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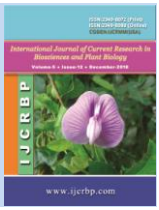

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Exoenzymatic Profile of Hydrocarbon-Degrading Bacteria Present in Oil-Contaminated Soils

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ABSTRACT

The presence in oil-contaminated soils of an abundant oil-degrading indigenous microbiota, able to hydrolyze additionally a diversity of bio-polymers by secreting specific enzymes, could explain in part the efficient bioremediation generally observed when oil-polluted soils are composted with a variety of bio-organic wastes. The present study achieved in 12 oil-contaminated soils, demonstrated in all cases their unsuitable nutrient content; however, their oil-degrading bacterial counts fluctuated mainly between 10^6-10^7 c.f.u./g soil. The exoenzymatic profile studied in 100 hydrocarbon-degrading bacteria isolated from such soils, gave a percent frequency of positivity of amylases 75, lipases 73, keratinases 67, caseinases 61, elastases 36, chitinases 34, lecithinases 30, chitosanases 25, DNases 19, pectinases 15, cellulases 7 and esterases 6. Most of these isolates (90%) produced 2-5, but some can secrete until 8-10 different exoenzymes.

Introduction

Organic refuses, particularly cellulosic ones, have been used for remediation of oil-contaminated soils by composting (Ro et al., 1998; Al Daher et al., 2001). Animal wastes including different manures, organic and inorganic substances such as urea, gypsum and water, are also added to achieve the appropriate carbon, nitrogen, phosphorus and mineral contents (Ravindran et al., 2017). This is done to obtain the adequate values of moisture and pH that favor the growth of hydrocarbon-degrading microorganisms present in the soil under treatment (Gupta & Tao, 1996; Guerin, 2001; Baudin et al., 1996). It is also worth noting that in most cases, independent of the type of waste used, the removal

of pollutants is successful. The explanations have been that such waste materials provide additional biota, thus contributing to the degradation of contaminants; that the aeration is improved due to an increase in the soil porosity, and that wastes represent additional nutrients sources for the indigenous soil microbiota. In the event that the results are not satisfactory, it is argued that some wastes are degraded so slowly that they become additional pollutants; that the absorption of the oil-hydrocarbons inside the waste particles obstruct their microbial biodegradation, and that being the wastes carbon/nitrogen sources, they are preferably degraded respect to the oil-hydrocarbons.

With regard to the importance of the exohydrolytic activity of the hydrocarbon-degrading indigenous microbiota, to explain successful remediations of oil-contaminated environments by composting with bio-organic wastes, it was found recently (Cervantes-González et al., 2008), that in drilling-waste polluted soils, there are bacteria able to produce chitinases and keratinases. When oil and keratinous wastes (milled chicken feathers) are present in submerged cultures of keratinolytic bacteria, the oil-hydrocarbons removal increased 60% and the cell concentration was 10 times greater, than that obtained when oil-hydrocarbons were the only carbon source. Besides, the oil removal, keratinous waste consumption and keratinase production were correlated. In the present paper the ability to produce three different proteases, five polysaccharidases, three lipo-esterases and one nuclease was evaluated in 100 hydrocarbon-degrading bacterial isolates, recovered from oil-contaminated soils. The results could be useful to know which wastes could be suitable for the composting of oil-polluted soils.

Materials and methods

Soil source and characterization

Soil samples were collected from a hydrocarbon-polluted site located in Tabasco, México. Six parameters were analyzed in 12 soil samples via the following processes: moisture was determined by drying 2 g of soil at 105°C until constant weight. The pH was measured with a pH-meter in a 10% aqueous soil suspension. The total organic carbon content was measured by burning 0.5 g of soil in a TOC-5000A Shimadzu Analyzer at 900°C. The nitrogen content was measured in a micro-Kjeldahl apparatus, using 0.3 g of soil (AOAC, 1970). The phosphorus content was determined by the stannous chloride method using 1 g of soil (APHA, 1974). The total petroleum-hydrocarbons (TPH) were measured by refluxing 10 g of soil in a Soxhlet apparatus using dichloromethane as solvent. The organic extract was concentrated and the residual TPH was determined by gravimetry (Fernández-Linares et al., 2007).

