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Organic Compounds and Inorganic Metals Removal from Wastewater Using *Klebsiella pneumoniae* and *Acinetobacter lwoffii*

EL-Gamal, M.S.[1], El-Shahawy, M.R.[2], Fouda, A.[1], Bekhit, H.A.[3]

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E.mail:hashim.arafa@yahoo.com

ABSTRACT

Wastewater contains high levels of organic material, numerous pathogenic microorganisms, as well as nutrients and toxic compounds. Wastewater samples were collected from influent of different wastewater treatment plant. *Klebsiella pneumoniae* and *Acinetobacter lwoffii* were isolated from the samples and these isolates were screened for the reduction of wastewater components which estimated by COD (Chemical Oxygen Demand), BOD (Biological Oxygen Demand), and TSS (Total Suspended Solids) values. Wastes reduction by the isolated bacteria using different pH values, incubation temperatures, inoculum volume, static and dynamic condition with different incubation periods and bioremediation the variable concentrations of metals in singles and mixtures states were studied. Our investigate show the optimum conditions at pH 7, 35˚C, dynamic condition, 0.5% standard inoculum volume for *K.pneumoniae* and 2% for *A.lwoffii*, 48 hours' incubation period, and metals concentrations 5ppm.

KEYWORDS

Abu Rawash Wastewater Plant, Klebsiella pneumonia, Acinetobacter lwoffii, Heavy Metal, Mineral Silica Oil (MSO), Tryptic Soy Agar (TSA).

1. Botany & Microbiology Department, Faculty of Science, Al-Azhar University, Cairo, Egypt.
2. Radiation Microbiology Dept, National Center for Radiation Research and Technology, Egyptian Atomic Energy Authority, Cairo, Egypt.
3. Reference Lab for Wastewater, Cairo, Egypt.

INTRODUCTION

Human sewage and waste from manufacturing industries was the main source for wastewater. The total volume of wastewater from industry is about 7 times that of domestic sewage. If untreated, and discharged directly to the environment, the receiving waters would become polluted and water-borne diseases would be widely distributed **(Davies, 2005).** Untreated wastewater generally contains high levels of organic material, pathogenic microorganisms, as well as nutrients and toxic compounds. It thus entails environment and health hazards, therefore, it must immediately transported away from its generation sources and treated appropriately before final disposal. The release of high amounts of heavy metals into water bodies creates serious health and environmental problems and may lead to an upsurge in wastewater treatment cost, to prevent the negative effects of heavy metals toxicity in wastewater, there is need for adequate treatment of effluents before discharge to receiving water bodies (**Oghenerobor *et al.*, 2014; Fouda *et al*., 2016)**. Egypt faces a rapidly increasing deterioration of its surface and groundwater due to increasing discharges of heavily polluted domestic and industrial effluents into its waterways. There are estimated to be some 24,000 industrial enterprises in Egypt, about 700 of which are major industrial facilities. Egyptian industry uses 638 Million m^3/ yr. of water, of which 549 Million m^3/ yr. is discharged to the drainage system. Industrial activities in the Greater Cairo and Alexandria regions use 40% of the total. The River Nile supplies 65% of the industrial water needs and receives more than 57% of its effluents **(Mohamed *et al.*, 2013)**. The domestic pollution affects water quality heavily depends on the way of disposal of pollutants. Approximately 65 percent of Egypt's population is connected to drinking water supply and only 24 percent to sewage services, although the latter percentage is expected to grow rapidly, due to works under construction. The population not connected to sewage systems relies on individual means of excreta and wastewater disposal such as latrines and septic tanks. The domestic wastewater spread into soil and groundwater by discharging and collecting wastewater in permeable septic tanks. The domestic wastewater is considered as the main source of pollution of groundwater. It contains many toxic and injurious chemical constituents that have serious effect on public health problems (**Easa and Abou-Rayan, 2010).** The ultimate goal of wastewater management is the protection of the environment in a manner commensurate with public health.**(ESCWA 2010)**. Different wastewater treatment methods or systems with minimum electric requirements and low maintenance costs were needed to overcome problems or hazardous of conventional methods currently used. **(Mara *et al.*, 1992; Brix, 1994; Vymazal, 2002; Bécares, 2006; Puigagut *et al.*, 2007).** In the early years of the twentieth century the method of biological treatment was devised, and now forms the basis of wastewater treatment worldwide. In the biological treatment the naturally occurring bacteria, together with some protozoa and other microbes, are collectively referred to as activated sludge. The concept of treatment is very simple. The bacteria remove small organic carbon molecules by 'eating' them. As a result, the bacteria grow, and the wastewater is cleansed. The treated wastewater or effluent can then be discharged to receiving waters normally a river or the sea **(Lin, 2007)**.

Therefore, this study aimed to:

1- Isolation the total bacterial consortia from different wastewater samples.

2- Screening and selection of the most potent isolates which play important role in wastes reduction in wastewater.

3- Identification of the most potent isolates and optimized the bacterial treatment conditions (pH, temperatures, inoculum size and incubation condition (static and shaking) at different incuba-

tion period) for enhanced wastewater treatment.

4- Study the effect of different concentrations of metals on isolates growth count, metals bioremediation and wastes reduction.

MATERIALS AND METHODS

Samples collection

Wastewater samples were collected from three different wastewater plant represented by Abu Rawash Wastewater plant (primary treatment), Egypt Alexandria desert road, giza, Egypt, El tanqia El sharqia Wastewater plant (Secondary treatment), the east Alexandria (beside Beirut university, El-Ramel Station), Egypt and Shobra El-khema Wastewater plant, ring road, Qalubia, Egypt.

The Wastewater samples were collected in sterile closed bottles; and dipped in the subsurface and transferred within 6 hours for Lab. and culturing immediately. The remaining samples from each one used for chemical analysis and total bacterial count.

Isolation and purification of total bacteria isolates

The collected wastewater samples were serially diluted using phosphate buffer. One ml of each dilution was inoculated on **Tryptic Soy Agar** (TSA) (containing g/L: Pancreatic digest casein, 15; Pancreatic digest soybean, 5; Sodium chloride, 5; Agar, 15; dis. H_2O, 1L) for 24h at 35±2˚C. The isolates were purified by re-streaking separately on TSA. Morphological characterization of purified isolates were be done.

