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ISOLATION AND CHARACTERIZATION OF A NOVEL STRAIN, *Bacillus sp* KJ629314, WITH A HIGH POTENTIAL TO AEROBICALLY DEGRADE DIESEL

AISLAMIENTO Y CARACTERIZACIÓN DE UNA NUEVA CEPA, *Bacillus sp* KJ629314, CON UN ALTO POTENCIAL EN LA DEGRADACIÓN AERÓBICA DE DIÉSEL

S. Cisneros-de La Cueva, M.A. Martínez-Prado*, J.A. Rojas-Contreras, H. Medrano-Roldán, and M.A. Murillo-Martínez

Technological Institute of Durango, Felipe Pescador Blvd. # 1830 East. Nueva Vizcaya, Zip Code 34080, Durango, Dgo., Mexico.

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Abstract

In this research, a diesel-degrading bacterium (strain KJ629314) was isolated from a mining soil contaminated with total petroleum hydrocarbons (TPH) and properly characterized using the polymerase chain reaction (PCR) molecular technique. The 16s rDNA sequence analysis allowed to identify KJ629314 as a strain of *Bacillus sp*. Experimental phase was conducted to assess the aerobic biodegradation of diesel; to determine the removal efficiency and the corresponding microbial growth; diesel was used as a substrate - electron donor - carbon source; and oxygen (via aeration) as the electron acceptor. Tests were conducted in microcosms with sterile sand with nutrients according to the Nitrogen:Phosphorus ratio of 15:1 at different diesel concentrations (10,000; 20,000; 30,000; 40,000 and 50,000 mg/kg soil). Results showed that the strain of *Bacillus sp* KJ629314 has a high potential in the biodegradation of diesel at the evaluated concentrations, and it was demonstrated that the removal efficiency was greater at low concentrations of diesel obtaining higher values for the microbial growth and diesel biodegradation rate constants. These promising results support the fact that *Bacillus sp* KJ629314 may be used as a novel biological resource to develop a bioprocess for the bioremediation of diesel-contaminated soil.

Keywords: aerobic degradation, *Bacillus sp* KJ629314, bioremediation, contaminated soils, mining industry, TPH.

Resumen

En esta investigación, una bacteria degradadora de diésel (cepa KJ629314) fue aislada de un suelo minero contaminado con hidrocarburos totales de petróleo (HTP) y debidamente caracterizada utilizando la técnica molecular de la reacción en cadena de la polimerasa (RCP). El análisis de la secuencia de 16s rDNA permitió identificar KJ629314 como una cepa de *Bacillus sp*. La fase experimental se llevó a cabo para evaluar la biodegradación aeróbica del diésel; para determinar la eficiencia de remoción y el crecimiento microbiano correspondiente; el diésel se utilizó como sustrato - donador de electrones - fuente de carbono, y el oxígeno (a través de la aireación) como aceptor de electrones. Las pruebas se realizaron en microcosmos empleando arena estéril con nutrientes de acuerdo con la relación Nitrógeno:Fósforo de 15:1 a diferentes concentraciones de diésel (10,000; 20,000; 30,000; 40,000 y 50,000 mg/kg de suelo). Los resultados mostraron que la cepa de *Bacillus sp* KJ629314 tiene un alto potencial en la biodegradación del diésel en las concentraciones evaluadas, y se demostró que la eficiencia de degradación fue mayor a bajas concentraciones de diésel; obteniéndose valores más altos para el crecimiento microbiano así como para las constantes de velocidad de degradación. Estos prometedores resultados apoyan el hecho de que el *Bacillus sp* KJ629314 puede ser utilizado como un recurso biológico novedoso para desarrollar un bioproceso para la biorremediación de suelos contaminados con diésel.

Palabras clave: *Bacillus sp* KJ629314, biorremediación, degradación aeróbica, HTP, industria minera, suelos contaminados.

*Corresponding author. *E-mail*: adriana.martinez@orst.edu

1 Introduction

Release of persistent, bioaccumulative and toxic chemicals has a detrimental impact on human health and the environment. These contaminants find their way into the tissues of plants, animals and human beings by the movement of hazardous constituents in the environment. Contamination of soils generally results from past industrial activities when awareness of the health and environmental effects connected with the production, use, and disposal of hazardous substances did not exist (Vidali, 2001).