Soil bacterial counts

Heterotrophic bacteria were determined by suspending 2.5 g of soil in 25 ml of nutrient broth and rotating at 180 rpm and 28°C for 2 h. Subsequently, 0.1 ml aliquots were diluted as required and 0.1 ml of the suitable dilutions were spread on nutrient agar plates. The colonies forming units (c.f.u./g soil) were counted after incubating at 28°C for 3 days. The hydrocarbon-degrading bacteria were counted similarly by using a mineral medium (MM) solidified with agar. The MM contained (g/l) KNO_3 1; $FeCl_3$ 0.02; $MgSO_4$ 0.2; $CaCl_2$ 0.1 and K_2HPO_4 1, at pH 7. After spreading the sample (0.1 ml of the appropriate dilutions of the fresh cultures grown in nutrient broth), filter paper circles impregnated with sterile crude oil were tightly placed on the underside of the plate covers and plates were sealed with parafilm tape; the volatile hydrocarbons accumulated inside served as carbon sources. Counting of c.f.u./g soil was carried out after incubating for 7 days at 28°C (Cervantes-González et al., 2008).

Oil-degrading bacterial isolates

The colonies grown on the plates used to count these particular bacteria were examined with respect to their colony and microscopic characteristics, and 100 representative isolates were selected. In this group some specimens of similar

appearance were included, because alike colonies were considered as different if they evolved from distinct soils.

Enzymatic assays

These evaluations were performed in both liquid and agar-solidified media, utilizing the MM added with particular carbon/nitrogen sources that also served as the enzyme inducers. The media previously adjusted to a pH of 6.8-7 was autoclaved at 121°C under a pressure of 15 psi for 15 min. For inoculations, fresh cultures of oil-degrading bacterial isolates grown in nutrient broth under rotation (180 rpm) at 28°C for 18 h were used.

For plate assays bacteria were inoculated by puncture with sterile toothpicks, followed by incubation at 28°C until visualization of the characteristic haloes produced by every exoenzyme. Additional data on the plate assays includes: for caseinase, 1% casein was dissolved with MM in a porcelain mortar and then heated in a boiling water bath for 30 min before the addition of 1.5% agar. After 48 h the resultant haloes showed whitish opaque external edges of coagulated casein in the first stage of hydrolysis, meanwhile their internal zones were transparent, due to an extensive degradation of casein into small peptides. Consequently, the size and aspect of the haloes gave an arbitrary approach to evaluate activity (Rojas-Avelizapa et al., 1999). For elastase the plates contained 0.4% elastin and the activity was observed after 96 h as clear haloes in a granular–opaque background (Kaur et al., 1988). For amylase, the plates contained 1% starch. Transparent or yellowish haloes were visible on a dark-violet background by flooding with lugol (Dahwale et al., 1982). Pectinase was assayed in plates with 1% pectin. After 96 h the plates were flooded with 5 % cetyltrimethyl ammonium bromide to precipitate the non-digested polysaccharide, and clear haloes on a whitish-opaque background were observed (Hubbel et al., 1978). For the chitinase assay, the plates contained 12% colloidal chitin prepared by swelling this substrate with phosphoric acid (Chávez-Camarillo & Cruz-Camarillo, 1984). After 6 days, transparent haloes on a white granular background were observed. For the chitosanase assay, the plates contained 3% colloidal chitosan, obtained by swelling with phosphoric acid and precipitating with cold ethanol (Cruz-Camarillo et al., 2004). Transparent haloes were observed after 144 h. For lipase detection, plates contained 5% butter and 0.015% resazurin as a pH-indicator. The fatty acids released by the enzyme action produced pink haloes on a violet background after 96 h (Tenorio-Sánchez, 2000). For the lecithinase assay, the medium contained 3% homogenized egg yolk. The production of opaque-haloes after 48 h indicated the action of type C lecithinases (Crisope et al., 1976). To detect esterases, 0.4% tween 80 and 0.05% calcium chloride were added to the medium. After 144 h opaque-granular haloes of precipitated calcium oleate were observed (Lovell & Bibel, 1977). For exonuclease, 0.03% salmon sperm DNA and 0.02% ortho-toluidine blue were added to the medium. After 24 h, positive colonies presented red haloes on a dark-blue background (Lachica et al., 1971). To validate plate assays the following positive controls were included: *Serratia marcescens* WF for protease, elastase, chitinase, lipase, lecithinase, esterase and DNase; *Bacillus thuringiensis* 132 for amylase and chitosanase; *Erwinia carotovora* for pectinase and *Trichoderma reesei* for cellulase.

