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Application of a continuously stirred tank bioreactor (CSTR) for bioremediation of hydrocarbon-rich industrial wastewater effluents

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ABSTRACT

A continuously stirred tank bioreactor (CSTR) was used to optimize feasible and reliable bioprocess system in order to treat hydrocarbon-rich industrial wastewaters. A successful bioremediation was developed by an efficient acclimatized microbial consortium. After an experimental period of 225 days, the process was shown to be highly efficient in decontaminating the wastewater. The performance of the bioaugmented reactor was demonstrated by the reduction of COD rates up to 95%. The residual total petroleum hydrocarbon (TPH) decreased from 320 mg TPH l^{-1} to 8 mg TPH l^{-1}. Analysis using gas chromatography–mass spectrometry (GC–MS) identified 26 hydrocarbons. The use of the mixed cultures demonstrated high degradation performance for hydrocarbons range n-alkanes (C10–C35). Six microbial isolates from the CSTR were characterized and species identification was confirmed by sequencing the 16S rRNA genes. The partial 16S rRNA gene sequences demonstrated that 5 strains were closely related to *Aeromonas punctata* (*Aeromonas caviae*), *Bacillus cereus*, *Ochrobactrum intermedium*, *Stenotrophomonas maltophilia* and *Rhodococcus* sp. The 6th isolate was affiliated to genera *Achromobacter*. Besides, the treated wastewater could be considered as non toxic according to the phytotoxicity test since the germination index of *Lepidium sativum* ranged between 57 and 95%. The treatment provided satisfactory results and presents a feasible technology for the treatment of hydrocarbon-rich wastewater from petrochemical industries and petroleum refineries.

1. Introduction

The petrochemical industry generates series of liquid effluents containing large amounts of priority pollutants during the petroleum-refining process. These effluents must be treated through depuration processes. The major pollutants found in these industries are petroleum hydrocarbons, specifically aliphatic hydrocarbons of 1–40 carbon atoms, along with cycloalkanes and aromatic compounds. Thus, it is a potentially dangerous waste product. Simply dumping these wastes or burning them with no previous treatment has serious environmental consequences and presents a threat to both ecosystems and human health [1]. Biodegradation by natural populations of microorganisms represents one of the primary mechanisms by which petroleum and other hydrocarbon pollutants can be eliminated from the environment [2,3].

Selective enrichment is the basic method by which individual microorganisms or a consortium of microorganisms capable of degrading a specific compound can be obtained [4–6]. The process involves providing conditions suitable for the growth of microorganisms capable of metabolising the desired compound [4]. Desired microorganisms can be obtained by providing a target substrate for biodegradation as the sole source of carbon.

The application of biotechnological processes involving microorganisms, with the objective of solving environmental pollution problems, is gradually growing. This has been especially in recent decades, when petroleum and its by-products are of great concern. Bioremediation processes, which take advantage of microbial degradation of organic compounds, can be defined as the use of microorganisms (especially bacteria) to detoxify and remove environmental pollutants from soils, waters and sediments. The bioremediation process, presenting countless advantages in relation to other processes employed, is an evolving method for the removal and the transformation of many environmental pollutants including those produced by the petroleum industry, being one of the most efficient methods to treat polluted environments [7–11]. However, it is known that hydrocarbon biodegradation in wastewater can be limited by many factors, including microorganism species, nutrients, pH, temperature, moisture, oxygen, soil properties, and contaminant concentration [12–16]. These parameters may influence the adaptation of microorganisms to the available substratum [17].

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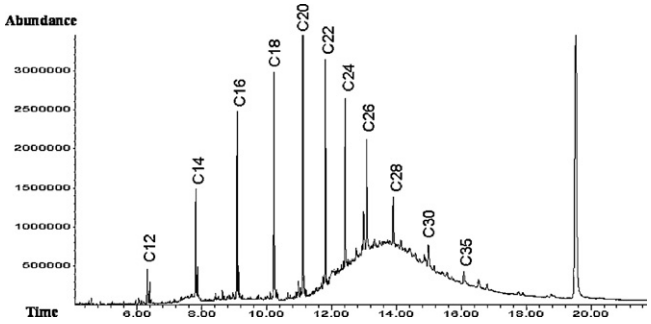

Fig. 1. Chromatogram of residual aliphatics fraction obtained from hydrocarbon-wastewater refinery. C12 to C35 indicate *n*-alkanes with the number of carbon atoms from 12 to 35.

In this context, an industrially wastewater contaminated by petroleum products was studied to demonstrate that bioremediation application is efficient for the process upgrade due to the availability of the bioaugmented specialized consortia.

Based on the laboratory scale assessment of the biodegradation performance within industrially contaminated wastewater polluted by petroleum, it was aimed to study the biodegradation of petroleum hydrocarbons in a bioreactor (CSTR) and to identify the dominant strains of this acclimatized consortium as candidates for application in the bioremediation of contaminated wastewater.

