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Vegetable oil plant wastewater treatment by bacterial isolates: A study in the city of Hila, Iraq

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Abstract: The present study was to reflect the use of some bacteria in the treatment and removal of pollutants in three selected wastewater sites, including a vegetable oil plant (viz. Al-Etihad Food Industries), the main wastewater treatment station in the city of Hila, and Al-Hila River water from October 2019 to January 2020. The bacterial isolates identified in these three sites were *Klebsiella pneumoniae*, *Escherichia coli*, *Enterobacteria cloacae*, *Pseudomonas aeruginosa*, *Thalasobacillus devorans*, *Acinetobacter baumannii*, and *Bacillus subtilis*. The molecular study of the bacterial isolates involved the detection of bacterial genera using the polymerase chain reaction (PCR). The results showed that water had a variable nature, depending on the substances in it. It recorded varying chemical and physical property values, ranging between 6.36 and 7.82 for pH and from 2500 to 7100 mg·dm^{-3} for total alkalinity. Additional values were 713–2051 µS·cm^{-1} for electrical conductivity (*EC*), 5.90–9.80 mg·dm^{-3} for chemical oxygen demand (*COD*), and 480–960 mg·dm^{-3} for total hardness. The given values were also 0.20–0.65 µg·dm^{-3}, 0.03-0.23 µg·dm^{-3}, and 0–107 mg·dm^{-3} for nitrite (NO_2), phosphate (PO_4) oils, respectively.

Keywords: biotreatment, polymerase chain reaction (PCR), sewage, vegetable oil plant, wastewater

INTRODUCTION

Water is an essential component for the sustenance of life. Management of water is thus the key to ensuring its efficient and equitable use and encouraging conservation of water resources [Ojekunle *et al.* 2016; Renuka *et al.* 2014].

Industries are major sources of pollution in all environments, based on the type of industry and various levels of pollutants discharged into the environment [Jagetiya, Porwal 2019; Obeta *et al.* 2019; Skrzypiec, Gajewska 2017]. Residences located near municipal sewage outfalls or contaminated water sources are, therefore, at the highest risk of illnesses due to increased microbial pathogens and deteriorating physicochemical parameters [Wakelin *et al.* 2008]. In this sense, food industries producing a large amount of vegetable and fruit waste can affect municipal landfills because of the high biodegradability of food waste [Misi, Forster 2002]. Industrial wastewater entering a water body can thus represent a heavy source of environmental pollution, influencing both water quality and microbial-aquatic flora [Kanu, Achi 2011].

The effluent mainly comes from the degumming, deacidification, and deodorisation steps taken in the vegetable oil industry. The effluent from the vegetable oil industry used to be discharged directly into soil or groundwater [Sridhar *et al.* 2002]. The use of fats and oils dates back into antiquity since their chemical composition and specific properties have allowed them to be used as foods, fuels, and lubricants. Their sources are also numerous, encompassing vegetables, animals, and marine. Fats and oils are naturally occurring substances, predominantly of mixtures of fatty acid esters of the trihydroxy alcohol or glycerol [Nwobi *et al.* 2006].

Biological treatment is an essential and integral part of any wastewater from either municipality or industry, having soluble organic impurities or a mix of the two types of wastewater sources [Mittal 2011]. In treatment methods in which the removal of contaminants is brought about by biological activity, such substances are converted into gases that can escape into the

atmosphere. The biological treatment is also practised in order to remove nutrients such as nitrogen (N) and phosphorus (P) in wastewater [Rosen *et al.* 1998].

MATERIALS AND METHODS

GENERAL INFORMATION

The present study was conducted from October 2019 to January 2020. For this purpose, pH, electric conductivity (*EC*) (by Hanna *EC* meter), and chemical oxygen demand (*COD*) was measured in the field in accordance with standard methods [Young *et al.* 2005]. Total water hardness along with nitrite (NO_2), phosphate (PO_4) was then determined according to American Public Health Association [APHA 1999]. Moreover, alkalinity and oil and grease were verified as reported by Wells *et al.* [1995].

