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Fungal contamination of stored automobile-fuels in a tropical environment

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Abstract

Because of the lack of reports, the base levels of microbial contamination on stored fuels are unknown in tropical regions and it is unclear whether these levels have some influence on fuel quality parameters. Therefore, fungal quality in automobile fuels stored across Costa Rican territory was evaluated during two years according to the standard ASTM D6974-04. For a total of 96 samples, counts and identification of molds and yeasts were performed on regular gas, premium gas and diesel taken from the bottom and superior part of the container tanks. The highest contamination was found on the bottom of the tanks, where an aqueous phase was usually identified, showing populations over the ones present in the hydrocarbon itself (up to 10^8 CFU/L). Diesel was the most contaminated fuel (up to 10^7 CFU/L); however, an alteration on the physicochemical parameters was not observed in any kind of fuel. Seventy-five mold strains were isolated, *Penicillium* sp. being the most common genus (45.8% of the samples), and ten yeast strains, from the genera *Candida* sp. and *Rhodotorula* sp. Four of the yeasts were able to grow on diesel as the sole carbon source, at concentrations ranging from 0.5% to 25%. Increasing the frequency of tank cleaning, adding antimicrobial agents and monitoring microbial populations are recommended strategies to improve microbial quality of stored fuels.

Key words: fuel; microbial contamination; biodegradation; molds; yeasts
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Introduction

Fuel's industry biggest microbiological problem is the contamination of the stored products, which may lead to quality loss, mud formation and spoilage of pipes and storage tanks (Gaylarde et al., 1999; Yemashova et al., 2007). Microorganisms arrive to the fuels from the ground, air and contaminated water or pipes (Gaylarde et al., 1999; ASTM, 2004a); once there, water is the most important requirement for microbial growth, a compound easily found in fuels due to multiple reasons (ASTM, 2004a; Yemashova et al., 2007).

Although reports on the microbial growth in this kind of hydrocarbons have increased, at present there are no official standards to regulate the microbiological quality of stored and expended fuels (Gaylarde et al., 1999). The easiest available indicators to evaluate microbial contamination in fuels are total counts of viable bacteria and fungi recovered directly from the fuels. However, they are usually lower than the counts found in the contaminated water usually present at the bottom of the containers (ASTM, 2004b), main site for microbial growth. Additionally, the water/fuel interface is the place where the highest microbial activity and fuel biodegradation develop (ASTM, 2004a).

Microflora may produce a negative effect on fuel properties through degradation processes, situation that could be a major concern in the case of kerosene (aviation fuel), whose deterioration, especially by molds, has been referred as the main cause of air collisions (Yang et al., 1992; Ferrari et al., 1998). From the studies related to biodegradation of oil derived products, more than 80% are dedicated to bacteria; nevertheless this ability has been described in molds and yeasts as well, but in a lower frequency (Atlas, 1981; Cerniglia and Crow, 1981; Gaylarde et al., 1999; Sutherland, 2004; Neilson and Allard, 2008).

Few reports deal with the biota of stored fuels (Edmonds and Cooney, 1967; Neihof and May, 1982; Pitcher, 1989; Haggett and Morchat, 1992; Gaylarde et al., 1999; Bento and Gaylarde, 2001; Rauch et al., 2006; Yemashova et al., 2007), and even less have been performed in tropical environments (Bento and Gaylarde, 2001). Due to this lack of reports, even worldwide, the base level of microbial contamination on stored fuels is unknown in tropical regions and it is unclear whether these levels have some influence on fuel quality parameters or keep these properties within the permissive values. Particularly, mycobiota of fuels in

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Costa Rica and its possible effects on deterioration in storage conditions have not been previously investigated.

The Costa Rican Petroleum Refinery, RECOPE, is the only organization in charge of fuel storage and distribution across the country. RECOPE manages four main oil distribution facilities, located in points where environmental conditions relative to mean temperature, precipitation and humidity are markedly different, even for a small country, which may have an effect on microbial populations. The purpose of the present investigation is to perform a fungal evaluation of the automotive fuels stored in the oil distribution facilities spread around Costa Rican territory. This constitutes a pioneer study that pretends to determine the levels of mesophilic fungi in the fuel storage tanks, their identification and the isolation of some strains that could grow on them, all with the purpose of establishing some recommendations to reduce their contamination and degradation.