2. Materials and methods

2.1. Material

2.1.1. Contaminated water

A wastewater contaminated with petroleum hydrocarbons was used as the source of naturally occurring microorganisms, which presumably had been well adapted to the target pollutants (Fig. 1). Wastewater was collected from a petroleum refinery industry site located in the north of Tunisia. The main characteristics of the hydrocarbon contaminated wastewater are described in Table 1. All collected samples were stored at 4 °C after collection until required for analysis.

2.1.2. Contaminated soil

The contaminated soil used in this study was a loamy sand soil, collected from adjacent areas of the same hydrocarbon-rich refinery industrial plant. The preparation soil contained (g^{-1}) 65 mg total dichloromethane extractible organics (TEO), this soil had a clay texture, pH of 7.8 (1:3 ratio of dry soil to wastewater). The amendment of the hydrocarbon contaminated soil in bioreactor was to use it as source of carbon and energy and as inoculum due to its rich content of indigenous microflora.

Table 1
Profile description of hydrocarbon-contaminated industrial wastewater.

Physical appearance	Hydrocarbon-contaminated wastewater
COD ($g\,l^{-1}$)	7–2.5
BOD_5 ($g\,l^{-1}$)	0.95–0.63
COD/BOD_5	7.4–3.9
pH	9–7.5
Nitrogen ($mg\,l^{-1}$)	338
Oil and greases ($g\,l^{-1}$)	0.17–0.11
Electric conductivity ($\mu S\,cm^{-1}$)	737
Total petroleum hydrocarbons ($mg\,l^{-1}$)	270

2.2. Analytical methods

The evolution of the parameters of pollution was followed by diverse measurements. *Biochemical oxygen demand* (BOD_5) was determined after 5 days by the manometric method with a respirometer [BSB Controller Model 620 T (WTW)] [18].

Chemical oxygen demand (COD) was estimated as described by Knechtel [19].

Electric conductivity (EC) and pH were measured using a conductivity-meter (Consort C 831) and a pH-meter (NeoMet pH-200 L), respectively.

Total nitrogen content (TKN) was analysed as described in Kjeldahl-N method [20].

Dry weight and moisture content were determined by weighing samples before and after drying overnight at 105 °C. *Biomass* was analysed, by loss on ignition at 600 °C for 2 h according to Standard Methods [21].

The characteristics of decantability of the activated sludge were measured by the Mohlman index (Im).

Phytotoxicity was estimated by the determination of the germination index (GI) according to Zucconi et al.'s method using *Lepidium sativum* seeds [22].

2.3. Bioreactor design

The biodegradation of hydrocarbons was studied in an aerobic CSTR (shown in Fig. 2) with a total volume of 20 l and a working volume of 12 l for continuous experiments. The reactor temperature was maintained at 30 °C with a thermostat jacket. Oxygenation was provided by injection, through a porous diffuser, of air at 0.5 vvm. For the microbial population growth, the bioreactor was fed continuously with the industrial wastewater, as the sole carbon source.

A masterflex peristaltic pump polytetrafluoroethylene (PTFE) tubing was used to pump slurry phase to the reactor (inoculum).

The monitoring of the reactor was carried out by analysis of the liquid phase for pH, chemical oxygen demand (COD), nitrogen content and biomass.

In the reactor, a dry soil/wastewater ratio of 1/3 was added during the first period (A: acclimatization phase: start up). In the second phase (B), only hydrocarbon-contaminated wastewater was used.

2.4. Chemicals and media

For microbiology analyses, a microbial consortium was isolated from an industrial wastewater contaminated with hydrocarbons and was enriched at 30 °C. The minimal medium (MM) used for subcultivating contained: 0.5 g KH_2PO_4, 0.4 g NH_4Cl, 0.4 g NaCl, 0.33 g $MgCl_2(6H_2O)$, and 0.05 g $CaCl_2(6H_2O)$ per litre of distilled water, supplemented with 1 ml of trace elements solution [23]. The pH of the medium was adjusted to 7. Wastewater was added as a carbon source at 1% (v/v). Solid MM plates presented the same composition supplemented with 18 $g\,l^{-1}$ of Agar. All media and solutions were prepared with distillate water and autoclaved at 121 °C for 20 min. For the working cell banks, the microbial suspension was resuspended in fresh minimal medium (MM) containing 15% of glycerol and stored in cryo-vials at −80 °C.

2.5. Isolation and identification of bacterial isolates

After an experimental period of 225 days, the enrichment mixing broths from the CSTR bioreactor were directly used for the isolation. Aliquots (100 μl) were carried out after 10-fold serial dilutions and spread-plate method on minimal medium (MM). Pure cultures were obtained by spreading each 100 μl of aliquots on the solid MM plates. Before use, 1% (v/v) of refinery wastewater was