BACTERIAL ISOLATES

Klebsiella pneumoniae, *Escherichia coli*, *Enterobacteria cloacae*, *Pseudomonas aeruginosa*, *Thalasobacillus devorans*, *Acinetobacter baumannii*, and *Bacillus subtilis* was the bacterial isolates from three sites, i.e., a vegetable oil plant, the main wastewater treatment station in the city of Hila, and Al-Hila River water. The bacterial isolates were also identified by the polymerase chain reaction (PCR).

POLYMERASE CHAIN REACTION

The PCR was performed to amplify the 16S ribosomal RNA (rRNA) gene of the bacterial isolates, using the universal 16S rRNA primer pairs. The forward primer was designed as 357F (5'-CTACGGGGGGCAGCAG-3'), while the reverse was designed as 806R (5'-GGACTACCGGGGTATCT) [Mori *et al.* 2014].

The PCR conditions were further met by initial denaturation at 95°C for 5 min, followed by 35 cycles of denaturation at 95°C for 30 s each. The annealing temperature of the primers at 60°C for 30 s and extension at 72°C for 40 s. The final extension was at 72°C for 5 min.

TREATMENT MECHANISM USING BACTERIAL ISOLATES

Four types of bacterial isolates were recruited for the treatment process, namely *P. aeruginosa*, *K. pneumoniae*, *E. coli*, and *B. subtilis*. To this end, 10 cm^3 bacterial isolates were inoculated into 200 cm^3 wastewater in 500-cm^3 plastic bottles, and then the treatment was incubated at 30°C for a week. The experiment was done according to Phong *et al.* [2014].

RESULTS AND DISCUSSION

The environmental parameters were recorded during this study for the three sites. The water temperature values ranged between 20 and 25°C, where the lowest and highest values were observed at sites 3 and 1, respectively. The pH ranged from 6.36 and 7.82, wherein the lowest value was seen at site 1, and the highest value was reported at site 3. Electrical conductivity (*EC*) also ranged between 713 and 2051 $\mu S \cdot cm^{-1}$, in which the lowest and the highest values were for site 3 and site 2, respectively. In addition, total water hardness ranged from 480 to 960 $mg \cdot dm^{-3}$, wherein the lowest value was reported at site 3, and the highest value was observed at site 1. Moreover, the total alkalinity value was between 2500 and 7100 $mg \cdot dm^{-3}$, in which the lowest and the highest values were reported in sites 3 and 2, respectively. Nitrite (NO_2^-) also had values from 0.20 to 0.65 $\mu g \cdot dm^{-3}$, where the lowest value was seen at site 1, and the highest value was reported at site 2. Furthermore, chemical oxygen demand (*COD*) had values between 5.90 and 9.80 $mg \cdot dm^{-3}$, wherein the lowest and highest values were observed at sites 3 and 1, respectively. Reactive phosphate (PO_4^{3-}) comparably ranged from 0.03 to 0.23 $\mu g \cdot dm^{-3}$, in which the lowest value was reported in site 1, while the highest value was seen in site 2. Oil and grease also had values ranging between 0 and 107 $mg \cdot dm^{-3}$, where the lowest and the highest values were observed in sites 3 and 1, respectively (Tab. 1).

The outcomes of bacterial isolation in this study revealed that the Gram-negative bacteria were dominant in all sites. The isolates included *K. pneumoniae*, *E. coli*, *E. cloacae*, *P. aeruginosa*, *T. devorans*, *A. baumannii*, and *B. subtilis* (Tab. 2). It should be noted that most of the isolated bacteria had a clinical importance and could pose a risk to human health. The present study's findings agreed with those of Yang *et al.* [2009], in which the clinical isolate of *E. coli*, *K. pneumoniae*, and *P. aeruginosa* was found to be common in wastewater. Moreover, the results were in accordance with Yamina *et al.* [2014], establishing that 75% of pathogenic wastewater bacteria were Gram-negative, including *E. coli* and *K. pneumoniae*.