1 Materials and methods

1.1 Sampling sites

The fuel storage tanks of the oil distribution facilities managed by RECOPE (located in La Garita 9°59′07″N, 84°20′17″W; Alto de Ochomogo 9°53′47″N, 83°56′45″W; Moín 9°59′55″N, 83°04′35″W and Barranca 9°59′41″N, 84°43′26″W), were sampled according to the norms established by the American Society for Testing and Materials (ASTM), using sterile Sample Thief (Bacon Bomb) containers (model K27790, Koehler, USA). A semiannual sampling was made in each facility during the years 2006 and 2007, where samples from regular gas (Bio Plus Gas), premium gas and diesel tanks were taken from the top as well as the bottom part of the containers. In total, 96 samples were taken, which correspond to 24 from each oil distribution facility. The samples from the bottom of the tanks often included a visible aqueous phase, which was separated and analyzed independently.

1.2 Physicochemical parameters

A physicochemical parameter determination used for quality control in Costa Rican fuels was made to each sample: distillation (ASTM D-86), Reid vapor pressure (ASTM D-323), sulfur content (ASTM D-4294), copper corrosion (ASTM D-130), gum content (ASTM D-381), lead content (ASTM D-3237), octane index and number IR correlation, color, water content (ASTM D-4176), density (ASTM D-1298), oxygenated compound content (ASTM D-5845), benzene content (ASTM D-6277) aromatic content (correlated to ASTM D-1319) and olefins content (correlated to ASTM D-1319) for regular gas and premium gas; distillation (ASTM D-86), cinematic viscosity (ASTM D-445), sulfur content (ASTM D-4294), combustion point (ASTM D-93), Conradson carbon residue (ASTM D-524), nitrogen content (ASTM D-3228), ashes content (ASTM D-482), color (ASTM D-1500), sediment and water content (ASTM D-1796), density (ASTM D-1298), total aromatic content (correlated ASTM D-1319), polinuclear aromatic content (correlated to ASTM D-1319) and cetane index (ASTM D-4737 and D-976) for diesel.

1.3 Mold and yeast quantification

The enumeration of molds and yeasts was performed following the methodology described by the standard ASTM D6974-04 (ASTM, 2004b). Briefly, three different fuel aliquots (200, 20, and 2 mL) were filtered in sterile nitrocellulose filters (0.45 μm). Each filter was washed by filtration with 10 mL Tween 80 of 0.1% (*V/V*) and three aliquots of 10 mL Ringer solution; the filters were placed on Potato Dextrose Agar (PDA) plates (pH 3.5) and incubated at 25°C for 5 days for their posterior count. The samples from the aqueous phase taken from the bottom of the tanks were separated and then the population of molds and yeasts was quantified by the plate count methodology using PDA. Only 14 of the samples presented this aqueous phase.

1.4 Mold and yeast identification

Molds and yeasts from PDA plates were purified onto new PDA plates. Yeasts identification was done according to carbohydrate assimilation patterns, using the API 20C AUX galleries (bioMérieux, France), which were used according to the manufacture's recommendations. The identification of molds was done by macroscopic examination of colonies in PDA slant tubes and microscopic examination (slide culture technique). The frequency (F) of each mold genus and yeast species was calculated with the following equation:

$$F = \frac{T_a}{T} \times 100 \quad (1)$$

where, T_a is the number of samples in which the taxon was found and T is the total number of samples. According to this equation, the species frequencies were classified as: rare < 10%, 10 ⩽ low ⩽ 25%, 25 < frequent < 35%, 35 ⩽ abundant ⩽ 50%; and very abundant > 50% (Azedo Loureiro et al., 2005).