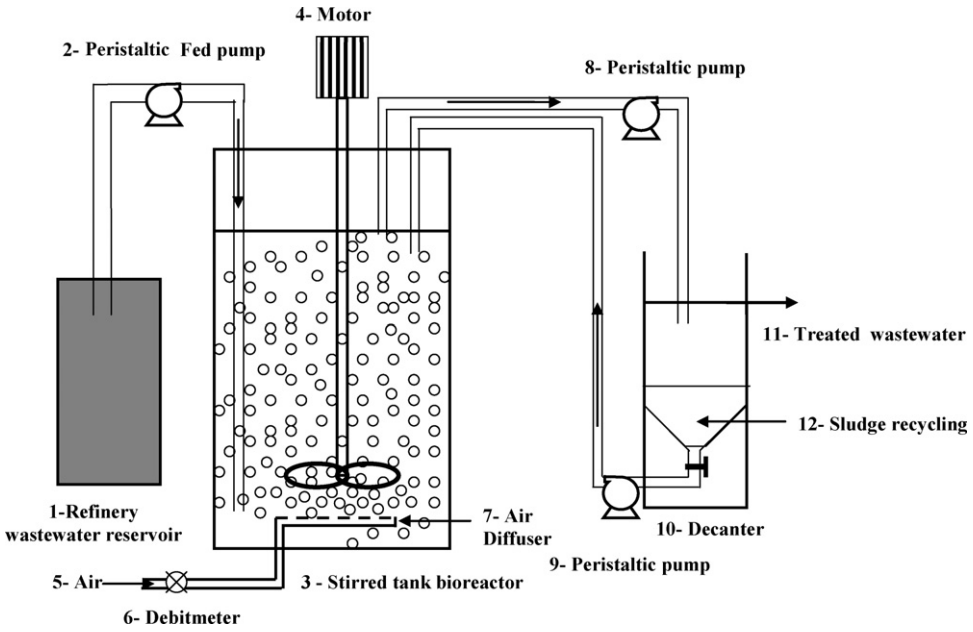

Fig. 2. Schematic diagram of the aerobic continuously stirred tank bioreactor (CSTR) used for continuous experiments.

dissolved in MM and spread on the surface of the plates as a sole source of carbon [24]. The plates were incubated at 30 °C, under aerobic conditions in duplicate for up 7 days until colony formation. Microbial growth was monitored every 2 days.

Isolates obtained were further streaked on to the surface of fresh plates and checked for purity prior to subsequent molecular identification. The most abundant colony, especially those forming clear morphology on the surface of the plates, was selected as the candidates for further investigation. Six single colonies were picked and used for screening. Individual colonies were purified by repeated streaking on agar basal medium containing 1% (v/v) of refinery contaminated wastewater. All isolates were stored at −20 °C in liquid cultures containing 15% glycerol (v/v).

The diversity of isolates on each agar plate was determined on the basis of colony morphology using a dissecting microscope. Preliminary identification of individual bacterial isolates was obtained by classical tests as outlined by Cowan and Steele's Manual for the Identification of Medical Bacteria [25] and Bergey's Manuals on Systematic Bacteriology [26]. Such identification includes the shape of cells, Gram-strain, the presence of spores, and colony morphology on solid MM plates.

Additional biochemical tests including catalase reaction, oxidase reaction, acid or gas production from carbohydrates and oxidation or fermentation from carbohydrates was determined by using the API 20NE and API 20E system according to the manufacturer's instructions (BioMerieux, France).

2.6. Characterization of the crude oil degradation potential

Degradation capacity for all isolated strains was evaluated in culture medium containing crude oil compound. The preliminary biodegradation assays were performed as described by Mandri and Lin [27] with modifications. Each single colony of the isolates was inoculated into 10 ml mineral medium at 30 °C overnight. The overnight culture was centrifuged for 15 min at 3500 rpm. The cell pellet was washed twice and was re-suspended with mineral medium until OD_{600} equivalent to 0.5. 1 ml of bacterial inoculum (0.5 OD_{600} equivalents) was transferred into 100 ml mineral medium with (1%) crude oil and was incubated at 30 °C at 170 rpm for 2 weeks. All experiments were performed in duplicate. Non-inoculated flasks were incubated at the same conditions and used as control to disregard abiotic hydrocarbon losses. All results of biodegradation were obtained with reference to sterile controls. The degradation of crude oil was studied by measuring OD at 600 nm, according to Barathi and Vasudevan [28]. Growth was then evaluated by comparison of optical density (OD_{600}) against sterile control.

2.7. 16S rRNA sequencing

The chromosomal DNA of the bacterial strains was isolated by the hexadecyltrimethyl ammonium bromide (CATB) method [29]. Bacterial 16S rRNA gene fragments were amplified by PCR using the primers fD1 (AGAGTTTGATCCTGGCTCAG) and rD1 (AAGGAGGTGATCCAGCC) [30]. PCR was performed in a total volume of 50 μl reaction mixture containing approximately 10 ng of genomic DNA, 5X GoTaq reaction buffer, 0.2 mM (concentration of each deoxynucleoside triphosphate), 2 mM of each primer (fD1 and rD1) and 1.25 units of GoTaq DNA polymerase (Promega). Thermal cycling consisted of an initial denaturation of 2 min at 94 °C, followed by 30 cycles of 1 min at 94 °C for, 1 min at 55 °C, 2 min at 72 °C, and a final extension of 10 min at 72 °C. The PCR obtained products were purified with illustra GFX PCR DNA and gel band purification kit (Amersham Biosciences, GE Healthcare) according to the manufacturer's protocol. Partial 16S rRNA gene sequences were performed using a BigDye® Terminator v3.1 Cycle Sequencing kit on the ABI PRISM 3100-Avant Genetic Analyser (Applied Biosystems) and the sequencing primers fD1 and rD1. All 16S DNA sequences of the strains were compared to all bacterial sequences available in the Gene Bank database using the BLAST program [31].