Diesel oil contains 2,000 to 4,000 hydrocarbons, a complex mixture of linear, branched and cyclic alkanes and aromatic compounds obtained from the middle distillate fraction during petroleum separation (Gallego *et al*., 2001). Soil pollution by total petroleum hydrocarbons (TPH) is very common and in most of the cases contamination occurs in industries by accidental spills during machinery and vehicles maintenance. Mining industry is not the exception; as for this research Goldcorp Mexico faces this problem; bioremediation with indigenous bacteria is believed to be a flexible and low cost method to achieve levels established by Mexican regulations (SEMARNAT, 2003).

Remediation of contaminated soil has been considered as an important environmental topic and it is studied worldwide. Among the different remediation techniques, bioremediation has been proven to have the most effective approach to alleviate the environmental problems associated with contaminated soil (Abbassi and Shquirat, 2008; Bento *et al*., 2005). Bioremediation involves the uses of indigenous or introduced autochthonous microorganisms to detoxify and degrade environmental contaminants. The majority of petroleum hydrocarbons can be biodegraded by several microbial strains; however, each one is capable of breaking down a specific group of molecules; biodegradation potential generally decreases by moving from n-alkanes to branched alkanes, low molecular weight n-alkyl aromatics, monoaromatics, cyclic alkanes and polynuclear aromatics (Leahy and Colwell, 1990; Huesemann, 1995; Van Hamme *et al*., 2003). However, the mineralization of complex hydrocarbon mixtures such as diesel usually requires the co-existence and effective cooperation of several specialized microorganisms with complementary substrate specificity (Richard and Vogel, 1999; Alexander, 1999).

Microbial consortia with such physiological and metabolic features might not exist in a soil, particularly if this was impacted with a spill by diesel and this is often the main cause of poor biotreatability of diesel fuel contaminated sites. In such cases, the inoculation of microorganisms characterized on contaminated soil with petroleum hydrocarbons (bioaugmentation) is one of the most promising options for getting its sustainable remediation.

Bioremediation is a biological technique that takes advantage of living organisms, free enzymes, and cellular components to initiate or accelerate the rate of natural degradation of organic compounds under different conditions and in different sites such as agricultural and industrial sludges and soils and mangrove soil (García Frutos *et al*., 2012, Megharaj *et al*., 2011, Martínez-Prado *et al*., 2011a; Martínez-Prado *et al*., 2014; Ruiz-Marín *et al*., 2013; Wan *et al*., 2002).

The major microorganisms responsible for biodegradation of petroleum hydrocarbons have been found to be bacteria and fungi (Zanaroli *et al*., 2010; Atlas and Cerniglia, 1995; Alexander, 1999). The genera to which hydrocarbon degrading bacteria belong are *Pseudomonas*, *Alcaligenes*, *Micrococcus*, *Nocardia*, *Corynobacterium*, *Rhodococcus*, *Enterobacter*, *Eschrechia*, *Arthrobacter*, *Bacillus*, *Streptomyces*, *Clostridium*, and *Proteus* (Strauss *et al*., 2000; Marchal *et al*., 2003; Ganesh and Lin, 2009; Hong *et al*., 2005; Kebria *et al*., 2009; Shukor *et al*., 2009; Ahari *et al*., 2012; Nisha *et al*., 2013; Panda *et al*., 2013). *Bacillus* strains have also been implicated in crude oil degradation; similarly, Bossert and Bartha (1984) stated that although *Bacillus* strains have been isolated from oil polluted soil, this is probably due to their persistence in soil and subsequent spore germination during enrichment and isolation procedures.

Few research works have been dedicated to investigate the kinetics of soil bioremediation in the use of the specialized portion of microflora of such sources; it would provide higher and more reproducible pollutant mineralization rates and extents with respect to those achievable with pure or tailored mixed cultures of specialized microorganisms (Alexander, 1999; Ghazali *et al*., 2004; Viñas *et al*., 2002). Information on kinetics is extremely important because it characterizes the concentration of the chemical remaining at any time and allows prediction of the levels likely to be present at some future time (Martínez-Prado and Williamson, 2011).

This investigation was conducted to determine the aerobic biodegradation capability of *Bacillus sp* KJ629314, isolated from a TPH mining contaminated soil; exposed to a wide range concentration of diesel, as a sole carbon source and electron donor under aerobic conditions.