In the case of keratinase, cellulase and chitinase (last one assayed also by plate), evaluations in submerged cultures were achieved using 125 ml Erlenmeyer flasks containing 25 ml of MM and 250 mg of one insoluble substrate that served as the enzyme inducer and also as the carbon source. Such substances included wool, acid-swelling filter paper and colloidal chitin that were firstly stained with Remazol Brilliant Blue, according to a previously described technique (Gómez-Ramírez et al., 2004). In this case, flasks were inoculated with 0.1 ml of the fresh bacterial cultures and incubated by rotating (180 rpm) at 28°C. Every 24 h one inoculated and one non-inoculated flask (negative control) were retired to evaluate in supernatants the absorbance at 595 nm of the dye released when the substrate was enzymatically hydrolyzed.

Results

Soils characterization

In table 1 it is shown that 8 of the 12 soils studied had less than 30% moisture. Their pH ranged from 6.8 to 10.2, however it predominantly fluctuated between 7.4 – 7.9. Their organic matter, considered according to Sundermeier (2005) as 57% of the carbon content was commonly low, illustrated by the fact that eight samples had less than 4% and only two soils contained 8-9%. Further data indicated that the nitrogen content was low; fluctuating between 0.01 and 0.13%, while the phosphorus values oscillated widely from 1.9 to 9.4%. The oil content was also variable, reaching values ranging from 582 to 174,173 mg/kg soil, which show the great variation of petroleum-pollution in the studied area. The above-mentioned data indicate that none of the soils studied had suitable C/N/P ratios to support a good microbial growth, since according to Bossert & Bartha (1984), such values should be close to 100/10/1. Additionally, the soils studied possessed a relative excess of phosphorus. Finally, the only adequate parameter was pH, whose values ranged within 6.5 to 8, and then it is advisable according to Baker & Herson (1994). Despite their unsuitable characteristics, in the studied soils the heterotrophic bacteria reached counts within 10^5 -10^8 cfu./g soil, being a variable proportion of them (1.4 to 86.4 %) able to use oil as the only carbon source.

Table 1. Characteristics of the soil samples collected around the oil well Paredón 31.

