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Evaluation of growth in diesel fuel and surfactants production ability by bacteria isolated from fuels in Costa Rica

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***Abstract*:** A total of 149 bacterial strains previously isolated from fuels stored in Costa Rica were selected in terms of their ability to grow aerobically in diesel and produce bioemulsifier active compounds. The diesel growth was evaluated by a redox-indicator based test, and surfactant production was estimated indirectly by both the emulsification index determination (E_{24}) and hemolytic activity. Twenty-six strains (16.8%) were considered as capable of growing in diesel, while surfactant production was detected in 22 (14.8%), estimated according to E_{24}. Seven strains showed high production of biosurfactants ($E_{24} \geq 50\%$), headed by *Pseudomonas aeruginosa* 148D-O, *P. aeruginosa* 87R-B and *Bacillus pumilus* 133S-B. No significant correlation was observed between hemolytic patterns and growth outcomes in diesel or E_{24}. Surfactant producing strains should be studied further to assess its potential applications.

Keywords: bioremediation, biosurfactant, emulsification index, fuel, diesel.

Evaluación de crecimiento en combustible diesel y capacidad de producción de surfactantes en bacterias aisladas de combustibles en Costa Rica

***Resumen*:** Un total de 149 cepas bacterianas previamente aisladas de combustibles almacenados en Costa Rica fueron seleccionadas en términos de sus habilidades para crecer aeróbicamente en diesel y producir compuestos con actividad bioemulsificante. El crecimiento en diesel fue evaluado por medio de un test basado en un indicador redox, y la producción de surfactantes fue estimada indirectamente con las determinaciones del índice de emulsificación (E_{24}) y la actividad hemolítica. Veintiseis cepas (16,8%) fueron consideradas como capaces de crecer en diesel, mientras que la producción de surfactantes fue detectada en 22 (14,8%), estimado de acuerdo con el E_{24}. Siete cepas mostraron alta producción de biosurfactantes ($E_{24} \geq 50\%$), encabezadas por *Pseudomonas aeruginosa* 148D-O, *P. aeruginosa* 87R-B y *Bacillus pumilus* 133S-B. No se observó correlación significativa entre los patrones de hemólisis y los resultados de crecimiento en diesel o E_{24}. Las cepas productoras de surfactantes deben ser estudiadas más a fondo para evaluar sus potenciales aplicaciones.

Palabras clave: biorremediación, biosurfactante, índice de emulsificación, combustible, diesel.

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Introduction

Bacterial microbiota from regular gas, premium gas and diesel was recently determined for the first time in Costa Rican fuels [1], although the ability of these isolates to grow in the hydrocarbons was not evaluated. To assess the importance of particular microorganisms in fuel deterioration, it is essential to determine their ability to grow, rather than merely exist in fuel systems [2]. The presence of microorganisms in fuel storage tanks and transportation systems may cause problems such as acid formation, increase in viscosity, suspended solids (sludge) and corrosion residues, which translate in filtration problems, deterioration of equipment and loss of fuel quality [3].

In addition, growth on fuels may be correlated to the production of biosurfactants, interesting compounds with potential industrial and biotechnological applications. Biosurfactants are microbial molecules which exhibit high surface and emulsifying activities, and their production is closely related to the ability to grow in hydrocarbon contaminated environments, since they are predominantly produced during growth on water-immiscible substrates [4-6]. Most surfactants produced today are synthetic; however, the use of biosurfactants has advantages such as

biodegradability, low toxicity and economically susteinable production cost [7], which makes them an attractive option for industrial purposes. The biosurfactants most commonly isolated are glycolipids containing sugars as rhamnose and trehalose [8], although other different kinds, produced by a wide variety of microorganisms, have been described [6]. Some biosurfactant applications include: oil recovery, additives in food industry, health care and cosmetic industry, microbial remediation of hydrocarbons and crude oil contaminated soils [6-9].

This work aimed to evaluate the ability to grow in diesel oil of bacteria isolated from fuels, and to find indigenous microorganisms from Costa Rican environments capable to produce emulsifier compounds.

