Acta Scientiarum

http://periodicos.uem.br/ojs/acta
ISSN on-line: 1807-863X
Doi: 10.4025/actascibiolsci.v40i1.35640

BIOTECHNOLOGY

Assessment of petroleum biodegradation for *Bacillus toyonensis* by the using redox indicator 2,6 dichlorophenol indophenol

Ferdyanne Beatriz Sabóia Peixoto[1,2*], Jean Charles da Cunha Peixoto[1,2], Diana Cibelli Lucas Motta[2], Ana Teresa Miranda Peixoto[2], José Odair Pereira[1,2] and Spartaco Astolfi Filho[1,2]

*[1]Rede de Biodiversidade e Biotecnologia da Amazônia Legal, Bionorte, Universidade Federal do Amazonas, Av. General Rodrigo Otávio, 6200, 69077-000, Coroado I, Manaus, Amazonas, Brazil. [2]Centro de Apoio Multidisciplinar, Universidade Federal do Amazonas, Manaus, Amazonas, Brazil. *Author for correspondence. E-mail: ferdyanne@gmail.com*

ABSTRACT. Petroleum degrading microorganisms have been isolated from different environments with the purpose of being used in bioremediation processes in areas impacted by petroleum spills. The objective of this study was to evaluate the ability of *Bacillus toyonensis* AM07 strain to metabolize petroleum compounds. The strain was isolated from the effluent dike of the Urucu Petroleum Province, Coari - Amazonas, Brazil. The degrading activity of *B. toyonensis* was evaluated by the colorimetric method using the redox indicator 2,6-dichlorophenol indophenol (DCPIP). Thus, the microorganism was inoculated into minimal medium with DCPIP, and with petroleum as the sole carbon source. The degradation potential of the microorganism was found by changing the DCPIP staining and absorbance readings 600_{nm}. The results obtained demonstrated that the bacterial strain was able to degrade petroleum by altering the color of the medium from blue to colorless and by reducing the concentration of the indicator in the absorbance readings. *B. toyonensis* AM07 strain has shown good performance in the petroleum degradation assays and may be used in the future in remediation technologies for hydrocarbon impacted environments.

Keywords: Amazon; *B. toyonensis*; biodegradability; DCPIP.

Avaliação da biodegradação do petróleo por *Bacillus toyonensis*, usando o indicador redox 2,6 diclorofenol indofenol

RESUMO. Microrganismos degradadores de petróleo têm sido isolados de diferentes ambientes com a finalidade de serem utilizados em processos de biorremediação de áreas impactadas com derrames de petróleo. O objetivo deste estudo foi avaliar a capacidade da linhagem de *Bacillus toyonensis* AM07, isolada do dique de efluente da Província Petrolífera de Urucu, Coari - Amazonas, Brasil, em metabolizar compostos do petróleo. A atividade degradadora do *B. toyonensis* foi avaliada pelo método colorimétrico, utilizando indicador redox 2,6-diclorofenol indofenol (DCPIP). Assim, o microrganismo foi inoculado em meio mínimo com DCPIP e petróleo como única fonte de carbono. O potencial de degradação do microrganismo foi constatado mediante a mudança de coloração DCPIP e leituras de absorbância 600_{nm}. Os resultados obtidos demonstraram que a cepa bacteriana foi capaz de degradar petróleo, alterando a coloração do meio de azul para incolor e reduzindo a concentração do indicador nas leituras de absorbâncias. A cepa de *B. toyonensis* AM07 mostrou bom desempenho nos ensaios de degradação do petróleo, podendo ser utilizada, no futuro, em tecnologias de remediação de ambientes impactados por hidrocarbonetos.

Palavras-chave: Amazônia; *B. toyonensis*; biodegradabilidade; DCPIP.

