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Full Length Research Paper

Improving the efficiency of involved bacteria in aeration tanks of waste water stations

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Advanced bioremediation in waste water and sewage treatments currently represents one of the important aspects of biotechnology. The removal of pathogenic microorganisms, complex hydrocarbons, heavy metals and nutrients were intensively investigated. The present work aims to improve the efficiency of involved bacteria in aeration tanks for maximizing mineralization process of organic substances and consequently reduce the time of the treatment process. Other target is the elimination of nutrients (N & P) to avoid their environmental and hazardous effects. In order to achieve these goals, isolation and identification of dominant microflora in aeration tanks were carried out and highly active strains were selected. Trails are newly done for hybridization or cloning one or more of dominant strains to increase their oxidizing efficiency. A pilot experiment was established in a green house to stimulate biological stage of municipal plant and to test the achieved genetically modified strains (Modell experiment). Also, monthly data were recorded of 20 parameters to highlight and controlling input and output of wastewater station of Taif city. Biological oxygen demand (BOD_5) and chemical oxygen demand (COD) clearly decreased in out fluent indicating lower organic load. The decrease of total organic carbon (TOC), total dissolved solids (TDS) and total suspended solids (TSS) assured the previous obtained data. Presence of large amount of dissolved oxygen (DO) in the out fluent means the efficiency of aeration pumping process. Nitrate content (NO_3^-) and nitrite content (NO_2^-) were sharply decreased in the out fluent indicating the higher requirement of H-acceptors. NH_3 (ammonia content) decrease, however total Kjeldahl Nitrogen (TKN) increase were due to intensive microbial bodies load. Total hardness (TH) decrease, which means lower conc. of Ca^{++} and Mg^{++} and better quality of output water. pH values were slightly decreased because of microbial acidic products. Turbidity was dramatically reduced because of different precipitation process. Phosphorous (P) content and Sulfate ($SO_4^=$) content decreased indicating consumption or fixion in microflora bodies. Otherwise, chloride (Cl^-) content were increased in outfluent because of chlorination process. Oil and grease were quite reduced in the outfluent. Finally, different heavy metals and hydrocarbons were found in the limit or lower than the permit levels globally. Total microbial count increased considerably in outfluent, especially in summer months; however, fermentative bacteria were very low because of enough O_2 present in outfluent. Only 15 strains of 280 isolates (about 5.4%) were found to be highly active in mineralizing organic substances which were completely identified. The most active one was used to modify the dominant strains by cloning technology and reinoculated in the pilot experiment

Key words: Waste water bioremediation, sewage microflora, mineralization of organic pollutants, eutrofication phenomena, microbial cloning, ligation reaction.

INTRODUCTION

Bioremediation of sewage wastes is considered the most effective, inexpensive and safe one from biotechnology views. The operations are based on different actions:

a) Mechanical remove of moderately sedimentable solids and scum.
b) Microbial mineralization of dissolved organic

pollutants in aeration tanks or in trickling filters.
c) Anaerobic digestion of sewage sludge to produce methane (Biogas).
d) Tertiary treatment to eliminate nutrient elements, that is, NO_3^-, NO_2^-, NH_3, PO_4, $SO_4^=$ to avoid Eutrophication phenomena.
e) Chlorination or ozonation to remove pathogenic microorganisms.
f) Biological chelating of heavy metals by Biomass of yeasts and algae, however, this removing is still temporary.

The paradox of sewage treatment depends essentially on:

1. Amount and type of easily decomposable organic matter, which considers the source of energy (H-donor) and supplies carbon for synthesis of microorganisms bodies (Burford and Bremner, 1975; Stanford et al., 1975; Reddy et al., 1982).
2. A sufficient oxygen concentration (pO_2) is necessary as H-acceptor for intensive mineralization. Moreover, bacteria could be switched to other acceptors such as nitrate or sulfate as O_2 alternative (Abou- Seada and Ottow, 1985; Ottow and Fabig, 1985).
3. pH: maximal mineralization usually occurred at neutral pH level (7.1-7.5). Highly acid or alkaline conditions lead to lower mineralization and less efficiency of biodegradation (Koskinen and keeney, 1982; Abou-Seada and Ottow, 1986).
4. The specific effect of quality of microorganisms in aeration tanks (Munch and Ottow, 1984, Abou-Seada and Ottow, 1987). With gene cloning technique, it could be managed and improved the efficiency of dominant bacterial strains in aeration tanks to optimize the treatment process (Oliver et al., 1977; Hickey and Smith, 1996).
5. Recently, Membrane Bioreactor (MBR) Technology, which provides a good alternative to conventional treatment by activated sludge followed by a setting tank in municipal waste- water plants. It combines the biological treatment with a membrane separation in one step, which 5 times smaller than activated sludge system. Moreover, the biomass concentration can be greater, which reduce reactor volume and also reduce operating coasts (Galil, 2003; Stephenson et al., 2002; Verma et al., 2006).

