Pollution, 5(1): 179-187, Winter 2019
DOI: 10.22059/poll.2018.254928.427
Print ISSN: 2383-451X Online ISSN: 2383-4501
Web Page: https://jpoll.ut.ac.ir, Email: jpoll@ut.ac.ir

Model for the Treatment of Refinery Wastewater and Expression of Catabolic Genes in Fluidized Bed Bioreactor using Mixed Bacterial Consortium

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Received: 22.03.2018 Accepted: 10.11.2018

ABSTRACT: This study was undertaken to evaluate a novel aerobic wastewater treatment model for the remediation of refinery effluents and to assess the removal efficiency of *Bulkholderia cepacia* strain AJI and *Corynebacterium kutscheri* strain AJ2 to clean oil waste from petrochemical company. Wastewater quality parameters including pH, BOD_5, COD, TDS, OIL & GREASE, PHENOL concentration, TPH and THC were monitored at 5, 10 and 15 days of treatment and the removal efficiencies were calculated. Results indicated that the raw oily wastewater effluents used during this study had extremely high levels of all the tested parameters. The mean values of all physicochemical parameters of the wastewater from primary tank at different treatment period were statistically different ($P<0.001$) After 15 days of biological treatment, BOD_5 ,COD, TDS, Phenol, TPH, Oil & grease level of the refinery wastewater were reduced by 95.60 %, 98.40 % , 66.34 % , 100 %, 97.60 % and 96.20 % respectively. The detection of the catabolic genes in the bacterial isolates recovered from primary tank using polymerase chain reaction revealed that both *Bulkholderia cepacia* strain AJ1 and *Corynebacterium kutsheri* strain AJ2 carried alk B and C23O but C12O was not detected in both isolates. Naphthalene dioxygenase was detected in *Bulkholderia cepacia* strain AJ1 but not found in *Corynebacterium kutscheri* strain AJ2. After treatment the waste water was filtered in the secondary tank. The results of physicochemical parameters in the outlet vessel essentially confirmed that the mixed culture in the two column model successfully carry out bioremediation of refinery wastewater. Therefore, aerobic treatment model for the bioremediation of refinery Petroleum refineries generate great amounts of wastewaters that may become seriously dangerous, leading to the accumulation of toxic products in the receiving water bodies with potentially serious long term effects to aquatic biota. Due to extreme toxicity of contaminants in refinery wastewater, there is a need to develop an economical technique to remove the pollutants from the wastewater is highly recommended owing its environmental friendliness.

Keywords: Refinery, Wastewater, Toxicity, Aerobic model, *Bulkholderia* strain AJ1, *Corynebacterium kutscheri* strain AJ2

INTRODUCTION

The method of refining crude oil requires a ton of water; subsequently, large volume volumes of wastewater are released into the environment (Coelho *et al*., 2006). In most developing countries, industries discharge their wastewater without treatment on account of the high cost of

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existing treatment developments. Refinery effluent is extremely toxic and poses an incredible threat to the nearby communities and a major source of environmental risk due to the presence of petroleum hydrocarbon. Therefore, Petroleum refining wastewater has to be sufficiently treated for quality to meet the established regulations before being discharged into the stream. (Musa *et al*., 2015; Santo *et al*., 2015).However, Physical and chemical methods of treating refinery wastewater are expensive and generation of large amount of sludge. Therefore, there is a need for a more robust and cost effective treatment technology (Musa *et al*., 2015). The Presence of pollutants in normal water alters the quality and often poses serious threats to aquatic life. Distinctive studies have shown positive correlation between pollution from petrochemical and refinery effluents and the health of aquatic organisms (Otunkunefor & Obiukwu, 2005). Hence, release of untreated petroleum refining wastewater into water bodies may pose both environmental and human health's hazard due to the release of their toxic contents (Duniya *et al*., 2016). As a result of the incapability of purification frameworks, wastewaters may become hazardous, leading to the accumulation of toxic products of the receiving water bodies of potentially major impact on the ecosystems (Beg *et al*., 2001; Beg *et al*., 2003; Otunkunefor & Obiukwu, 2005). Numerous studies have already shown the presence of large and different populations of microbes with diverse metabolic capacities of petroleum systems (Magot *et al*., 2000; Aitken *et al*., 2004). Knowledge of the bacterial composition and catabolic genes involved in the degradation of hydrocarbons, description of microbial metabolic pathway and additionally a superior application of particular microorganisms in biodegradation or bioremediation processes is desirable (Verde *et al*., 2013). Horizontal gene transfers enhance the biodegradation process by creating the development of an efficiently degrading microbial community. Hence, the demonstration of specific degradative genes on mobile genetic elements or the determination of genes that are able to promote HGT is evidence that a bioremediation process has been successful (Wilson *et al*., 2003; Nie *et al*., 2014). There are several catabolic genes that play a major roles as well as markers for characterizing the microbial community of petroleum hydrocarbons polluted site (Shahi *et al*., 2016).