Introduction

The microorganisms capable of degrading petroleum have been isolated from different environments with the objective of being used in bioremediation processes of impacted areas (Olajire, & Essien, 2014; Das, & Chandran, 2011). The presence of hydrocarbons on the environment selects microbial populations able to use them as substrate for their maintenance and survival (Darsa, Thatheyus, & Ramya, 2014; Bujang, Ibrahim, & Eh Rak, 2013).

The species of the genus Bacillus have been reported as petroleum compounds degraders, such as pyrene, naphthalene and n-alkenes (Darsa et al., 2014; Sorkhoh, Ibrahim, Ghannoum, & Radwan, 1993). In this genus, a new specie was described by Jiménez et al. (2013a) as part of the phylogenetic group *Bacillus cereus*, named *B. toyonensis*. The group consists of Gram-positive, endospore-forming bacteria found in soils, covering the bacterial species *B. cereus*, *Bacillus thuringiensis*, *Bacillus anthracis*, *Bacillus mycoides*, *Bacillus pseudomycoides*, *Bacillus*

weihenstephanensis, B. *toyonensis* and *Bacillus cytotoxicus* (Jiménez, Blanch, Tamames, & Rosselló-mora, 2013b).

The *B. toyonensis* was isolated for the first time in Japan, in 1966, as *B. cereus var. toyoi* (BCT- 7112 strain), and it has been used as probiotics in the animal nutrition since 1975, when it was officially approved by Japan´s Ministry of Agriculture, Forestry and Fisheries as Toyocerin® (Jiménez et al., 2013a-b). Okaiyeto, Nwodo, Mabinya, and Okoh (2015) recorded the occurrence of *B. toyonensis* in samples of sediments from marine environment in South Africa. Adewale, Goh, Lim, and Ting (2015) isolated *B. toyonensis*, as an endophytic bacteria, from medicinal plants *Mentha spicata*, with highlighting commercial interest for Asian communities. However, the use of *B. toyonensis* as a petroleum degrading agent has been little explored.

The method of fast selection of microorganisms that degrade petroleum by using redox indicator DCPIP has been broadly accepted, once it detects an oxidation of NADH to NAD+ (Hanson, Desai, & Desai, 1993; Kubota, Koma, Matsumiya, Chung, & Kubo, 2008). In practice, DCPIP molecule act as electrons acceptor, receiving them from the process of oxidation, mediated by microorganisms which use hydrocarbons as substrate. A test is considered positive when DCPIP changes the original color, from blue (oxidized state) to colorless (reduced state), in the presence of hydrocarbons (Hanson et al., 1993; Bidoia, Montagnolli, & Lopes, 2010). The present study had the aim to assess the petroleum degrading potential by a strain of *B. toyonensis,* isolated from an effluent dike of Urucu Petrol Basin, Coari – Amazonas, Brazil, by using the technique of rapid selection with redox indicator DCPIP.

Material and methods

The research was performed with the strain of *B. toyonensis* isolated from samples of water from the dike effluent of Urucu Petrol Basin, located in the Municipality of Coari, Amazonas-Brazil. The bacterial strain AM07 is deposited in the culture collection of the Laboratory of Bacterial Genetics of *Universidade Federal do Amazonas* - UFAM, Manaus.

Enrichment and isolation

The water samples were enriched in 500 mL Erlenmeyer flasks, containing 90 mL of Bushnell Haas Broth -BH mineral medium (Difco ™) (magnesium sulphate 0.2 g L^{-1}, calcium chloride 0.02 g L^{-1}; potassium nitrate 1.0 g L^{-1}, ferric chloride 0.05 g L^{-1}, monopotassium phosphate 1.0 g L^{-1}, diammonium hydrogen phosphate 1.0 g L^{-1}), 10 ml of water, and 1% of petroleum as the only source of carbon. The vials were incubated at 30°C, and at 180 rpm per minute in an orbital shaker (Thermo Scientific MaxQ ™ 4000) for 21 days. The microorganism was isolated on plates containing BH agar medium plus petroleum. The pure culture was transferred to plates containing tryptone medium soybean (TSC) (Difco™) (15 g L^{-1} casein hydrolyzate; sodium chloride 5 g L^{-1}; papaya soybean hydrolyzate 5 g L^{-1}; 15 g L^{-1} agar). The petroleum used in the experiments came from the Coari Petroleum Province, and before being used, it was previously filtered in Millipore™ membranes of 0.22 µm.

