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Molecular Characterization and Evaluation of Oil-degrading Native Bacteria Isolated from Automotive Service Station Oil-contaminated Soils

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In this study, a hydrocarbon-degrading mixed inoculum which is able to use used oil as sole carbon source, was selected from 15 bacterial isolates obtained from automotive service station oil-contaminated soils. Degrading microorganisms were isolated using different oils as sole carbon source and identified by the amplification and sequencing of the 16s rRNA sequences. In addition, the presence of hydrocarbon-degrading genes such as catechol 2,3 dioxygenase (*nahH*), alkane monooxygenase (*alkB*), Gram-negative (GN-RHDα) and Gram-positive PAH-Ring Hydroxylating Dioxygenase alpha (GP-RHDα) was analyzed by PCR and the molecular diversity by LSSP-PCR methods. Four (4) out of fifteen (15) isolates corresponding to *Stenotrophomonas maltophilia*, *Pseudomonas aeruginosa* and *Klebsiella pneumoniae* showed significant differences regarding oil/grease removal in liquid culture after 72 hours. Subsequently, a degrading mixed inoculum composed of these isolates was constructed and its degrading potential tested in a two-liter bioreactor containing unsterile liquid oily wastes with 0.8 % (w/v) Total Petroleum Hydrocarbons (TPHs) concentration for 42 days. The use of the mixed inoculum led to a decrease of 98.4 % Biological Oxygen Demand (BOD), 97.5 % Chemical Oxygen Demand (COD) and 97.2 % TPHs after 40 days. Further scale-up of the process to five liters using 0.2 % (w/v) unsterile oily wastes produced similar results, with a reduction of 85 % BOD, 39 % COD and 87 % TPHs after 38 days. The degrading mixed microbial inoculum presented high potential for the treatment of impacted soils at automotive service stations and sites polluted with oily wastes due to its elevated growth at high hydrocarbon concentrations and its capacity to utilize oils as energy source.

1. Introduction

Automotive service stations are important elements in the functioning of our society. However, they are also important sources of soil and groundwater contamination due to the activities taking place and the products involved, which in most cases are pollutants such as liquid petroleum fuels (gasoline, diesel, kerosene) and oils. It is important to consider that under the current development models, the consumption of automotive fuels (mainly gasoline and diesel fuel) increases the environmental risks associated with the contamination of soil and groundwater and their impacts have increased in recent years, taking into account the growing demand for energy. In automotive service stations, fuels that affect soil and groundwater come from leaks and spills from underground storage tanks and piping systems. Leaks are frequently produced by defective installations or storage/transport structures, while spills usually occur on the surface and they can reach the ground and nearby underwater sources through cracks in the concrete or by means of the soil continuous exposure. The continuous episodes of contamination with crude oil and related wastes favour the deposition and accumulation of xenobiotics and potentially toxic compounds in soils. Thus, the management of used oil and oily wastes from automotive service stations represents an important environmental problem.
Bioremediation uses the metabolic versatility of microorganisms to degrade different hazardous pollutants, including hydrocarbons. The main objective of bioremediation is to transform organic pollutants into non-toxic metabolites or mineralize them to carbon dioxide and water (Haritash and Kaushik, 2009). Native soil

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microbial populations established in a polluted environment possess a high degree of stability and resilience towards introduced microorganisms, being an extremely important factor in the long-term success of a bioaugmentation process (Zafra et al. 2014). Microorganisms such as bacteria, fungi and algae possess specific catabolic activities that can be exploited for the remediation of soil impacted with low and high molecular weight hydrocarbons. Hydrocarbon microbial degradation pathways tend to exhibit a broad substrate specificity, being driven by different oxidoreductase enzymes including monooxygenases, dioxygenases, peroxidases and laccases, and occur either aerobically or anaerobically (Haritash and Kaushik, 2009). Thus, the aim of this work was to isolate and evaluate bacterial isolates from automotive service station oil-contaminated soils with potential of hydrocarbon degradation, as well as stablish their metabolic potential for hydrocarbon degradation in order to construct a highly degrading mixed inoculum being able to degrade hydrocarbons and oil wastes in soils.