Screening and selection of the most potent bacteria

The purified bacterial isolates were growing on **Mineral Silica Oil** (MSO) containing diesel oil as only carbon source **(EL Shahawy, 2007).** Five ml of each separately bacterial isolates (adjusted at $O.D._1$) were inoculated in 500 ml wastewater and incubated for 7 days at 35ºC. Bacterial isolates have the ability to grow on diesel oil as only carbon source selected for growing on 500 ml wastewater to select most potent isolates according to COD, BOD, TS, TSS and pH a triplicate for each organism on each sample. Control was raw wastewater sample and sterilized raw wastewater without inoculation.

Molecular identification of most potent bacterial isolates

The most potent bacterial isolates were identified by 16S rRNA gene as the following:

- **DNA extraction using protocol of GeneJet genomic DNA purification Kit (Thermo K0721).**

  The bacterial cell (up to $2x10^9$) was harvested in a 1.5 or 2 ml micro centrifuge tube by centrifugation for 10 min at 5000 x g. the cell was completely digested and lysed. The prepared lysate transferred to a GeneJET™ Genomic DNA Purification Column inserted in a collection tube. The purified DNA was used immediately in PCR.

- **PCR using Maxima Hot Start PCR Master Mix (Thermo K1051).**

  Gently vortex and briefly centrifugation Maxima® Hot Start PCR Master Mix (2X) after thawing. The following components were added for each 50µl reaction at room temperature: Maxima® Hot Start PCR Master Mix (2X) 25µl, 16SrRNA Forward primer 1ul (20uM), 16SrRNA Reverse primer (of each 8 primer) 1ul(20uM), Template DNA 5ul and Water, nuclease-free 18µl where total volume was 50µl. Gently vortex the samples and spin down. PCR performed using the recommended thermal cycling conditions F: AGAGTTTGATCCTGGCTCAG R:GGTTACCTTGTTACGACTT

- **PCR clean up to the PCR product using GeneJET™ PCR Purification Kit (Thermo K0701).**

  Added a 45ul of Binding Buffer to completed

PCR mixture and Mix thoroughly. The mixture was transferred from step 1 to the GeneJET™ purification column and centrifugation for 30-60 s at >12000 x g. Discard the flow-through, then 100ul of Wash Buffer was added to the GeneJET™ purification column and centrifugation for 30-60 s. Discard the flow-through and the purification column back was placed into the collection tube, the purified DNA at -20°C. Finally sequencing to the PCR product was made on GATC Company by use ABI 3730xl DNA sequencer by using forward and reverse primers. Only by combining the traditional Sanger technology with the new 454 technologies, can genomes now be sequenced and analyzed in half the usual project time, with a considerable reduction in the number of coatings and gaps. In addition, considerable cost advantages now make genome sequencing with the 454 technology accessible to the research community.

Optimization of culture conditions for enhanced wastewater treatment by most potent bacterial isolates

The effect of various culture conditions such as pH, temperature, inoculum size, and incubation period at different condition (static and shaker status) on wastewater treatment by the most potent bacterial strains was examined.

Control (raw wastewater without inoculation) was running with each experiments.

Effect of different incubation temperature, pH and inoculum size on wastewater treatment by two bacterial isolate

In order to test the effect of different incubation temperature on the wastewater treatment process, the two bacterial isolates were allowed to grow on 500 ml raw wastewater. The microbial isolates were incubated for 7 days at different pH values (5, 6, 7, 8 and 9) a triplicate for each organism on each sample. Similarly, the effect of different incubation temperature 20, 25, 30, 35 and 40˚C on wastewater treatment was tested. Separately, the wastewater treatment percentages were evaluated under the effect of different bacterial inoculum sizes were applied as 0.5%, 1%, 2% and 3 % (v/v) in 500 ml of wastewater samples.

At the end of each experiment, the following parameter were analyzed (COD, BOD, TS, TSS, TDS, pH, Conductivity and Ammonia)

Effect of different incubation period and incubation condition (static and shaking status) on wastewater treatment

This experiment was carried out in order to investigate the effect of different incubation period and condition on wastewater treatment process. The two potent bacterial isolates were allowed to separately grow on 500 ml of wastewater, allowed to grow at previous optimum pH value, incubation temperature and inoculum size for 24, 48, 72, 96, 120, 144 and 168 hours at static and shaking (150 rpm) a triplicate for each organism on each sample to determine the best incubation conditions and time. At the end of experiment, the following parameters were analyzed (COD, BOD, TS, TSS, TDS, pH, Conductivity and Ammonia).

Effect of different metals present in wastewater on growth of two potent isolates

Wastewater containing different type of metal such as Cr, Pb, Fe, Zn and Cu. Therefore, it's important to detect the effect of this metal on microbial growth used in wastewater treatment process.

Effect of different concentrations of single metals on microbial growth count

Two most potent bacterial isolates were inoculated in 200 ml of TSB containing different concentration of metals in single form and mixture (Cr, Pb, Fe, Zn, Cu applied as 5, 10 and 15 mg/l) in separate conical flask. The flasks were incubated at 35°C for 48h. The ability of bacterial isolates to tolerate metal concentration were be detected by bacterial count at the end of experiment.

Effect of different concentrations of metals mixture on wastewater treatment process by bacterial isolates

The optimum inoculum of each most potent bacterial isolates were inoculated in 500 ml of wastewater containing different concentration of metal mixture (Cr, Pb, Fe, Cu & Zn at 5, 10 and 15 mg/l) and incubated at optimum previous condition. The metals reduction were detected by Plasma Emission Spectroscopy system and wastes reduction were detected by measuring COB, BOD, TSS and ammonia.

Analyses of treated wastewater

The values of pH, TDS (Total Dissolved Solids), TSS (Total Suspended Solids), TS (Total Solid), COD (Chemical Oxygen Demand), BOD (Biological Oxygen Demand), ammonia and TOG (Total oil & Grease) were measured for wastewater samples before and after the different treatments. The measures were carried out according to the standard methods recommended by **FEW and APHA, 2012, Eugene, *et al*., 2012.**

Metals were measured by Plasma Emission Spectroscopy. The equipment's used were ICP-OES 7300DV (Perkin Elmer, U.K), Microwave (Anton Paar, Europe) and Electronic balance (Sartouriu, Germany) according to **FEW and APHA, 2012,** ^{22}th Edition, **method No 3120B.**

Statistical Analysis

Data were statistically analyzed by SPSS v17, one-way and two way analyses of variance (ANOVA) test were used for multiple sample comparison, when normality and homogeneity of variance were satisfied, followed by multiple comparison Tukey test.