The target of this work is to improve the efficiency of involved bacteria in aeration tanks and so maximizing elimination of organic substances and also nutrient elements

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Abbreviations: BOD_5, Biological oxygen demand; **COD,** chemical oxygen demand; **TOC,** total organic carbon; **TDS,** total dissolved solids; **TSS,** total suspended solids; **DO,** dissolved oxygen; **TKN,** total Kjeldahl nitrogen; **TH,** total hardness; **MBR,** membrane bioreactor.

MATERIALS AND METHODS

This study was conducted during 16 months. Representative samples were monthly taken from influent and out fluent of municipal sewage plant in Taif city and analyzed bacteriologically and physico-chemically (20 parameters) to recognize the efficiency of treatment process.

Bacteriological analysis

Representative sewage samples were carefully and serially diluted in sterilized distilled water and densities of total microbial flora and fermentative bacteria were determined by plate count technique using Difco nutrient agar as described by Page et al. (1982). 280 pure cultures were picked up and further tested for their abilities to degrade the complex organic substances (cellulose, oil derivatives and phenols) under sterilized conditions.

The used synthetic mineral medium contains (per liter) 1.4 g KH_2PO_4; 2.2 g $NaHPO_4$; 0.5 g $(NH4)_2SO_4$; 0.2 g $MgSO_4$, 10 mg $CaCl_2$, 5.0 mg $FeSO_4$ 2.5 mg $MnSO_4$, 2.5 mg Na_2McO_4, 1.0 g KNO_3 and 5% source of C, trace element solution 10 ml, pH 7.2 as described by Sperl and Hoare (1971). The test was carried out in 5 replicates (tubes) for each strain. The tubes were inoculated and incubated at 30°C for 48 h. The active strains were able to degrade the organic matter (no turbidity) in all replicates. Control tubes without inoculation were simultaneously run.

To identify the most active strains, standard morphological, cultural and physiological tests were carried out as described by Naveke and Trepper (1979) and Sussmuth et al. (1987). Classification of isolates was followed according to Bergy,s manual of systematic bacteriology (Krieg and Holt, 1984; Sneath et al., 1986).

Chemical and physical determinations

1) Dissolved oxygen (DO) levels were determined by electrometric method using an oximeter basing on diffusion rate of molecular O_2 a cross membrane. The procedure was described in details by Tortora et al. (1986).
2) Biological oxygen demand (BOD_5) was detected by measuring dissolved O_2 in sewage samples before and after incubation at 20°C for 5 days. The difference is the amount of consumed oxygen (mg/L) in oxidizing decomposable organic pollutants by present microflora during 5 days. The higher organic C, The higher O_2 consumed (Jenkins, 1981).
3) Chemical oxygen demand (COD) is a rapid method for measuring consumed oxygen in oxidation of total organic compounds using concentrated dichromate digestion solution ($K_2CrO_7 + H_2SO_4$) under heating (Himebaugh and Smith, 1979).
4) Total organic carbon (TOC) and total dissolved solids (TDS) were measured as described by Waring and Gilliam (1983).
5) Nitrate, nitrite, ammonia and total nitrogen were measured as described in details by Page et al. (1982). Nitrate content was calorimetrically determined after reduction by cadmium and reaction with Na-salicylate, nitrite content was also calorimetrically evaluated by reaction with sulfanil acid and – naphthylamine, ammonia content was measured by Nessler's reagent, while total nitrogen was determined by Kjeldahl method.
6) Total hardness (TH) was measured by soap precipitation chiefly in presence of Ca^{++} and Mg^{++} ions. The procedure was described by Goetz and Smith (1959).

Figure 1. Pilot plant set-up.

7) Alkalinity is the sum of all titratable bases, that is, carbonate, bicarbonate, hydroxide, borates and silicates as reviewed by Jenkins and Moore (1977).
8) pH values were measured electrometrically with WTW-pH-meter.
9) Turbidity is caused by suspended and colloidal matter such as clay, silt, fine divided organic and inorganic matter as well as plankton and microscopic organisms that causes light to be scattered. The higher the intensity of scattered light, the higher the turbidity. The procedure was described by Hernandez et al. (1991).
10) Chloride ion (Cl^-) was quantitively detected by silver nitrate titration to precipitate silver chloride (white color) till red silver chromate is formed in range of pH 7 to 10 as referred by Cooper et al. (1982) and Paustian (1987).
11) Heavy metals (that is, Cad, Mo, Zn, Cr, M, Ag, Hg, Pb, Ni, Ba, Se) and hydrocarbons (phenols, pesticides, and detergents) were spectrophotometrically determined after filtration of sample using atom absorption as described by Page et al. (1982).
12) The samples were taken in July and August 2009 to represent summer season and December 2009 and Jan 2010 to represent winter season.