This work is a pilot scale aiming to using aerobic treatment model for the bioremediation of refinery effluents.

MATERIALS AND METHODS

The bacterial strains *Bulkholderia cepacia* strain **AJ1** and *Corynebacterium kutsheri* strain **AJ2** isolated from refinery wastewater samples as reported previously (Ajao *et al*., 2013; Ajao *et al*., 2014) were grown and maintained in oil agar. The experimental set up for the bioremediation of refinery effluent was adopted from the model suggested by Porwal *et al. (*2015); the schematic diagram of the experimental set-up is shown in Fig. 1.

All the plastic containers used for the bioremediation process were washed with alcohol to make it sterile and then rinsed with sterile distilled water. The laboratory scale 1.5 L reactor (primary tank) was then fed with 1 L autoclaved untreated refinery wastewater. The autoclaved effluent was cooled to room temperature and then added to the reactor supplemented with About 2 % molasses and mineral medium with following, composition of mineral medium (MM) as described by Porwa *et al*. (2014) (g/L): Na_2HPO_4 – 2.2; KH_2PO_4 – 1.4; $MgSO_4.7H_2O$ – 0.6; $(NH_4)_2SO_4$ – 3; yeast extract – 1; NaCl – 0.05; $CaCl_2.7H_2O$ – 0.02; $FeSO_4.7H_2O$ – 0.01. The medium was supplemented with 1 mL of the trace elements solution Porwal