RESULTS

Samples

The collected wastewater samples were undergo chemical analysis to detect their content before treatment as shown in Table 1. Data showed that the highest content of heavy metal especially Pb and Fe and total bacterial count were in shobra El-Khema plant. During this study, thirty-one bacterial isolates were isolated from three plant and classified as the following, eleven isolates from Abu Rawash, eleven isolates from El Tanqia and nine from Shobra El-Khema.

Thirty-one isolates were inoculated in MSO containing diesel oil as a sole carbon source. From this isolates, fifteen isolates have the ability to grow on MSO and classified as the following, four isolates from Abu Rawash, three isolates from El Tanqia and eight from Shobra El-Khema. Therefore, fifteen bacterial isolates inoculated in wastewater sample to select most potent bacterial isolates according to result of TSS, COD and BOD as shown in Table 2. According to the results of TSS, COD and BOD for different bacterial isolates which detected the best treatment, the bacterial isolates A4 and A5 were least can be compared by other isolates. Therefore, selected isolates A4 and A5 as most potent

The molecular identification based on 16S rRNA gene amplification showed that the bacterial strains A4 and A5 have similarity to *Klebsiella pneumonia* and *Acinetobacter lwoffii,* respectively. The topology of *Klebsiella pneumonia* and *Acinetobacter lwoffii* strains A4 and A5 was retrieved from the phylogenetic tree including various bacterial 16S rRNA gene sequences of the common bacterial families (Fig.1).

Table (1) : *Chemical analysis and total bacterial count of the collected wastewater samples.*

Parameters\Samlpe types	Units	Abu Rawash	ElTanqia El Sharqia	Shobra Elkhema
pH		7.35 ± 0.09	7.33 ± 0.09	7.41 ± 0.09
Conductivity	µhmos	768 ± 3	1845 ± 5.57	846 ± 6.08
TDS	ppm	461 ± 1.15	1107 ± 3.46	510 ± 4.04
TS	ppm	709 ± 3.21	1408 ± 8.14	710 ± 5
TSS	ppm	193 ± 4.73	200 ± 2.65	184 ± 2.52
Ammonia	ppm	26.9 ± 0.12	32.3 ± 0.58	20.6 ± 0
COD	ppm	348 ± 1.53	502 ± 2	548 ± 1.73
BOD	ppm	116 ± 1.53	244 ± 3.51	243 ± 3.51
TOG	ppm	55 ± 1.53	58 ± 2	61 ± 1
Pb	ppm	0.0040 ± 0.0001	0.2051 ± 0.0001	2.1667 ± 0.0012
Cd	ppm	0.0000 ± 0.0000	0.0020 ± 0.0000	0.0030 ± 0.0001
Zn	ppm	0.1167 ± 0.0015	0.3737 ± 0.0012	0.2430 ± 0.0017
Cu	ppm	0.0000 ± 0.0000	0.2760 ± 0.0000	0.0990 ± 0.0000
Ni	ppm	0.0050 ± 0.0000	0.0457 ± 0.00115	0.0400 ± 0.0000
Fe	ppm	0.4520 ± 0.0000	0.6700 ± 0.0000	1.8167 ± 0.0116
Cr	ppm	0.0080 ± 0.0006	0.2493 ± 0.00058	0.1080 ± 0.0017
Total bacterial count on TSA	cell/ml	$26.2 *10^5 \pm 3.8 *10^3$	$11.4 *10^5 \pm 4.04*10^2$	$62.1*10^5 \pm 11.5 *10^3$
Total bacterial count on MSO	cell/ml	3657 ± 14	3998 ± 13	4975 ± 22

CFU= Colony Forming Unit *ppm= Part Per million*

**All data represented means of three replica ± Stander Deviation (SD)*

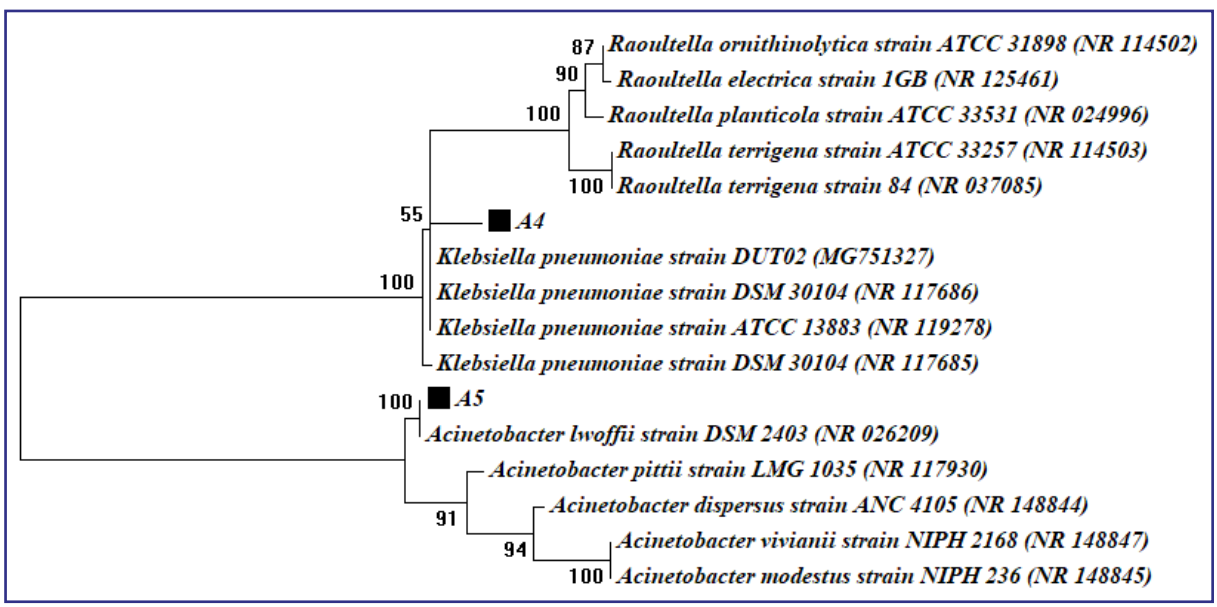

Fig. (1): Phylogenetic analysis of 16S rRNA sequences of the bacterial isolates with the sequences from NCBI. Symbol ■ refers to 16S rRNA gene fragments retrieved from this study. The analysis was conducted with MEGA 6 using neighbor-joining method.