2.8. GC–MS analysis

Gas chromatography–mass spectrometry (GC–MS) analysis was monitored in order to evaluate the potential biodegradation of total hydrocarbons in the wastewater. Before each analysis, samples of the mixture were extracted with dichloromethane by adding 20 ml of solvent and 20 ml of each sample into a 125 ml separation funnel three times (v/v). After separation phase, a sample of the aqueous phase was removed and put in a sealed flask for sub-

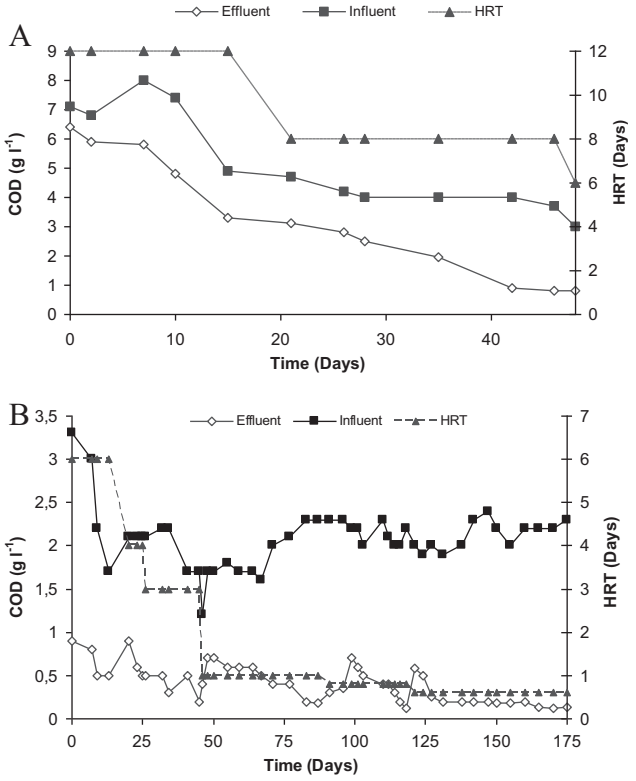

Fig. 3. Variation in filtered COD of the feed material (■) and at the outlet (◊), during the tests with effluents. Acclimatizing process (A); continuous treatment in the CSTR (B).

sequent analysis. The aqueous phase was concentrated to 1 ml by rotary evaporation, dissolved in equal volume of dichloromethane and further cleaned through a column filled with florisil and then analysed by gas chromatography–mass spectrometry. After evaporation of the solvent, the amount of residual TPH was determined gravimetrically [32].

GC–MS analysis was performed with a HP model 5975B inert MSD, equipped with a capillary DB-5MS column (30 m length; 0.25 mm i.d.; 0.25 μm film thickness (Agilent Technologies, J&W Scientific Products, U.S.A.). The carrier gas was the helium used at a 1 ml min^{-1} flow rate. The oven temperature program was as follows: 2 min at 70 °C, ramped from 70 to 230 °C at 20 °C min^{-1}, then from 230 °C to 300 °C at 40 °C min^{-1} and 10 min at 300 °C. The chromatograph was equipped with a split/split less injector used in the split mode. The split ratio was 100:1. Chromatogram peaks were identified by comparing their mass spectra with Wiley and NIST library database and standards of the main components and quantified using the retention time and response factors of these compounds, correlating chromatographic areas to molar concentrations.

3. Results and discussion

3.1. Bioreactor functional performance

The CSTR bioreactor was run over a total period of 225 days under the operating conditions. As indicated, the operational treatment was divided into two stages according to the dilution rate and the type of influent fed into the bioreactor. The purpose of these stages was to evaluate the functional performance of the bioreactor in terms of hydrocarbon degradation, when the microbial culture was baseline operating (A), and the addition of wastewater as a sole source of carbon (B). Fig. 3 shows the evolution of the operational performance of the effluent wastewater after treatment in the bioreactor.

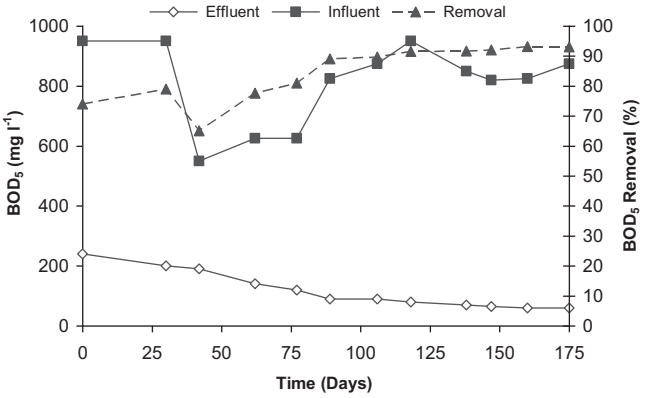

Fig. 4. Evolution of the BOD_5 in the feed (■), at the outlet (◊), and BOD_5 removal (▲) as a function of time during biodegradation wastewater treatment in the CSTR.