1.5 Yeasts ability to grow in diesel

In order to evaluate the yeasts capacity to grow in fuels, suspensions with a turbidity equal to the pattern 2 of Mc Farland were prepared, and 1 mL of each suspension was added to flasks with 20 mL of Bushnell-Hass broth (Bushnell and Haas, 1941), supplemented with 100 μL of aseptically filtered diesel (final concentration of 0.5%). As controls, flasks containing 20 mL of Bushnell-Hass broth without diesel and 1 mL of each yeast suspension, and one flask containing 20 mL Bushnell-Hass broth with 100 μL diesel without yeast suspension were used. All of the flasks were incubated at 28°C with constant shaking at 120 r/min for 17–25 days. Every two days, both determination of turbidity at 620 nm and PDA plate counts were conducted simultaneously. Similar growth curves using higher diesel concentrations (2%, 5%, 10%, 25%, 50%, 75% and 90%) were performed employing the four strains being able to grow in 0.5% diesel. The inoculum employed in this case was 1 mL of each yeast suspension, with a population of

10^7 cells approximately, previously grown in Bushnell-Hass broth with 0.5% diesel, in order to obtain initial populations of 10^5 CFU/mL at the beginning of the curves.

1.6 Statistical analyses

The fungal contamination levels at the top and the bottom of the tanks were compared by a *t*-test analysis for paired samples employing the logarithms of the counts from the pooled data of the three kinds of fuel; a similar analysis was performed to compare the contamination in the aqueous and organic phases from the bottom of the tanks. The differences among the counts from each kind of fuel were compared by an ANOVA test employing the logarithms of the pooled data; similarly, contamination levels in the four oil distribution facilities were compared. All analyses were performed with the statistical software SPSS version 16.0.

2 Results and discussion

This work is the first mycological survey on fuels in Costa Rica, as a first approach to determine normal fungal diversity and levels, which could eventually lead to the establishment of microbiological standards relative to the quality of automotive fuels sold in tropical regions. Microbiological parameters have never been taken into account in such quality matters before.

The mold and yeast counts obtained from the three kinds of fuel from RECOPE's oil distribution facilities are shown in Table 1. In general, higher fungal contamination was present in the fuel from the bottom of the storage tanks, if compared to the samples from the top ($t = 3.263$, $p < 0.05$); nevertheless, the results obtained from the aqueous phase accumulated in 14.6% of the tanks were even higher, 2.9 logarithms on average ($t = 2.706$, $p < 0.05$, Table 2). These results correlate with previous reports, where microbial populations tend to increase as depth of stored fuel tanks also increases (Pitcher, 1989; Yemashova et al., 2007). In such big containers, microbial growth is favored when water accumulation is present at their base which usually leads to a continuous contamination of the fuel itself.

Results revealed differences between the three kinds of fuel ($p < 0.05$); diesel showed the highest degree of fungal contamination, since 17 (53.1%) out of 32 samples presented counts ranging from 10^2 and 10^5 CFU/L, whereas only 9 (28.1%) of regular gas and 4 (12.5%) of premium gas presented similar count levels. This finding agrees

Table 2 Mold and yeast counts in accumulated water found at the bottom of fuel storage tanks in oil distribution facilities of RECOPE, Costa Rica

Oil distribution facility	Water phase origin	Mold and yeast count (CFU/L)
Ochomogo (I-2006)[a]	Diesel	2.0×10^6
Ochomogo (I-2006)	Regular	< 10
Ochomogo (I-2006)	Premium	< 10
Ochomogo (II-2006)	Diesel	6.1×10^6
Ochomogo (II-2006)	Regular	3.0×10^5
Moín (II-2006)	Regular	1.0×10^4
Moín (II-2006)	Premium	2.0×10^4
Ochomogo (I-2007)	Diesel	3.7×10^5
Ochomogo (I-2007)	Regular	1.0×10^4
Ochomogo (I-2007)	Premium	< 10
Ochomogo (II-2007)	Regular	< 10
Ochomogo (II-2007)	Diesel	4.5×10^7
Barranca (II-2007)	Diesel	5.0×10^4
Moín (II-2007)	Premium	1.1×10^8

[a] Code for correspondent sampling is shown in parenthesis.

