in heterozygotes, polyploids or hybrids compared

to their genetically divergent (often homozygous) parents. Despite the importance of heterosis, its molecular bases are still enigmatic. Several genetic models have been proposed but fail to give mechanistic insights (**Veitia and Vaiman, 2011)**. A specific problem associated with heavy metals polluted the liquid industrial wastes resulted from these industry in the environment is the accumulation of metals in the food chain and persistence in the environment. This study aimed to evaluate hybrid effectiveness of bacterial recombinants in removing the toxicity of heavy metals from factory effluents resulted from chemical fertilizer industry.

2. Materials and Methods

Genetic Material

Organism and culture conditions:-

Ten bacterial strains (Table 1) belong to four genera were used in this study. They are kindly obtained from National Center for Agriculture Utilization Research, USA. All strains used in this investigation are wild type strains.

Table 1: Bacterial strains used in this study

Strains	Designation
Citrobacter amalonaticus	NRRL B-41228
Citrobacter freundii	NRRL B-2643
Bacillus subtilis var *niger*	NRRL NRS-213
Bacillus subtilis	NRRL B-642
Bacillus licheniformis	NRRL B-571
Bacillus licheniformis	NRRL B-1584
Bacillus licheniformis	NRRL NRS-1264
Bacillus licheniformis	NRRL B-358
Micrococcus luteus	NRRL B-287
Kocuria rhizophila	NRRL B-4375

Factory effluents: The present study was undertaken using wastewaters resulted from the ammonia unit of Chemical Fertilizer Industry (CFI) located in Dakahlia Governorate. Polluted water was collected from the main pipe of the factory before being mixed with the water in the river.

3. Methodology

Antibiotic susceptibility assays: Antibiotic susceptibility was measured by a plate diffusion method according to **Collins and Lyne (1985**) with cultures grown to logarithmic growth phase in nutrient agar medium for each microbe. Antibiotics designation was listed in Table 2. All antibiotics were used at a concentration of 100 µg/ml according to **Roth and Sonti (1989).** Genetic selectable markers were identified as antibiotic resistance and or sensitive as listed in Table 3.

Table 2: Designation of antibiotics used for genetic marking against bacterial strains used in this study.

Antibiotics	Designation
Flucamox	*flu*
Streptomycin	*Str*
Tetracycline	*Tc*
Neomycinsulphate	*Nm*
Ampicillin	*Ap*
Erythromycin	*Erth*
Amoxycillin and flucloxacillin	*Am-Fluc*
Cephalexim	*Cp*
Ibiamox	*Ibim*
Amoxycillin	*Amoxy*
Ibidroxil	*Ibid*
Haiconcil	*Hico*
Velosef	*Velo*
Epicocillin	*Epico*
Nystatin	*Nyst*
Erythrocin	*Ery*
Duricef	*Duri*
pencillin	*pen*

4. Conjugation

Nutrient broth cultures, in the late of exponential growth phase were used in conjugation process. Qualitative spot of conjugal transfer were carried out according to Lessel *et al.* (1993) by inoculating 10 µl of the donor culture onto the surface of selective medium, previously seeded with 100 µl of recipient culture. A single colony of transconjugants appeared on selective medium was picked up and transferred to slant nutrient agar medium. Conjugation was carried out between strains carrying the opposite genetic markers as shown in Table 3. Two different isolates were selected from each mating to be used in heavy metals uptake tests.

Table 3: Mating between bacterial strains carrying the opposite genetic markers.