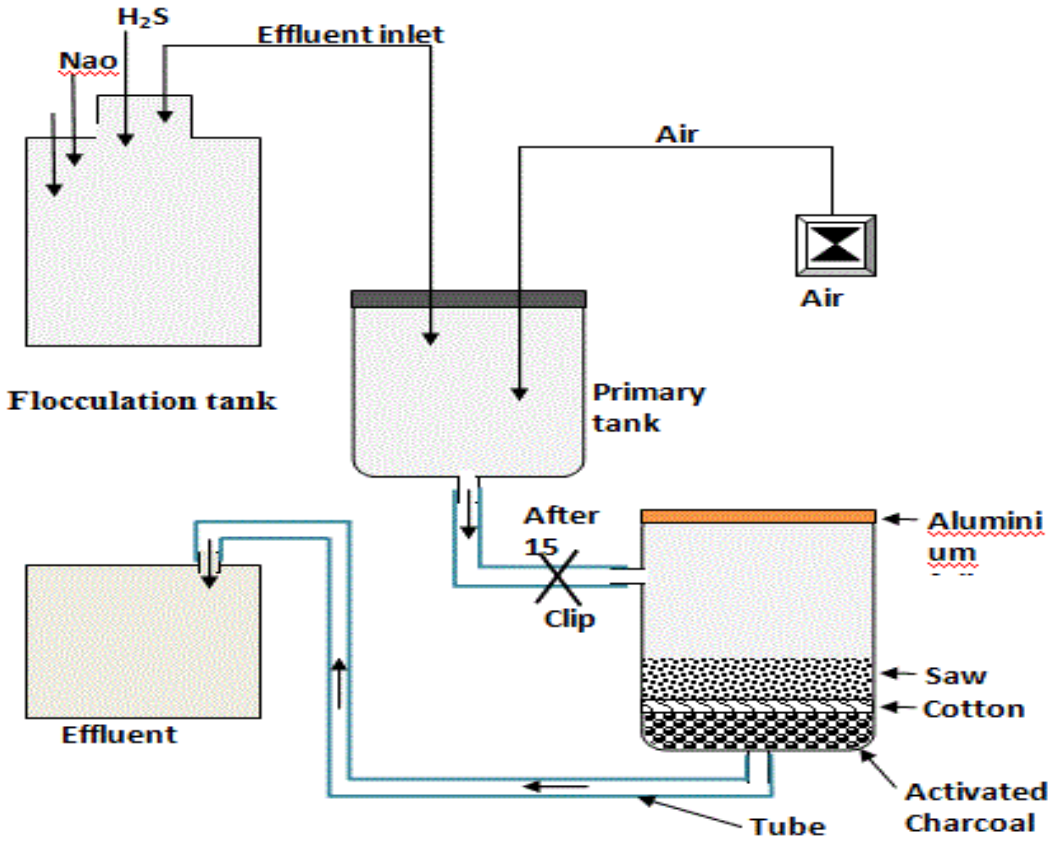

Fig. 1. Schematics of the experimental setup for bioremediation of refinery effluent

*et al. (*2015) (mg/L): $ZnSO_4$.7H2O – 50; $MnCl_2.4H_2O$ – 400; $CoCl_2.6H_2O$ – 1; $CuSO_4.5H_2O$ – 0.4; H_3BO_2 – 2; $Na_2MoO_4.2H_2O$ – 500. The experimental flasks were inoculated following the method of (Ajao *et al*., 2014). Experimental set up was left for 20 days. Samples were taken aseptically from the primary tanks and outlet tank at 5, 10 and 15 days interval for pH, BOD_5 COD, Oil and Grease, Phenol concentration, Total Dissolved Solid, Total Petroleum Hydrocarbon and Total Viable Count for the assessment of the performance of the set up for a period of 15 days.

Several protocols from different Authors were followed for the determination of total dissolved solid (TDS) Tufekci *et al*. (1998), Dissolved oxygen (DO) of the effluent samples were measured by Hanna Instruments: H19829. The pH was determined by using Hanna Instruments pH meter, Model pH-600AQ. BOD and COD were determined by 5-Day BOD test and Closed Reflux, Titrimetric Method, respectively Anonymous and APHA 1998; APHA 2005. The phenol concentration was determined using spectrophotometric method APHA (2005); Banerjee & Ghoshal (2016). Oil & grease was determined by Liquid-liquid extraction procedure as reported by Adewuyi & Olowu (2012) and Method of Chikere & Ekwuabu 2014 was adopted to carry out Total heterotrophic Counts (THC). Molecular characterization of the bacterial isolates was done by sequencing of their 16S rRNA gene. The 16S rDNA was amplified using PCR with Taq polymerase (Qiagen) and the universal primer pair of 27F (5-AGAGTTTGATCCTGGCTCAG-3) and 1523R (5- GGTTACCTTGTTACGACTT-3) (Weisburg *et al*., 1991). The 16S rDNA

sequences of the bacterial isolates were BLAST (Basic local alignment search tool) searched against the sequences of 16S rRNA of bacterial isolates available in the Genbank Nucleotide Database (http://www.ncbi.nih.gov/blast) Sequences with more than 98 % identify with a GenBank sequence were considered to be of the same species as the highest score-matching sequence on the public sequence databases (Altschul *et al.* 1997).

Related sequences were obtained from the GenBank database (National Center for Biotechnology Information, NCBI) using the BLASTN search program. The 16S rRNA sequences determined and reference sequences obtained from GenBank databases were aligned using the multiple sequence alignment software CLUSTAL W ver. 1.81. Phylogenetic tree was constructed with MEGA 6.0 based on the 16S rDNA sequences of some selected strains and **AJ1** and **AJ2**. The sequences were submitted to the GenBank database and assigned an accession number of MH382819 and MH382820 respectively.