Table 1 Mold and yeast counts in automotive fuels from storage tanks in four oil distribution facilities of RECOPE, Costa Rica

Oil distribution facility	Sample type	Mold and yeast count (CFU/L)[a]			
		I-2006	II-2006	I-2007	II-2007
Ochomogo	Regular, top	1.5×10^1	< 5	< 5	5.5×10^4
	Regular, bottom	1.0×10^1	8.4×10^3	1.0×10^1	6.0×10^2
	Premium, top	< 5	1.6×10^3	< 5	< 5
	Premium, bottom	< 5	1.5×10^3	1.0×10^3	< 5
	Diesel, top	< 5	4.8×10^2	1.2×10^3	8.2×10^4
	Diesel, bottom	3.0×10^3	2.5×10^4	1.5×10^3	8.4×10^4
La Garita	Regular, top	3.3×10^1	< 5	1.0×10^1	< 5
	Regular, bottom	2.2×10^1	1.0×10^1	< 5	< 5
	Premium, top	< 5	< 5	< 5	< 5
	Premium, bottom	5.0×10^0	< 5	< 5	< 5
	Diesel, top	5.0×10^0	< 5	2.5×10^1	< 5
	Diesel, bottom	8.7×10^3	1.5×10^2	1.7×10^2	< 5
Barranca	Regular, top	< 5	< 5	2.5×10^1	< 5
	Regular, bottom	1.8×10^2	< 5	3.3×10^1	6.7×10^3
	Premium, top	1.0×10^1	< 5	2.5×10^1	< 5
	Premium, bottom	4.3×10^1	5.0×10^0	7.0×10^1	< 5
	Diesel, top	< 5	5.0×10^1	< 5	< 5
	Diesel, bottom	< 5	3.0×10^2	< 5	1.0×10^2
Moín	Regular, top	1.8×10^2	< 5	2.2×10^2	< 5
	Regular, bottom	< 5	2.4×10^4	7.3×10^2	< 5
	Premium, top	< 5	< 5	1.0×10^1	5.0×10^1
	Premium, bottom	1.0×10^2	1.0×10^1	5.0×10^1	< 5
	Diesel, top	3.7×10^3	3.3×10^2	1.0×10^1	< 5
	Diesel, botttom	6.0×10^2	1.0×10^2	2.7×10^3	5.0×10^1

[a] Sampling codes: I-2006, first of 2006; II-2006, second of 2006; I-2007, first of 2007; II-2007, second of 2007.

with previous studies in Brazil, where the most important fungal problems related to fuels were shown in diesel storage tanks due to accumulation of biomass in the water-fuel interface (Gaylarde et al., 1999; Bento and Gaylarde, 2001). Likewise, the lower fungal levels were obtained from the premium gas, since 18 (56.3%) out of 32 samples showed negative counts (< 5 CFU/L), compared with 14 (43.8%) and 10 (31.3%) of regular gas and diesel samples, respectively. Besides, premium gas presented 28 samples (87.5%) with counts of less than 10^1 CFU/L. Such results could be coincident with the stricter quality parameters that regulate premium gas, on a merely physicochemical level. The presence of a wide range of carbon sources (including different kinds of additives such as stabilizers or surfactants), less sulfur-compound content and the higher molecular weight of diesel components (which do not act as membrane solvents), may enhance microbial growth and could explain the major microbiological problems attributed to diesel (Leahy and Colwell, 1990; Gaylarde et al., 1999).

Considering the location of the four oil distribution facilities, fungal counts also differ ($p < 0.05$); results show that fuels from Ochomogo were the most contaminated, since 13 (54.2%) of the samples presented counts over 10^2 CFU/L, whereas only 8 (33.3%) presented negative counts; the facility of Moín, was slightly less contaminated (10 samples, 41.7%, with counts > 10^2 CFU/L and 8 samples, 33.3%, with negative counts). On the contrary, La Garita presented the "cleanest" fuels, where 14 (58.3%) out of 24 samples showed negative counts and only 3 (12.5%) had counts over 10^2 CFU/L. Despite the variation in climatologic conditions of storage, an association among these results and weather conditions as temperature and rainfall was not found: Ochomogo, located in the mountain region crossing the center of the country, presents the lowest mean temperatures (16.6°C) while Barranca, in the Pacific coast, the highest (26.6°C). Meanwhile, the lowest and highest annual precipitations are reported for Ochomogo (1354.4 mm) and Moín (3158.4 mm), respectively, the latter located in the rainy Caribbean coast (National Weather Institute, Environment, Energy and Telecommunication Department, Costa Rica). Nonetheless, results seem to correlate more with the presence of water at the bottom of the containers, since most of the aqueous-phase containing samples were found in Moín and mainly in Ochomogo, facility where 83.3% of the samples presented aqueous phase. This finding could be partially explained because both facilities are located in higher relative humidity places (87% and 86%, respectively) if compared to La Garita (76%) and Barranca (81%), for which only one sample presented water. Since climatologic parameters surrounding the storage tanks do not seem to have an impact as important as the accumulated water, it is highly suggested promoting tank drainage and increasing cleaning frequency of fuel containers in high humidity places.