Table (2) : *Selection of the most potent bacterial isolates.*

Sample	pH	TS (mg/l)	TSS (mg/l)	COD (mg/l)	BOD (mg/l)
Raw sample	7.11 ± 0.01	810.3 ± 7.2	204.5 ± 3.6	438 ± 2.1	214 ± 0.9
Control	7.1 ± 0.15	828.21 ± 13.35	56.39 ± 3.12	114.80 ± 5.65	54.38 ± 0.78
Isolate A1	8.0 ± 0.21	807.45 ± 7.21	40.28 ± 2.13	75.63 ± 3.27	36.25 ± 1.19
Isolate A2	7.82 ± 0.12	788.83 ± 11.23	24.17 ± 5.29	76.53 ± 4.34	36.25 ± 2.18
Isolate A3	8.21 ± 0.09	809.15 ± 17.5	48.34 ± 2.12	95.67 ± 2.11	45.32 ± 4.76
Isolate A4	7.63 ± 0.13	787.11 ± 13.2	16.11 ± 1.23	73.51 ± 5.23	35.25 ± 1.57
Isolate A5	7.93 ± 0.23	801.19 ± 9.66	32.22 ± 6.53	57.30 ± 7.56	27.19 ± 1.23
Isolate A6	7.77 ± 0.14	796.67 ±12.31	40.28 ± 7.29	94.86 ± 5.82	45.32 ± 6.11
Isolate A7	7.91 ± 0.05	778.73 ± 20.9	19.08 ± 5.26	80.03 ± 3.19	29.20 ± 7.23
Isolate A8	8.36 ± 0.21	775.5 ± 14.23	28.20 ± 6.17	90.03 ± 2.62	42.29 ± 2.11
Isolate A9	8.19 ± 0.17	776.63 ±13.72	25.18 ± 1.23	88.20 ± 5.17	27.19 ± 1.25
Isolate A10	8.2 ± 0.07	775.27 ±11.15	28.20 ± 0.9	94.26 ± 4.36	44.31 ± 4.73
Isolate A11	8.05 ± 0.13	799.57 ±15.83	40.28 ± 4.75	87.91 ± 3.17	41.29 ± 6. 12
Isolate A12	8.02 ± 0.05	774.59 ± 9.38	32.06 ± 3.17	80.29 ± 6.15	33.23 ± 2.57
Isolate A13	8.11 ± 0.13	804.37 ± 17.1	44.31 ± 7.85	91.84 ± 2.13	43.30 ± 0.9
Isolate A14	7.83 ± 0.18	782.13 ±15.23	20.14 ± 3.22	91.33 ± 2.94	43.30 ± 1.13
Isolate A15	7.69 ± 0.12	785.13 ± 11.2	22.15 ± 4.21	91.53 ± 4.63	59.20 ± 3.5

**All data represented means of three replica ± Stander Deviation (SD)*

Optimized factor affecting wastewater treatment

Effect of pH, temperature, and inoculum size on wastewater treatment process

Data of COD, BOD, TSS and ammonia for treated wastewater by two most potent bacterial isolates revealed that the maximum treated was recorded at pH 7 and any increase or decrease in pH from the optimal value reduces the treatment process. Our result showed that COD, BOD, TSS and ammonia for wastewater treated by *Klebsiella pneumonia* and *Acinetobacter lwoffii* were 46.63±0.69 and 43.53±0.65, 21.97±0.33 and 20.97 ± 0.31, 9.98±0.15 and 8.99 ±0.13, 29.76±0.44 and 32.05 ±0.48 respectively when compared with control (**Table 3**).

The results showed that the highest treatment values were found at temperature 35°C for both the two most potent bacterial isolates according to data of COD, BOD, TSS and ammonia value showed in **Table 4** when compared to control. Data also showed that the maximum waste reduction in treated wastewater was achieved at inoculum size represented by 0.5% and 2% for *Klebsiella pneumoniae* and *Acinetobacter lwoffii* respectively (**Table 5).**

Table (3) : *Effect of different pH values on the wastewater treatment process.*

Sample	Conditions	TS (mg/l)	TDS (mg/l)	COD (mg/l)	BOD (mg/l)	TSS (mg/l)	Ammonia (mg/l)
Raw sample		820.13 ± 15.02	552.81 ± 10.12	441.03 ± 7.05	217.49 ± 4.43	208.07 ± 4.7	24.58 ± 0.62
Control at different initial pH values	**pH5**	949.41 ± 17.23	839.74 ± 12.45	84.47 ± 1.25	40.94 ± 0.61	27.96 ± 0.41	32.55 ± 0.48
	pH6	908.68 ± 16.88	806.79 ± 11.96	80.68 ± 1.2	38.94 ± 0.58	22.97 ± 0.34	38.04 ± 0.56
	pH7	608.03 ± 11.41	539.19 ± 7.99	67.60 ± 1.3	32.95 ± 0.49	17.97 ± 0.27	31.35 ± 0.53
	pH8	679.42 ± 14.15	600.10 ± 8.9	67.20 ± 1.92	31.95 ± 0.47	19.97 ± 0.3	35.45 ± 0.53
	pH9	846.75 ± 18.59	752.87 ± 11.16	66.10 ± 0.98	31.95 ± 0.47	17.97 ± 0.27	34.65 ± 0.51
***K.pneumonia* at different initial pH values**	**pH5**	936.62 ± 17.71	832.75 ± 12.35	68.50 ± 1.02	32.95 ± 0.49	21.97 ± 0.33	33.35 ± 0.49
	pH6	878.67 ± 13.21	802.79 ± 11.9	47.23 ± 0.7	22.97 ± 0.34	7.99 ± 0.12	33.85 ± 0.50
	pH7	629.61 ± 16.48	560.16 ± 8.3	46.63 ± 0.69	21.97 ± 0.33	9.98 ± 0.15	29.76 ± 0.44
	pH8	647.74 ± 10.83	584.12 ± 8.66	55.72 ± 0.83	26.96 ± 0.40	11.98 ± 0.18	36.15 ± 0.54
	pH9	829.53 ± 13.2	748.87 ± 11.1	58.61 ± 0.87	27.96 ± 0.41	15.98 ± 0.24	34.75 ± 0.52
***A.lwoffii* at different initial pH values**	**pH5**	931.55 ± 15.69	833.74 ± 12.36	59.81 ± 0.89	28.96 ± 0.43	19.97 ± 0.3	33.15 ± 0.49
	pH6	883.42 ± 15.45	798.80 ± 11.84	55.62 ± 0.82	26.96 ± 0.40	9.98 ± 0.15	39.64 ± 0.59
	pH7	610.03 ± 9.69	553.17 ± 8.2	43.53 ± 0.65	20.97 ± 0.31	8.99 ± 0.13	32.05 ± 0.48
	pH8	663.94 ± 15.62	590.11 ± 8.75	49.73 ± 0.74	23.96 ± 0.36	12.98 ± 0.19	37.34 ± 0.55
	pH9	832.61 ± 18.36	749.87 ± 11.12	64.50 ± 0.96	30.95 ± 0.46	17.97 ± 0.27	34.75 ± 0.52