Polymerase chain reaction: For screening of catabolic genes viz. alkB, C12O, C23O and nahAc in the isolates of *Bulkholderia cepacia* strain **AJ1** and *Corynebacterium kutscheri* strain **AJ2** primers specific for those genes were used in PCR assays.Alkane monooxygenase (alkB), Catechol dioxygenase (*C12O* and *C23O*), and Naphthalene dioxygenase (nahAc) genes were listed in Table 1. PCR conditions were initial denaturation for 5min at 95 ^{0}C, 35 cycles with 40 s at 94 ^{0}C, 40 s at 55 ^{0}C, 60 s at 72 ^{0}C, and final elongation for 7min at 72 ^{0}C for all genes *alkB, C12O, C23O and nahAc* Rajaei *et al*., 2013. However, PCR was performed for Catechol 1,2- dioxygenase (*C12O*) and Catechol 2,3-dioxygenase (*C23O*) genes with initial denaturation for 5min at 95 ^{0}C, 35 cycles with 20 s at 94 ^{0}C, 30 s at 63 ^{0}C, and 45 s at 72 ^{0}C, and final elongation for 5min at 72 ^{0}C. The PCR products were analyzed in 1.5 per cent agarose gel prepared in 1X TAE buffer (40mM Tris-acetate and 1mM EDTA, pH 8.0) containing ethidium bromide (0.3 µg/ml). Electrophoresis was done at 7 volts/cm for 1 h 30 min. Finally, photograph of the gel was taken with digital camera.

RESULTS AND DISCUSSIONS

The mean values of the physicochemical characteristics such as BOD_5 , COD, TDS, Phenol concentration, TPH and Oil & grease level of refinery wastewater prior to the treatment were 236, 875, 446, 68.13, 41.2 and 56.12 mg/L respectively shown in Table 2. The mean values of all physicochemical parameters of the wastewater from primary tank at different treatment period were statistically different ($P<0.001$). The efficiency of the treatment after 15 days of biological treatment the BOD_5 ,COD, TDS, Phenol, TPH, Oil & grease level of the refinery wastewater were 95.60 %, 98.40 % , 66.34 % , 98.5 %, 97.60 % and 96.20 % respectively. The results of physicochemical parameters in the outlet tank essentially confirmed that the mixed culture in the two column model successfully carried out bioremediation of refinery wastewater.

The phylogenetic relationship among the strains **AJ1**, **AJ2** and other related bacteria found in the GenBank database. The homology assay result indicated that the strains **AJ1** and **AJ2** were in the phylogenetic branches of the *Burkholderia and Corynebacterium* spp. **AJ1** exhibited maximum identities (100 %) to *B. cepacia strain ATCC 25416* while **AJ2** showed maximum identities (100 %) to *Corynebacterium strain CIP* 103423 and CIP 103423 showed in Fig. 2. The bacterial isolates clustered with members of the genera *Burkholderia and Corynebacterium*, thus differentiating the bacterial isolates on the genetic basis.

Table 1. Primer sequence, expected PCR products and amplified internal fragments of catabolic genes in bioremediation of refinery effluent.

Gene	Sequence Primers	Fragment size (bp)	A	B
alkB-	F :5'-TGGCCGGCTACTCCGATGATCGGAATCTGG-3' R:5'-CGCGTGGTGATCCGAGTGCCGCTGAAGGTG -3'	870	+	+
C12O	F: 5'-GCCAACGTCGACGTCTGGCAGCA-3' R: 5'-CGCCTTCAAAGTTGATCTGCGTGGTTGGT-3	350	-	-
C23O	F: 5'-AAGAGGCATGGGGGCGCACCGGTTCGA-3' R: 5'-TCACCAGCAAACACCTCGTTGCGGTTGCC-3'	900	+	+
nahAc	F:5'-TGGCGATGAAGAACTTTTCC-3' R:5'-AACGTACGCTGAACCGAGTC-3'	487	+	-