Pilot plant set-up

In order to optimize nutritional and environmental parameters to improve efficiency of wastewater treatment at Taif WWT plant, a set-up was designed and executed using local materials.

The location for the set-up was selected, inside Taif Univ. comps as an isolated piece of land constructed with walls and roof. This shall provide safety to surrounding environment and facilitate execution of the work under controlled conditions.

The set –up consists of several vessels connected to each other using 4-way valves (Figure 1). These type of valves provide flexibility and enable changing water flow forwards and backwards according to its composition, type of additives, degree of aeration and type of microorganisms to be used.

Experimental procedure

It is an attempt to divide biodegradation activities within several vessels such as accommodates certain microbial group, which is specific for degrading of particular component (s) present in wastewater. Therefore, the treated sewage coming out from certain vessel is pumped to the next vessel for further degradation of one or more of remaining organic pollutants. It believes that a number of feeding scenarios to the above-mentioned set-up have to be tested before to reach the optimal scenarios

Tested organisms

This work will be two- prolonged; either with naturally microflora exists in Taif wastewater plant or with the genetically modified strain (s) to confirm its highly improved capabilities in biodegradation of organic pollutants. For both directions, wastewater entering the system has to pretreat with manual removal of course and floating particles. A special vessel could be adapted for such task. A second vessel could be also used to optimize the conditions of biodegradation (temperature, O_2 potential, pH value, etc.) before pumping to the following vessels. Because of some technical problems and missing of peristaltic pumps, we hope, as soon to be finished and continue this work.

Cloning the EDTA and nitrilotriacetic acid (NTA) degradation gene *(emoB)* from the Bacterium BNC1 (Wild Type)

Used bacterial strains

Bacterium BNC1 (Wild Type) – *E. coli* BL21 (DE3) *E. coli* BL21 (DE3)

Table 1. Chemicals and enzymes.

No.	Item	Amount
1-	Taq-DNA polymerase + $Mgcl_2$ + 10Xbuffer	200 Units
2-	Pfu-DNA polymerase + $Mgcl_2$ +10Xbuffer	100 Units
3-	dNTP's mix	100 mM each
4-	T4-DNA ligase	50 Units
5-	NdeI	200 Units
6-	NotI	200 Units

Primers

EmoB (F): 5' - GAT GAC GAC GAC CAT ATG ACC TAC TCC - 3'
(Contained an additional *NdeI* recognition site).
EmoB (R): 5' - TCA AGT GAT GTG CGG CCG CGC GCG – 3'
(Contained an additional *NotI* recognition site).
Plasmids: pET30-LIC (expression vector) (Table 1)

Preparing the construction harboring the EDTA and nitrilotriacetic acid (NTA) degradation gene

Using PCR, two specific primers were used to amplify a 300 bp represent the coding sequence of the x gene from the genomic DNA of the strain of *E. coli* [Bacterium BNC1 (Wild Type)].
- The sequence of the specific primers was as the following:

F: 5' - GAT GAC GAC GAC CAT ATG ACC TAC TCC - 3'
R: 5' - TCA AGT GAT GTG CGG CCG CGC GCG – 3'

The PCR amplification reaction

The PCR was performed in a 50 ul reaction volume containing 1X PCR buffer (10 mM Tris-HCl pH 8.3, 50 mM KCl, 2 mM $MgCl_2$, 0.01% (w/v) gelatine), 250 uM each of dGTP, dATP, dCTP and dTTP (dNTPs), 2.5 units of Taq DNA polymerases, 100 pmol of each primer and the DNA template, was released from the bacterial cells by boiling in a water bath for 5 min to lyses the cells and then the tubes were spun briefly to collect the condensate (Carozzi et al.,1991). PCR reaction conditions were: 94ºC for 3 min; 35 cycles of denaturation at 94ºC; annealing at 52ºC; and extension at 72ºC for 2 min each, followed by 7 min extension at 72ºC. After performing the PCR reaction, the amplified 300 pb fragment was eluted and purified from the gel and ligated to the pGEM T-easy cloning vector.

