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# Effect of Drying on Bioremediation Bacteria Properties

F. WEEKERS,[1] PH. JACQUES,[2] D. SPRINGAEL,[3] M. MERGEAY,[3] L. DIELS,[3] AND PH. THONART,*,[1]

[1]*University of Liege, Walloon Center for Industrial Biology, Bat. B 40 - 4000 Sart-Tilman, Liege, Belgium;* [2]*Faculty of Agricultural Sciences of Gembloux, Bio-industries Unit, Passage des Déportés, 2-5030 Gembloux; and* [3]*Flemish Institute for Technological Research (VITO) Boerentag, 200 - B2400 Mol. Belgium*

## ABSTRACT

Bioremediation bacteria with drought-resistance characteristics were selected and compared to a collection of 10 strains selected only for their bioremediation properties. Twenty-six strains were selected from dried diesel-polluted soil, and they exhibit a better level of survival during drying, compared to collection bioremediation strains (two orders of magnitude difference). The lyophilization process does not affect the strains' ability to grow on xenobiotic compound when measured immediately after drying. However, collection bioremediation strains selected only for their bioremediation properties lose up to 80% of their properties when stored at 25°C for 15 d, but the strains selected for their drought resistance lose their properties to a lesser extent during the same period. The maximal growth rate and the rate of xenobiotic degradation of the still-active cells are not affected by the drying process.

**Index Entries:** Biodegradation; drought resistance; selection; maintenance of properties.

## INTRODUCTION

The estimated number of contaminated industrial sites in the European Union is significant (150,000), as well as in the rest of the world (over 1,500,000 leaking underground storage tanks estimated in the United States alone) *(1)*. Different techniques have been developed for the remediation of these sites *(2)*. *In situ* bioremediation is one that has already been applied, but that deserves further development. The use of microbial products in

* Author to whom all correspondence and reprint requests should be addressed.

the bioremediation processes, however, is controversial and, in most cases, is being abandoned. These products usually have high efficiency in vitro, but competition, predation, lag phase, heavy metals copollution, and so on, make them less competitive than autochthonous strains when used *in situ (3,4)*. However, in some cases of specific recalcitrant compound pollution, the use of appropriate starter cultures can readily boost the clean-up process *(5)*. These starter cultures are mostly available in ready-to-use dry form, commercially distributed.

In these cases, it is important to have good knowledge of suitable techniques for the production and the conditioning of the starters. The drying process and its direct influence on the properties of the product constitute a bottleneck between the production chain and the *in situ* use of the bacteria. The final product must have a high survival ratio and maintain a high level of biodegradation activity.

To ensure a high level of survival after drying, the technique must be adapted to respect the cells' integrity. The kinetics of water activity ($a_w$) variation is a very important factor *(6–8)* for the viability of bacteria subjected to a drying process. Since slower decrease of the $a_w$, down to a limit threshold, affords higher survival ratio, the drying methods should be designed to allow slow water depletion.

Survival after drying and stability over time of the surviving fraction are necessary, but not sufficient, conditions for a starter culture to be competitive *(9)*. In addition, the degradation properties must be maintained in the surviving cells. Actually, ensuring genetic stability after drying and during preservation is a problem, since the viability of the cells after preservation may not correlate with the full maintenance of all properties. Plasmid-encoded degradation activities may be lost at high frequencies during drying of a culture, although little loss of viability occurs *(10)*. Changes in various properties have been reported, especially during inadequate lyophilization *(11–13)*. Although Lang and Malik *(10)* found a loss of properties in their strains, they could not detect any plasmid loss.

To quantify the biodegradation ability of a bacterium, the rate of substrate uptake ($-dS/dt$), i.e., degradation rate, is an important parameter to monitor *(14)*. When comparing the influence of drying on biodegradation properties, one should also look at the maximal growth rate in different instances, because significant bacterial growth is of prime importance in a bioremediation process.

This laboratory specializes in large-scale drying of sensitive microorganisms of industrial interest. In this context, the fundamental phenomena accompanying the drying of the cells are studied. This paper reports the selection of bioremediation strains according to their resistance to the drying process, the characterization of their degradation properties, and the influence of the drying process on their survival, as well as on the maintenance of their degradation properties.

## MATERIALS AND METHODS

### Culture Medium Composition

Minimum (mineral) medium: 10 m*M* buffer, $Na_2HPO_4{\cdot}2H_2O/KH_2PO_4$ pH 7.0; 1 mM $FeSO_4{\cdot}7H_2O$; (100 m*M*) $CaCl_2{\cdot}2H_2O$; (8.5 m*M*) NaCl; (1 m*M*) $MgSO_4{\cdot}7H_2O$; (18 m*M*) $(NH_4)_2SO_4$; 500 $\mu L/L$ of an aqueous vitamin solution (20 mg/L folic acid, 50 mg/L pentothenic acid, 50 mg/L riboflavin, and 30 mg/L pyridoxal), sterilized by filtration, are added after autoclave sterilization of the medium. One % of diesel (or other tested hydrocarbon), sterilized by filtration, is added as carbon C source before inoculation.

869 rich medium: 10 g/L Peptone from casein; 5 g/L yeast extract; 5 g/L NaCl; 1 g/L glucose and 0.345 g/L $CaCl_2$; pH 7.0.

### Collection Strain Origin

The bioremediation reference strains come from Dr L. Diels' VITO (Mol, Belgium) strain collection.