Within this locus, a total of 22 (S1–S22) samples were included, which showed about 598 bp amplicon in length. The sequencing reactions also indicated the confirmed identity of the amplified products by performing the Basic Local Alignment Search Tool (BLAST), showing high sequences of similarities between the samples and seven genera of high relative bacterial sequences, namely *K. pneumonia*, *E. coli*, *E. cloacae*, *P. aeruginosa*, *T. devorans*, *A. baumannii*, and *B. subtilis*. The NCBI BLAST engine correspondingly confirmed the presence of about 98–100% of homology with the expected bacterial target that covered a portion of the 16-S rRNA region. By comparing the observed DNA sequences of these local samples with the retrieved DNA ones, the exact positions and other details of the retrieved PCR fragments were identified. After positioning the 598 bp amplicon sequences within the ribosomal sequences, the details of these sequences were highlighted within the amplified 16-S rRNA sequences.

The alignment results of the 598 bp samples similarly revealed the detection of 13 nucleic acid variations with the corresponding reference bacterial sequences. These sequences were prepared by aligning the samples investigated in the present study with the most relative sequences deposited in the NCBI database (GenBank accession number MT604895.1 of *K. pneumonia* for S2, S6, S11, S14, and S21; GenBank accession number MK714220.1 of *E. coli* for S3, S12 and S20; GenBank accession number MT520149.1 of *E. cloacae* for S18 and S23; GenBank accession number MT646431.1 of *P. aeruginosa* for S1, S5, S7, S8, and S9; GenBank accession number MT605373.1 of *T. devorans* for S4 and S10; GenBank accession number CP053098.1 of *A. baumannii* for S15 and S22; and GenBank accession number MN515097.1 of *B. subtilis* for S16, S17, and S19).

Table 1. Environmental parameters of wastewater in three sites before and after treatment

Parameter	Site No.	Value before treatment	Value after treatment				Significance
			Pseudomonas aeruginosa	*Klebsiella pneumonia*	*Escherichia coli*	*Bacillus subtilis*	
		mean ±*SD*					
Water temperature	1	25±4.0	23±0.0	23±0.0	23±0.0	23±0.0	0.9000
	2	23±3.5	23±0.0	23±0.0	23±0.0	23±0.0	0.9100
	3	20±1.7	23±0.0	23±.0	23±0.0	23±0.0	0.8700
pH	1	6.36±1.12	6.20±1.02	6.92±1.30	6.90±0.08	6.72±0.90	0.8600
	2	7.76±1.90	8.56±8.02	8.45±0.55	8.50±0.77	8.57±1.12	0.8360
	3	7.82±1.02	8.50±1.11	8.14±1.42	8.15±0.84	8.10±1.22	0.9460
Electrical conductivity	1	2000±30.5	1344±23.1	2000±20.1	1915±47.2	1716±44.9	0.0001
	2	2051±2.12	1235±40.8	1998±90.7	1864±55.1	1494±42.7	0.0002
	3	713±33.7	315±50.4	689±78.5	588±80.1	429±64.7	0.0001
Total water hardness	1	960±100.1	200±12.3	500±44.5	700±77.8	900±90.4	0.0004
	2	900±105.4	160±16.7	600±37.4	700±57.1	860±78.1	0.0005
	3	480±54.7	60±12.1	80±11.8	100±20.1	80±10.7	0.0001
Total alkalinity	1	4600±120.5	2200±122.8	4000±200.4	3900±347.6	3100±100.4	0.0001
	2	7100±99.8	5000±106.7	7000±150.7	5800±130.7	4800±123.5	0.0001
	3	2500±177.5	1200±140.6	2000±120.8	2000±200.0	1900±178.4	0.0001
Nitrite (NO_2^-)	1	0.20±0.08	0.04±0.01	0.19±0.02	0.18±0.07	0.07±0.07	0.0001
	2	0.65±0.02	0.14±0.08	0.64±0.11	0.55±0.14	0.40±0.02	0.0001
	3	0.37±0.05	0.13±0.01	0.36±0.04	0.31±0.03	0.29±0.07	0.0003
Chemical oxygen demand	1	9.80±1.4	6.50±1.2	8.40±2.4	7.90±1.5	7.70±1.7	0.0280
	2	6.30±2.1	3.60±0.9	6.30±1.4	5.60±1.7	4.70±1.1	0.0340
	3	5.90±1.1	1.00±0.1	5.20±1.2	4.80±1.2	3.00±0.6	0.0010
Reactive phosphate (PO_4^{3-})	1	0.03±0.001	0.04±0.001	0.03±0.002	0.03±0.001	0.02±0.001	0.8930
	2	0.23±0.1	0.09±0.001	0.22±0.02	0.20±0.01	0.18±0.04	0.0001
	3	0.19±0.04	0.05±0.001	0.18±0.002	0.17±0.009	0.11±0.04	0.0002
Oil and grease	1	107±12.0	50±10.5	100±14.7	91±7.7	70±9.7	0.0001
	2	86±10.4	44±7.9	86±4.6	80±5.7	67±11.1	0.0001
	3	0±0	0±0	0±0	0±0	0±0	