In order to determine a possible relationship between fungal counts and fuel quality properties, the samples were analyzed for different parameters, depending on the kind of product. All of the samples showed physicochemical parameter values considered as acceptable according to national standards, with the exception of four premium gas samples, that presented octane numbers below the permitted limit, and five diesel samples, whose sulfur content surpassed the upper limit. Nevertheless, those variations were small if compared to the standard. Permitted values for each parameter as well as the range of obtained values are shown in Tables 3 and 4.

Even though no acceptable standards for fuel microbial

Table 3 Physicochemical parameters of regular and premium gas, stored in four oil distribution facilities of RECOPE, Costa Rica

Parameter	Permitted values (national normative)	Value range	
		Regular gas	Premium gas
Initial distillation temperature (°C)	I.P.	31.1–38.3	31.4–37.0
Distillation temperature at 10% (°C)	⩽ 70	51.0–60.6	50.5–59.7
Distillation temperature at 50% (°C)	77–140	94.2–112.3	82.4–115.1
Distillation temperature at 90% (°C)	⩽ 190	149.5–173.2	144.9–178.6
Distillation final point temperature (°C)	⩽ 225	182.9–213.0	178.2–215.2
Distillation residue (%)	⩽ 2	0.4–1.0	0.6–1.0
Reid vapor pressure at 37.8°C (kPa)	⩽ 69	49.5–67.6	51.0–68.7
Sulfur content (%, *W/V*)	0.10	0.027–0.081	0.020–0.085
Copper corrosion	Standard 1 maximum	1 A	1 A
Gum content (mg/100 mL)	5	< 1	< 1
Lead content (g/L)	0.013	0.00023	0.00023
Octane index	⩾ 83 (regular) ⩾ 89.0 (premium)	84.6–89.0	89.0–90.6
Octane number	⩾ 88 (regular) ⩾ 95.0 (premium)	89.0–95.0	94.0–96.5
Color	Orange (regular) Red (premium)	Orange	Red–reddish
Water (%)	None	No detected[a]	No detected[a]
Density at 15°C (kg/m^3)	I.P.	733–751	733–756
Oxygenated content (%)	⩽ 2 (regular) I.P. (premium)	0.00–3.01	0.85–2.61
Benzene content (%)	I.P.	0.94–1.19	0.70–1.83
Aromatic content (%)	I.P.	20.6–29.2	23.4–31.5
Olefins content (%)	I.P.	15.5–23.2	15.5–26.6

[a] Aqueous phase present in some samples of the bottom of the containers was not consider. I.P.: inexistent parameter.

Table 4 Physicochemical parameters of diesel stored in four oil distribution facilities of RECOPE, Costa Rica

Parameter	Permit values	Value range
Initial distillation temperature (°C)	I.P.	170.9–196.9
Distillation temperature at 10% (°C)	I.P.	212.9–233.2
Distillation temperature at 50% (°C)	I.P.	277.2–290.6
Distillation temperature at 90% (°C)	⩽ 360	329.8–349.3
Distillation final point temperature (°C)	I.P.	359.4–375.0
Distillation residue (%)	⩽ 2	1.1–1.4
Cinematic viscosity at 40°C (cSt)	1.9–5.3	2.97–3.42
Sulfur content (%, *W/V*)	0.35	0.16–0.46
Inflammation point (°C)	⩾ 52	62.3–80.5
Conradson carbon residue (%)	⩽ 0.2	0.0011–0.0031
Nitrogen content (%, *W/W*)	I.P.	0.03–0.35
Ashes content (%)	⩽ 0.01	0.001–0.003
Color	Standard 3	1.0–2.0
Water and sediment content (%, *V/V*)	⩽ 0.05	< 0.05[a]
Density at 15°C (kg/m^3)	I.P.	852–866
Total aromatic content (%, *W/W*)	I.P.	20.8–26.1
Polinuclear aromatic content (%, *W/W*)	I.P.	3.9–7.1
Cetane Index, ASTM D-976	⩾ 45	46.4–49.1
Cetane Index, ASTM D-4737	⩾ 45	45.0–48.6