2. Materials and Methods

2.1 Soil samples

A soil polluted with used oil residues from automotive service stations grease traps, collected from a landfill site located in Floridablanca, Colombia, (7°3'55.15"N -73°7'36.5268"W, 29°C annual average temperature) was used in this study. Composite samples were obtained at three sampling moments (day 0, month 3 and month 6) in a random zig-zag strategy, following the procedures described by Brady and Weil (2008). Soil samples were conserved at 4 °C until physicochemical analyses were conducted. Table 1 shows the main physicochemical characteristics of soil samples.

2.2 Microbial isolation from soil

Bacterial populations were isolated from contaminated soils by diluting 10 g of each soil in 90 ml of Basal Saline medium (BSM) containing (grams per L): $(NH4)_2HPO_4$, 2.7; Na_2HPO_4, 4.3; K_2HPO_4, 4.2; $MgSO_4.7H_2O$, 5.5; $CaCl_2*2H_2O$, 0.06; using 0.5 % crude oil as sole carbon source. Cultures were incubated at 30 °C for 40 days with constant agitation at 200 rpm. One hundred microliters of the culture supernatant were used to inoculate plates of BSM. Plates were incubated at 30 °C for 4 days and individual colonies were picked and transferred to new plates of BSM with crude oil.

2.3 Molecular identification and characterization

Genomic DNA extraction from bacterial isolates was performed by using the Wizard Genomic DNA Purification Kit (Promega, USA). Isolates were identified by 16S rRNA sequencing as described by Lane (1991), using the universal primers 27F (5′-AGAGTTTGATCMTGGCTCAG-3′) and 1525R (5′-AAGGAGGTGWTCCARCC-3′) to amplify a 1,450-bp fragment of the bacterial 16S rRNA gene. PCR products were analyzed by agarose gel electrophoresis and purified using the Column-pure DNA Gel Recovery Kit (ABM inc., Canada). DNA sequencing was performed using the BigDye Terminator v3.1 Cycle Sequencing Kit (Invitrogen, USA), and an Applied Biosystems ABI 3100 genetic analyzer using oligonucleotides 27F/1525R as sequencing primers. BLAST (Altschul et al. 1990) was used for homology searching and for the identification of microorganisms. Phylogenetic analysis was performed using the MEGA 6 software package (Tamura et al. 2013). Molecular detection ad characterization of genes involved in hydrocarbon degradation was performed by PCR and Low-Stringency Single specific Primer PCR (LSSP-PCR). The catechol 2,3 dioxygenase (*nahH*), alkane monooxygenase (*alkB*), Gram negative (GN-RHDα) and Gram positive PAH-Ring Hydroxylating Dioxygenase alpha (GP-RHDα) genes were detected by PCR as reported previously (Cebron et al. 2008, Powell et al. 2006). *nahH* gene-specific signatures were generated by LSSP-PCR as previously described by Pena et al., (1994), using the primer Cat2,3R (CGGTCGTGGTAAAAGATCG) as driver. The resulting amplified fragments were analyzed on 4% low melting point agarose gels, stained with SafeView nucleic acid stain (ABM inc., Canada) and photographed.

Table 1: Physicochemical characteristics of contaminated soils

Soil (sampling date)	TPH content (mg kg^{-1})	pH (mean)	Texture
Initial sampling (Day 0)	115,500	7,6	Loam clay soil
Month 3	93,900	7,5	Loam clay soil
Month 6	99,700	7,5	Loam clay soil

2.4 Evaluation of microbial hydrocarbon degradation

Individual bacterial isolates were evaluated for their potential to degrade used motor oil, obtained from automotive service stations grease traps, in liquid culture assays. Glass flasks with 50 ml of BSM containing 0,5 % and 1 % used oil as sole carbon source were inoculated with $3x10^8$ Colony Forming Units (CFU) of each

isolate and incubated at 30 °C for 24 h under constant agitation at 150 rpm. Four isolates showing the highest removal ability were used to construct a degrading mixed inocula. Total Petroleum Hydrocarbon (TPH) degradation of this consortium was evaluated in a two liter bioreactor using 1.25 L of BSM containing 0,8 % (v/v) used motor oil. Microbial isolates were grown separately on BSM with 0.05 % (v/v) crude oil as sole carbon source, and used to inoculate in the bioreactor at a final concentration of 10^9 CFU each (1:1 ratio). The process was carried out for 42 days at 30 ºC with constant aeration. TPH removal was assessed according to Standard Methods 5520D protocol (Sohxlet extraction). Biochemical (BOD_5) and Chemical Oxygen Demand (COD) were determined according to Standard Methods 5520C and 5210B protocols. Abiotic controls were included to compensate for adsorption losses. Assays were carried out in triplicate.