2 Materials and methods

2.1 Diesel

Diesel was supplied by PEMEX (Mexican Petroleum Corporation) and was used as target compound for the degradation experiments. The diesel oil consisted of alkanes (42.7%), cycloalkanes (33.4%) and aromatics (23.9%) as described in the material and safety data sheet (MSDS) provided by PEMEX.

2.2 Soil

Sand was collected and sterilized in an oven at 200°C for 48 h to remove all microorganisms present, organic matter was quantified to make sure that the soil contains no (or negligible) organic content.

2.3 Isolation of bacteria

Bacteria were isolated from petroleum hydrocarbon contaminated soil of a mining site in San Dimas Tayoltita, Durango. Soil dilution technique was done by the pour plate method; with Agar-Agar media and mineral salt solution (Makula and Finnerty, 1972) containing $(NH_4)_2SO_4$ (2 g/L), KH_2PO_4 (4 g/L), Na_2HPO_4 (6 g/L), $CaCl{\cdot}2H_2O$ (0.05 g/L), $MgSO_4{\cdot}7H_2O$ (0.2 g/L), $FeSO_4{\cdot}7H_2O$ (0.01 g/L). An aliquot of 0.2 mL of diesel was added to the inner surface of the Petri dishes covered with sterile filter disk according to Ridgway *et al*. (1990) and Vecchioli *et al*. (1990). Plates were incubated at 35ºC for 72 h and examined at intervals of 12 h for colony formation.

2.4 Bacteria identification

2.4.1 Extraction of DNA

Genomic DNA was extracted from the pellets resulting from centrifugation of 2.0 mL aliquot of active mixed and pure bacterial cultures using Hoffman and Winston (1987) method.

2.4.2 Polymerase chain reaction (PCR)

The 16S rRNA gene was amplified using primers NVZ-1 (5'-GCG GAT CCG CGG CCG CTG CAG AGT TTG ATC CTG GCT CAG-3') and NVZ-2 (5'-GGC TCG AGC GGC CGC CCG GGT TAC CTT GTT ACG ACT T-3') (Relman, 1993). The PCR reaction mixture (25 μL) contained: 0.25 μL Taq DNA polymerase of 5 units/μL (PROMEGA Bio, EUA), 1 μL Deoxynucleoside triphosphate (10 mM), 1 μL $MgCl_2$ (25 mM), 0.5 μL of each primer, 5 μL Buffer (5X), 0.5 μL of DNA template (38.7 ng/μL) and 16.25 μL of nuclease free water. Reaction conditions were as follows: initial DNA denaturation at 95°C for 1 min followed by 10 cycles of denaturation at 95°C for 30 s and annealing from 65 to 60°C for 30 s, lowering temperature 0.5°C each cycle, and followed by an extension at 72°C for 1 min. In addition, 20 cycles at 95°C for 30 s, 60°C for 30 s and 72°C for 1 min; with a final extension at 72°C for 7 min were performed with a T-gradient Thermo cycler (Techne TC-5000). Finally, the PCR product was purified using ZR DNA sequencing Clean-up Kit by Zymo Research (ZR).

2.4.3 Phylogenetic analysis

Sequences were analyzed with the MEGA software version 5.1 and compared to the database of the National Center for Biotechnology Information (NCBI) using Blast on sequences 16S rRNA with a higher percentage of similarity of 98%, the phylogenetic tree construction was performed using the Neighbor Joining method with 100 replicates (Salemi and Vandamme, 2003).

2.5 Experimental phase

2.5.1 Microcosm experiments

Biodegradation tests were carried out in 250 mL rubber sealed glass flask containing 160 g of sterilized sand; mixed thoroughly with specific amounts to reach diesel concentrations (10,000; 20,000; 30,000; 40,000 and 50000; mg/kg soil or parts per million), the addition of mineral salt $(NH_4)_2SO_4$ and KH_2PO_4 adjusted to (N:P ratio of 15:1), and bacterial inoculum was added to microcosm in a concentration of 10^7 cells/g soil, approximately. Water content of soil was adjusted to 70% of the field capacity, since it is the ideal for microbial activities. All glass flasks were incubated at 30°C, in the dark for 90 days, with an air flow of 6.27 cm^3/h to guarantee aerobic conditions (USEPA, 2003).