[a] Aqueous phase present in some samples of the bottom of the containers was not consider. I.P.: inexistent parameter.

contamination have been published (Gaylarde et al., 1999), according to Allsopp et al. (2004), crude oil or oil products can be classified as slightly or highly contaminated by the number of microorganisms present in the residual water bottoms; about 10^6–10^7 molds and yeasts/L are characteristic for slight contamination and 10^7–10^9 molds and yeasts/L for high contamination. According to this, only 14.3% of the samples with accumulated water were considered as slightly contaminated, while an equal amount was highly contaminated. Similarly, oil products can be considered as "clean" if they contain less than 5.0×10^4 microorganisms/L product, which indicates that only 2.1% of the fuels analyzed in this study were "not clean", all of them from the oil distribution facility of Ochomogo. These results, representing a small proportion of contaminated fuels, are consistent with the absence of significant alterations in the physicochemical parameters, and also reveal that maximal microbial burdens such as the ones found in the present study are not enough to promote fuel alteration, at least based on the analyzed parameters.

From the 96 fuel samples, 75 mold strains were isolated, to an average of 0.8 strains per sample; 56 (74.7%) of them corresponded to *Penicillium* sp., 10 (13.3%) to *Cladosporium* sp., 8 (10.7%) to *Aspergillus* sp. and one (1.3%) to *Paecilomyces* sp. Isolation frequencies of these taxons were 45.8% (44 samples), 10.4% (10 samples), 8.3% (8 samples) and 1.0% (1 sample), respectively. Therefore, *Penicillium* sp. was classified as abundant and *Cladosporium* sp. of low frequency, whereas the remaining genera were considered as rare (Azedo Loureiro et al., 2005). On the other hand, 10 strains of yeasts were isolated (0.1 strains per sample). Seven of them belonged to the genus *Candida* sp. (isolation frequency: 7.2%, the most frequent), distributed as follows: two strains of *Candida tropicalis* (2.1%), three of *C. guilliermondii* (3.1%), one of *C. famata* (1.0%) and one of *C. parapsilosis* (1.0%). The remaining species were identified as *Rhodotorula mucilaginosa* (3.1%). All of the yeasts species were considered as rare (< 10%) due to their low isolation frequency in fuel. All of the genera of both molds and yeasts have been previously found in studies performed in automotive fuels of different latitudes (Bushnell and Haas, 1941; Edmonds and Cooney, 1967; Neihof and May, 1983; Kartavtseva et al., 1989; Gaylarde et al., 1999; Bento and Gaylarde, 2001; Bento et al., 2005; Yemashova et al., 2007), as well as aviation fuel tanks (Rauch et al., 2006), where abundance of *Cladosporium* sp. is remarkable.