Biodegradability test using the DCPIP redox indicator

The qualitative assay was performed in sterile multiwell (24 wells) microplates in triplicate. 1.5 mL of DCPIP [0.01 g L^{-1} BH] was added to each well, along with 10 µL of petroleum and 25 µL of bacterial inoculum previously cultured in nutrient broth medium (HIMEDIA) at 30°C for 12 hours, at 150 rpm in an orbital shaker (Thermo Scientific MaxQ™ 4000). The cells were centrifuged for 10 min, washed with saline solution (0.9%), and then standardized in a UV-VIS spectrophotometer (Thermo spectronic Biomate 3) at 600_{nm} O.D = 1 (10^9 cells mL^{-1}). The control (1) was prepared with 1.5 mL of DCPIP and 2.5 µL of BH, the control (2) was prepared with 1.5 mL of DCPIP, 2.5 µL of BH, and 10 µL of petroleum. The plates were incubated at 30°C, and the evaluations were performed every 12 hours until the color of the medium changed, from blue (oxidized) to colorless (reduced), indicating a positive result (Hanson et al., 1993; Bidoia et al., 2010; Peixoto et al., 2017).

Quantitative analysis

The quantitative assay was performed in 250 mL Erlenmeyer flasks, in triplicate, incubated at 30°C at 180 rpm in an orbital shaker (Thermo Scientific MaxQ ™ 4000). Aliquots of 1 mL of the culture were collected to read the absorbance at 600 nm in a UV-VIS (Thermo spectronic Biomate 3) spectrophotometer at 24, 48 and 72 hours intervals. For this test, the DCPIP solution was prepared in the concentration of 1 g L^{-1} in sterile BH medium. The composition of this assay is shown in Table 1.

The quantitative analysis was performed by reading the absorbance, converted to mg of DCPIP, using the standard curve (R2 = 0.9998) (Figure 1), according to Equation 1: [DCPIP] = ($Abs._{600}$ + 0.0037) / 0.0154 where: [DCPIP] = concentration of redox indicator DCPIP in mg L^{-1}; $Abs._{600}$ = absorbance of the sample at wavelength 600_{nm}. The standard curve was drawn from five dilutions of the DCPIP indicator in BH broth, which were read ($Abs._{600}$ nm) on a UV-VIS (Thermo spectronic Biomate 3) spectrophotometer (Table 2).

Table 1. Composition of the quantitative assay.

Test	Composition	BH	DCPIP	Petroleum	AM07	H_2O
Control 0	BH	90.000 mL	-	-	-	10.000 mL
Control 1	BH + DCPIP	90.000 mL	4.761 mL	-	-	5.239 mL
Control 2	BH+DCPIP+Petroleum	90.000 mL	4.761 mL	0.595 mL	-	4.644 mL
Control 3	BH+DCPIP+AM07	90.000 mL	4.761 mL	-	2.380 mL	2.859 mL
Inoculum	BH+DCPIP+Petroleum+AM07	90.000 mL	4.761 mL	0.595 mL	2.380 mL	2.264 mL

Table 2. Absorbance readings at 600_{nm} and dilutions of redox indicator DCPIP.