Explanations: *SD* = standard deviation.
Source: own study.

Table 2. Distribution of isolated pathogens based on study sites

Pathogen	Site 1	Site 2	Site 3
Klebsiella pneumonia	–	+	+
Escherichia coli	+	+	–
Enterobacteria cloacae	–	–	+
Pseudomonas aeruginosa	+	+	+
Thalasobacillus devorans	–	–	+
Acinetobacter baumannii	–	+	+
Bacillus subtilis	+	–	–

Source: own study.

The 16S rRNA gene sequences, is by far the most commonly used housekeeping genetic marker, has also been used to study bacterial phylogeny and taxonomy for several reasons. Namely, the 16SrRNA sequence is present in almost all bacteria, often existing as a multigene family or operons. The function of the 16S rRNA gene does not change over time, suggesting that random sequence changes are a more accurate measure of time (i.e., evolution). The 16S rRNA gene (1,500 bp) is large enough for informatics purposes [Patel *et al.* 2001].

Highly interesting differences were observed in the current nucleic acid substitution, as detected in the analysed samples. These accounted for 13 nucleic acid substitutions, in which *K. pneumonia*, *P. aeruginosa*, *A. baumannii*, and *B. subtilis* exhibited six, four, two, and one nucleic acid substitutions,

respectively. However, the sequencing chromatograms of the identified variation region and its detailed annotations were verified and documented. The chromatograms of these sequences were shown according to their positions in the PCR amplicons.

Of note, mutations are heritable changes in the genetic coding instructions of DNA. They are also essential to studying genetics and useful in many other biological fields of base substitutions, as the simplest type of gene mutation and the alternation of a single nucleotide in the DNA. In this regard, base substitutions are of two types. The first, in transition, the purine is replaced by a different type of purine or pyrimidine and is substituted for a different type of pyrimidine. Second, in a transversion, a purine is replaced by a pyrimidine or a pyrimidine is substituted for a purine. Mutations also result from both internal and external factors. Those that are the outcomes of natural changes in the DNA structure are termed spontaneous mutations. In contrast, the ones that result from changes caused by environmental chemicals or radiations are named induced mutations [Al-Nuaimi 2020].

The 16SrRNA gene sequence was further analysed among some local isolates of *K. pneumonia*, and the results were compared with the standard data of similar registered strains in the NCBI and in agreement with Ibrahim *et al.* [2019]. The study results also confirmed the reports of Senthilraj *et al.* [2016]. Accordingly, it is concluded that 16S rRNA sequence-based identification reduces time by circumventing biochemical tests and increases specificity and accuracy. In addition, clinical and environmental samples can be identified and compared, and the source of microbes can be determined.