Materials and methods

Growth on diesel test: Diesel degradation ability was evaluated in 149 bacterial strains previously isolated from storage tanks of automotive fuels from Costa Rican oil distribution facilities, identified by miniaturized biochemical-test galleries as described by Rodríguez-Rodríguez *et al.* [1]. Diesel oil is an excellent model for hydrocarbon biodegradation studies [5] and also it may act as a substrate for biosurfactant production. A modified version of a methodology based on a redox-indicator change, developed by Hanson *et al*. [10] was used; briefly, 125 µL suspension of each strain (30% T, λ = 600 nm) was added separately onto duplicate test tubes containing 7.5 mL of sterile Bushnell-Hass medium (which lacks of carbon sources) [11] supplemented with triphenyl tetrazolium chloride (TTC, 0.225 gL^{-1}) and 50 µL of diesel-oil. Tubes were incubated at 25 °C for 21 days, and daily monitored for the production of a reddish color, considered as a positive result. Tubes without bacterial inoculum were used as negative controls. This metod is based on the use of TTC as an electron acceptor, which receives electrons from microbial oxidation of hydrocarbons and changes from the colorless (oxidized-form) to the red-reddish (reduced-form).

Hemolyitic activity: Hemolytic activity was also assessed on bacterial strains by plating cells (a loop) onto duplicate blood agar plates and subsequent incubation (25 °C and 35 °C respectively). Hemolytic activity is considered by some authors as indicative of biosurfactant production and used as a rapid method for bacterial screening [5,12,13].

Surfactant production screening: Potential diesel-degraders were screened for surfactant production by determination of the Emulsification Index (E_{24}) [4,5,14-16]. Previously, Correa Bicca *et al*. [5] designed a yeast extract-containing medium called M2, and demonstrated its improved performance for biosurfactant production. Inocula for E_{24} tests were prepared by adding a loop of each strain into flasks containing 50 mL of M2 medium supplemented with 1% of diesel and subsequent incubation in agitation (200 rpm) at 28 °C for 48 h; then O.D. was adjusted at 0.650±0.030 (λ=600 nm). Cultures for E_{24} tests were performed in triplicate, adding 5 mL of the inoculainoculum into flasks containing M2 medium, to obtain a final volume of 50 mL and a diesel concentration of 1%; flasks were incubated at 28 °C in agitation at 200 rpm. Negative controls without bacteria were used by triplicate. At times 0, 1, 2, 3, 4, 7, 9 and 10 days, aliquots of 2 mL were withdrawn from the cultures and mixed in vortex for 2 minutes with 2 mL of sterile diesel in flat bottom tubes (diameter 1.5 cm); the tubes were allowed to stand for exactly 24 h. E_{24} index was given as the percentage of emulsified layer height (mm) divided by the total height of the liquid column (mm) [17].

Results and discussion

A set of 149 bacterial strains, previously obtained from storage tanks of the main automobile fuels (gasoline, *premium* gasoline and diesel), was evaluated for a fuel degradation test. A total of 25 strains (17%) showed a positive result in the diesel-degrading screening test. The time necessary to produce a color change in TTC differed among the strains, as it is observed on table 1, ranging from one day for *Pseudomonas aeruginosa* 149B-D, to 8 days for *Bacillus pumilus* 133S-B, with a mean of 5 days. These results suggest that only a fraction of the microorganisms previously isolated from stored Costa Rican fuels are able to exert some degree of diesel degradation and consequently reduce the quality. It is remarkable that all the strains of *P. aeruginosa* were able to grow on diesel, as well as *P. pseudoalcaligenes*, but not the remaining strains of this genus. *Pseudomonas* metabolic diversity related to hydrocarbon assimilation has been described, and the use of some strains for the removal of fuel from polluted sites has been documented [6,8,18,19]. Additionally Gram-negative diesel-degraders were only represented by *Burkholderia cepacia*, best known for its ability to degrade polycyclic aromatic hydrocarbons [20]. Among Gram-positive bacteria, the most frequently diesel-degrading genera were *Bacillus* (5 strains) and *Micrococcus* (4 strains), representing 18% and 15% respectively. The ability to degrade some fuel components has been reported for species such as *Micrococcus* sp., *Bacillus* sp., *B. cereus*, *B. sphaericus*, *B. pumilus* and *B. subtilis* [21-23]. On the other hand, only two out of 32 *Staphylococcus* strains showed positive results in the screening test. Arthrobacter stood out among the remaining isolates, since it is considered as one of the main fuel degraders in soil [21]; *Rhodococcus*, a metabolic-diverse nocardioform microorganism [5] and *Deinococcus* or *Kurthia*, are unusual hydrocarbon-degraders too.