Table (4) : *Effect of different incubation temperatures on the wastewater treatment process.*

Sample	Condition	TS (mg/l)	TDS (mg/l)	COD (mg/l)	BOD (mg/l)	TSS (mg/l)	Ammonia (mg/l)
Raw sample		841.34 ± 15.38	576.13 ± 8.54	463.80 ± 6.88	221.67 ± 3.29	194.71 ± 2.89	18.52 ± 0.27
Control at different incubation temperatures	**20 °C**	736.64 ± 14.32	654.02 ± 9.7	84.37 ± 1.25	39.94 ± 0.59	19.97 ± 0.3	38.78 ± 1.15
	25 °C	623.82 ± 9.25	691.96 ± 10.26	82.72 ± 2.71	38.87 ± 1.29	20.97 ± 0.31	37.54 ± 0.56
	30 °C	764.54 ± 11.39	685.97 ± 10.17	80.63 ± 1.2	37.94 ± 0.56	21.97 ± 0.33	43.93 ± 0.65
	35 °C	771.32 ± 14.54	685.97 ± 10.17	73.09 ± 1.08	34.95 ± 0.52	19.97 ± 0.3	47.03 ± 0.70
	40 °C	767.27 ± 13.46	687.96 ± 10.20	80.08 ± 1.19	37.94 ± 0.56	15.98 ± 0.24	37.34 ± 0.55
***K.pneumonia* at different incubation temperatures**	**20 °C**	660.87 ± 11.05	592.11 ± 8.78	77.78 ± 1.15	36.94 ± 0.55	15.98 ± 0.24	36.20 ± 0.98
	25 °C	615.12 ± 13.94	667.00 ± 9.89	69.10 ± 1.02	33.5 ± 0.94	17.97 ± 0.27	34.05 ± 0.50
	30 °C	681.78 ± 15.03	605.59 ± 8.98	45.78 ± 1.68	21.97 ± 0.33	15.48 ± 0.23	35.05 ± 0.52
	35 °C	699.57 ± 10.44	638.04 ± 9.46	40.14 ± 0.6	18.97 ± 0.28	9.98 ± 0.15	37.84 ± 056
	40 °C	691.87 ± 13.96	618.07 ± 9.16	48.63 ± 0.72	22.97 ± 0.34	13.98 ± 0.21	30.15 ± 0.45
***A.lwoffii* at different incubation temperatures**	**20 °C**	783.78 ± 15.5	695.95 ± 10.32	78.18 ± 1.16	36.94 ± 0.55	19.97 ± 0.30	45.83 ± 0.68
	25 °C	720.17 ± 16.98	777.83 ± 11.53	71.79 ± 1.06	34.89 ± 0.67	18.97 ± 0.28	39.44 ± 0.58
	30 °C	792.87 ± 15.65	708.93 ± 10.51	63.37 ± 0.94	29.95 ± 0.44	15.98 ± 0.24	49.92 ± 0.74
	35 °C	816.11 ± 17.24	733.90 ± 10.88	45.43 ± 0.67	20.97 ± 0.31	9.98 ± 0.15	46.03 ± 0.68
	40 °C	844.03 ± 14.78	757.86 ± 11.23	58.91 ± 2.87	27.96 ± 0.41	16.97 ± 0.25	43.53 ± 0.65

Table (5) : *Effect of different inoculum size on treatment process.*

Sample type		TS (mg/l)	TDS (mg/l)	COD (mg/l)	BOD (mg/l)	TSS (mg/l)	Ammonia (mg/l)
Raw water		841.34 ± 15.38	576.13 ± 8.54	463.80 ± 6.88	259.61 ± 3.85	194.71 ± 2.89	18.52 ± 0.27
Control		775.35 ± 13.6	690.96 ± 10.24	70.39 ± 1.04	38.94 ± 0.58	20.97 ± 0.31	38.04 ± 0.56
Klebsiella pneumoniae	Inoculum 0.5 %	711.38 ± 12.83	642.03 ± 9.52	39.44 ± 0.58	21.97 ± 0.33	9.98 ± 0.15	36.94 ± 0.55
	Inoculum 1 %	711.38 ± 12.83	641.03 ± 9.5	40.04 ± 0.59	21.97 ± 0.33	10.98 ± 0.16	37.94 ± 0.56
	Inoculum 2 %	719.44 ± 11.34	650.02 ± 9.64	42.04 ± 0.62	22.97 ± 0.34	12.98 ± 0.19	39.04 ± 0.58
	Inoculum 3 %	710.68 ± 10.61	647.03 ± 9.59	42.94 ± 0.64	23.96 ± 0.36	10.98 ± 0.16	39.04 ± 0.58
Acintenobater lwoffii	Inoculum 0.5 %	787.80 ± 13.51	711.93 ± 10.55	48.23 ± 0.71	26.96 ± 0.4	11.98 ± 0.18	40.14 ± 0.6
	Inoculum 1 %	801.26 ± 13.46	726.91 ± 10.78	45.03 ± 0.67	24.96 ± 0.37	9.98 ± 0.15	43.14 ± 0.64
	Inoculum 2 %	798.55 ± 12.02	729.90 ± 10.82	43.14 ± 0.60	23.96 ± 0.36	8.99 ± 0.13	41.64 ± 0.62
	Inoculum 3 %	820.83 ± 17.74	733.90 ± 10.88	42.94 ± 0.64	23.96 ± 0.33	13.98 ± 0.21	42.14 ± 0.6