No. of mating	Mating	Revelant geneotype of mating
1	NRRL B-571 X NRRL B-1584	*Erth*$^+$, *Ap*$^+$, *Ibim*$^+$, *Amoxy*$^+$, *Hico+*, *Epico*$^+$,*Cp*$^-$ X *Erth*$^-$, *Ap*$^+$, *Ibim*$^-$, *Amoxy*$^-$, *Hico*$^-$, *Epico*$^-$,*Cp+*
2	NRRL B-571 X NRRL B-358	*Erth*$^+$, *flu*$^+$, *Hico+ Epico*$^+$,*Cp*$^-$ X *Erth*$^+$, *Flu*$^-$, *Hico*$^-$, *Epico*$^-$,*Cp+*
3	NRRL B-571 X NRRL B-2643	*Erth*$^+$, *flu*$^+$, *Epico*$^+$, *Velo*$^-$, *Duri*$^-$, *Cp*$^-$, *Ibid*$^-$ X *Erth*$^-$, *flu*$^-$ *Epico*$^-$, *Velo*$^+$, *Duri*$^+$, *Cp*$^+$, *Ibid*$^+$
4	NRRL B-1584 X NRRL NRS-213	*Ap*$^+$, *Cp*$^+$, *Am-Fluc*$^+$, *pen*$^+$, *Amoxy*$^-$ X *Ap*$^-$, *Cp*$^-$, *Am-Fluc*$^-$, *pen*$^-$, *Amoxy*$^+$
5	NRRL NRS-1264 X NRRL B-2643	*Erth*$^+$, *Tc*$^+$, *Ibim*$^+$, *flu*$^+$, *Ibid*$^+$, *Velo*$^-$, *Duri*$^-$ *X Erth*$^-$, *Tc*$^-$, *Ibim*$^-$, *flu*$^-$, *Ibid*$^+$, *Velo*$^+$, *Duri*$^+$
6	NRRL B-358 X NRRL B-642	*Ap*$^+$, *Cp*$^+$, *Am-Fluc*$^+$, *pen*$^+$, *Ibim*$^+$, *Amoxy*$^+$, *Hico*$^-$, *Epico*$^-$ X *Ap*$^-$, *Cp*$^-$, *Am-Fluc*$^-$, *pen*$^-$, *Ibim*$^-$, *Amoxy*$^-$ *Hico*$^+$, *Epico*$^+$
7	NRRL B-2643 X NRRL B-642	*Ap*$^+$, *Cp*$^+$, *Am-Fluc*$^+$, *pen*$^+$, *Ibim*$^+$, *Amoxy*$^+$, *Ibid*$^+$, *Velo*$^+$, *Duri*$^+$, *Epico*$^-$ *X Ap*$^-$, *Cp*$^-$, *Am-Fluc*$^-$, *pen*$^-$, *Ibim*$^-$, *Amoxy*$^-$, *Ibid*$^-$, *Velo*$^-$, *Duri*$^-$, *Epico*$^+$
8	NRRL B-41228 X NRRL B-642	*Cp*$^+$, *Am-Fluc*$^+$, *pen*$^+$, *Ibim*$^+$, *Amoxy*$^+$, *Epico*$^-$ *X Cp*$^-$, *Am-Fluc*$^-$, *pen*$^-$, *Ibim*$^-$, *Amoxy*$^-$ *Epico*$^+$
9	NRRL B-642 X NRRL B-4375	*Hico+*, *Epico*$^+$, *Am-Fluc*$^-$, *pen*$^-$ X *Hico-*, *Epico*$^-$, *Am-Fluc*$^+$, *pen*$^+$
10	NRRL B-642 X NRRL NRS-213	*Hico+*, *Epico*$^+$, *Amoxy*$^-$ *X Hico-*, *Epico*$^-$, *Amoxy*$^+$
11	NRRL B-4375 X NRRL NRS-213	*Am-Fluc*$^+$, *pen*$^+$, *Amoxy*$^-$ x *Am-Fluc*$^-$, *pen*$^-$, *Amoxy*$^+$

+ , - = Resistant and sensitive to antibiotic , respectively .