RESULTS AND DISCUSSION

Data recorded in Tables 2 to 5 Show the different parameters, which were measured to control the final product of municipal plant of Taif city and recognize the efficiency of treatment process. The following results were obtained:

1) Mineralization of organic pollutants indexed by BOD_5 (Biological oxygen Demand) and COD (Chemical Oxygen Demand) leads to removing ca 80% of organic load. The values of BOD^5 and COD (as means of 16 months, Table 2) clearly deceased from 353.3 and 759.1 mg O_2/L in influent to 1.43 and 6.82 mgO_2/L in out influent, respectively. Basing on the lower BOD^5 and COD, desto lower organic load

2) TOC (Total Organic Carbon%), TDS (Total Dissolved Solids%) and TSS (total Suspended Solids%) decreased from 148.9, 72.4 and 324.7 in influent to 6.89, 64.87 and 1.62 in outfluent, respectively. These results assured the previous obtained values of BOD and COD.

3) DO (Dissolved Oxygen) in plant influent was around Nell throughout the entire period (16 months) of the experiment; however, it increased in outfluent to 4.92 mg/L. It means that efficiency of O_2- pumping was enough

4) Mineral nutrients as indexed by NO_3^-, NO_2^- and NH_3 in the influent were (as means of 16 months measurements) 31.1, 19.4 and 64.9 ppm, respectively. Other side, in the outfluent, they are sharply decreased to 10.9, 0.0 and 5.8 ppm, respectively. It seems that intensive mineralization of organic pollutants enhanced the demand for H-acceptors and consequently leads to more reduction of nitrate and nitrite. The decrease of Ammonia may be due to highly assimilation in microflora bodies. Total Nitrogen relatively increased from 76.1% in influent to 91.9% in outfluent. This is due to of mineralization of protein of pollutants and N release.

5) Hardness decreased clearly from 154.1 mg $CaCO_3$/L (mean of 16 months) in influent into 135.3 mg $CaCO_3$/L in outfluent. It indicates lower concentration of Ca^{++} and Mg^{++} (ions), which improve the quality of output wastewater.

6) Alkalinity and acidity (pH values): Alkalinity means all of bases such as carbonate, bicarbonate, hydroxide, borates and silicates. It was noticed that alkalinity decreased from 362.7 influent to 224.3 mg/L in outfluent of the plant. Otherwise, pH values were slightly decreased from 7.67 influent to 7.56 in out fluent. It is easily to explain, because of acid production by microbial metabolisms.

7) Turbidity, which caused by suspended and colloidal substances, was dramatically reduced from 342.9 into 31.2. The main reason is various precipitation processes during the entire treatments.

8) Phosphorous and Sulfate: P and $SO_4^=$: P and S such like N compounds lead to enrich of photoplankton and microflora exhausting dissolved O_2 and lead to biological death of lakes and reveres (eutrophication phenomena).

Table 2. Determinations of Biological Oxygen Demand (BOD),Chemical Oxyge Demand (COD), Dissolved Oxygen (DO)And Total Dissolved Solids (TDS).

Month	Year	BOD5		COD		DO		TOC		TDS	
		Plant fluent		Plant fluent		Plant fluent		Plant fluent		Plant fluent	
		input	output	Input	output	input	output	output	input	Output	input
1	2009	326.34	1.45	693.61	6.95	0	5.1	8.55	139.61	77.1	78.71
2	2009	332.02	1.48	702.18	7.1	0	5.9	8.1	179.04	75	80.28
3	2009	329.44	1.5	691.74	6.85	0	5.6	7.8	200.97	73.65	74.61
4	2009	336.06	1.53	694.37	6.73	0	4.85	7.65	189.07	70.9	73.9
5	2009	332.25	1.33	720	6.9	0.03	4.7	7.4	153.55	68.55	64.51
6	2009	348.48	1.35	769.2	6.65	0.01	4.4	7.1	150.23	64.6	74.93
7	2009	356.63	1.25	812.23	6.5	0.01	4.5	6.95	136.87	60.95	75.91
8	2009	360.09	1.44	818.1	7.35	0	4.35	6.7	134.45	60.7	71.81
9	2009	352.63	1.49	794.27	7.28	0.01	4	6.42	137	58.01	74.01
10	2009	418.12	1.49	922.83	6.88	0	4. 80	6.3	140.87	55.3	75.13
11	2009	407.08	1.45	817.3	6.4	0.01	4.9	6.15	143.33	54.15	94.17
12	2009	358.88	1.43	816.42	6.15	0.01	4. 75	6.01	189.45	60.45	62.73
1	2010	341.4	1.47	802.97	6.83	0.02	5.1	5.67	205.71	61.9	67.03
2	2010	340.12	1.44	665.04	6.86	0.01	4.98	5.71	164.14	66.24	56.69
3	2010	350.92	1.5	690.74	6.94	0.01	4.9	6.43	199.77	64.35	60.02
4	2010	362.17	1.37	735.9	6.7	0.01	4.83	7.29	205.8	66.1	65.27
	Mean values	353.3	1.43	759.1	6.82	nell	4.92	6.89	148.9	64.87	72.48