Effect of shaking and static condition at different incubation period

The wastewater treatment process capacity of two potent bacterial isolates was estimated under shaking and static state at different incubation time and at optimum temperature, pH and inoculum size for each isolate. Time is a critical factor in treatment process in a large scale. Therefore, shaking condition was favorable for treatment process compared to static condition and the minimum time with accepted result was at 48h, where the additional time meaning higher cost and space of wastewater plants.

The higher ability of the two most potent bacterial strains for wastewater treatment in shaking condition compared to static condition suggest that shaking status is favorable to enhance bacterial biomass and oxygen transfer between bacterial cells and the surrounding substrates. By increasing time of treatment process, data showed that static condition was favorable for treatment compared to shaking, this may be due to bacterial cell enter in death phase and the highest treatment in static regarded to settling process (**Table 6**).

Table (6) : *Effect of static and shaking state at different incubation time on wastewater treatment.*

	Time (h)	COD		BOD		TSS		ammonia	
		Static	Shaker	Static	Shaker	Static	Shaker	Static	Shaker
Control	zero	449.13 ± 8.94	449.13 ± 8.94	267.94 ± 5.97	267.94 ± 5.97	246.68 ±5.52	246.68 ±5.52	23.01 ± 0.45	23.01 ± 0.45
	24	112.01 ± 2.65	98.85 ± 2.57	64.78 ± 1.57	56.68 ± 1.53	49.59 ± 1.51	54.65 ± 1.52	22.98 ± 0.42	11.74 ± 0.22
	48	90.75 ± 2.54	95.81 ± 2.56	52.63 ± 1.52	54.65 ± 1.51	33.39 ±1.51	50.60 ± 1.51	24.60 ± 0.46	13.06 ± 0.24
	72	81.64 ± 2.51	83.67 ± 2.52	46.56 ± 1.5	47.57 ± 1.5	26.31 ± 1.54	46.56 ± 1.5	25.81 ± 0.48	10.53 ± 0.2
	96	78.60 ± 2.51	81.64 ± 2.51	40.48 ± 1.5	42.51 ± 1.5	24.28 ± 1.55	39.47 ± 1.5	28.65 ± 0.54	9.51 ± 0.19
	120	75.57 ± 2.5	83.67 ± 2.52	38.46 ± 1.5	43.52 ± 1.5	22.26 ± 1.56	36.43 ± 1.5	33.71 ± 0.64	8.30 ± 0.17
	144	75.57 ± 2.5	86.70 ± 2.55	38.46 ± 1.50	44.53 ± 1.51	20.74 ± 1.57	32.38 ± 1.51	35.63 ± 0.68	7.19 ± 0.16
	168	73.54 ± 2.5	86.70 ± 2.71	37.44 ± 1.5	44.53 ± 1.65	20.23 ± 1.57	29.35 ± 1.23	39.07 ± 0.75	6.78 ± 0.17
Klebsiella pneumoniae	zero	449.13 ± 8.94	449.13 ± 8.94	267.94 ± 5.97	267.94 ± 5.97	246.68 ±5.52	246.68 ±5.52	23.01 ± 0.45	23.01 ± 0.45
	24	129.94 ± 4.91	67.17 ± 3.83	75.26 ± 2.4	38.81 ± 1.73	47.26 ± 2.96	41.18 ± 2.87	23.62 ± 0.57	12.89 ± 0.35
	48	106.65 ± 4.49	61.10 ± 3.73	62.10 ± 2.15	34.77 ± 1.66	20.94 ± 2.58	36.12 ± 2.79	25.14 ± 0.6	11.27 ± 0.31
	72	58.06 ± 3.68	54.01 ± 3.62	33.75 ± 1.64	30.72 ± 1.59	15.88 ± 2.51	32.07 ± 2.73	26.25 ± 0.62	10.26 ± 0.29
	96	43.89 ± 3.47	58.06 ± 3.68	22.62 ± 1.46	29.70 ± 1.58	10.81 ± 2.45	30.05 ± 2.7	30.81 ± 0.72	7.32 ± 0.24
	120	42.88 ± 3.45	62.11 ± 3.75	21.60 ± 1.45	31.73 ± 1.61	9.80 ± 2.44	26.00 ± 2.65	33.34 ± 0.77	5.91 ± 0.21
	144	41.86 ± 3.44	69.20 ±3.86	21.60 ± 1.45	35.78 ± 1.68	9.80 ± 2.44	18.91 ± 2.55	34.56 ± 0.79	2.16 ± 0.14
	168	40.65 ± 3.42	70.21 ± 3.87	20.59 ± 1.43	35.78 ± 1.68	9.80 ± 2.44	16.89 ± 2.52	36.68 ± 0.84	1.86 ± 0.14
Acintenobater lwoffii	zero	449.13 ± 8.94	449.13 ± 8.94	267.94 ± 5.97	267.94 ± 5.97	246.68 ±5.52	246.68 ±5.52	23.01 ± 0.45	23.01 ± 0.45
	24	137.36 ± 4.54	89.78 ± 3.67	79.47 ± 2.25	52.14 ± 1.72	43.94 ± 1.71	40.91 ± 1.66	24.16 ± 0.48	12.28 ± 0.24
	48	87.75 ± 3.63	76.62 ± 3.44	51.13 ± 1.7	44.04 ± 1.56	5.47 ± 1.11	28.76 ± 1.45	25.17 ± 0.46	11.14 ± 0.36
	72	50.30 ± 3.01	56.37 ± 3.1	28.86 ± 1.29	32.91 ± 1.36	3.45 ± 1.08	21.67 ± 1.34	26.86 ± 0.5	8.47 ± 0.4
	96	41.18 ± 2.87	47.26 ± 2.96	20.76 ± 1.16	23.80 ± 1.21	3.45 ± 1.08	19.65 ± 1.31	31.72 ± 0.62	3.71 ± 0.43
	120	40.17 ± 2.85	54.35 ± 3.07	20.76 ± 1.16	27.85 ± 1.28	3.25 ± 1.08	15.60 ± 1.25	35.13 ± 0.64	2.33 ± 0.33
	144	40.17 ± 2.85	57.38 ± 3.12	20.76 ± 1.16	29.87 ± 1.31	3.25 ± 1.08	15.60 ± 1.25	37.89 ± 0.77	1.58 ± 0.27
	168	38.15 ± 2.82	63.46 ± 3.22	19.75 ± 1.14	32.91 ± 1.36	3.25 ± 1.08	14.59 ± 1.23	41.2 ± 0.86	0.32 ± 0.33