Uptake experiments: In heavy metals uptake test, precultured cells were suspended in 250 ml conical flasks containing 150 ml minimal medium supplemented with factory effluents without any dilution and incubated under a

static conditions at 30 °C for 48 h. Thereafter, the cells were collected by filtration on membrane filter (pore size 0.45 µm). Amounts of metals uptake by the bacterial cells were determined according to **Nakajima and Sakaguchi (1986).**

Determination of heavy metals concentration: The samples were collected and filtered using Millipore filters of 0.45 µm pore size. The filtrate was collected to be used for heavy metals analysis. The concentration of heavy metals in solution was determined using the atomic absorption spectrophotometer at the Atomic Absorption Unit, Department of Chemistry, Faculty of Science, Mansoura University. Heavy metals under investigation in this work were as follows; Cu , Co, Fe, Cd and Pb.

Data evaluation (Langmuir isotherms): The uptake of the metals (mg of metal/g of dry cell weight) was calculated according to **Liu *et al*. (2004**) using the following formula:

$Q = v (C_i - C_f)/m$

Where Q is the metal uptake (mg metal per g biosorbent), *v* the liquid sample volume (ml), Ci the initial concentration of the metal in the solution (mg/L), Cf the final (equilibrium) concentration of the metal in the solution (mg/L) and *m* the amount of the added biosorbent on the dry basis (mg).

Measuring transconjugant efficiency (TE): Transconjugant efficiency (TE) was calculated according to **Bakker (2006**) using the following formula;

TE MP (Mid parents) = Average PF_1 - Average PP/ mid parents, measured in units of the trait.

TE BP (Better parent) = Average PF_1 - Average Better parent / Better Parent, measured in units of the trait.

P_{F1}= Average performance of crossbreds.

P_p = Average performance of parents lines = P1+P2/2.

5. Results and Discussion

Transconjugant efficiency of bacterial recombinants

Environmental pollution has been recognized as one of the major problems of the modern world. Industrial development results in the generation of industrial effluents, and if untreated it was resulted pollution in water, sediment and soil pollution (**Fakayode, 2005**). Industrial wastes and emission contain toxic and hazardous substances, most of which are detrimental to human health (**Rajaram and Ashutost, 2008**). The key pollutants include heavy metals, chemical wastes and oil spills etc. Heavy metal resistant bacteria have significant role in bioremediation of heavy metals in wastewater. The biosorption is basically at lab scale in spite of its development for decades (**Wang and Chen, 2006**). Microbial biomass can be used to decontaminate metal bearing wastewaters ,as well as, to concentrate metals. Biological methods such as biosorption or bioaccumulation strategies for the removal of metals ions may provide an attractive alternative to existing technologies (**Preetha and Viruthagiri, 2005**).

Bacterial transconjugants were used in this work as the biosorbents for Cu, Co, Fe, Cd and Pb. All bacterial strains used herein were grown in minimal medium containing 100% factory effluents.

As shown from the results in Table 4, most of bacterial transconjugants appeared higher levels in heavy metals uptake than their parental strains. Higher positive efficiency was achieved in transconjugant treatment in relation to the mid parents, as well as, the better parent. These transconjugants were as follows, Tr1 resulted from NRRL B-571 x NRRL B-1584, NRRLB-571 x NRRLB-2643 and NRRL B-358 x NRRL NRS 642. In addition, Tr1 and Tr2 resulted from NRRL NRS-1264 x NRRL B-2643, NRRLB-1584 x NRRL NRS-213, NRRL B-2643 x NRRL B-642, NRRL B-41228 x NRRL B-642 and NRRL B-642 x NRRL B-4375 appeared the same trend. The results obtained herein agreed with those reported by **John Milton and Reetha (2012**), who found that among the five strains of bacteria, *Bacillus* HMB1 was high efficient than the others strains in removal of heavy metals from the solution of waste water containing 100 mg/L. In addition, **Ting and Choong (2009)**, found that three strains of *Pseudomonas* isolated from heavy metal contaminated soil accumulated 29, 25 and 26 mg g^{-1} dry weight of cells, respectively at the zinc concentration of 1.6 mM. **Ahmad *et al*. (2005)** found that gram negative bacteria showed higher bioaccumulation capacity of heavy metals than Gram positive counter parts due to their higher level of intrinsic metal resistance. **Brierley (1990)** reported that bacteria make excellent biosorbents because of their high surface-to-volume ratios and a high content of potentially active chemosorption sites such as on teichoic acid in their cell walls. **Churchill *et al*. (1995)** used two gram-negative strains (*Escherichia coli* K-12 and *Pseudomonas aeruginosa*) and a gram-positive strain (*Micrococcus luteus*) to demonstrate biosorption of Cu^{2+}, Cr^{3+}, Co^{2+} and Ni^{2+}. Their sorption binding constants suggested that *E. coli* cells were the most efficient at binding copper, chromium and nickel and *M. luteus* sorbed cobalt most efficiently. Microbial species, such as *Pseudomonas*, have been shown to be relatively efficient in bioaccumulation of uranium, copper, lead, and other metal ions from polluted effluents (**Gupta *et al*., 2001**).