2.5 Data Analysis

Microbial isolates were identified according to the results of the 16S rRNA sequencing. For LSSP-PCR, the molecular weights of amplified bands were calculated using Photo-CaptMw v10.01 software (Vilber Loumat, France) and a binary data matrix of 0 and 1 were created to encode the absence or presence of bands, respectively, in each electrophoretic mobility level. LSSP-PCR profiles were then analyzed using Pyelph v1.4 software and the clustering tree was generated using the UPGMA option. Numerical data were analyzed by analysis of variance (ANOVA) followed by a multiple comparison test (LSD) with SPSS Statistics Software version 19 (IBM), considering statistically significant differences those with a p value < 0.05.

3. Results and Discussion

A total of 15 Gram-negative and Gram-positive bacterial organisms with bacillary and filamentous morphologies were obtained from oil-contaminated soils, based on their capacity to use crude oil as sole carbon source. Homology analyses of 16S rRNA sequences with BLAST showed that isolates corresponded to five different genera and eight species with genetic similarity values close to 100 % compared with those reported in GenBank (Table 2). Previously reported hydrocarbon-degrading species were isolated, including *Pseudomonas aeruginosa*, *Pseudomonas stutzeri*, *Enterobacter cloacae*, *Stenothrophomonas maltophilia*, *Bacillus cereus* and *Bacillus pumilus* (Borah and Yadav 2014, Zafra et al. 2014). Isolates belonging to the same species, i.e. *Klebsiella pneumoniae* and *Enterobacter cloacae*, were found to have 100 % similarity in their 16S rRNA sequences. These sequences were aligned with the corresponding sequences of several known hydrocarbon-degrading organisms, and the resulting phylogenetic tree indicated that these isolates were grouped into Proteobacteria (six isolates) and Firmicutes (three isolates) being Gammaproteobacteria the most represented class (Figure 1). Proteobacteria isolates are closely related between them and with previously reported aliphatic and aromatic hydrocarbon-degrading organisms.

Table 2: Molecular identification and PCR-detection of hydrocarbon-degrading genes in isolates from oil-contaminated soils. +: detected; -: Not detected.

Isolate	Closest related sequence (GenBank accession number)	Identity	PCR detection			
			nahH gene	*alkB* gene	GN-RHD gene	GP-RHD gene
PTRF01	*Stenotrophomonas maltophilia* BAC2031 (HM355622.1)	99 %	-	+	+	-
PTRF02	*Pseudomonas aeruginosa* ANF2 (KR809373.1)	99 %	-	+	+	-
PTRF03	*Pseudomonas aeruginosa* S7PS5 (KR349493.1)	99 %	-	-	+	-
PTRF04	*Klebsiella pneumoniae* VRBG-77 (KR265470.1)	99 %	+	-	-	-
PTRF05	*Klebsiella pneumoniae* VRBG-77 (KR265470.1)	99 %	+	-	-	-
PTRF06	*Enterobacter cloacae* RJ20 (KC990811.1)	99 %	+	-	-	-
PTRF07	*Klebsiella pneumoniae* VRBG-77 (KR265470.1)	99 %	+	-	-	-
PTRF08	*Bacillus subtilis* SCDB1470 (KM922588.1)	99 %	-	-	-	-
PTRF09	*Klebsiella pneumoniae* VRBG-77 (KR265470.1)	99 %	+	-	-	-
PTRF10	*Pseudomonas stutzeri* 46DMVR (KR140186.1)	100 %	-	-	+	-
PTRF11	*Pseudomonas stutzeri* ARO3 (KP744123.1)	99 %	-	-	+	-
PTRF12	*Bacillus pumilus* FeRB-FL1404 (KM405294.1)	99 %	+	-	-	-
PTRF13	*Klebsiella pneumoniae* VRBG-77 (KR265470.1)	99 %	+	-	-	-
PTRF14	*Enterobacter cloacae* VITPSSJ (KP305908.1)	100 %	+	-	-	-
PTRF15	*Bacillus firmus* strain Z1-7 (GQ927170.1)	99 %	-	-	-	-