Effect of variable concentrations of different metals on K.pneumonia *and* A.lwoffii *growth counts*

Growth activities of *Klebsiella pneumoniae* and *Acinetobacter lwoffii* were affected by metals in single or mixture form present in wastewater. Therefore, it is necessary to study the effect of metals on bacterial growth and hence on metal removal.

Data in **Table 7** showed that the growth of *Acinetobacter lwoffii* was more affected by metal than *Klebsiella pneumonia*. The growth of *Klebsiella pneumonia* was highest at 5,10,15 ppm of Fe, 5,10 ppm of Cr and 5 ppm of Zn, respectively compared with control. While the growth of *Acinetobacter lwoffii* not affected by Cr, Pb, Fe and Zn metal at concentration 5 ppm. The growth of *Klebsiella pneumonia* in presence of metal in mixture form has partially affected by increasing mixture concentration. While growth of *Acinetobacter lwoffii* was more affected at high concentration of metal mixture. Data in **Figure 2&3** revealed that the ability of *Klebsiella pneumoniae* and *Acinetobacter lwoffii* to removal

Table (7) : *Effect of different concentrations of single and mixtures metals on bacterial growth count.*

Sample / Metals	Heavy metal concentration	*K.pneumonia*	*A.lwoffii*
Control	**Without metals**	$(280 \pm 7) * 10^8$	$(216 \pm 9) * 10^6$
Cr at different concentration	**5 ppm**	$(278 \pm 13) * 10^8$	$(188 \pm 4) * 10^6$
	10 ppm	$(270 \pm 15) * 10^8$	$(280 \pm 9) * 10^4$
	15 ppm	$(225 \pm 15) * 10^8$	$(83 \pm 17) * 10^3$
Pb at different concentration	**5 ppm**	$(113 \pm 8) * 10^8$	$(300 \pm 11) * 10^5$
	10 ppm	$(230 \pm 7) * 10^7$	$(283 \pm 8) * 10^4$
	15 ppm	$(121 \pm 10) * 10^7$	$(252 \pm 12) * 10^3$
Fe at different concentration	**5 ppm**	$(280 \pm 2) * 10^8$	$(108 \pm 10) * 10^5$
	10 ppm	$(280 \pm 3) * 10^8$	$(63 \pm 11) * 10^5$
	15 ppm	$(280 \pm 3) * 10^8$	$(48 \pm 3) * 10^5$
Zn at different concentration	**5 ppm**	$(241 \pm 14) * 10^8$	$(188 \pm 3) * 10^4$
	10 ppm	$(273 \pm 14) * 10^7$	$(103 \pm 18) * 10^4$
	15 ppm	$(221 \pm 7) * 10^7$	$(70 \pm 3) * 10^4$
Cu at different concentration	**5 ppm**	$(185 \pm 6) * 10^8$	$(64 \pm 17) * 10^5$
	10 ppm	$(220 \pm 11) * 10^7$	$(81 \pm 7) * 10^4$
	15 ppm	$(135 \pm 8) * 10^7$	$(93 \pm 6) * 10^3$
different concentration of mixtures metals (Cr+Pb+Fe+Zn+Cu)	**5 ppm**	$(180 \pm 1) * 10^8$	$(230 \pm 15) * 10^5$
	10 ppm	$(180 \pm 7) * 10^8$	$(118 \pm 7) * 10^3$
	15 ppm	$(173 \pm 14) * 10^8$	$(108 \pm 12) * 10^3$

of metal mixture and enhanced the treatment process in wastewater at different concentrations. Our results showed that the maximum mixture removal with highest treatment process were at concentration 5 ppm by two most potent isolates while less remove for metal mixture and treatment process were achieved at high concentration.

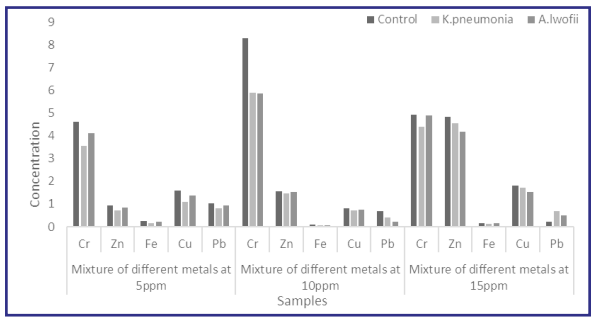

Fig. (2): Metals bioremediation in mixtures of metals (Cr, Zn, Fe, Cu & Pb).

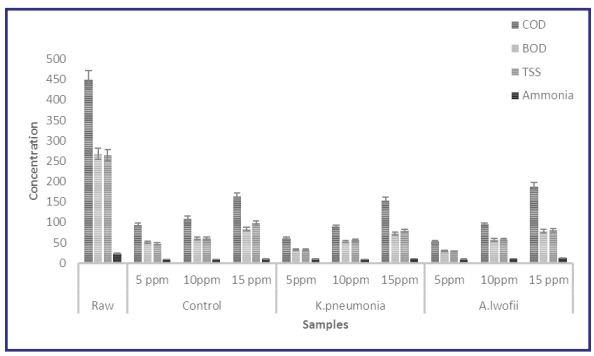

Fig. (3): Waste reduction in wastewater contained different concentrations of metals mixtures.