Erlenmeyer Flasks	Volume (mL)			[DCPIP]	Abs_{600} nm
	BH	DCPIP [1 g L^{-1}]	H_2O	(mg L^{-1})	u.a
0	90.000	0.000	10.000	0.000	0.0000
1	90.000	0.595	9.405	5.950	0.0880
2	90.000	1.190	8.810	11.900	0.1730
3	90.000	2.380	7.620	23.800	0.3620
4	90.000	3.571	6.429	35.710	0.5490
5	90.000	4.761	5.239	47.610	0.7270

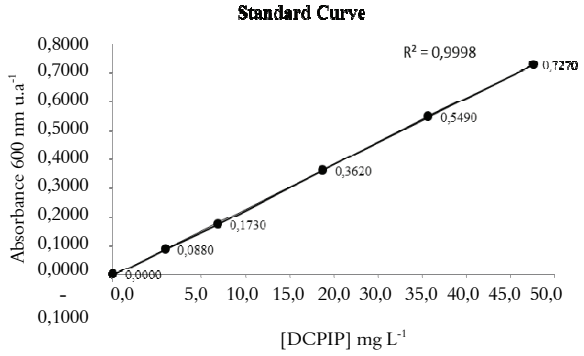

Figure 1. Standard curve - [DCPIP].

Biochemical characterization of bacterial isolate

The bacterial isolated was characterized according to its morphology, tinctorial property, and then submitted to biochemical tests having as base Bergey's Manual of Determinative Bacteriology (Holt, Krieg, Sneath, Staley, & Williams, 1994).

Amplification, sequencing and phylogenetic analysis of rRNA 16S gene

A bacterial pre-inoculum from pure culture was inoculated in 20 mL of nutrient broth (HIMEDIA™) at 35ºC, at 150 rpm overnight. The isolation and purification of DNA genomic was made based on extraction kit PureLink™ Genomic DNA Mini Kit, from Invitrogen™, using 3 mL of bacterial culture, according to the manufacturer´s instructions. The reaction of amplification was performed by using primers 530F (5′-TGACTGACTGAGTGC CAGCMGCCGCGG-3´) and 1495R (5' TGACTGACTGAGAG CTCTACCTTGTTA-3). The total volume of the reaction was 25 μL having (2.5 μL $MgCL_2$ [25 mM], 2.5 μL dNTPs [2.5 mM]; 2.0 μL of each primer [5 pMol μL^{-1}]; 0.3 μL of *Taq* DNA polymerase [5 U μL^{-1}]; 2.5 μL of buffer 10X). The amplification took place during 35 cycles in Thermal Cycler, Veriti® 96-Well - Applied Biosystems. The thermal profile from the reaction of PCR constituted of denaturation of initial cycle at 95ºC per 1 minute; followed by 35 cycles to 95ºC for 40 seconds, annealing of primers at 58ºC for 40 seconds, and polymerization at 72ºC for 40 seconds (35 cycles); and at final extension step at 72ºC for 7 minutes. For the sequencing of amplicon, it was used the sequencing kit named BigDye® Terminator v3.1 in a capillary sequencer (model 3500 ABI PRISM® Genetic Analyzer, Applied Biosystems). The sequences generated were treated in programs phred/phrap (Ewing, Hillier, Wendl, & Green, 1998) and CAP 3 available in http://www.biomol.unb.br/phph/, and then, the sequences were analyzed in the program BLASTn National Center for Biotechnology Information –NCBI. The phylogenetic analysis was undertaken based on the evolution distances calculated by algorithms Neighbor-Joining and Jukes-Cantor, by using the program MEGA 7.0. After the building of the phylogenetic tree, it is possible to determine the taxonomical position of the isolate in relation to the species whose corresponding sequences were obtained in NCBI (Kumar, Stecher, & Tamura, 2016; Jukes & Cantor, 1968; Felsenstein, 1985).