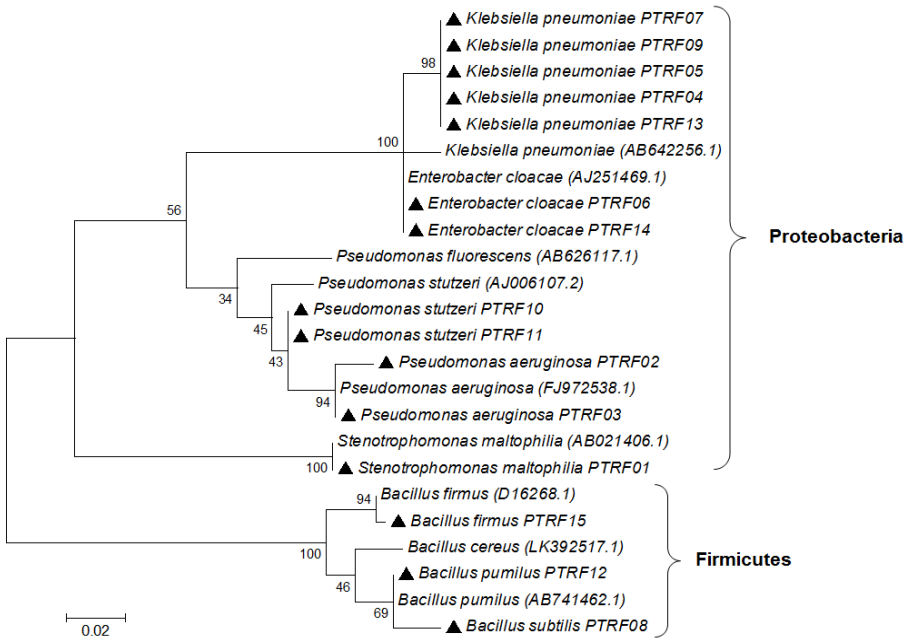
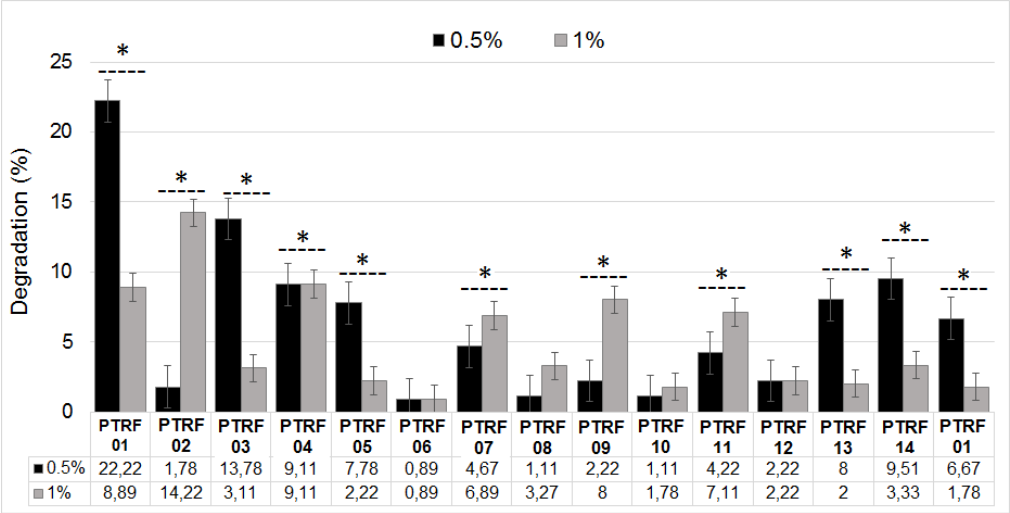


Figure 1: Consensus phylogenetic tree based on partial bacterial 16S rRNA sequences, inferred by using the Maximum Likelihood method. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) is shown next to the branches. Black triangles indicate the native organisms isolated in this study. GenBank accession numbers of reference sequences are indicated.