Regarding the production of bioemulsifier compounds, tested by monitoring E_{24} in 10 day-cultures supplemented with diesel, 22 strains from the 25 potential diesel-degraders (88%) yielded positive results. This is not surprising, since biosurfactant production is correlated to the ability to grow in polluted environments with highly hydrophobic substrates [6]. However, E_{24} results for most of the strains were below

Table 1. Emulsification index E_{24} and hemolytic activity in potential diesel oil degrading strains isolated from stored Costa Rican fuels.

Strain	Diesel-degrading ability screening test, time for color change (days)	E_{24} (maximum, %)	Time to maximum E_{24} (days)	Hemolytic activity[a]	
				25 °C	35 °C
Arthrobacter cumminsii 25D-B	5	17	10	G	G
Bacillus sp. 86S-B	6	0	-	G	G
Bacillus sp. 125S-G	4	0	-	G	G
Bacillus laevolacticus 89S-B	6	17	10	G	G
Bacillus megaterium 122R-G	6	57	10	G	B
Bacillus pumilus 133S-B	8	62	10	B	B
Burkholderia cepacia 111D-G	6	12	10	G	G
Deinococcus radiodurans 54R-M	4	56	10	G	G
Kocuria varians 101S-G	6	17	9	G	B
Kurthia gibsonii 102S-G	6	19	10	B	B
Kytococcus sedentarius 82S-O	6	0	-	G	N.G.
Micrococcus sp.76D-O	6	39	9	G	A
Micrococcus sp. 78R-O	5	23	4	G	G
Micrococcus sp. 92S-B	5	50	10	G	G
Micrococcus lylae 131S-B	4	19	10	G	B
N.I. 110D-G [b]	5	15	7	G	G
N.I. 114R-O [b]	5	58	10	G	G
Pseudomonas aeruginosa 87R-B	5	65	7	B	B
Pseudomonas aeruginosa 88R-B	5	48	10	B	B
Pseudomonas aeruginosa 148D-O	4	77	9	G	N.G.
Pseudomonas aeruginosa 149D-B	1	11	10	G	B
Pseudomonas pseudoalcaligenes 105D-G	5	15	9	G	G
Rhodococcus sp. 139R-O	4	23	10	G	G
Staphylococcus haemolyticus 144S-B	3	21	9	G	G
Staphylococcus hominis 146R-M	4	8	1	G	B

[a] A: alpha-hemolysis; B: beta-hemolysis; G: gamma-hemolysis (no hemolysis); N.G.: no growth. [b] Non-identified strains; both correspond to Gram-positive aerobic bacilli.

50% (Table 1). Therefore, and since most published data reported E_{24}>50% for potential biosurfactant producing microorganims in the present work we have identified seven promising microbial candidates for biosurfactant production. Nonetheless, it should be taken into account that the maximum E_{24} value was obtained in most cases after 10 days of culture (Table 1, figure. 1), which indicates that higher values might be accomplished by longer periods than those tested here. Those promising strains are headed by *P. aeruginosa* 148D-O (E_{24}=77%, 9 d), *P. aeruginosa* 87R-B (E_{24}=65%, 7 d) and *B. pumilus* 133S-B (E_{24}=62%, 10 d). E_{24} values ranging from 70 to 78% have been reported for hexadecane and diesel oil in surfactant-producing *P. aeruginosa* strains [14,15,17]. Similarly, values of 59% for diesel and values ranging from 66 to 76% for other hydrocarbons, have been obtained with *B. pumilus* [4,5]. Reports of surfactant-producing strains of *B. megaterium* have been published [13], although no results of E_{24} are available; in this work, *B. megaterium* 122R-G yielded an E_{24} of 57%. Several studies report the ability of both bacterial genus, *Pseudomonas* [24-26] and *Bacillus* [24,27-30], to produce diverse biosurfactants as well.