Since no important alterations were observed in physicochemical characterization of fuels, the authors decided to test the ability of the yeasts to grow on diesel as the sole carbon source, on the basis of their better growth in oxygen-limiting conditions such as the bottom of the tanks, if compared to molds. Diesel oil is an excellent model for hydrocarbon biodegradation studies, since it is constituted by a wide and complex mixture of these kinds of compounds (Correa Bicca et al., 1999; Pinto Mariano et al., 2008). Four out of the 10 yeast strains were able to grow on diesel as the sole source of carbon and energy, as it was determined by the growth in 0.5% diesel supplemented medium (data not shown): two of *C. guilliermondii* (strains 124R-O and 128D-O) one of *C. famata* (strain 73R-B) and one of *C. parapsilosis* (148S-M). These results corresponded to 40% of the isolated yeasts, which surpasses the 11% previously determined in yeast hydrocarbon assimilation studies (Kogamata et al., 1964), where the majority corresponded to the genus *Candida* sp., as it also occurred in the present case. Previously, the ability to grow in hydrocarbons has been identified in *C. guilliermondii* and *C. famata* (Gaylarde et al., 1999; Neilson and Allard, 2008). For *C. parapsilosis*, it has been reported the ability to grow at the expense of hydroxybenzoates, through oxidative descarboxylation metabolism (Neilson and Allard, 2008). For other species such as *C. lipolytica*, *C. silvicola* and *C. tropicalis* the ability to degrade different hydrocarbons has been also reported (Cerniglia and Crow, 1981; Bento and Gaylarde, 2001; Neilson and Allard, 2008), as well as the capacity to uptake hydrocarbons by means of previous emulsification (Goma et al., 1973; Kappeli and Fletcher, 1981; Singer and Finnerty, 1984). In the case of *Rhodotorula* sp., none of the isolates was capable to grow in diesel. However, previous investigations demonstrated the degradation of other hydrocarbon sources by some strains of *R. aurantiaca* (Miranda et al., 2007), *R. glutinis* and *R. minuta* (Romero et al., 2002). The metabolic mechanisms employed by yeasts and other microorganisms to degrade hydrocarbons have been reviewed elsewhere (Van Hamme et al., 2003; Singh, 2006; Neilson and Allard, 2008).

Figure 1 shows the growth of these strains at different diesel concentrations. *C. famata* 73R-B and *C. guilliermondii* 124R-O (Fig. 1a, b.) presented a similar behavior, growing at concentrations ranging from 2% to 25% diesel, during the study period of 16 days, reaching maximum populations of 10^8–10^9 CFU/mL. However, the growth was only achieved at concentrations up to 5% and 0.5% for *C. guilliermondii* (Fig. 1c) and *C. parapsilosis* 148S-M