Many genera of microbes like *Bacillus, Enterobacter, Escherichia, Pseudomonas* and also some yeasts and fungi help in bioremediation of metals contaminated soil and water by bio-absorption. Heavy metal resistance genes are often found on plasmids and transposons **(Chu *et al*., 1992).** Plasmids also assist bacteria to acquire tolerance and resistance mechanisms against heavy metals or other toxic substances in the polluted environment (**Boronin 1992**).

Table 4: Transconjugant efficiency percentage for heavy metals uptake (mg per g biosorbent) by bacterial recombinants growing on minimal medium supplemented with wastewaters.

Biocontrol agents		ppm				
		Cu	Co	Fe	Cd	Pb
NRRL B-571 X NRRL B-1584	P1	793	724	13793	862	758
	P2	631	368	7017	368	736
	MP	712	546	10405	615	747
	Tr1	1085	1200	22685	1028	1771
	TE MP	52.4	120	118	67	137
	TE BP	36.8	66	64	19	134
	Tr2	403	456	7070	403	543
	TE MP	-43.4	-16	-32	-34	-27
	TE BP	-49.2	-37	-49	-53	-28

NRRL B-571 X NRRL B-2643	P1	793	724	13793	862	758
	P2	549	167	6061	244	76
	MP	671	445	9927	553	417
	Tr1	371	268	4123	185	432
	TE MP	-45	-40	-58	-67	4
	TE BP	-53	-63	-70	-79	-43
	Tr2	351	120	4362	241	307
	TE MP	-48	-73	-56	-56	-26
	TE BP	-56	-83	-68	-72	-59
NRRL B-571 X NRRL B-41228	P1	793	724	13793	862	758
	P2	514	495	7561	533	666
	MP	653	609	10677	697	712
	Tr1	234	71	4153	224	428
	TE MP	-64	-88	-61	-68	-40
	TE BP	-70	-90	-70	-74	-44
	Tr2	675	525	10000	450	775
	TE MP	3	-14	-6	-35	9
	TE BP	-15	-27	-27	-48	2