In the case of *Micrococcus* sp., values up to 50% were obtained, while Adebusoye *et al*. [14] reported 49% with diesel and Toledo *et al*. [16]values ranging from 65 to 73% with other hydrocarbons. The finding of E_{24}=56% for *D.*

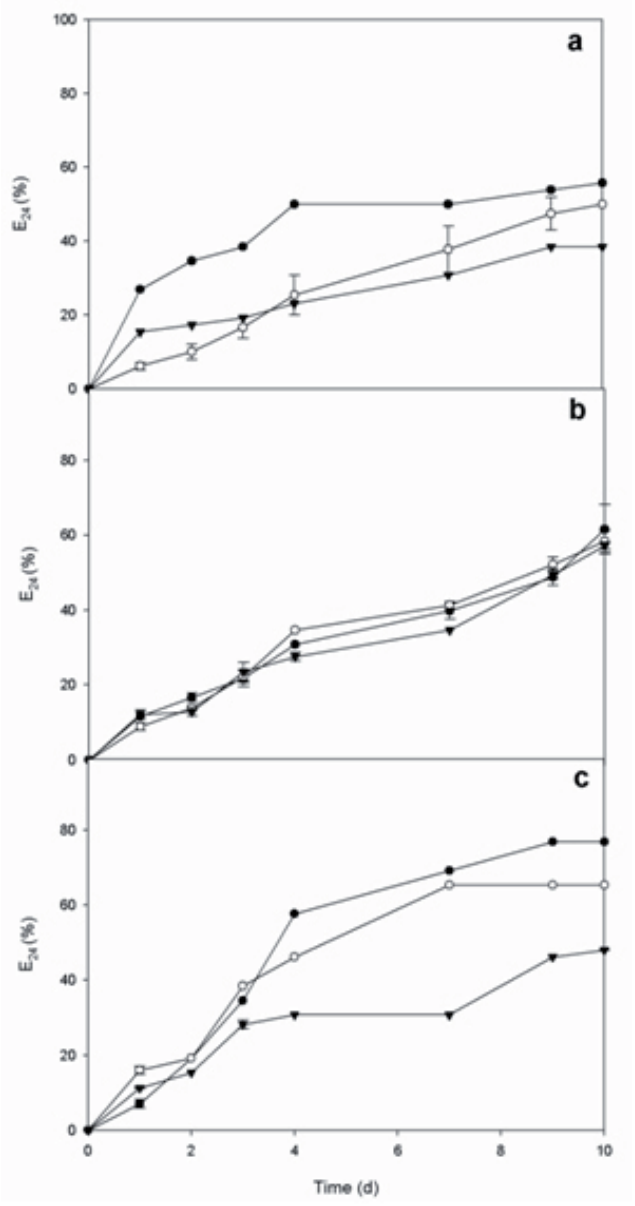

Figure 1. Time-course of E_{24} for cultures of the main emulsifier-producing strains ($E_{24} \geq 39\%$). Cultures were prepared in M2 medium supplemented with 1% diesel. Values plotted are means ± SD for triplicate cultures. **A**: Gram-positive cocci, *Deinococcus radiodurans* 54R-M (●), *Micrococcus* sp. 92S-B (○), *Micrococcus* sp. 76D-O (▼); **B**: Gram-positive bacilli, *Bacillus pumilus* 133S-B (●), NI 114R-O (○), *B. megaterium* 122R-G (▼); **C**: Gram-negative bacilli, *Pseudomonas aeruginosa* 148D-O (●), *P. aeruginosa* 87R-B (○), *P. aeruginosa* 88R-B (▼). "d" denotes time of incubation in days.

radiodurans 54R-M is highly remarkable, since it is, as far as the authors know, the first report of surfactant-production evidence by this specie. Members of the genus *Deinococcus* are known for their remarkable tolerance towards ionizing radiation and desiccation, and have been isolated from very diverse environments, including exposed to extreme physical conditions [31].