2.5.2 Abiotic hydrocarbon losses

Control bottles were prepared with sterile sand and no addition of bacterial inoculum, mixed thoroughly with diesel oil at a concentration of 10,000 mg/kg dry soil (or parts per million, ppm) with mineral salt solution. Operating conditions used were the same as in previous microcosm experiments described.

2.5.3 Diesel quantification

Concentration of TPH in the artificially contaminated soil samples were determined by soxhlet extraction using 10 g of soil with hexane and acetone ratio of 1:1 (100 mL each). All three extracted portions were pooled and dried at room temperature by evaporation of solvent in a fume hood. After solvent evaporation, the amount of residual TPH was determined gravimetrically (USEPA, 2003).

2.5.4 Microbial monitoring

Microbial monitoring was performed at 0, 5, 10, 15, 30, 45, and 60 days. To monitor cell count 1 g of soil was removed from each microcosm at set times and resuspended in 9 mL of sterile saline solution in sterile centrifuge tubes. The mixture was vigorously shaken on a vortex mixer for 5 min and then the soil particulates were allowed to settle for 1 min before 0.1 mL of fluids were sampled for colony forming unit (CFU) counts. The population of strain isolated used for seeding purposes was calculated using Eq. 1 (Farinazleen *et al*., 2004).

$$CFU = \frac{\text{number of colonies x dilution factor}}{\text{volume of inoculum used}} \quad (1)$$

2.6 *Analysis of results*

The experimental design was a single factor with five levels and two replicates having as response variable the concentration of TPH. Statistica 7® software was used to validate the differences between treatments, using one-way ANOVA by Fisher LSD (Least Significant Differences) test at an alpha (α) value of 0.05%.

3 Results and discussion

3.1 *Long chain hydrocarbons degrading bacteria: Isolation and identification*

The genus of microorganism was identified from the isolates of mining soil contaminated with TPH, using diesel as a sole carbon source/electron donor and oxygen as electron acceptor in agar-agar. Results showed that the microorganism belong to *Bacilli* genera. Fig. 1 shows phylogenetic analysis of *Bacillus sp* KJ629314 by BLASTN using the variable region of 16S rRNA and registered in The Gen Bank at the National Center for Biotechnology Information (NCBI).

According to Fig. 1 the strain isolated shared a 16S rRNA gene similarity of 100% corresponding to several strains of the genus Bacillus: *Bacillus thuringiensis*, *Bacillus cereus*, and *Bacillus amyloliquefaciens*; for this reason it was identified as *Bacillus sp* KJ629314. Main colony forms were identified as *Bacillus sp*, which is comprised within the genus Bacillus Gram positive bacteria with low levels of G+C (Kingdom Bacteria; Phylum Firmicutes; Class Bacilli; Order Bacillales; Family Bacillaceae) and it is most closely related to the genera *Listeria*, *Streptococcus*, and *Staphylococcus*. Fig. 2 shows the microscopic observation of Gram stain test to confirm the morphology of the bacteria. *Bacillus* species are ubiquitous in nature, having been isolated from environments as diverse as freshwater, saline water, soil, plants, animals, and air. The phenotypic diversity encompassed by members of the *Bacillus* genus is spectacular: high temperatures, extreme salinity, acidic conditions and the immune systems of many animals pose little challenge to some members (Maughana and Van der Auwera, 2011). Drobniewski (1993) reported three broad groups for the genus *Bacillus* based on the morphology of the spore and sporangium. In group 1 are classified the large-cell subgroup (bacillary cell width ≥1 μm) comprised by *Bacillus cereus*, *Bacillus anthracis*, *Bacillus megatenium*, *Bacillus thuringiensis*, and *Bacillus cereus var. mycoides*; which are gram-positive rods that produce central or terminal ellipsoid or cylindrical spores that do not distend the sporangia; and where protoplasmic inclusions of poly-beta-hydroxybutyrate are found in these large-celled species. On the other hand *Bacillus subtilis*, *Bacillus pumilus*, and *Bacillus licheniformis* form a separate small-celled subgroup.