Table 3. Determinations of Total Suspended Solids (TSS%), Nitratecontent (NO_3^- ppm), Nitrite content (NO_2^- ppm), Ammonia (NH_3ppm) andTotal Kjeldahl Nitrogen (N_2%)

Month	Year	TSS		NO_3^-		NO_2^-		NH_3		TKN	
		Plant fluent		Plant fluent		Plant fluent		Plant fluent		Plant fluent	
		Output	input	output	input	output	input	output	input	Output	input
1	2009	1.33	234.03	10.3	33.76	0	18	5.1	96.2	95.5	108
2	2009	1.15	249.61	12.5	28.28	0	14.7	4.88	83.8	88.4	98.7
3	2009	1.65	261.61	14.2	25.56	0.01	12.7	5.7	80	85.6	95.6
4	2009	1.5	258.13	14.6	23.29	0.01	10.6	5.04	60.4	88.4	75.3
5	2009	1.8	328.68	12.2	26.1	0.01	12.6	5. 75	57.8	89.9	68.9
6	2009	1.35	375.27	10.1	25.43	0	17.7	5.55	56.5	95.3	65.2
7	2009	2.28	370.48	8.87	23.41	0	16.9	6.35	63.7	92.2	73.2
8	2009	2.7	372.77	9.11	25.26	0	19.5	6.9	68.9	94.5	76.3
9	2009	1.93	351.63	6.45	34.18	0	26.6	6. 80	66.3	95.8	75.4
10	2009	1.77	404.17	8.75	41.18	0	32.5	6.68	61.6	99.4	73.3
11	2009	1.93	386.93	9.9	30.39	0	24	5.85	54.4	98.7	67.6
12	2009	1.58	341.13	9.57	32.21	0	24.1	5.1	58.1	90.6	70.7
1	2010	1.52	334.77	9.45	32.44	0	24.1	6.46	58	88.7	70.2
2	2010	1.75	287.82	15.3	39.9	0	23.6	6.8	57.2	90.1	65.5
3	2010	1.65	305.58	12.9	38.8	0	17.1	5.61	57.4	88.9	65.5
4	2010	1.5	331.6	11.4	36.78	0	15.5	4.7	58.7	87.4	67.3
	Mean values	1.62	324.7	10.9	31.1	0	19.4	5.8	64.9	91.9	76.1

Therefore, The clear decrease of P and $SO_4^=$ content from 24.6 and 50.8 ppm influent to 5.1 and 7.76 mg/L in outfluent, respectively. It may be due to consumption or fixion in microflora bodies. These results has another benefit to avoid eutrophication.

9) Chloride Ion Cl^-: This parameter was found to be increased from 146.2 ppm in influent into 170.9 ppm in outfluent because of chlorination treatment to remove

Table 4. Determinations of Total Hardness (TH), Alkalinity, pH values, Turbidity and Phosphorous compounds (P).

Month	Year	Total Hardness		Alkalinity		PH		Turbidity		P	
		Plant fluent		Plant fluent		Plant fluent		Plant fluent		Plant fluent	
		output	Input	output	input	output	input	output	input	output	input
1	2009	122.3	142.3	315.4	348.4	7.55	7.69	24.6	254.8	4.9	20.94
2	2009	111.2	141.2	323.6	339.8	7. 60	7.7	25.8	265.7	5.64	19.43
3	2009	118.5	138.8	303.4	333.2	7.75	7.78	29.4	274.9	5.37	20.37
4	2009	126.1	146.9	310.8	334.8	7.65	7.77	21.5	274.7	5.55	24.5
5	2009	129.8	146.1	301.5	331.1	7.65	7.7	38.4	348.6	5.8	22.91
6	2009	130	150.1	312.4	343.8	7.6	7.88	32	392.8	4.46	22.16
7	2009	147.5	167.9	326.6	366.4	7.62	7.88	33.8	389.3	6.43	22.49
8	2009	145.5	155.6	343.5	362.5	7.64	7.9	30.5	390.8	5.86	22.66
9	2009	140.9	150.3	330.8	374.5	7.72	7.92	38.8	372.8	5.64	23.69
10	2009	134.7	154.2	333.5	346.5	7.61	7.91	45.4	425.4	5.7	23.73
11	2009	139.8	159.8	324.6	354.5	7.64	7.88	42.6	402.4	5.75	25.22
12	2009	143.5	163.5	355	385.5	7.52	7.82	36.2	460.2	5.25	25.35
1	2010	147.7	170.4	239.8	420.5	7.56	7.77	30.6	352.9	5.13	26.73
2	2010	149.8	169.2	242.1	385.4	7.3	7.43	37.5	309.6	5.24	30.09
3	2010	141.4	152.2	248.9	385.3	7.42	7.5	38.7	326.4	5.45	30.51
4	2010	137.1	157.5	250	390.6	7.35	7.65	39	351.6	5.9	32.71
Values	mean	135.3	154.1	244.3	362.7	7.56	7.76	31.2	342.9	5.1	24.64