As previously mentioned, hemolytic activity was included in this work as it is widely used to screen biosurfactant production [13]. Moreover, a possible association between hemolytic activity and bioemulsifier-compounds has been proposed [12]. Nonetheless, as it is shown in table 1, a correlation between hemolytic patterns and the ability to grow on diesel oil or E_{24} values could not be determined, given that only 40% of the strains that were able to grow on diesel showed either beta or alpha hemolysis at 25 °C and/or 35 °C. Similarly, only 45% of the strains with positive results in the E_{24} test presented some kind of hemolysis, and only half of the strains with E_{24}>40% were able to produce lysis on blood agar plates. These results suggest that hemolytic activity assay may lead to misrecognize many good biosurfactant producers and therefore it should not be used as the only screening method for this purpose.

Given the origin of the strains analyzed, the findings of this study suggest the presence of potential risks caused by bacterial growth in stored fuels. However, for a more accurate assessment of such risks, further studies should be conducted in situations that resemble storage conditions. Results also led to the identification of potential candidates for the production of bioemulsifier compounds, with potential biotechnological applications such as the bioremediation of polluted sites.

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References

1. Rodríguez-Rodríguez CE, Rodríguez-Cavallini E, Blanco R. Bacterial contamination of automotive fuels in a tropical region: the case of Costa Rica. Rev Biol Trop. 2009; 57:489-504.
2. Menezes Bento F, Gaylarde CC. Biodeterioration of stored diesel oil: studies in Brazil. Internat Biodet Biodegrad. 2001; 47:107-12.
3. Yemashova NA, Murygina VP, Zhukov DV, Zakharyantz AA, Gladchenko MA, Appana V, Kalyuzhnyi SV. Biodeterioration of crude oil and oil derived products: a review. Rev Environ Sci Biotechnol. 2007; 6:315-37.
4. Menezes Bento F, de Oliveira Camargo FA, Okeke BC, Frankenberger Jr WT. Diversity of biosurfactant producing microorganisms isolated from soils contaminated with diesel oil. Microbiol Res. 2005;160: 249-55.
5. Correa Bicca F, Colombo Fleck L, Záchia Ayub MA. Production of biosurfactant by hydrocarbon degrading *Rhodococcus ruber* and *Rhodococcus erythropolis*. Rev Microbiol. 1999; 30:231-6.
6. Desai J, Banat IM. Microbial production of surfactants and their commercial potential. Microbiol Mol Biol Rev. 1997; 61:47-64.
7. Kosaric N. Biosurfactants and their application for soil bioremediation. Food Technol Biotechnol. 2001; 39:295-304.
8. Christofi N, Ivshina IB. Microbial surfactants and their use in field studies of soil remediation. J Appl Microbiol. 2002; 93:915-29.