Soil samples	Moisture (%)	pH	Organic matter (%)	N (%)	P (%)	TPH (mg/kg)	Heterotrophic bacteria (cfu./ g soil)	Hydrocarbon-degrading bacteria (cfu./ g soil)	Proportion of hydrocarbon-degrading bacteria (%)
1	12.0	7.6	2.6	0.09	5.8	13,020	$5.1X10^7$	$1.2X10^7$	23.5
2	16.7	7.5	1.6	0.06	6.5	49,477	$4.7X10^7$	$2.6X10^7$	55.3
3	44.5	8.2	3.5	0.04	5.1	62,139	$8.5X10^7$	$1.3X10^7$	15.3
4	25.7	6.8	ND	ND	ND	ND	$5.4X10^7$	$4.1X10^7$	75.9
5	29.3	7.9	9.0	0.04	4.3	174,753	$7.5X10^7$	$7.7X10^6$	10.3
6	12.0	7.4	ND	0.03	1.9	2,465	$1.1X10^6$	$9.5X10^5$	86.4
7	22.2	7.8	3.8	0.01	2.7	10,809	$4.2X10^5$	$5.7X10^3$	1.4
8	22.7	7.4	1.6	0.03	1.7	1,183	$7.6X10^6$	$5.9X10^6$	77.6
9	32.8	7.6	3.2	0.02	2.9	54,139	$9.4X10^7$	$7.1X10^6$	7.6
10	26.6	7.8	8.0	0.13	9.4	17,128	$3.3X10^8$	$5.3X10^7$	16
11	35.5	10.2	1.6	0.02	3.9	72,521	$2.5X10^6$	$4.9X10^5$	19.6
12	14.8	7.5	0.4	0.02	2.4	582	$7.4X10^6$	$5.7X10^6$	77

TPH, total petroleum hydrocarbons; N, nitrogen; P, phosphorous; ND, non-determined.

General characteristics of the oil-degrading bacteria

When counting the oil-degrading microbiota in the soil samples, bacteria were by far the main population, with only one fungus and a few actinomycetes colonies being found otherwise. Related to the 100 bacterial isolates chosen for this study, their cell-shape corresponded mainly to small non-sporulated bacilli (70% Gram-negative, and 15% Gram-positive). The Gram-positive bacilli of median size were 13%; whereas 2% corresponded to Gram-positive small cocci. That aside, the bacterial colonies were usually small, round and wet-bright with a convex surface, pigmented in white, yellow or orange colors. Regarding their identification, in other parallel work where only the most keratinolytic and chitinolytic isolates were studied by 16S rDNA, the following six bacterial genera were found: *Bacillus*, *Brevibacillus*, *Micrococcus*, *Nocardia*, *Nocardiodes*, *Pseudomonas* and *Stenotrophomonas* (Cervantes-González et al., 2008).

Table 2. Production of 12 different hydrolytic enzymes by hydrocarbon-degrading bacterial isolates recovered from oil-polluted soils.

Enzyme	Substrate characteristics	Frequency (%)	The 3 more active isolates*	Enzyme activity of the best isolates with respect to the reference strain values.
Caseinase	Casein; milk phospho-protein which contains 15 different aminoacids, MW 375 kDa, P.I. 4.7. Sparingly soluble in water.	61	EW1-A-d EW4A-i EW6A-d	As much 80 % of the activity of *S. marcescens WF*.
Elastase	Elastin; chromoprotein from conective tissues, rich in valine. It is an insoluble and elastic molecule.	36	EW4-A-k EW6A-d EW4A-h	3 times more activity than *S. marcescens WF*.
Keratinase	Keratin; insoluble structural protein from wool, hair, nails, feathers and horns, with a high content of cystine.	67	EW1-A-d EW4A-h EW5A-c	25 % more active than *S. marcescens WF*.
Amylase	Starch; plant storage polymer of glucose, composed by 27% of amylose (lineal) and 73% amylopectin (branched) molecules.	75	EW-2A-c EW-2A-d EW-5A-f	30 % more active than *B. thuringiensis* Bt-132
Cellulase	Cellulose; plant structural and insoluble polymer, formed by glucose units joined by β1-4 glycosidic bonds.	7	EW-5A-a EW-6A-b EW-2A-d	90 % less active than *Trichoderma reesei*
Chitinase	Chitin, structural and insoluble polymer of N-acetylglucosamine joined by β 1-4 glycosidic bonds. Present in insect and crustacean shells, and in fungi cell walls.	34	EW-1A-c EW-6A-d EW-11A-d	50 % less active than *S. marcescens WF*.
Chitosanase	Chitosan, soluble at acid pH. Polymer of glucosamine and N-acetilglucosamine joined by β 1-4 glycosidic bonds, present in the cell wall of some fungi.	25	EW-7A-c EW-4A-d EW-9A-a	Similar activity to that found in *B. thuringiensis* Bt-107.
Pectinase	Pectin; mixture of methylesterified galacturonan, galactan and araban; found in roots, stems and fruits of plants, where is acting as a cementing substance.	15	EW-8A-b EW-4A-h EW-2A-d	10-20 % less active than *E. carotovora*.
Lipase	Lipids; triglycerides where the alcoholic groups of glycerol are esterified by fatty acids. Widely distributed in all living beings.	73	EW-1A-c EW-4A-d EW-6A-a	Similar activity to that produced by *S. marcescens WF*.
Lecithinase	Lecithins; where two alcoholic groups of glycerol are esterified with fatty acids, and the other one with phosphorylcholine. Present in all living beings.	30	EW-8A-c EW-6A-b EW-12A-a	100 – 300 % more active than *S. marcescens WF*.
Esterase	Esters; compounds where alcoholic groups are esterified with organic acids. All lipidic substances are esters.	6	EW-4A-k EW-6A-d EW-4A-h	Similar activity than *S. marcescens WF*.
DNase	DNA; the genetic material, polymer of deoxyribonucleotides joined by 3´, 5´phosphodiester bonds.	19	EW-5A-b EW-9A-e EW-6A-b	As much 80 % of the activity of *S. marcescens WF*.