3.1.1. Acclimatization phase: start-up

During the start-up period, the CSTR was firstly fed with low feeding flow of 1 l d^{-1} and 1.5 l d^{-1} during 20 and 27 days, respectively. These feeding flows corresponded to hydraulic retention times (HRTs) of 12 and 8 days, respectively.

During the first few days of the start-up, the average of $COD_{effluent}$ of hydrocarbon-contaminated wastewater was 6.4 g l^{-1} this value decreased to 1.95 g l^{-1} on day 35 (Fig. 3A). The $COD_{effluent}$ values remained below 0.8 g l^{-1} during the rest of the start-up period. Thus, it can be concluded that, the aerobic consortium was acclimatized to use the contaminated wastewater as carbon source in the experiment conditions. Indeed, start-up is the period during which the microorganisms are progressively acclimatized to new environmental conditions and substrate. This relatively long period of adaptation and stabilization of the bioreactor was necessary because of the variability of the hydrocarbon-wastewater refinery composition.

After the start-up period, the OLR introduced into the bioreactor was increased in order to study its influence on process efficiency.

3.1.2. Performance of the CSTR treatment

In the second phase, after an acclimatization period of 48 days, the feeding flow was progressively increased from 2 to 4, 8, 12, 15 and 18 l d^{-1}, corresponding to a HRT decrease from 12 days to 16 h. Because of the variation of carbonaceous pollution manifested by fluctuations in $COD_{influent}$ values, the parameters of the effluent were characterized by significant fluctuations.

In order to study the influence of OLR on bioprocess efficiency, ORL values between 0.36 and 3.45 g COD l^{-1} d^{-1} were applied. Mainly, at the beginning of each phase, when the OLR increased, there was a decrease in the removal efficiency but the system recovered shortly and adapted to the new conditions with time.

Initial organic matter in the second phase is represented by average $COD_{effluent}$ and $BOD_{5effluent}$ concentrations which were 900 mg l^{-1} and 240 mg $l,^{-1}$ respectively corresponding to an ORL about 0.36 g COD l^{-1} d^{-1} at a HRT of 6 days. Average residual $COD_{effluent}$ and $BOD_{5effluent}$ concentrations at a HRT of 16 h were 130 and 60 mg l^{-1}, respectively. These values were under the required Tunisian standards for the reject in hydraulic public domain [33] and for wastewater reuse in irrigation [34]. Indeed, after continuous treatment in the CSTR, the $COD_{effluent}$ and $BOD_{5effluent}$ average removals were high reaching 96% and 93%, respectively (Fig. 3B and 4).

As seen in Fig. 5, the increase of biomass concentration in the CSTR was observed during this period. During the first 40 days,

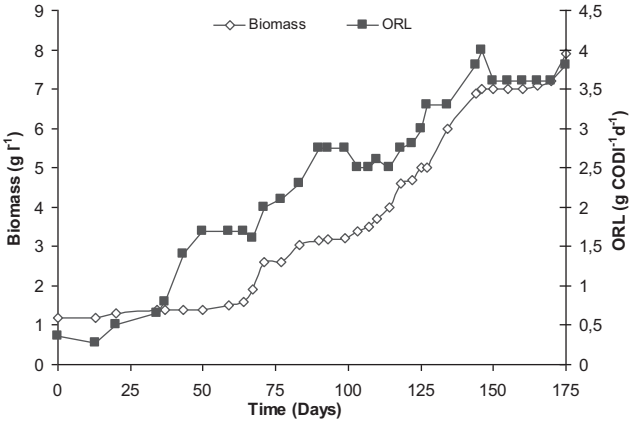

Fig. 5. The biomass in the CSTR (◊) and organic load evolutions (■) during the biodegradation process.

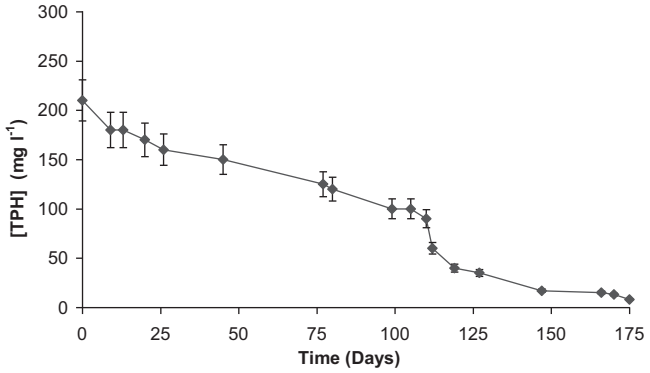

Fig. 7. Evolution of total petroleum hydrocarbon (TPH) degradation profile of mixed culture in microcosm experiments. Error bars indicate the SD of the mean of three replicates.

when the biomass was recycled after decantation, the biomass concentration increased slightly and reached 1.4 g VSS l^{-1}. Then, it increased with time and reached 7.9 g l^{-1} in terms of VSS, at the end of the treatment.