Results and discussion

The biodegradability test using DCPIP is a low complexity and low-cost test with proved efficiency by different authors, which highlight the important role of this indicator in the detection of microorganisms, such as fungi, bacteria and yeasts with the potential for degrading petroleum and derivatives (Hanson et al., 1993; Bidoia et al., 2010; Luz, et al., 2011; Mariano, Bonotto, De Angelis, Pirôllo, & Contiero, 2008). When selecting microorganisms with the purpose of using them in bioremediation processes, the DCPIP assay allows the confirmation of the degradation capacity,

and in this study, the degradation activity of the bacterial strain AM07 can be observed in the qualitative test, after 16 hours of incubation (Figure 2). The quantitative test of the biodegradability of petroleum, carried out by absorbance readings, showed a reduction in the concentration of DCPIP due to the degrading activity of the bacterial strain AM07 after 24 hours of incubation (Figure 3, Table 3). The controls were stable due to the lack of carbon source and/or bacterial inoculum. The results obtained represent a remarkable activity when compared to the results described in Varjani and Upasani, (2013). The authors tested the efficiency of the use of petroleum by 69 bacterial strains isolated from enrichment of soil samples. Among the isolates tested, fifteen strains discolored the medium, and six strains recorded the shortest discoloration time of the medium in 120 hours, out of a total of 144 hours. Afuwale and Modi (2012) found hydrocarbon degradation in seven of the 36 bacterial isolates tested, and two of these completely discolored the DCPIP containing medium.

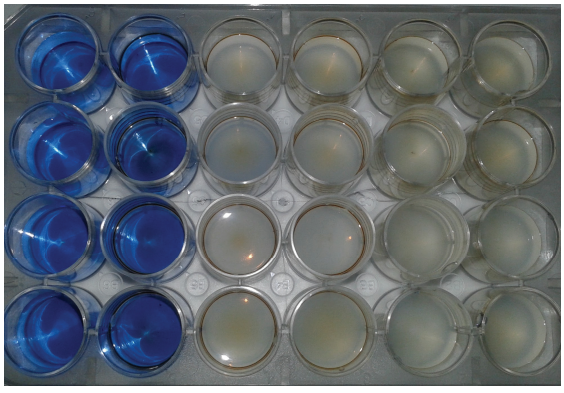

Figure 2. Biodegradation test with DCPIP – plate after 16 hours of incubation.

The bacterial isolate showed Gram-positive staining, endospore formation, cell diameter > 1 µm, hydrolysis in the starch tests, positive Voges-Proskauer reaction, motility, positive activity for catalase, and its growth patterns in blood agar presented the β-hemolytic pattern. In view of the presented results, the microorganism was classified as *B. cereus*. To confirm the species, the 16S rRNA gene was sequenced, which classified the microorganism as belonging to *B. toyonensis* species with 99% identity and 100% coverage when compared to the *B. toyonensis* sequence (BCT-7112) available in the database from GenBank. The nucleotide sequence was deposited on GenBank as *B. toyonensis* AM07 under accession number KX686603. In order to confirm the taxonomic position of this strain, the phylogenetic analysis of the MEGA 7.0 program (Felsenstein, 1985; Kumar, et al., 2016; Saitou, & Nei 1987), aligning the 16S rRNA gene sequence of the strain isolated in this study, *B.toyonensis* AM07, along with eight other sequences of the 16S rRNA gene, obtained in Genbank, from different species belonging to the genus Bacillus. The phylogenetic analysis showed that the sequence belonging to the strain isolated in this study, KX686603 *B. toyonensis* AM07, grouped with robustness to the strain 972704713 in LN995772.1 *B. toyonensis*, presenting 100% bootstrap, when compared to the sequences of the other species belonging to the Group *B. cereus*, as shown in Figure 4.

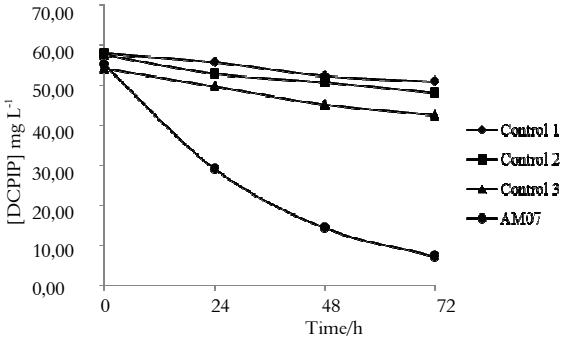

Figure 3. Concentration of DCPIP as a function of time.