Table 4 continued

Biocontrol agents		ppm				
		Cu	Co	Fe	Cd	Pb
NRRL B-1584 X NRRL NRS-213	P1	631	368	7017	368	736
	P2	653	428	8163	428	387
	MP	642	398	7590	398	561
	Tr1	1600	1050	20150	1100	1250
	TE MP	149	164	165	176	123
	TE BP	154	185	187	199	70
	Tr2	677	225	13000	677	1161
	TE MP	5	-43	71	70	107
	TE BP	7	-39	85	84	58
NRRL NRS-1264 X NRRL B-2643	1264	326	456	8630	456	391
	2643	549	167	6061	244	76
	MP	437	311	7345	350	233
	Tr1	612	193	12806	483	903
	TE MP	40	-38	74	38	288
	TE B P	88	-58	48	6	131
	Tr2	1600	1300	20150	1300	1550
	TE MP	266	318	174	271	565
	TE BP	391	185	133	185	296
NRRL B-358 X NRRL B-642	P1	590	180	6557	245	590
	P2	301	198	2830	188	179
	MP	446	189	4694	217	385
	Tr1	426	280	5373	266	520
	TE MP	-4	48	14	23	35
	TE BP	-28	56	-18	9	-12
	Tr2	39	33	839	43	75
	TE MP	-91	-83	-82	-80	-80
	TE BP	-93	-82	-87	-82	-87
NRRL B-2643 X NRRL B-642	P1	549	167	6061	244	76
	P2	301	198	2830	188	179
	MP	425	182	4445	216	127
	Tr1	225	258	3166	175	325
	TE MP	-47	42	-29	-19	156
	TE BP	-59	54	-48	-28	328
	Tr2	298	168	5220	194	324
	TE MP	-30	-8	17	-10	155
	TE BP	-46	1	-14	-20	326
NRRL B-41228 X NRRL B-642	41228	514	495	7561	533	666
	642	301	198	2830	188	179
	MP	407	346	5195	360	422
	Tr1	10800	10800	160000	8800	8800
	TE MP	2554	3021	2980	2344	1985
	TE BP	2001	2082	2016	1551	1221
	Tr2	2750	5250	100000	3750	7750
	TE MP	576	1417	1825	942	1736
	TE BP	435	961	1223	604	1064
NRRL B-642 X NRRL B-4375	P1	514	495	7561	533	666
	P2	301	198	2830	188	179
	M.P.	407	346	5195	360	422
	Tr1	10800	10800	160000	8800	8800
	TE MP	2554	3021	2980	2344	1985
	TE BP	2001	2082	2016	1551	1221
	Tr2	2750	5250	100000	3750	7750
	TE MP	576	1417	1825	942	1736
	TE BP	435	961	1223	604	1064
NRRL B-642 X NRRL NRS-213	P1	301	198	2830	188	179
	P2	653	428	8163	428	387
	MP.	477	313	5496	308	283
	Tr1	410	205	5038	230	25
	TE MP	-14	-35	-8	-25	-91
	TE BP	36	4	78	22	-86
	Tr2	1250	1750	33333	1833	3250
	TE MP	162	459	506	495	1048
	TE BP	315	784	1078	875	1716
NRRL B-4375 X NRRL NRS-213	P1	335	167	4188	188	293
	P2	653	428	8163	428	387
	MP	494	297	6175	308	340
	Tr1	597	675	10311	597	1246
	TE MP	21	127	67	94	266
	TE BP	78	304	146	218	325
	Tr2	640	420	7860	320	760
	TE MP	30	41	27	4	124
	TE BP	91	151	88	70	159

MP = Mid parents, TEMP= Transconjugant efficiency related to mid-parents, TEBP = Transconjugant efficiency related to better parent.

Most treatments with bacterial recombinants exceeded one hundred percentage than their parental strains in their efficiency of heavy metals uptake from waste waters. This reflected the important role of bacterial recombinants to be used in bioremediation experiments in industrial sites. The data obtained herein agreed with **Brierley *et al*. (1986) ,** who suggested that a metal loading capacity greater than 15% of biomass could be used as an economic threshold for practical applications of biosorption as compared with alternative techniques.

In conclusion, the removal of heavy metals from industrial waters has become an important application in water and wastewater treatment systems. Many bacterial strains contain genetic determinants to heavy metals uptake such as Hg, Ag, Ci, Cd and undoubtedly others. These resistance determinants are often found on plasmids**.** Biosorption is being an alternative to conventional methods for the removal of toxic heavy metals from chemical industrial effluents. The results obtained in this study indicated the potential use of recombinant bacteria for efficient removal of heavy metals from industrial effluents containing higher concentration of heavy metals.

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