Understanding the community structure and the genetic relatedness among the strains of *P. aeruginosa* present in nature (that is, sea, river, soil, plants, and animals) is thus essential for gaining greater insights into the ecology and distribution of this bacterium. There have even been debates on the extent to which they share common niches in the environment and the degree to which environmental and clinical strains are genetically distinct. Khan *et al.* [2008] had suggested that *P. aeruginosa* strains isolated from Ushubetsu River water in Japan had originated primarily from the environments of human activity. Others had also observed that environmental *P. aeruginosa* isolates were genotypically, chemotaxonomically, and functionally indistinguishable from clinical ones. Together, these works had indicated that the populations present in different environments were highly intermingled or that high rates of genetic recombination could lead to relatively homogeneous genetic structures among the strains [Aoi *et al.* 2000].

Numerous studies focusing on the diversity of *Acinetobacter spp*., published over the last few years, also refer to the potential of some members of this genus to act as opportunistic pathogens and develop antibiotic resistance or discuss their role in wastewater biotreatment systems. However, drinking water can harbour different *Acinetobacter* species, consistent with Narciso-da-Rocha *et al.* [2013]. Therefore, the 16S rRNA gene partial sequences have been further deposited in the GenBank under the accession number MN515097.1 of *B. subtilis* for S16, S17, and S19, which were in line with Zaccardelli *et al.* [2020]. To summarise all the results obtained from the sequenced 598 bp fragments, the exact positions and annotations of the observed nucleic acid substitution mutations were described in the NCBI reference sequences, as shown in Table 3.

Table 3. The pattern of observed single-nucleotide polymorphisms in 598 bp amplicons of 16S rRNA sequences of currently investigated bacterial isolates compared with reference NCBI sequences

Organism	Sample No.	Native	Allele	Position in PCR fragment
Klebsiella pneumonia	S6, S11, S14, S21	G	A	161
	S2, S6, S11, S14, S21	C	T	187
	S6	G	A	198
	S6	A	T	210
	S6	T	G	228
	S6	C	T	242
Pseudomonas aeruginosa	S5	T	C	335
	S7	T	G	363
	S7	C	T	384
	S5, S8	C	G	399
Acinetobacter baumannii	S15, S22	G	A	205
	S15, S22	T	G	249
Bacillus subtilis	S16, S17, S19	C	T	189

Explanation: PCR = polymerase chain reaction.
Source: own study.

It should be noted that spontaneous gene mutations result from errors during DNA replications and those occurring in recombination, spontaneous lesions, and transposable elements. There are four gene mutation classes at the DNA level, including substitutions, deletions, insertions, and inversions. A substitutional mutation is also called a point mutation, which changes a single nucleotide base pair in the DNA molecule. There are also two purine bases, i.e., adenine (A) and guanine (G), and two pyrimidine bases, that is, cytosine (C) and thymine (T). In the normal double-stranded DNA molecule, A on one strand is paired with T on the complementary strand, and this occurs for C with G. Therefore, substitutional mutations generally replace the base pairs rather than the base on only one strand. In fact, the whole nucleotide consisting of phosphate (PO_4^{3-}) group, sugar, and the base, is typically substituted rather than simply the base. Substitutional mutations are either transitions or transversions. In this sense, transitions replace a purine with a purine or a pyrimidine with a pyrimidine. The four types of transition mutation are thus AT-GC and CG-TA. Transversions are also substitutions of a purine for a pyrimidine or vice versa.

CONCLUSIONS

All the sites selected in this study were polluted due to high concentrations of different types of pathogenic Gram-positive and Gram-negative bacteria. The study results also established that *P. aeruginosa* had high efficiency in removing nutrients (here, nitrite – NO_2^- and phosphate PO_4^{3-}) from wastewater, as the highest nitrite (NO_2^-) removal rate by 78.8% was recorded in

site 2 and the highest phosphate (PO_4^{3-}) removal rate by 71.1% was observed in site 3. Additionally, the results indicated that the *Bacillus subtilis* had the lowest efficiency, with its removal rate being 59.2%.

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