It has been stated that in the presence of excess of nutrients and oxygen, the pH values of a wastewater under microbial degradation would drop [35]. In our study, the initial pH value of the industrial wastewater was 7.3 and varied between 7.5 and 7.9 by the end of the half period and between 7.5 and 7.2 by the end of the bioremediation treatment (Fig. 6). These results showed that the pH was stabilized during the remediation period. This implied that the nutrient and the oxygen levels in the wastewater were not in excess. The final pH range (i.e. 7.3) did not fall out of the range 7–8, which is known to be the optimal range for degrading micro-flora [36,37].

3.2. Identification and quantification of hydrocarbon compounds

The study of the biodegradability of petroleum hydrocarbons contaminated wastewater was carried out in an enlarged scale, using the microbial culture. Total petroleum hydrocarbon (TPH) values estimate the amount of petroleum hydrocarbon waste coming into the system. This was one of the important measured parameters to provide the removal efficiency of the treatment process. At the end of the treatment process, the system showed overall hydrocarbon waste removal efficiency (about 94%, Fig. 7). Biodegradation behaviour suggests a preferential consumption of the more easily degradable compounds during the first 20 days. After 45 days, there was an increase in the rate at which TPH concentration was reduced from 150 to 8 mg l^{-1} suggesting a change in either specific enzymatic systems or a change in the efficiency of the applied system. Results of biodegradation experiment indicate that biodegradation rate was accelerated during the treatment and the TPH load was decreased efficiently. This high removal efficiency suggests that the microbial consortium from the bioreactor had already been exposed to contaminants Okerentugba and Ezeronye stated that microbiological communities exposed to hydrocarbons adapt to the exposure through selective enrichment and genetic changes, resulting in an increase in the ratio of hydrocarbon-degrading versus non degrading bacteria [38]. To degrade hydrocarbons, it is advantageous to use native microorganisms cultured from areas with historical contamination. This approach is likely to reduce or eliminate the initial lag phase and optimize overall process time.

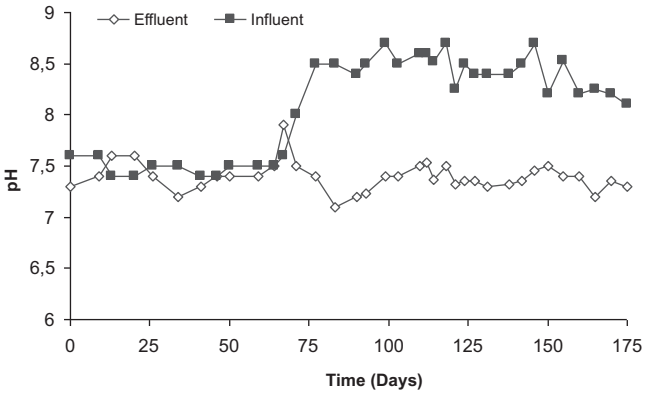

Fig. 6. Evolution of pH during bacterial growth and biodegradation.

3.3. Biodegradability of n-alkanes

Gas chromatography/mass spectrometry analysis was performed to identify the presence of the heavier petroleum TNA (total n-alkanes) compounds in the influent and effluent streams of the system. The identification of individual hydrocarbon compounds was conducted on the initial sample (time = 0 day) and a treated sample (after 225 days of total continuous feeding) using a gas chromatography mass spectrometry apparatus (GC–MS) (Fig. 8). Influent produced water typically consists mainly of n-alkanes C10 through C35 (>70%), with intermediate branched chain hydrocarbons and other petroleum-based compounds. As can be seen in Fig. 8, from comparing the beginning and the end of the treatment (T = 0 day and T = 225 days), the GC–MS abundance and area values of most substances in the effluent decreased greatly. This means that a significant enhancement of the effluent quality is achieved, confirming the ability of the system to decompose the organic compounds in the contaminated wastewater. In addition, GC/MS analysis showed that petroleum hydrocarbons were almost completely removed by biodegradation after 225 days of a total continuous feeding (Fig. 8). These results prove the further performance of the microbial consortium in degrading petroleum hydrocarbon and the good degradation ability for a wide range of n-alkanes. Such consortium could be used for bioremediation and for wastewater treatment in bioreactors.

3.4. Crude oil biodegradability potential of the isolated strains

Growth of strains HC2, HC5, HC6, HC7, HC8, and HC9 on enriched liquid medium was tested (Fig. 9). The growth of the different strains on crude oil was followed by measuring the OD_{600} nm at culture's time. The majority of isolated strains from enrichment