Several species of the genus *Bacillus* has been reported to aerobically degrade a broad type of hydrocarbon compounds. Toledo *et al*., (2006) reported *Bacillus pumilus* and *Bacillus subtilis* as able to grow and efficiently utilize polycyclic aromatic hydrocarbons (PAH) such as naphthalene, phenanthrene, fluoranthene, and pyrene. Previous researchers also reported capability of *Bacillus cereus* to degrade pyrene (Kazuga and Aitken, 2000).

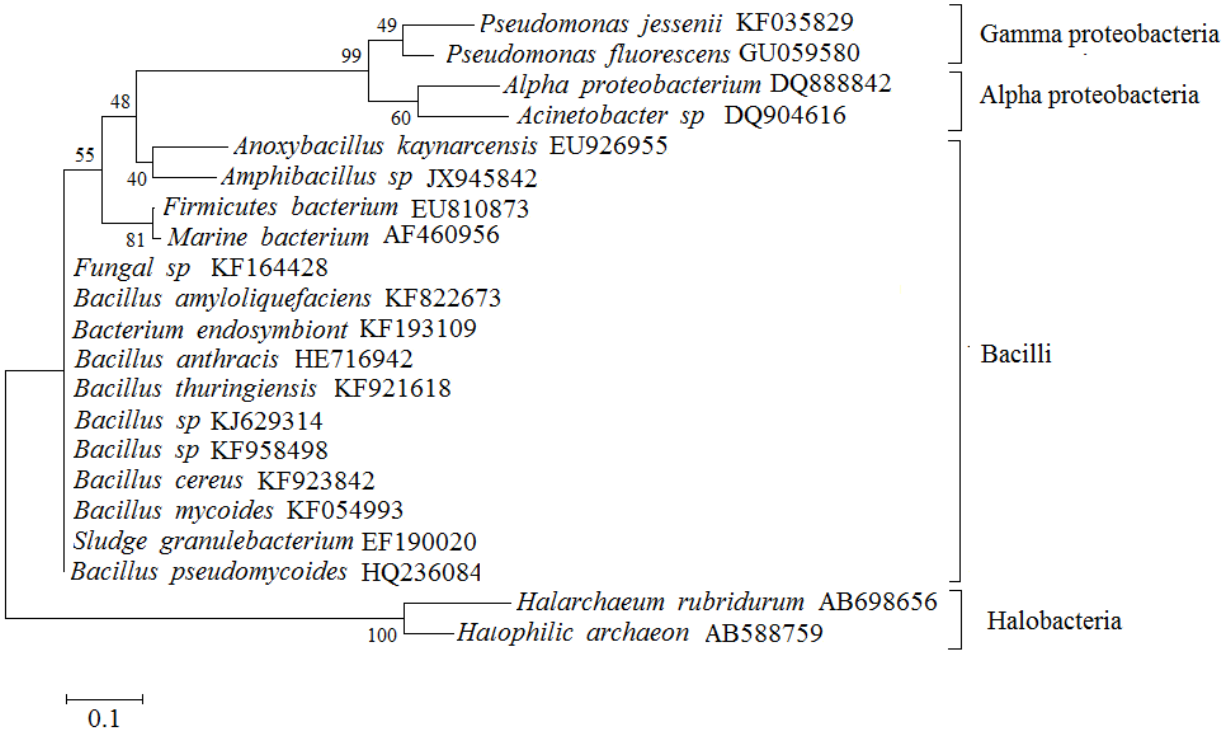

Fig. 1. Phylogenetic tree based on 16S rRNA sequences showing the positions of the strain KJ629314, *Bacillus* species and representatives of other species using Neighbor Joining method.

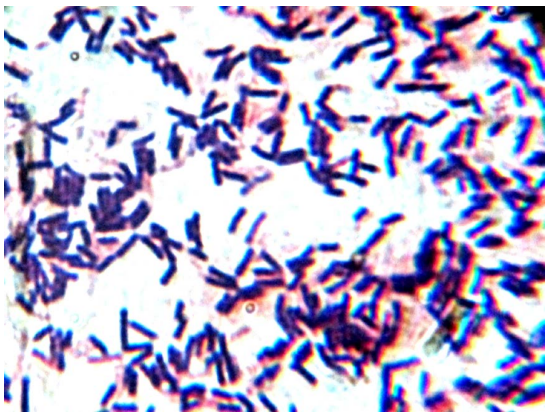

Fig. 2. Morphology of *Bacillus sp* KJ629314: Microscopic observation of Gram Stain test.