Alk B Alkane monooxygenase; C12O Catechol-1,2-dioxygenase; C23O Catechol-2,3-dioxygenase, nahAc Naphthalene dioxygenase; A : *Bulkholderia cepacia* B: *Corynebacterium kutscheri*

Table 2. Physicochemical characteristics of refinery wastewater fed into Fluidized bioreactor

Parameters	Mean ± Standard deviation
pH	8.00 ± 0.25
BOD5 (mg/L)	236 ± 0.40
COD (mg/ L)	875 ± 3.92
TDS (mg/ L)	446.4 ± 5.25
Phenol (mg/ L)	68.13 ± 0.36
TPH (mg/ L)	41.2 ± 0.64
Oil & Grease (mg/ L)	56.12 ± 3.91
THC (cfu/ml)	6.2×10^3

BOD_5 Biochemical oxygen demand; COD Chemical oxygen demand TDS Total dissolved solid THC Total heterotrophic count.

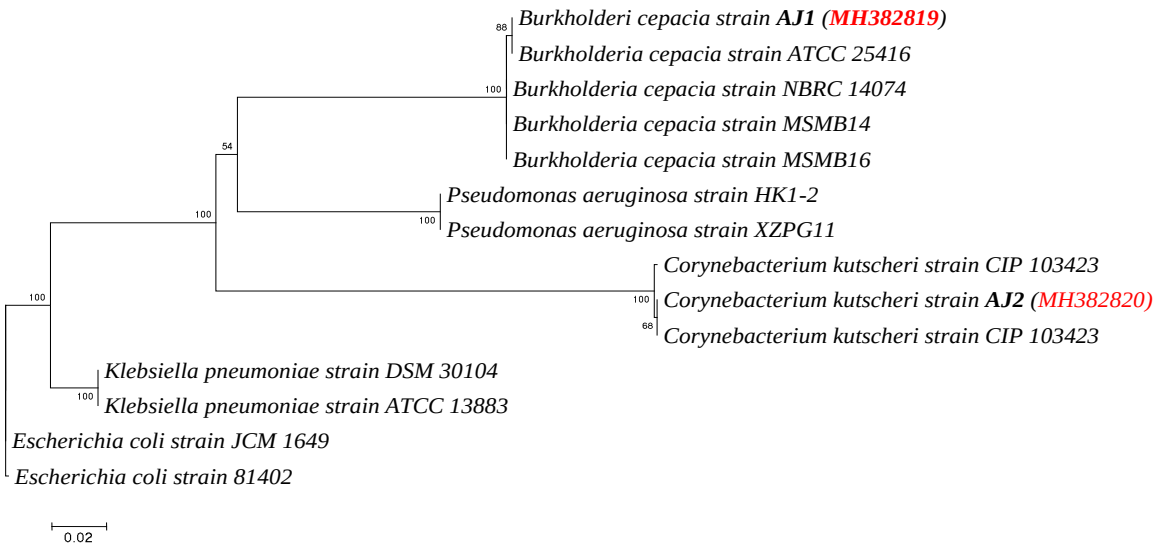

Fig. 2. Phylogenetic tree constructed by Neighbor-Joining method derived from analysis of the 16S rRNA gene sequences of native isolates and related sequences obtained from NCBI. Scale bar, 0.2 substitutions per nucleotide position and numbers in parenthesis represent GenBank accession numbers.

The mean values of the raw refinery wastewater was 8.0 while, the highest pH values in the secondary tank were in ranges of (6.43-7.88). The mean values of the pH tend to fluctuate during bioremediation process, such fluctuations patterns is an evidence of chemical changes due to microbial enzyme activities (Atlas and Bartha 1992). The biochemical oxygen demand (BOD_5) in both the reaction tank and outlet as presented in Table 2.0 ranged from (10-150) mg/ml. Mean values of BOD_5 found to reduce as treatment progressed. The efficiency of the treatment was statistically significant ($p< 0.001$). The levels of Biological Oxygen Demand were high in raw wastewater shown in Table 1 which tends to reduce as the treatment progressed. Comparison of the mean values were statistically significant ($P<0.01$).This shows the capacity of the mixed culture to reduce BOD_5 in the effluent with 95.70 %. Biological oxygen demand (BOD) test represents waste loading and higher degree therefore indicates the presence of large amount of organic pollutant and relatively higher level of microbial activities with consequent depletion of oxygen content.