Table 3. DCPIP Concentrations.

Time/h	Control 1	Control 2	Control 3	AM07
0	57.97	57.77	54.27	55.20
24	55.89	52.97	49.79	29.16
48	52.38	50.82	45.24	14.35
72	51.02	48.16	42.58	7.23

Species belonging to the genus Bacillus isolated from different environments have often been described as degrading hydrocarbons (Darsa et al., 2014; Sorkhoh et al., 1993). Rajasekar et al. (2007) reported the biodegradation of diesel oil and manganese oxidation by B. cereus. Bidoia et al. (2010) verified the potential of isolated strain of *B. subtilis* in degrading lubricating oil, using the DCPIP colorimetric method. Liu, Liu, Ju, Li, and Yu (2016) isolated strains belonging to the species *B. licheniformis* that demonstrated their ability to degrade petroleum and produce biosurfactant. Kreischer and Silva (2017) isolated *B. toyonensis* from soil samples contaminated with agrochemicals and verified the production of biosurfactant. The production of biosurfactant by microorganisms is of paramount importance in the process of biodegradation, as it assists in emulsification, reducing recalcitrance and facilitating the oxidation of hydrocarbons. The present study carried out a preliminary analysis that verified by the colorimetric method with the redox indicator DCPIP the potential of *B. toyonensis* in degrading petroleum, however no previous records of the association of this related species degradation of this carbon source were found.

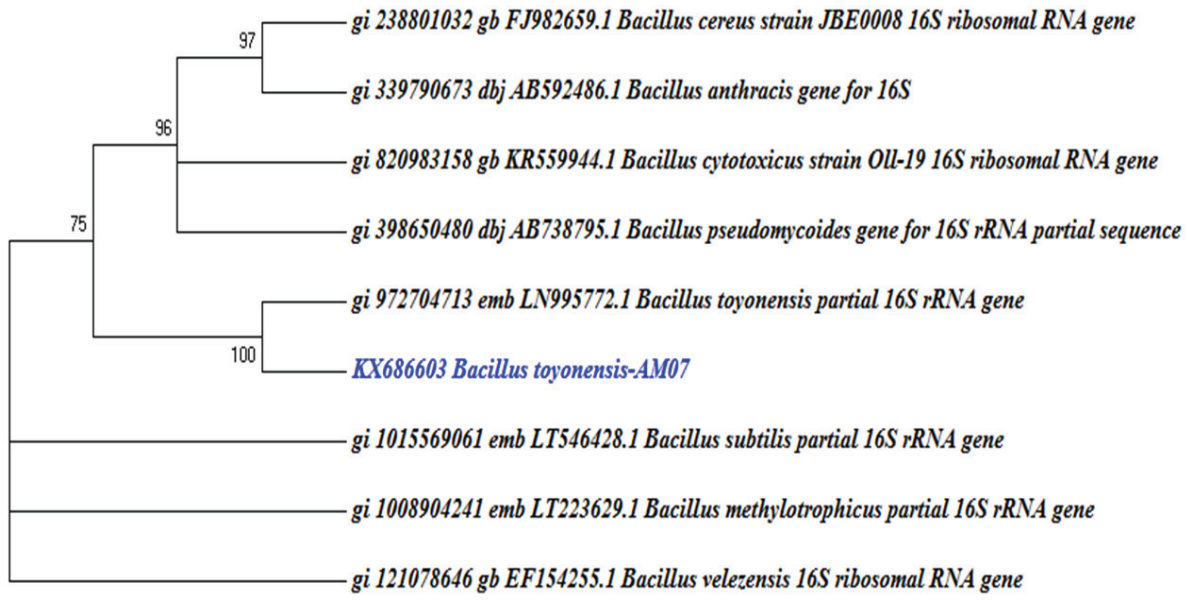

Figure 4. Phylogenetic analysis of the specie *B. toyonensis* AM07 by using partial sequences from gene rRNA 16S bacterial strains from the GenBank-NCBI.