As shown in Figure 2, four (4) out of fifteen (15) isolates corresponding to *Pseudomonas aeruginosa* PTRF01, *Stenotrophomonas maltophilia* PTRF02 and PTRF03 and *Klebsiella pneumoniae* PTRF04 showed the highest TPH removal after 72 hours, compared with the other 11 isolates. These species have been previously described as excellent hydrocarbon degraders, able to metabolize a wide variety of aliphatic and aromatic compounds. On the other hand, *Enterobacter cloacae* PTRF06 and *Bacillus pumilus* PTRF12 showed the lowest removal values, reaching only 2 % removal. As described, bacterial degradation of hydrocarbons can occur either by aerobic or anaerobic pathway, although aerobic mechanisms are the most studied. The metabolism of aliphatic and aromatic hydrocarbons by aerobic bacteria is usually initiated by either monooxygenase or dioxygenase enzymes (i.e. alkane monooxygenase, ring-hydroxilating dioxygenases, catechol dioxygenase), which incorporate one or two atoms of O_2 into the hydrocarbon molecule to form one or more metabolites (Seo et al. 2009). Dihydrodiols and other intemediates are reduced by dihydrodiol dehydrogenases to form dihydroxylated aromatic intermediates (catechols), which then may serve as substrates for *ortho* and *meta* ring fission dioxygenases. The hydrocarbon products are further metabolized to tricarboxylic acid cycle intermediates and eventually mineralized to CO_2 (Seo et al. 2009).

Taking into account the above, the detection of hydrocarbon-degrading genes producing these enzymes is important to assess the potential of isolated microorganisms for hydrocarbon degradation and further use for bioremediation purposes. Thus, we screened the presence of four genes involved in the degradation of aliphatic and aromatic hydrocarbons by aerobic bacteria by PCR (Table 2). We detected the *nahH* gene, encoding the catechol-2,3 dioxygenase, in eight isolates (PTRF04, PTRF05, PTRF06, PTRF07, PTRF09, PTRF12, PTRF13 and PTRF14) corresponding to *Klebsiella pneumoniae*, *Enterobacter cloacae* and *Bacillus pumilus*. Interestingly, these isolates did not remove TPHs at the highest extent, except for the isolate PTRF04 (*Klebsiella pneumoniae*). On the other hand, in isolates PTRF01, PTRF02 and PTRF03 -showing in turn the highest degradation activity- only the *alkB* gene, the GN-RHDα gene or a combination of both were detected. According to our results, the presence of the *alkB* and GN-RHDα genes and not *nahH* influenced at a higher extent the degrading ability of these bacterial native isolates. As observed in Figure 3, the molecular characterization of the *nahH* gene by LSSP-PCR revealed different genetic signatures between similar isolates that could explain the different degradation rates in isolates from the same genus (i.e. *Klebsiella* and *Enterobacter*). As expected, the LSSP-PCR signature from *Bacillus pumilus* PTRF12 is notoriously different, revealing marked differences in *nahH* gene which could influence catechol dioxygenase enzyme activity and affinity.



	PTRF 01	PTRF 02	PTRF 03	PTRF 04	PTRF 05	PTRF 06	PTRF 07	PTRF 08	PTRF 09	PTRF 10	PTRF 11	PTRF 12	PTRF 13	PTRF 14	PTRF 01
0.5%	22,22	1,78	13,78	9,11	7,78	0,89	4,67	1,11	2,22	1,11	4,22	2,22	8	9,51	6,67
1%	8,89	14,22	3,11	9,11	2,22	0,89	6,89	3,27	8	1,78	7,11	2,22	2	3,33	1,78

Figure 2: TPH degradation by 15 native bacterial isolates in liquid cultures containing 0,5 and 1% used motor oil. Asterisk denotes significant differences between treatments.