3.2 Mechanisms of diesel aerobic degradation

Biodegradation of diesel is quite complex, since it is a mixture of alkanes, cycloalkanes, and aromatics. Formation of metabolites and diesel degradation mechanisms by *Bacillus sp* KJ629314 were not followed in this research. Metabolic pathways are reported for diesel/hydrocarbons aerobic degradation by several microorganisms. Degradation of n-alkanes (which are well known to be easily degraded) by *Pseudomonas putida* Gpo1 is well documented; the mechanism consists of the conversion of an alkane to an alcohol where a membrane bound monooxygenase and soluble rubredoxin and rubredoxin reductase serve to transfer electrons through NADH (reduced form of nicotinamide adenine dinucleotide, NAD) to the hydroxylase. In the next step the alcohol is oxidized to an aldehyde and acid, and in the last stage carbon dioxide and water are produced by the metabolic pathway of the tricarboxylic acid (Van Hamme *et al*., 2003). The mechanism for the biodegradation of cycloalkanes, as naphthalene, carried out by different bacterial strains such as *Pseudomonas, Mycobacterium, Corynebacterium, Aeromonas, Rhodococcus*, and *Bacillus*, has been reported. In this metabolic pathway it is suggested that bacteria incorporates both atoms of molecular oxygen into naphthalene while oxidation occurs to form cis-1,2-dihydroxy-1,2- dihydronaphthalene. Further metabolites in the breakdown reported are 1,2-dihydroxynaphthalene, cis-o-hydroxybenzalpyruvic acid, salicylaldehyde, salisylic acid, catechol/gentisic acid in studies conducted with *Pseudomonas sp*.

NCIB 9816 and *Pseudomonas putida* ATCC 17484 (Mrozik *et al*., 2003). Annweiler *et al*. (2000) reported metabolites such as 2,3-dihydroxynaphthalene, 2-carboxycinnamic acid, phthalic and benzoic acid; additionally to typical metabolites for naphthalene degradation in studies conducted with *Bacillus thermoleovorans*. Microbial degradation of monocyclic and polycyclic aromatic hydrocarbons has been extensively studied (Cerlinga 1993). Aliphatic hydrocarbons are converted to alcohols, and then are sequentially oxidized to carboxylic acids which are β-oxidized. The monocyclic aromatic compounds, such as benzene, is first hydroxylated by an oxygenase enzyme to cis-1,2-dihydroxy-1,2-dihidrobenzene which is then converted to catechol. The subsequent metabolism of catechol can follow two routes: ortho scission leading to cis, cis-muconate while meta cleavage results in 2-hydroxymuconic semialdehyde. Both routes lead to compounds that can enter the Krebs cycle.

3.3 *Specific microbial growth rate*

Growth rate (μ) was estimated using Eq. 2 (Sadouk *et al*., 2008) which describes the population change as a function of time, where $X(t)$ is the bacterial abundance at time t, expressed as CFU/kg soil in a logarithmic scale, and $X_o = X(t = 0)$.

$$X(t) = X_o e^{\mu t} \tag{2}$$

Results for the kinetic constants in the microcosm experiments ranged from 0.01 to 0.13 1/day, with an inhibitory effect as a function of hydrocarbon concentration. The highest constant rate for microbial growth ($0.13 \pm 4.9\times10^{-4}$ 1/day) was observed at 10,000 ppm microcosms, and as concentration increased (20,000; 30,000; 40,000 and 50,000 ppm) the rate constant of microbial growth decreased ($0.073 \pm 5\times10^{-3}$, $0.069 \pm 1\times10^{-3}$, $0.033 \pm 1\times10^{-4}$, and $0.010 \pm 7\times10^{-4}$ 1/day, respectively). Same behavior has been demonstrated in studies performed by Admon *et al*. (2001), a major factor affecting the rate of biodegradation is the concentration of hydrocarbons in a soil system. Extremely high TPH concentrations have proven to be lethal to microbial activity, thus limiting the biodegradation potential. Similarly, extremely low TPH concentrations; even if not lethal to organisms, it can limit biodegradation because the carbon source may be too low to support microbial growth (Leahy and Colwell, 1990).