9. Lai CC, Huang YC, Wei YH, Chang JS. Biosurfactant-enhanced removal of total petroleum hydrocarbons from contaminated soil. J Hazard Mater. 2009; 167:609-14.
10. Hanson KG, Desai JD, Desai AJ. A rapid and simple screening technique for potential crude oil degrading microorganisms. Biotechnol Tech. 1993; 7:745-8.
11. Bushnel LD, Hass HF. Utilization of certain hydrocarbons by microorganisms. J Bacteriol. 1941; 41:653-73.
12. Carrillo PG, Mardaraz C, Pitta-Alvarez SI, Giulietti AM. Isolation and selection of biosurfactant-producing bacteria. World J Microbiol Biotechnol. 1996; 12:82-4.
13. Thavasi R, Jayalakshmi S, Balasubramanian T, Banat IM. Production and characterization of a glycolipid biosurfactant from *Bacillus megaterium* using economically cheaper sources. World J Microbiol Biotechnol. 2008; 24:917-25.
14. Adebusoye SA, Amund OO, Ilori MO, Domeih DO, Okpuzor J. Growth and biosurfactant synthesis by Nigerian hydrocarbon-degrading estuarine bacteria. Rev Biol Trop. 2008; 56:1603-11.
15. Lotfabad TB, Shourian M, Roostaazad R, Najafabadi AR, Adelzadeh MR, Noghabi KA. An efficient biosurfactant-producing bacterium *Pseudomonas aeruginosa* MR01, isolated from oil excavation areas in south of Iran. Colloid Surf B-Biointerfaces. 2009; 69:183-93.
16. Toledo FL, Calvo C, Rodelas B, González-López J. Selection and identification of bacteria isolated from waste crude oil with polycyclic hydrocarbons removal capacities. Syst Appl Microbiol. 2006; 29:244-52.
17. Raza ZA, Khan MS, Khalid ZM, Rehman A. Production kinetics and tensoactive characteristics of biosurfactant from a *Pseudomonas aeruginosa* mutant grown on waste frying oils. Biotechnol Lett. 2006; 28:1623-31.
18. Emtiazi G, Shakarami H, Nahvi I, Mirdamadian SH. Utilization of petroleum hydrocarbons by *Pseudomonas* sp. and transformed *Escherichia coli*. Afr J Biotechnol. 2005; 4:172-6.
19. Pinto Mariano A, Bonotto DM, de Franceschi de Angelis D, Santos Pirôllo MP, Contiero J. Biodegradability of commercial and weathered diesel oils. Braz J Microbiol. 2008; 39:133-42.
20. Palleroni N. Genus I. *Burkholderia*. In: Brenner DJ, Krieg NR, Staley JT editors. Bergey`s Manual of Systematic Bacteriology. New York: Springer; 2005. p. 575-600.
21. Atlas RM. Microbial degradation of petroleum hydrocarbons: an environmental perspective. Microbiol Rev. 1981; 45:180-209.
22. Gaylarde CC, Menezes Bento F, Kelley J. Microbial contamination of stored hydrocarbon fuels and its control. Rev Microbiol. 1999; 30:1-10.
23. Menezes Bento F, de Oliveira Camargo FA, Okeke B, Frankenberger-Junior WT. Bioremediation of soil contaminated by diesel oil. Braz J Microbiol. 2003; 34 Suppl 1:S65-8.
24. Pornsunthorntawee O, Arttaweeporn N, Paisanjit S, Somboothanate P, Abe M, Rujiravanit R, Chavadej S. Isolation and comparison of biosurfactants produced by *Bacillus subtilis* PT2 and *Pseudomonas aeruginosa* SP4 for microbial surfactant-enhanced oil recovery. Biochem Eng J. 2008; 42:172-9.
25. Pornsunthorntawee O, Wongpanit P, Chavadej S, Abe M, Rujiravanit R. Structural and physicochemical characterization of crude surfactant produced by *Pseudomonas aeruginosa* SP4 isolated from petroleum-contaminated soil. Bioresour Technol. 2008; 99:1589-95.
26. Yin H, Qiang J, Jia Y, Ye J, Peng H, Qin H, Zhang N, He B. Characteristics of biosurfactant produced by *Pseudomonas aeruginosa* S6 isolated from oil-containing wastewater. Process Biochem. 2009; 44:302-8.
27. Altmajer Vaz D, Gudiña EJ, Jurado Alameda E. Performance of a biosurfactant produced by a *Bacillus subtilis* strain isolated from crude oil samples as compared to commercial chemical surfactants. Colloid Surf B-Biointerfaces. 2012; 89:167-74.
28. Joshi S, Bharucha C, Desai AJ. Production of biosurfactant and antifungal compound by fermented food isolate *Bacillus subtilis* 20B. Bioresour Technol. 2008; 99:4603-8.
29. Najafi AR, Rahimpour MR, Jahanmiri AH, Roostaazad R, Arabian D, Ghobadi Z. Enhancing biosurfactant production from an indigenous strain of *Bacillus mycoides* by optimizing the growth conditions using a response surface methodology. Chem Eng J. 2010; 163:188-94.
30. Sousa M, Melo VMM, Rodriguez S. Screening of biosurfactant-producing *Bacillus* strains using glycerol from the biodiesel synthesis as main carbon source. Bioprocess Biosyst Eng. 2012; 35:897-906.
31. Daly MJ. A new perspective on radiation resistance based on *Deinococcus radiodurans*. Nature Rev. 2009; 7:237-45.