Conclusion

The present study demonstrated, through the colorimetric method with DCPIP indicator, the potential of the *B. toyonensis* AM07 strain isolated from an effluent dike, to use petroleum as a single source of carbon with the possibility of using this strain in remediation technologies of environments impacted with hydrocarbons.

Acknowledgements

The authors thank to the *Conselho Nacional de Desenvolvimento Científico e Tecnológico* (CNPq), to the *Coordenação de Aperfeiçoamento de Pessoal de Nível Superior* (CAPES) and to the *Fundação de Amparo à Pesquisa do Estado do Amazonas* (FAPEAM), for the financial support. The authors are also grateful to the *Universidade Federal do Amazonas* (UFAM)/Program of Post-Graduation in Biodiversity and Biotechnology of Legal Amazonia (BIONORTE).

References

Adewale, A. M., Goh, J. K., Lim, S. P., & Ting, A. S. Y. (2015). Antimicrobial and enzymatic activities of endophytic bacteria isolated from Mentha spicata (MINT). *Malaysian Journal of Microbiology*, *11*(2), 102-108.

Afuwale, C., & Modi H. A. (2012). Study of bacterial diversity of crude oil degrading bacteria isolated from crude oil. *Life Sciences Leaflets*, *6*(June), 13-23.

Bidoia, E. D., Montagnolli, R. N., & Lopes, P. R. M. (2010). Microbial biodegradation potential of hydrocarbons evaluated by colorimetric technique : a case study. *Appleid Microbiology and Microbial Biotechnology*, *3*(290-295), 1277-1288.

Bujang, M., Ibrahim, N. A., & Eh Rak, A. (2013). Biodegradation of oily wastewater by pure culture of *Bacillus cereus*. *ARPN Journal of Agricultural and Biological Science*, *8*(2), 108-115.

Darsa, K. V., Thatheyus, A. J., & Ramya, D. (2014). Biodegradation of petroleum compound using the bacterium *Bacillus Subtilis*. *Science International*, *2*(1), 20-25.

Das, N., & Chandran, P. (2011). Microbial degradation of petroleum hydrocarbon contaminants: An overview. *Biotechnology Research International*, *2011*, 1-13. doi: 10.4061/2011/941810.

Ewing, B., Hillier, L., Wendl, M., & Green, P. (1998). Base-calling of automated sequencer traces usingPhred. I. Accuracy assessment. *Genome research*, *1998*(8), 175-185.

Felsenstein J. (1985). Confidence limits on phylogenies: an approach using the bootstrap. *Evolution*, *39*(4), 783-791.

Hanson, K. G, Desai, J. D., & Desai, A. J. (1993). A rapid and simple screening crude oil degrading technique for potential microorganisms. *Biotechnology Techniques*, 7(1), 745-748.

Holt, J. H., Krieg, N. R., Sneath, P. H. A., Staley, J. T., & Williams, S. T. (1994). *Bergey's manual of determinative bacteriology ninth edition*. Baltimore, Maryland, USA: Williams & Wilkins.

Jiménez, G., Urdiain, M., Cifuentes, A., López-López, A., Blanch, A. R., Tamames, J., ... Rosselló-Mora, R. (2013a). Description of *Bacillus toyonensis* sp. nov., a novel species of the *Bacillus cereus* group, and pairwise genome comparisons of the species of the group by means of ANI calculations. *Systematic and Applied Microbiology*, *36*(6), 383-391.

Jiménez, G., Blanch, A. R., Tamames, J., & Rosselló-mora, R. (2013b). Complete genome sequence of *Bacillus toyonensis* BCT-7112 T , the active ingredient of the feed additive preparation Toyocerin. *Announcements*, *1*(6), 1-2.