DISCUSSION

Environmental condition play an important role in wastewater treatment and heavy metal removal by microorganisms. A number of studies have indicated that the microbial wastewater treatment influenced by treatment conditions **(Ravi *et al.,* 2013; Rajesh *et al.,* 2013, Hassan *et al.,* 2015).** In this regard, this study focusing on effect of pH, temperature, inoculum size, incubation condition (static and shaker) at different incubation time and heavy metals concentrations affecting on wastewater treatment process. **Ravi *et al.,* (2013)** reported that optimized factors of treatment process lead to highly efficiency of microbial cell in treatment.

BOD is the amount of oxygen consumed by microorganisms during degrading organic matter in aqueous systems, therefore, decreasing in BOD values during treatment process its positive result for treatment. This decreasing in BOD of the samples may be as a result of the metabolic activities of microorganisms either those indigenous to the wastewater samples or those exogenously added. **Kessington *et al.,* (2014)** showed that BOD value of samples was varied with remediation time after adding microbial cell, also reported slightly decreasing in BOD for control related to the activity of the indigenous microorganisms. **Amenaghawon *et al.*, (2013)** investigated the treatment of domestic wastewater supplemented with inorganic fertilizers. They reported reductions in BOD for wastewater and attributed this observation to the activity of the stimulated indigenous microorganisms. The most significant reductions in BOD were obtained for the samples with initial pH value of 6 followed by that with an initial pH value of 5 both of which are slightly acidic. On the other hand, very slight BOD reduction was observed for samples with initial pH 8 and 10, both of which are alkaline. The same trend was observed for the samples with initial pH 3 which is an acidic condition. These results show that biodegradation was highly inhibited in the very alkaline and acidic conditions. In our study *Klebsiella pneumonia* and *Acinetobacter lwoffii* could be waste reduce at pH range 5 to 9 but the optimum removal at pH 7, where the values of COD, BOD and TSS for wastewater treated by *Klebsiella pneumonia* and *Acinetobacter lwoffii* were 46.63±0.69 and 43.53±0.65, 21.97±0.33 and 20.97 ± 0.31, 9.98 ± 0.15 and 8.99 ± 0.13 respectively when compared with control.

Ravi *et al.,* (2013) mentioned the removal efficiency of pollutant in terms of BOD in the domestic wastewater at various concentration of inoculum were tabulated, they have noticed that, the biore-

mediation capabilities of consortium inoculation in terms of BOD reduction was 56.12%, 61.55%, 63.80%, 64.44%, 65.13, and 66.16% at consortium inoculum size 0.05%, .1%, 0.2%, 0.3%, 0.4% and 0.5%, respectively. As the quantity of wastewater is more, it is found that using more than 0.5% inoculum is not feasible. Hence, 0.2% of inoculum is considered as optimized concentration. It is opined that instead of using high concentration of inoculum we have to screen, isolate and enumerate high efficiency strains of microorganisms. **Nadirah *et al.* (2008)** reported that 61% removal of BOD, 97% COD, 86% removal of ammonia, 71% removal of total suspended solids, 50% removal of nitrate and 53% removal of oil and grease using *Pseudomonas putida*, *Pseudomonas fluorescence, Xanthobacter sps*., and *Rhodoccus sps.,* for treatment of domestic wastewater. Which agreed with our results which reported that the different inoculum size influences on waste removal where the optimum inoculum volume was 0.5% for *Klebsiella pneumonia* and the values of COD, BOD and TSS for wastewater treated were 39.44 ± 0.58, 21.97 ± 0.33 and 9.98 ± 0.15, respectively, while the optimum inoculum size was 2% for *Acinetobacter lwoffii* and the values of COD, BOD and TSS for wastewater treated were 43.14 ± 0.60, 23.96 ± 0.36 and 8.99 ± 0.13, respectively, when compared with control. **Balaji *et al*. (2005)** reported that 71% of BOD removal using cow dung as the source of microorganisms with dosing of 3% for 18 hours Hydraulic Retention Time (HRT) during the experiments conducted for treatment of tannery industry wastewater. **Imran, (2007)** reported the mean removal efficiency of COD was 87% after 24 hours of treatment using activated sludge. **Chuang *et al.*, (1997)** reported that high HRT may help in the production of heterotrophic biomass and finally results in readily biodegradable COD from the sewage. In our study the dynamic aeration with different incubation time affected on the treatment process and the optimum dynamic aeration time was 48 hours for both isolates, where the biodegradation activity in optimum dynamic aeration time lead to the reduction in the values of COD, BOD, TSS and ammonia to 61.10 ± 3.73, 34.77 ± 1.66, 36.12 ± 2.79 and 11.27 ± 0.31 by *Klebsiella pneumonia,* respectively, while by *Acinetobacter lwoffii* the values of COD, BOD, TSS and ammonia were 76.62 ± 3.44, 44.04 ± 1.56, 28.76 ± 1.45 and 11.14 ± 0.36, respectively.

Microbial communities play an importance role in metal removal in wastewater due to it is less cost, with non-hazardous end products **(Oghenerobor *et al*., 2014).** During pollutant removal, the microbe(s) alter the metal chemistry and mobility through either reduction, accumulation, mobilization or immobilization **(Faryal and Hameed, 2005).** The microbial remediation of toxic metals is said to occur in two ways: direct and indirect reduction **(Sinha *et al*., 2009).** In this investigation the optimum metals removal with best treatment process was at 5 ppm for both isolates where the values of treated Cr, Zn, Fe, Cu and Pb by *Klebsiella pneumonia* and *Acinetobacter lwoffii* were 3.566 ± 0.025 and 4.108 ± 0.074, 0.706 ± 0.002 and 0.856 ± 0.013, 0.172 ± 0.002 and 0.209 ± 0.003, 1.102 ± 0.002 and 1.383 ± 0.026, 0.801 ± 0.003 and 0.939 ± 0.003 respectively, while the values of COD, BOD, TSS and ammonia of treated wastewater by *Klebsiella pneumonia* were 60.7 ± 3.51, 32.3 ± 2.52, 33.7 ± 0.58 and 10.3 ± 0.17 and by *Acinetobacter lwoffii* were 53 ± 3, 29.3 ± 0.58, 28.7 ± 0.58 and 9.4 ± 0.14 respectively.

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