One way to overcome the numerous barriers in the degradation of hydrocarbons, especially those of high molecular weight, is the use of defined co-cultures (mixed inocula or consortia) (Boonchan et al. 2000). These barriers include the inability of bacteria to transport high molecular weight hydrocarbons inside the cell due to the molecular size, the hydrocarbon not being a substrate for the available enzymes or not being a suitable inductor for transport or degrading enzymes (Juhasz and Naidu, 2000). There is also evidence for a cooperative degradation of aromatic hydrocarbons by bacterial-bacterial and fungal-bacterial consortia (Anastasi et al. 2009; Zafra et al. 2015). Subsequently, a degrading mixed inoculum composed of isolates PTRF01, PTRF02, PTRF03 and PTRF04 was constructed and its degrading potential tested in a two-liter bioreactor containing unsterile liquid oily wastes with 0.8 % (w/v) Total Petroleum Hydrocarbons (TPHs) concentration for 40 days. No microbial antagonism was detected between isolates PTRF01, PTRF02, PTRF03 and PTRF04 (data not shown). Overall, the use of the mixed inoculum led to a decrease of 98.4 % BOD, 97.5 % COD and 97.2 % TPHs after 42 days (Table 3). Even though control reactors also presented high degradation of COD, BOD and TPH, the use of the mixed inocula promoted a significantly faster degradation of TPHs especially at days 14 and 28. This, together with the presence of hydrocarbon-degrading genes confirm the high potential of these microorganisms to degrade hydrocarbons and remediate soils contaminated with waste oils.

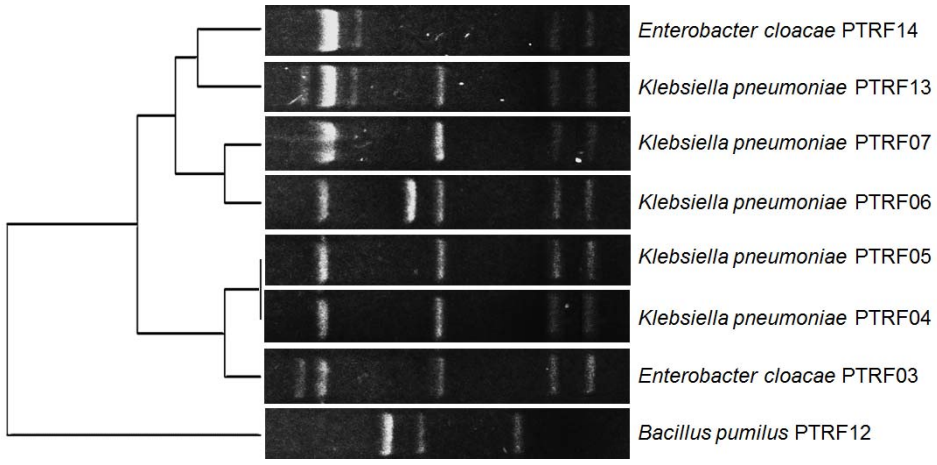


Figure 3: LSSP-PCR signatures of nahH gene sequences from eight hydrocarbon-degrading bacterial isolates. Clustering was performed using the UPGMA method.

Table 3: COD, BOD_5 and TPH levels monitoring during the treatment of oily wastes in liquid culture by a mixed microbial inocula.

Day	COD (mg O_2 L^{-1})		BOD_5 (mg L^{-1})		TPH (mg L^{-1})	
	Control (removal %)	Inoculated (removal %)	Control (removal %)	Inoculated (removal %)	Control (removal %)	Inoculated (removal %)
0	50125	50125	6,255	6,255	3,260	3,260
7	36,373.7 (27.4)	38,916,7 (22,4)	4,878.7 (22)	4,183 (33.1)	1,797.3 (44.9)	1,056 (67.6)
14	29,175 (41.8)	20,921 (58,3)	4,317 (31)	1,297 (79.3)	1,800.5 (44.8)	534.3 (83.6)
28	8,950 (82.14)	3,856 (92,3)	1,129.7(82)	483 (92.3)	719.3 (77.9)	161.2 (95.1)
42	3,306.7 (93.4)	1,267,7 (97,5)	623 (90)	103 (98.4)	375.7 (88.5)	92.3 (97.2)

4. Conclusions

The degrading mixed microbial inoculum described in this study presented high potential for the treatment of impacted soils at automotive service stations and sites polluted with oily wastes due to its elevated growth at high hydrocarbon concentrations, its capacity to utilize used motor oils as energy source and its ability to rapidly reduce BOD, COD and TPH contents. Further studies testing the treatment of polluted soils are necessary to better address the bioremediation potential of this microbial consortium.

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