* In the naming code of the isolates, EW means exploratory well; the arabic numeral and the capital indicates the soil sample; the small letter is refered to one of the various isolates obtained.

Exoenzymatic profile of the oil-degrading bacteria

The table 2 shows that 12 substances, embracing four of the more important bio-molecules, represented by 3 different proteins, 5 various polysaccharides, 3 distinct ester-like substances and one polynucleotide, were used as the substrates to assay the hydrolytic ability of the isolates studied. Additional data on the chemical structure, location and biological function of these substrates are also indicated to emphasize their notable differences. It was thus interesting, that the assayed substrates were all enzymatically attacked, although to varying extents. Consequently, the frequencies of positivity were in a descending order: amylases 75, lipases 73, keratinases 67, caseinases 61, elastases 36, chitinases 34, lecithinases 30, chitosanases 25, DNases 19, pectinases 15, cellulases 7 and esterases 6%. This result was noticeable in that the enzymatic levels produced by the more active isolates in the cases of amylases, lipases and keratinases, were as high as those found with the reference strains. Furthermore, the isolates showed particular profiles, with respect to the number and type of hydrolases that each one was able to produce. Almost 90% of the isolates produced at least 2-5 of the studied enzymes, but only one secreted 10 of the 12 studied enzymes, although not all at a high level.

Discussion

The The restoration of oil-polluted soils by composting with diverse bio-organic wastes has generally been successful. This is provided that the adjustments of such factors as moisture, pH and C/N/P ratios promote the growth of a myriad of heterotrophic indigenous microorganisms able to degrade simultaneously petroleum hydrocarbons and the organic wastes. These studies have been focused on the evaluation of physico-chemical parameters to find simple and reliable indicators of maturity and to improve the efficiency of the composting process, while very little is known about the indigenous microbiota which determine the rate of composting, affect the quality of the product, and produce most of the physical and chemical changes in the compost (Tichia et al., 2002). It is also known that the oil-degrading bacteria isolated more frequently in oil-polluted soils include in a descending order: *Pseudomonas*, *Arthrobacter, Alcaligenes, Corynebacterium*, *Flavobacterium, Achromobacter, Micrococcus*, *Nocardia* and *Mycobacterium* (Englert et al., 1993). Unfortunately, the information about their hydrolytic exoenzymes is scarce.