3.4 *Diesel biodegradation and bacterial growth*

Bacillus sp KJ629314 growth resulted from using the mixture of compounds present in the diesel as the sole carbon source (substrate) under aerobic conditions (oxygen as electron acceptor). A histogram of diesel degradation (Average ± Std. Dev.,%) for all five different concentrations of diesel evaluated, is shown in Fig. 3; bars indicate the total diesel degraded by *Bacillus sp* KJ629314 over the 60 days of experimental phase.

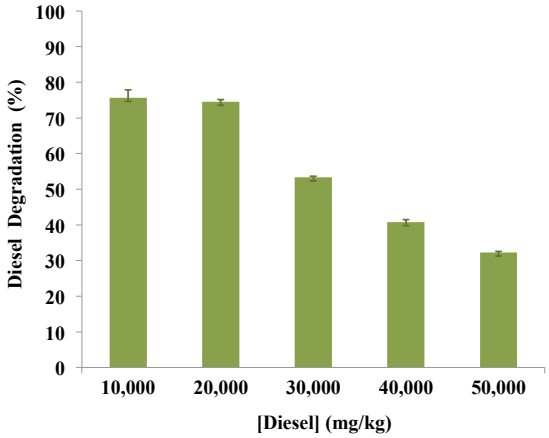

Fig. 3. Histogram of diesel degradation removal by *Bacillus sp* KJ629314. Overall Average values ± Std. Dev. (%) for all diesel concentrations [Diesel] evaluated, in mg/kg; for the 60 days period that experimental phase lasted.

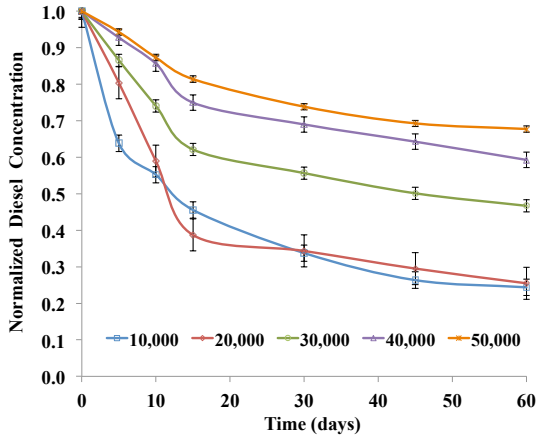

Fig. 4. Comparison of diesel degradation by *Bacillus sp* KJ629314 (Average values ± Std. Dev.) in of all five concentrations (10,000; 20,000; 30,000; 40,000 and 50,000 mg/kg) evaluated, based on normalized diesel concentration.

Fig. 4 shows degradation of diesel vs time using normalized concentration (Average values ± Std. Dev.) to easily compare the behavior for each residual diesel concentration at any given time with respect to the initial concentration, for all five concentrations evaluated. Trend of microbial growth kinetics for *Bacillus sp* KJ629314 was similar for all concentrations; a lag phase was observed from 0 to 5 days, exponential phase took place from this point until day 15, followed by a stationary phase and a well-defined endogenous phase at the end of experimentation; probably due to the bioavailability of hydrocarbons present in the soil. Diesel consists mostly of linear and branched alkanes with different chain lengths and contains a variety of aromatic compounds; many of these compounds, especially linear alkanes, are known to be easily biodegradable. However, due to their low water solubility, the biodegradation of these compounds is often limited by slow rates of dissolution, desorption, or transport. In general, the bioavailability of hydrophobic compounds is determined by their sorption characteristics and dissolution or partitioning rates and by transport process to microbial cell (Márquez-Rocha *et al*., 2001). It is believed that the kinetic trend of the diesel biodegradation at different concentrations by *Bacillus sp* KJ629314 is due to the biodegradation of PAH contained in the diesel accumulate forming toxic metabolites or recalcitrant compounds which decrease the viability of degraders (Viñas *et al*., 2005).

Table 1 summarizes results obtained experimentally, showing the biodegradation behavior by *Bacillus sp* KJ629314 at concentrations evaluated. For the 10,000 ppm concentration a diesel reduction up to 76 ± 2.2% was achieved corresponding to the highest degradation rate constant, $0.0214 \pm 1.7 \times 10^{-3}$ 1/day. For the 20,000 ppm concentration there was a diesel reduction of 61 ± 4.4% during the first 15 days with a constant rate value of $0.0212 \pm 7.8 \times 10^{-4}$ 1/day; from this point and up to 60 days an extra 13% reduction took place for a 74 ± 0.60% of total biodegradation.