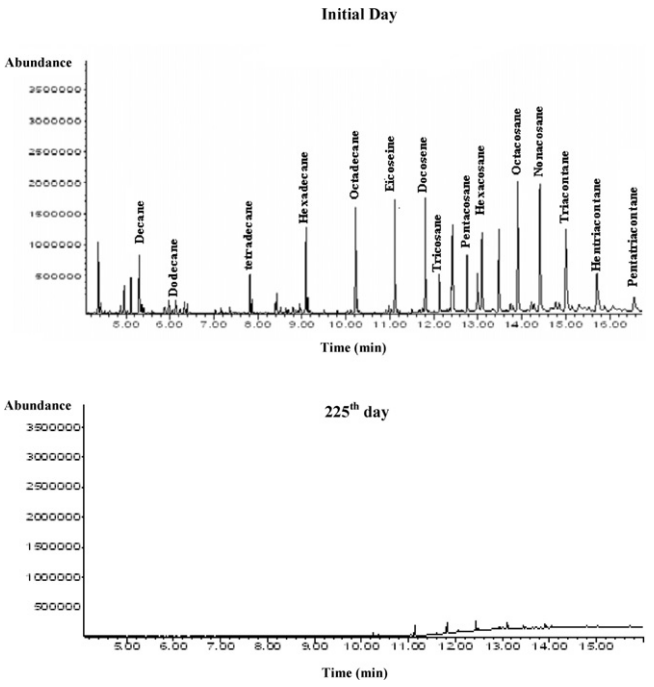

Fig. 8. GC–MS profiles of the hydrocarbon-contaminated wastewater effluent on bioreactor on day 0 and indigenous wastewater population after 225 days of continuous treatment.

process on CSTR were able to degrade crude oil (1%, v/v) in liquid mineral medium and solid media as sole carbon and energy source.

Enrichment culture with crude oil was the successful method in terms of abundance and diversity of culturable bacteria. Crude oil is a medium distillate of petroleum containing: n-alkanes, branched alkanes, olefins and small concentrations of aromatic polycyclic compounds.

3.5. Cress seed germination test

The germination index of *L. sativum* (GI) is commonly used as a phytotoxic assay for treated and untreated wastewaters as well as for soil. The choice of this plant is due to its high sensitivity to organics as well as mineral pollutants [39]. The seed germination test was used to evaluate changes in the phytotoxicity of the industrial wastewater during the treatment process. The GI of five samples of industrial refinery wastewaters taken at different times

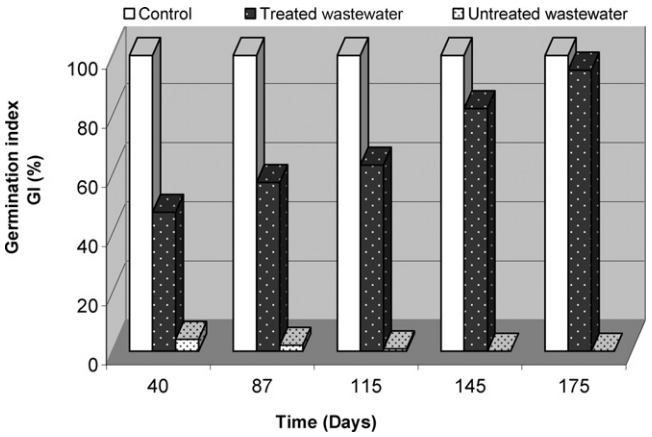

Fig. 10. Germination index (GI) of treated and untreated samples of refinery wastewater contaminated by hydrocarbons.

ranged between 0 and 4% revealing a strong phytotoxic character of the effluent (Fig. 10). During treatment by bioremediation process, a significant phytotoxicity removal was achieved, explaining the increase efficiency of the GI up to 47%, 57%, 63%, 82% and 95%, proportionally to the evolution of the treatment for the tested samples of treated hydrocarbon contaminated wastewaters.

3.6. Decantability of the activated sludge

The decantability of the activated sludge was evaluated by the measurement of the Mohlman index (Im). The evolution of this parameter is given in Fig. 11. The average values of this parameter were included in an interval of 50–100 ml g^{-1}, characteristic of a sludge having excellent properties of decantability.

3.7. Isolation and identification of petroleum-degrading bacteria from enrichment process

Six aerobic bacteria were isolated from petroleum hydrocarbon-contaminated water using industrial wastewater as a sole carbon source. The isolated bacterial cultures were characterized by their morphological and biochemical properties (Table 2). According to the data obtained using light and electron microscopy, the isolated bacteria had the form of rods or cocci, were spore-forming or non-spore-forming, occurred as single cells or were integrated in chains, and were immotile or motile with flagella. Two strains are Gram-positive and four strains are Gram-negative.

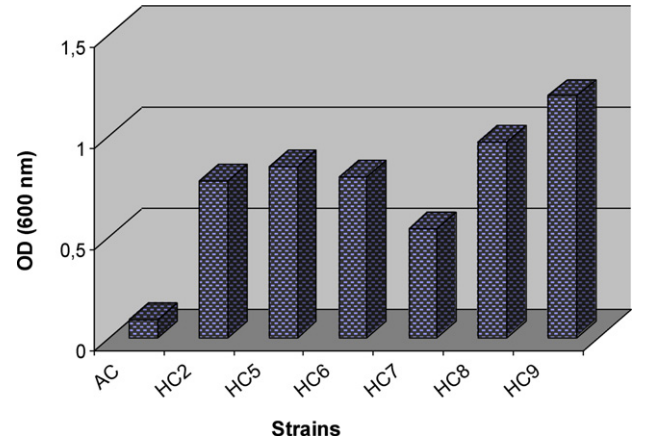

Fig. 9. Biodegradation of crude oil by acclimatized isolated strains. Cells were cultivated in mineral medium supplemented with (1%) crude oil. AC: abiotic control.