The mean values of chemical oxygen demand (COD), Total dissolved solid (TDS), phenol concentration, total petroleum hydrocarbon (TPH) and oil and grease in secondary tank during bioremediation process ranged as (14.2-196), (150-312), (1-33.41), (2.0-28.60) and (3.11-31.91) mg/ml with the following efficiency of 98.4 % , 66.34 %, 98.50 %, 97.6 % and 96.2 % respectively. All the mean values of the parameters recorded after 15 days of treatment and the mean values of outlet effluent were significantly not different ($p< 0.001$). All the mean values of the parameters obtained at the every sampling duration between 5 to 15 days were significantly different. Total heterotrophic count was recorded after 10 days of treatment followed by 15 days. While, the least bacterial count was recorded in the outlet effluent this may be as a result of entrapment on the cotton wool and saw dust.

Table 3. Physicochemical Characterization of Treated refinery Wastewater

Duration (days)	pH	BOD	COD	TDS	Phenol	TPH	Oil/Grease	THC
5	7.13 ± 0.02^{a}	150.00 ± 4.68^{a}	196.06 ± 5.16^{a}	312.20 ± 8.00^{a}	33.41 ± 1.41^{a}	28.60 ± 1.24^{a}	31.91 ± 5.03^{a}	1.5×10^{5}
10	6.73 ± 0.41^{ab}	85.12 ± 2.00^{b}	89.38 ± 3.16^{b}	266.00 ± 14.0^{b}	14.71 ± 1.11^{b}	12.11 ± 1.70^{b}	15.42 ± 1.18^{b}	4.6×10^{9}
15	6.48 ± 0.51^{ab}	10.11 ± 1.11^{c}	26.92 ± 0.00^{c}	177.10 ± 10.00^{c}	1.16 ± 0.01^{c}	2.00 ± 0.51^{b}	3.11 ± 0.08^{bc}	7.4×10^{7}
Outlet	7.88 ± 0.61^{c}	10.00 ± 0.00^{c}	14.20 ± 1.60^{d}	150.19 ± 1.42^{cd}	0.00 ± 0.12^{c}	0.95 ± 0.06^{b}	2.51 ± 0.00^{bc}	2.6×10^{3}
Efficiency (%)	-	95.70	98.40	66.34	100	97.60	96.20	-

BOD_5 Biochemical oxygen demand; COD Chemical oxygen demand TDS Total dissolved solid; THC Total Heterotrophic count; TPH Total petroleum hydrocarbon. Mean values with different superscripts in the same column are significant different. Mean values were separated using Duncan Multiple Range Test (DMRT).

COD is often used as indicator of water quality; The Chemical Oxygen Demand values recorded in the raw effluent was 875 mg/L which was drastically reduced to 14.20 mg/L in the outlet tank. The mean values obtained at different sampling time were statistically significant using Duncan multiple comparison at ($P<0.01$).In similar study Najafi *et al.* (2012) reported that (COD), PAHS and TPH reduction was increased by an increase in the reaction's duration from 1 day to 10 days and the percentage removal of (COD) reached to 100 % in 10 days with the initial concentration of