The present work involved the characterization of 12 soils collected next to an oil-well in disuse and the study of the exoenzymatic profile of 100 oil-degrading bacterial isolates recovered from these soils. Concerning to the first point, the soils showed widely varying oil content, because some areas were preferred to discard wastes after oil-extraction. Other parameters showed that with the exception of pH, they were all inappropriate to support abundant microbial growth. The C/N/P ratios were also unsuitable because they should be close to 100/10/1 (Bossert & Bartha, 1984) and in this case low nitrogen and high phosphorus contents were found. Nevertheless, in most soils the counts of heterotrophic bacteria ranged between 10^6-10^7 cfu./g soil, with proportions of hydrocarbon-degrading bacteria of 1.4 to 86.4%. However, no correlation was found between both types of bacterial populations with some of the parameters evaluated. Apparently, the type of microorganisms and their cellular concentrations could depend on multiple environmental factors, many more than those examined in this study, which could explain the great variation of results reported by different authors. As a reference, in non-contaminated, well nutrient balanced soils, the microbial population reached values as high as 10^8-10^9 cfu./g dry soil (Prescott et al., 2004). In oil-polluted soils, some have reported 6 x 10^4 and 1.7 x 10^2 cfu./g soil of heterotrophic and oil-degrading bacteria, respectively (Pala et al., 2002). Others have found counts for heterotrophic bacteria of 10^6 to 10^7 cfu./g soil and similar values for the oil-degrading ones. The counts for heterotrophic remained constant, but decreased significantly for the oil-degrading bacteria along the bioremediation process (Al-Daher, 2001). Other behavior was reported by Cardona & Iturbe (2003) indicating that counts

changed from 10^5 to 10^7 c.f.u./g soil for heterotrophic bacteria in oil-polluted soil before and after its bioremediation by composting. The lack of correlation found in the present work between the oil content in the soils and their counts of oil-degrading bacteria is in agreement with a previous report (Margesin et al., 2003).

Concerning to the exohydrolytic profile of the oil-degrading bacterial isolates, the enzymes more frequently found and as well produced at higher levels were: amylases 75, lipases 73, keratinases 67 and caseinases 61%. A second group included elastases 36, chitinases 34, lecithinases 30 and chitosanases 25 %. The lower frequencies corresponded to DNases 19, pectinases 15, cellulases 7 and esterases 6 %. Most isolates (92 %) produced 2-5 different enzymes, but only one secreted 10, and two 8-9 of the 12 assayed enzymes. In this respect, only two of the studied enzymes have been reported in hydrocarbon-degrading bacteria, those being lipases and proteases. In the first case it was observed during bioremediation of an oil-polluted soil, previously fertilized, that the count of the oil-degraders increased from 10^5 to 10^7 cfu./g soil, which correlated with the increase of the soil lipase activity, whereas the oil content was reduced from 5 to 0.5 mg/g soil after 30 days. Similar correlations occurred during decontamination by natural attenuation in a non-fertilized soil (Margesin et al., 1999). With respect to proteases, it was observed that *Nocardiopsis* sp. NCIM 5124 isolated from an oil-contaminated marine environment could be cultured in a defined sea water medium containing alkanes, glucose, casein and starch. In this scenario, the actinomycete degraded the hydrocarbons and simultaneously secreted proteases (Dixit & Pant, 2000). Something similar was observed recently when an oil-degrading bacterial consortium able to produce extracellular keratinase was cultured in a synthetic liquid medium containing crude oil and milled chicken feathers as the carbon/nitrogen sources. In such conditions, the kinetics outlines of growth and keratinase correlated with the oil removal and the feathers consumption (Cervantes-González et al., 2008).

Conclusion

The present study indicates that in oil-polluted soils there is an abundant oil-degrading bacterial microbiota, as well able to hydrolyze polymeric molecules as polysaccharides, proteins and lipids. Therefore, some agro-industrial wastes rich in starch, lipids or proteins such as potatoes and banana discards, rest of oleaginous seeds and chicken feathers, could be useful to enhance the removal of oil-hydrocarbons by composting.

Conflict of interest statement

Authors declare that they have no conflict of interest.

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