Table 5. Determinations of oil-grease content, sulfate content (SO4=), Chloride ion (Cl-) , Setting solids and temperature degrees.

Month	Year	Oil and Grease		$SO_4^=$		Chloride		Settling solid		Temperatue	
		Plant fluent		Plant fluent		Plant fluent		Plant fluent		Plant fluent	
		output	input	output	input	output	input	output	input	output	input
1	2009	0	28.78	7.1	61.2	171.3	139.8	ND	4.07	21.6	22.8
2	2009	0	26.36	7.22	57.9	172.6	134.6	ND	3.73	21.8	22.9
3	2009	0	28.77	7.96	54.2	170.6	132.4	ND	3.66	22.5	23.3
4	2009	0	28.67	7.64	48.7	168.8	140.5	ND	4.01	22.9	23.5
5	2009	0	28.84	7.13	47.9	173.4	135.4	ND	4.29	25	25.9
6	2009	0	27.77	8.15	47.1	175.6	140.9	ND	4.34	26.4	27.4
7	2009	0	27.74	9.56	44.6	174.2	150.9	ND	4.39	26.6	28
8	2009	0	27.06	7.68	51.8	164.6	147.9	ND	4.39	27.7	28.3
9	2009	0	34.93	7.6	49.8	173.5	144.9	ND	4.25	28	29.1
10	2009	0	37.33	7.6	47.4	174.6	139.3	ND	7.68	27.5	28.7
11	2009	0	34.83	9.06	49.8	168.6	190.1	ND	4.44	26.4	27.8
12	2009	0	32.45	6.6	51.1	164.6	148.1	ND	5.01	22.4	25.4
1	2010	0	29.65	7.1	49.8	173.3	146.2	ND	5.58	21.8	24.2
2	2010	0	29.14	7.16	49.2	172.6	141.9	ND	3.99	22.4	24.9
3	2010	0	28.74	9.96	51.7	172.6	142.8	ND	3.12	23.3	25.5
4	2010	0	27.5	6.64	50.1	164.6	163.6	ND	3.07	24.2	26.3
Values	Mean	0	29.9	7.76	50.8	170.9	146.2	ND	4.37	24.4	27.5

pathogenic flora.

10) Oil and Grease content: The removal efficiency of oil-grease was quit completely, which it was 29.9 mg/L in influent and reached Nell in the outfluent.

11) Temperature values of treated wastewater ranged between 27.5℃ at the begin to 24.4℃ at the end of treatment process, being clearly higher in summer months than winter months.

12) The contents of different heavy metals (that is, Cad, Mo, Zn, Cr, M, Hg, Ag, Pb, Ni, Ba, Se) and hydrocarbons (phenols, pesticides detergents) were found throughout the investigation period in the limit or lower than the

Table 6. Determinations of total microbial flora and fermentative microorganisms (Cell /L).

Month	Year	Total microorganisms Cell /ml		Fermentative bacteria Cell / ml	
		Output	input	Output	Input
1	2009	$4.5x10^6$	$3.5x10^5$	$2.1x10^2$	$4.9x10^2$
2	2009	$4.1x10^6$	$2.8x10^5$	$3.3x10^2$	$4.5x10^2$
3	2009	$5.9x10^6$	$3.9x10^5$	$3.5x10^2$	$4.2x10^3$
4	2009	$8.4x10^6$	$4.1x10^5$	$3.1x10^2$	$5.3x10^3$
5	2009	$9.1x10^6$	$5.5x10^5$	$4.1x10^2$	$6.3x10^3$
6	2009	$6.3x10^7$	$6.1x10^6$	$5.3x10^3$	$8.5x10^4$
7	2009	$8.5x10^8$	$9.2x10^6$	$7.6x10^3$	$9.0x10^4$
8	2009	$8.8x10^8$	$9.9x10^6$	$8.1x10^3$	$8.2x10^4$
9	2009	$7.9x10^7$	$7.4x10^6$	$7.2x10^3$	$6.8x10^4$
10	2006	$7.4x10^6$	$6.9x10^5$	$2.3x10^3$	$6.5x10^3$
11	2009	$5.9x10^6$	$5.4x10^5$	$6.8x10^2$	$4.6x10^3$
12	2009	6.5x105	$5.3x10^4$	$5.3x10^2$	$3.4x10^3$
1	2010	$5.4x10^5$	$4.7x10^4$	$3.1x10^2$	$4.4x10^2$
2	2010	$3.9x10^5$	$2.4x10^4$	$1.8x10^2$	$5.1x10^2$
3	2010	$4.5x10^5$	2.3x105	$2.4x10^2$	5.8x103
4	2010	$6.3x10^6$	$3.3x10^5$	$4.1x10^2$	$6.8x10^3$
	Mean	$121x10^6$	$2.3x10^6$	$2.2x10^3$	$23.1x10^3$