Analysis of results showed significant differences, on the removal of diesel, for 10,000 and 20,000 (mg/kg soil) with respect to the other concentrations ($p < 0.095$), indicating that at these concentrations *Bacillus sp* KJ629314 had a greater affinity for diesel. Regarding to the degradation constant rate, the 10,000 ppm concentration was statistically different ($p < 0.095$) as compared to the other concentrations; indicating that the assimilation of diesel by *Bacillus sp* KJ629314 on this concentration is more accelerated causing an increase in microbial growth and degradation because the substrate is more bioavailable (Wutzler and Reichstein, 2013). Results on the 30,000 ppm concentration showed a 53 ± 0.39% hydrocarbon reduction having its maximum degradation during the first 30 days with a degradation rate constant of $0.0118 \pm 7.1 \times 10^{-5}$ 1/day; from this time onwards there were no gradual change in the concentration of hydrocarbons. Results for 40,000 and 50,000 ppm concentrations showed the lowest values in the biodegradation of diesel, 41 ± 0.78% and 32 ± 0.25%, respectively; both also presented the lowest values in the kinetic rate constants of which were $0.00835 \pm 7.1 \times 10^{-5}$ and $0.00645 \pm 2.12 \times 10^{-4}$ 1/day, respectively. These results indicate that this species, *Bacillus sp* KJ629314, have efficient catabolism of hydrocarbons in comparison with other studies performed by Nwaogu *et al*. (2008) and Singh and Lin (2008) in which different species of *Bacillus* were used.

Table 1. Degradation rate constants and removal efficiency (Average values ± Std. Dev.) of diesel by *Bacillus sp* KJ629314 at different concentrations

[Diesel] (mg/kg soil)	Degradation rate constants (1/day)	Diesel removal (%)
10,000	$0.0214 \pm 1.70 \times 10^{-3}$	76 ± 2.25
20,000	$0.0212 \pm 7.78 \times 10^{-4}$	74 ± 0.60
30,000	$0.0118 \pm 7.07 \times 10^{-5}$	53 ± 0.39
40,000	$0.0084 \pm 7.07 \times 10^{-5}$	41 ± 0.78
50,000	$0.0065 \pm 2.12 \times 10^{-4}$	32 ± 0.25

3.5 Abiotic control

Abiotic microcosm showed 0.22 ± 0.02% loss by volatilization of diesel, which is not attributable to microbial activity and a negligible amount relative to the total mass in the system.

Conclusions

Studies conducted in this research with *Bacillus sp* KJ629314, bacteria isolated from mining soil contaminated with TPH showed that this pure culture was able to grow on diesel as a source of carbon and energy (electron donor, ed^-) at aerobic conditions using oxygen as electron acceptor (ea^-). The biodegradation kinetics of *Bacillus sp*

KJ629314 on diesel showed that low concentrations of 10,000 and 20,000 ppm caused a positive stimulatory effect on microbial growth with a higher efficiency on hydrocarbon biodegradation; as diesel concentration increased (30,000; 40,000; and 50,000) the hydrocarbon biodegradation rate decreased. Results obtained in this research support the hypothesis that *Bacillus sp* KJ629314 can be used effectively to bioremediate diesel-contaminated soil. Efficiency of *Bacillus sp* KJ629314 to degrade diesel suggests that the strain possesses a substantial potential for the cleaning up of oil sludge in storage tanks and the bioremediation of sites contaminated with TPH, as in the case of Goldcorp Mexico, via bioaugmentation. However, it is recommended to conduct further experiments to estimate the biokinetic constants such as maximum utilization rate ($K_{\max}$) and the half-saturation or affinity constant (k_s) to determine the concentration at which the maximum removal is achieved (Martinez-Prado & Williamson, 2011).

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Nomenclature

CFU	Colony Forming Unit
$X(t)$	bacterial abundance at time t, CFU/kg soil.
X_o	bacterial population at time zero (t = 0), CFU/kg soil.
t	time, days.
μ	growth rate constant, 1/day.

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