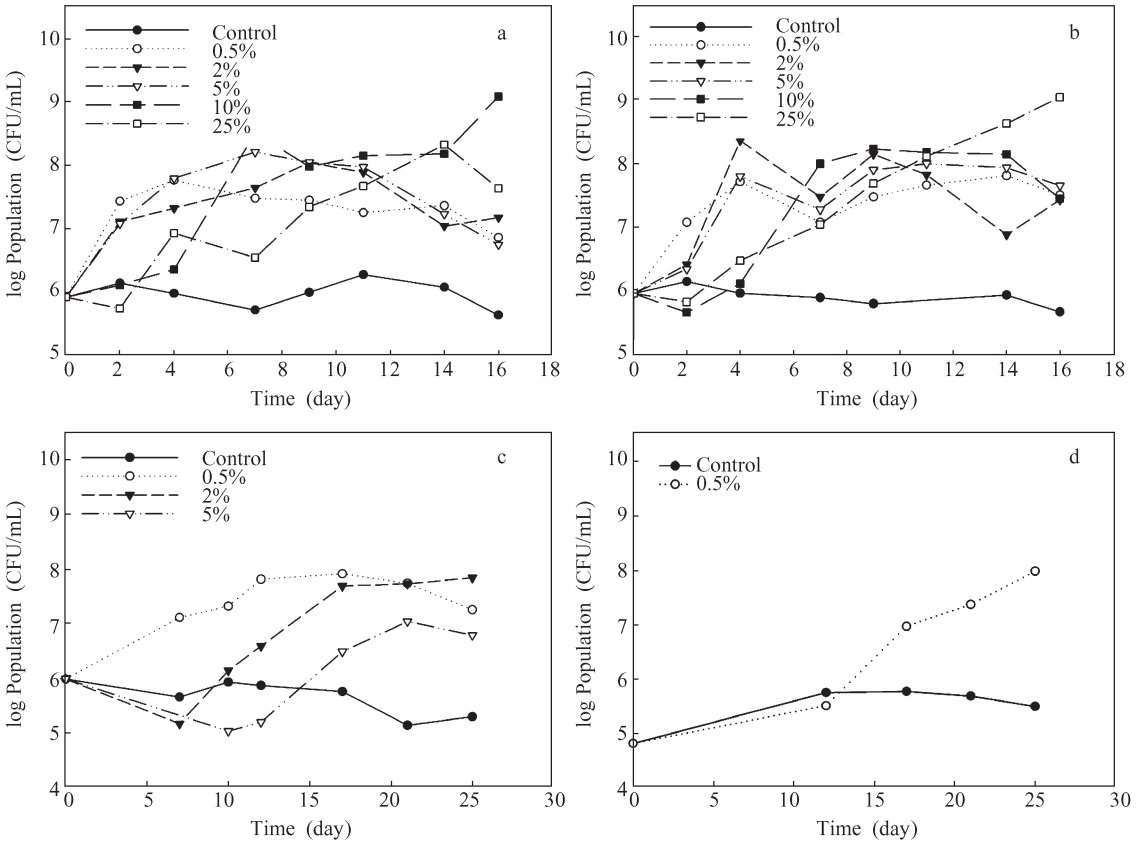

Fig. 1 Growth of yeasts at different diesel concentrations. (a) *Candida famata* 73R-B; (b) *Candida guilliermondii* 124R-O; (c) *Candida guilliermondii* 128D-O; (d) *Candida parapsilosis* 148S-M.

(Fig. 1d), respectively. Nevertheless, in every single case, the initial growth was first observed at the lower diesel concentrations, demonstrating that lag phase increases as diesel concentration increases. For diesel concentrations over the ones previously indicated, yeast growth was not observed, which could be due to inhibitory effect of fuel or because lag phase was not finished by the end of the experiment. These results strongly suggest that continuous water removal from the bottom of the tanks would prevent the appropriate growth of yeast populations in the containers. Besides, the extent of biodegradation by microorganisms is a function of the ecosystem and local environmental conditions; therefore in such locations, the length of the lag phase could be increased for these yeasts, since the rates of hydrocarbon degradation are reduced in sites with low oxygen reduction potential (Singh, 2006).

Although it is difficult to prevent microbial contamination of this kind of products, due to the impossibility of maintaining sterile conditions during their storage and transportation, its negative effects can be diminished. Since most of the microbial flora of fuels is located in the aqueous phase or the water-hydrocarbon interface, the main way to avoid growth and fuel spoilage is removing the accumulated water. Therefore, in the case of Costa Rica, three well defined suggestions are stated to improve microbial quality in fuel storage conditions: (1) increasing the frequency of tank cleaning (including removal of water residues and vapor and/or detergent application), since this is not a clearly regulated practice; (2) the implementation of antimicrobial agents as fuel additives (Haggett and Morchat, 1992; Yemashova et al., 2007), not employed in the country at present; and (3) microbial population monitoring, particularly at the bottom of the containers.

Although this study is not enough to establish microbiological quality standards to classify Costa Rican fuels, it is a first approach to demonstrate the importance of conducting continuous research on this field. Particularly, it is now required to repeat these analyses at gas stations, where the products are finally acquired by consumers. The continuous microbial monitoring could be even more relevant at the near future, since a progressive addition of ethanol to regular and premium gas, up to 10% for 2012, will be implemented by RECOPE throughout the country, a situation that might change the amounts of dissolved water on fuels and subsequently microbial proliferation profiles. This study provides a foundation for identifying fungal flora and its possible effect on stored fuels in tropical regions.

3 Conclusions

Fungal evaluation of stored fuels in Costa Rican territory revealed that higher contamination appeared at the bottom of the storage tanks, if compared to the upper part. The difference was especially marked if the tanks contained an aqueous phase, which occurred often in the samples from the more humid locations. Among the studied fuels, diesel showed the highest degree of contamination. Although a few samples were considered as slightly or

highly contaminated, alteration on the physicochemical parameters was not observed in any kind of fuel. *Penicillium* sp. and *Candida* sp. were the most frequent genera of molds and yeasts found in the fuels, respectively, and several strains of yeasts were able to grow on diesel as the sole carbon source. Results suggest that strategies such as increasing the frequency of tank cleaning, addition of antimicrobial agents and monitoring microbial populations should be applied to improve the quality of the stored fuels.

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