permit levels globally. It may be due to those sources of influent introduced to Taif municipal plant mainly urban sewage or agricultural drainage or rainfalls, which rarely contain heavy metals or complex hydrocarbons in comparison with industrial wastewater.

Microbiological densities and selection of highly active strains

Data given in Table 6 show that densities of total microbial flora present in Tarif WWW plant increased con-siderably in out fluent ($121x10^6$ cell/ml liquor as mean of 16 tested months) in comparison with influent (only $2.3x10^6$ cell/ ml liquor). Moreover, the total count was clearly noticed higher in summer months than in winter months. This result could be attributed to suitable conditions in aeration tanks for bacterial growth beside effect of high temperature in summer. Other side, fermentative bacteria were lower in outfluent ($2.2x10^3$ cell/ml as a mean of 16 months) in comparison with influent ($23.1x10^3$ cell /ml), being also higher in summer months . This result may be due to negative effect of residual O^2 present in outfluent comparing with absent O_2 in influent as recorded before (Table 2). This result is in agreement with the findings of Abou-Seada and Bardtke (1989).

Selection test of active strains:

Only 15 strains of 280 isolates were found to be highly active in degradation of organic substances and fate residuals in pure cultures within 2 days incubation at 25°C. They constituted a very small fraction (only 5.4%) of total isolates. This may be attributed to the difference between biochemical properties of mixed and pure cultures, which reflects the importance of microbial association to increase their efficiency. The active strains were completely identified according to Watanabe et al. (1981) Bergey,s manual (Krieg and Holt, 1984; Sneath et al., 1986). They were found to belong to two groups:

Group I: Gram negative, short rods, motile, oxidase negative and fermentative of glucose (*Enterbacter* spp).
Group II: Gram positive endospores forming single or in chain long rods, which are motile, oxidase positive and non fermentative (*Bacillus* spp.).

Cloning the EDTA and nitrilotriacetic acid (NTA) degradation gene (emoB) from the Bacterium BNC1 (Wild Type)

Elution and purification of PCR fragments

The 300 bp DNA band was excised from ethidium bromide –stained agarose gel with a razor blade, weighted and transferred to a plastic tube. Three volumes of binding buffer was added to agarose gel piece, the tube placed in a 45 – 55°C water bath and incubated for 5 min or until the agarose is melted. The High pure filter tube was combined to the collection tube and the sample was pipted to the upper one. Centrifugation was