Jukes, T. H., & Cantor, C. R. (1969). Evolution of protein molecules. In Munro, H. N. (Ed.), *Mammalian Protein Metabolism* (p. 21-123). New York, NY: Academic Press.

Kubota, K., Koma, D., Matsumiya, Y., Chung, S. Y., & Kubo, M. (2008). Phylogenetic analysis of long-chain hydrocarbon-degrading bacteria and evaluation of their hydrocarbon-degradation by the 2,6-DCPIP assay. *Biodegradation*, *19*(5), 749-757.

Kreischer, A. C., & Silva, L. P. (2017). Bioprospecção de bactérias produtoras de biossurfactantes a partir de solo contaminado por agrotóxicos**.** *Revista Eletrônica Estácio Saúde*, *6*(1), 35-46.

Kumar, S., Stecher, G., & Tamura, K. (2016). MEGA7: Molecular Evolutionary Genetics Analysis version 7.0 for bigger datasets. *Molecular Biology and Evolution*, *33*(7), 54.

Liu, B., Liu, J., Ju, M., Li, X., & Yu, Q. (2016). Purification and characterization of biosurfactant produced by *Bacillus licheniformis* Y-1 and its application in remediation of petroleum contaminated soil. *Marine Pollut Bulletin*, *107*(1), 46-51.

Luz, C. C., Santos, E. A., Santos, M. O. S., Mussy, M. Y., Yamashita, M., & Bastos, W. R. (2011). Estudos de biodegradação de óleo diesel por consorcio microbiano coletado em Porto Velho- Ro, Amazônia. *Quimica Nova*, *34*(5), 775-779.

Mariano, A. P., Bonotto, D. M., De Angelis, D. D. F., Pirôllo, M. P. S., & Contiero, J. (2008). Biodegradability of commercial and weathered diesel oils. *Brazilian Journal of Microbiology*, *39*(1), 133-142.

Olajire, A. A., & Essien, J. P. (2014). Aerobic degradation of petroleum components by microbial consortia. *Journal of Petroleum & Environmental Biotechnology*, *5*(5), 1-22.

Okaiyeto, K., Nwodo, U. U., Mabinya, L. V., & Okoh, A. I. (2015). *Bacillus toyonensis strain AEMREG6*, a bacterium isolated from South African marine environment sediment samples produces a glycoprotein bioflocculant. *Molecules*, *20*(3), 5239-5259.

Peixoto, F. B. S., Peixoto, J. C. C., Assunção, E. N., Peixoto, E. M., Pereira, J. O., & Astolfi Filho, S. (2017). Petroleum biodegrading and co- resistance to antibiotics by *Serratia marcescens* strain isolated in Coari, Amazonas. *Acta Scientiarum. Biological Sciences, 39*(4), 489-496.

Rajasekar, A., Babu, G., Pandian, S. K., Maruthamuthu, S., Palaniswamy, N., & Rajendran, A. (2007). Biodegradation and corrosion behavior of manganese oxidizer *Bacillus cereus* ACE4 in diesel transporting pipeline. *Corrosion Science*, *49*(6), 2694-2710.

Sorkhoh, N. A., Ibrahim, A. S., Ghannoum, M. A., & Radwan, S. S. (1993). High-temperature hydrocarbon degradation by *Bacillus stearothermophilu*s from oil-polluted Kuwaiti desert. *Applied Microbiology and Biotechnology*, *39*(1), 123-126.

Saitou, N., & Nei, M. (1987) The Neighbor-joining method: A new method for reconstructing phylogenetic trees. *Molecular Biology and Evolution*, *4*(4), 406-425.

Varjani, S., & Upasani, V. (2013). Comparative studies on bacterial consortia for hydrocarbon degradation. *International Journal of Innovative Research in Science, Engineering and Technology*, *2*(10), 5377-5383.

Received on March 16, 2017.
Accepted on September 5, 2017.