440 mg MI^{-1}. Banerjee and Ghoshal (2016) in their study reported the efficiency of *Bacillus cereus* in reducing the COD level and phenolic compounds by 95 % in the refinery wastewater using continuous mode of operation. The present findings have also demonstrated that the aerobic treatment model can reduce all the physicochemical parameters measured for specific duration of treatment in agreement with the work of Gargouri *et al*. (2011). The residual total petroleum hydrocarbon (TPH) decreased from 320 mgTPH l^{-1} to 8mg TPH l^{-1}. *Bulkholderia cepacia* and *Corynebacterium kutscheri* proved to have enzymatic and degradative capacity to synergistically degrade hydrocarbon component of the refinery wastewater, this assertion is in agreement with the work of Hamza *et al*. (2012) who reported that demonstrated 56.19 % and 52.43 % reduction in COD by *Bacillus subtilis and Micrococcus luteus respectively* with higher growth in the Bushnell-Haas medium supplemented with the refinery wastewater. Rapid degradation of crude oil in refinery effluent could be attributed to constant aeration provided during bioremediation. Thus, bio augmentation and nutrient amendment (Molasses) in the flocculation tank might also have contributed to biodegradation potential of the cultures in the primary tank. In a similar study, Agamuthu *et al*., 2013 reported rapid biodegradation (82 %) of used lubricant oil was achieved in a soil amended with sewage sludge. Also, Gang *et al*. (2013) also affirmed the assertion that supplementing PHs-contaminated soil with rice straw biochar resulted in the 84.8 % of TPHs degradation. In another study, Madedela *et al*., 2016 also reported that bio augmentation and nutrient amendment (glucose and NPK) resulted in the removal of 79.47 % PHs from crude oil treated soil after 30 days. Detection of the catabolic genes in the bacterial isolates recovered from primary tank revealed that both *Bulkholderia cepacia* and *Corynebacterium kutsheri* carry alk B and C23O but C12O was not detected in both isolates. Naphthalene dioxygenase was detected in *Bulkholderia cepacia* but not found in *Corynebacterium kutscheri*.

xylE gene was detected in both isolates which may have contributed to their synergistic degradation of the crude oil. Both isolates lack C12O but C23O were detected in them which in turn imply that both isolates used meta cleavage for degradation. Genes that are lacking in one isolate is present in the second isolate which allows to them exhibit synergism in the bioremediation of refinery effluent (Lappin *et al.,* 1985) .The results also confirmed the presence of *alkB* and *xylE* genes in *both Bulkholderia cepacia* and *Corynebacterium kutsheri* the hypothesis that can explain this phenomenon may be attributed to the conservation of the genes among hydrocarbonoclastic organisms. The naphthalene dioxygenase (*nahAc*) gene was detected in *Bulkholderia cepacia* but not detected in *Corynebacterium kutsheri.* This gene has been reported to play a significant role in the degradation of naphthalene and other PAHs compounds (Guo *et al*., 2010).The degradation of aliphatic and polyaromatic hydrocarbons degradation have been attributed to the expression of catabolic genes such as monooxygenase (*alkB* and *alkB1*), dioxygenase (*nahAc*) and Catechol dioxygenase (C23O) genes which were detected in the current study.

Duniya *et al. (*2016) also demonstrated the presence of the genes for the hydrocarbon enzymes Catechol 2, 3 dioxygenase (C2,3O) in *Enterobacter hormaechei*, *Escherichia coli* and *Shigella flexneri*, from soil samples from Mechanic Workshops. The detection of these genes in *Bulkholdria cepacia* and *Corynebacterium kutscheri* confirms the genetic potential of such organisms to serve as bioremediation agents. It has been reported that the *alkB* gene is in a mobile form and it is; therefore, very unstable Gerhardt (1981). Some alkane degradation genes may either be located on transposons (Van *et al*.,

2001), or on plasmids (Van *et al*., 1994; Sekine *et al.*, 2006) which may clearly facilitate their horizontal transfer. Matsumiya and Kubo (2007) reported that short chain alkanes are especially difficult for microorganisms to degrade in nature. *Burkholderia cepacia* has been widely reported in terms of degradation of the low-molecular-weight PAHs, naphthalene and phenanthrene (Laurie and Jones 2000).

CONCLUSIONS

In conclusion, Crude petroleum is a complex mixture of hydrocarbon components; assemblies of mixed populations with overall broad enzymatic capacities are required for the biodegradation and bioremediation of crude oil contaminated environment. Therefore, an in-depth understanding of diversity and distribution of catabolic genes among microorganisms involved in degradation process would provide more chance towards bioremediation of refinery effluents.

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