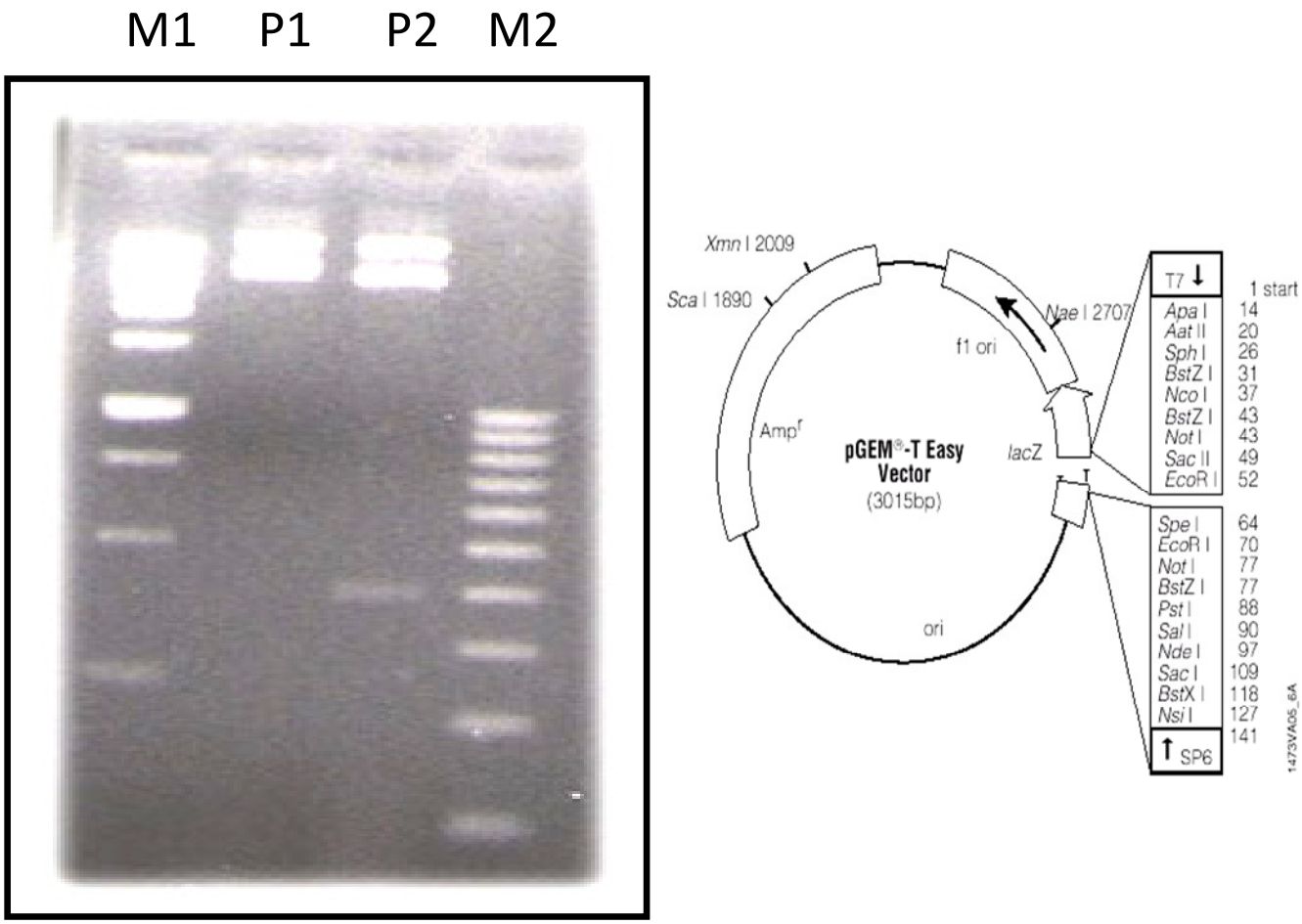

Figure 2. Ligation reaction.

performed for 30 s at maximum speed (approx. 13,000 g) in a standard tabletop centrifuge. The flow through was discard and the filter tube combined again with the same collection tube. A 500 µl binding buffer added into the upper reservoir, and incubated at room temperature for 1 min and centrifugation again for 30 s. The flow through was discard and the filter tube combined again with the same collection tube. A 500 µl wash buffer added to the upper reservoir and centrifugation for 30 s (Biospin Gel Extraction Kit).

The wash buffer flow was discarding and the filter tube combined again with the same collection tube. A 200-µl wash buffer was added, and centrifugation for 30 s. The collection tube was discarding, and the filter tube inserted in a clean 1.5-ml reaction tube. 50 –100 µl elution buffer or sterile distilled water (pH 8-8.5) used for the elution of the DNA.

Ligation reaction

In a clean tube the following components were add: 1 µl 10x ligation buffer, 2 µl PCR vector (50 ng), 1 µl T4 DNA ligase, 3 µl purified PCR product and sterile distilled water up to 10 µl.

The ligation tube was mixed briefly and spined down to collect the contents in the tube. Incubation was performed at 14°C for 16 h. The ligation was checked and part of this mixture was used to transform high efficiency competent cells of *E. coli* strain JM109 (Figure 2).

Genetic modification of dominate strains

The group team will introduce this construct to the genetic background of the most dominant strains. Thereafter, the modified strains will be checked in the laboratory for their containing of the desired gene using specific PCR primers. The final step will be moving the genetically engineered bacteria to the pilot experiment.

The pilot experiment

The genetically modified microorganisms were inoculated to the treated wastewater in the designed vassals and

samples were analyzed till finishing the mineralization process. In comparison with the control (uninoculated) vassals, the processing time will be reduced from 36 to 28 h, which represent 22% time downgrading. This result is very important, which means increasing capacity and efficiency of treatment station and so decreasing treatment cost.

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