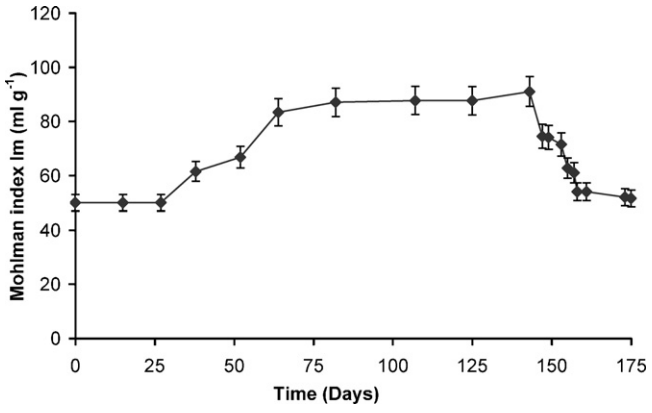

Fig. 11. Variations of the Mohlman index (Im). (Error bars indicate the SD of the mean of three replicates.)

Table 2
Identification of bacteria isolates on the basis of biochemical tests and 16S rDNA sequencing.

Strains	Relevant characteristics[a]	Identification[b]	% Similarity
HC2	Gram (−), Catalase (+), motile, O^+/F^+, Rhodamine (+)	*Aeromonas punctata*	99
HC5	Gram (+), Catalase (+), unmotile, O^-/F^-, Sporulente, Rhodamine (−)	*Bacillus cereus*	99
HC6	Gram (−), Catalase (+), motile, O^-/F^-, Rhodamine (−)	*Ochrobactrum intermedium*	99
HC7	Gram (−), Catalase (+), motile, O^+/F^-, Rhodamine (−)	*Achromobacter* spp.	99
HC8	Gram (−), Catalase (+), motile, O^+/F^-, Rhodamine (−)	*Stenotrophomonas maltophilia*	99
HC9	Gram (+), Catalase (+), motile, O^+/F^-, Rhodamine (+)	*Rodococcus* sp.	99

O/F: oxidative/fermentative.
[a] As determined by biochemical tests (API).
[b] As confirmed by 16S rDNA sequencing.

Species identification was performed using biochemical test (API) and confirmed by sequencing the 16S rRNA genes of the six isolates (Table 2). The partial 16S rRNA gene sequences of HC2, HC5, HC6, HC7, HC8 and HC9 strains were determined (data not shown). The sequence comparison demonstrated the affiliation of most strains (HC2, 6, 7 and 8) to Proteobacteria phylum. HC2, HC5, HC6, HC8 and HC9 strains were closely related to *Aeromonas punctata* (*Aeromonas caviae*), *Bacillus cereus*, *Ochrobactrum intermedium*, *Stenotrophomonas maltophilia* and *Rhodococcus* sp., respectively. HC7 isolate was affiliated to genera *Achromobacter* with 99% similarity. Bacteria of the genera *Aeromonas*, *Achromobacter*, *Rhodococcus, Stenotrophomonas*, *Ochrobactrum* and *Bacillus*, isolated in this study, belonged to bacteria, playing an important role in the degradation of petroleum hydrocarbons in the environment, as previously reported [2,40–45].

The most successful process for the removal or the elimination of hydrocarbon from the environment is the microbial transformation and biodegradation. The rate of biodegradation of petroleum hydrocarbon in the environment depends on different factors such as pH, temperature, oxygen, microbial population and chemical structure of the compounds [2,40]. Many studies are focused on the isolation and characterisation of microorganisms degrading hydrocarbon components. Numerous microorganisms, namely bacteria, yeast and fungi have been reported as good degraders of hydrocarbons [2,40–42,46]. Many of these microorganisms were applied in bioremediation processes to reduce the concentration and the toxicity of various pollutants, including petroleum products [24,47,48].

4. Conclusion

This study proposes the application of an aerobic CSTR for the treatment of hydrocarbon-contaminated wastewater. From results, it was observed that, during the treatment process, the degradation of hydrocarbons was enhanced, implying that the aerobic treatment is an effective bioremediation technology. These encouraging results are mainly due to the development of an efficient microbial consortium and to the optimization of specific hydrodynamic conditions of the bioreactor. Laboratory scale experiments are very useful in determining optimal operating conditions in the CSTR.

In summary, biodegradation has an edge over other treatment methods because it can efficiently destroy the present hydrocarbons pollutants and does not allow the contaminant to accumulate [49]. For this reason, our strategy was developed to obtain highly efficient acclimatized consortium defined as inoculants. Therefore, isolated strains could be useful for their application in bioremediation of hydrocarbon-contaminated wastewater technologies. This approach could represent an interesting alternative with high potential benefits. However, further experiments are still needed to evaluate its performance *in situ*, as factors affecting the ability of the microorganisms to degrade hydrocarbons in natural environments.

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