### Selection of Strains with Drought Resistance Characteristics

Soil samples, collected on a diesel-polluted site, were dried in a (Niro-Aeromatic AG Budendorf, CH) fluidized bed for 20 min with an inlet air temperature of 45°C and outlet temperature ranging from 21 up to 28°C. The dry matter of the final dried product reached 98%. Duplicate samples were lyophilized. The freezing occurred in liquid nitrogen (−198°C), and the sublimation was carried out for 24 hr in a Leybold-Heraeus Lyovac GT2 (Köln, Germany) at a pressure of $10^{-5}$ bar, and with a trap temperature of −40°C. The samples were rehydrated and plated-out on mineral medium, with diesel as sole source of C. The survivors were isolated, and were used to inoculate liquid mineral cultures with diesel as C source.

### Standardized Drying Procedure

The strains were grown in rich medium (869), harvested, and washed with 2 vol of magnesium sulfate ($10^{-2}$ *M*). The sample were resuspended in 1 vol of a 0.5% (w/v) trehalose solution as protective agent, frozen in liquid nitrogen (−198°C), and dried under conditions described above.

### Other Drying Method

Slow dehydration was achieved by the method described by Mattimore *(15)*. The water activity of the atmosphere around the samples is set at 0.49 by the use of silica gel (Nerk, Darmstadt, Germany). The samples were analyzed after 48 hr and 14 d of dehydration at a temperature of 25°C.

Some samples were dried in the fluidized bed described above, with silica as a solid substratum, on which the $MgSO_4$ cell suspension is sprayed with 15% (w/v) maltodextrine as protective agent.

In the course of the determination of the maintenance of the degradation properties, the lyophilizations were made after freezing at −60°C. The samples were washed in the same manner as in the standard lyophilization and resuspended in 1 vol of $MgSO_4$ ($10^{-2}$ *M*), either with 0.5% trehalose or without protector.

## Rehydration Procedure

All dried samples were rehydrated in $MgSO_4$ ($10^{-2}$ *M*) at 30°C under shaking for 30 min.

## Determination of Maintenance of Biodegradation Properties

Before and after drying, cells were plated-out on plate-count agar and simultaneously on mineral medium, with decane as sole source of C supplied via the gas phase. The survival ratio was determined by the ratio of the plate-count agar (PCA) count before and after treatment. The still-active fraction was measured by the ratio between the mineral medium count and PCA count, both done at the same time after drying.

## Kinetic Factor Determination

Five-mL mineral medium cultures were inoculated with 100 µL of a $MgSO_4$ suspension of cells centrifugated after 24 h of culture in 869 medium. To ensure reproductibility, hydrocarbon extractions and colony counts were done in duplicate every 24 h on the total volume of the tightly closed culture vials. To measure the kinetic factors after the lyophilization process, 5 mL of cell suspensions in $MgSO_4$ were lyophilized, rehydrated in an equal volume, and used to inoculate the mineral cultures as before drying.

The generation time (g [h]), which measures the time required to double the cell concentration of the culture, is directly correlated to the growth rate by the equation below. It was calculated from the maximal growth rate ($\mu_{max}$) values. The $\mu_{max}$ was evaluated from the experimental data from the steepest slope of the growth curve. The rate of hydrocarbon consumption ($-dS/dt$) was measured from the steepest slope of the hydrocarbon consumption curve.

$$g = \log (2x) - \log (x)/\mu = \log (2)/\mu$$

## Hydrocarbon Dosage

The hydrocarbons were extracted from the liquid culture medium at regular intervals. The extraction was made with 1 vol of cyclohexane. The organic phase was analyzed by gas chromatography on a Chrompack capillary column WCOT fused silica CP-Sil8CB. The carrier gas was helium and the head column pressure was 60 kPa. The injector temperature was

maintained at 200°C and the flame ionization detector (FID) temperature at 300°C. The column temperature was raised from 50°C to 280°C, with a gradient of 7°C/min. 50 μL of a 5000 ppm heptanol standard solution were added to 500 μL of the sample before injection. One μL was injected for each measurement. The extraction efficiency was measured by the addition of a known quantity of hexadecane to the liquid culture just before extractions; its average value was 87%. The removal of hydrocarbons caused by evaporation was evaluated by the determination of the quantity left in a reference sterile, closed vial at the time of the last sampling. It was measured to be less than 5% with decane and less than 1% with diesel.

## RESULTS AND DISCUSSION

### Drying Resistance of Selected Collection Strains

Collection strains were selected for their ability to decompose rather recalcitrant compounds (e.g., polyaromatic hydrocarbons [PAH]), and were submitted to a standardized lyophilization procedure.

Table 1 shows the characteristics of strains representative of different genera, and the result of the lyophilization test. The bacteria have good remediation potentialities, but withstand poorly the drying procedure.

### Screening for Drought-Resistant Microorganisms

In order to find drought-resistant bioremediation bacteria, soil samples from a hydrocarbon-polluted site were dried according to the procedure described above, and 26 different strains were collected. The strains named T901–T907, T981–T987, and TF1–TF7 were selected from fluidized soil samples. The strains named TFL1–TFL5 were selected from fluidized soil samples that were subsequently rehydrated and lyophilized.

Those strains selected for their ability to resist drying techniques were then tested for their growth in liquid mineral medium, with diesel as sole source of C (*see* Fig. 1).

Four different strains (TF5 and TFL1 were identified to be the same one) exhibited good growth levels in these conditions. They were submitted to the same standardized drying procedure as the reference strains, and identified (*see* Table 2).

The strains selected from a dried soil survive with a higher proportion to the same drying technique than the collection bacteria. Thus, selecting the bacteria according to their drought resistance yields new strains more suitable to the technological conditioning treatment. However, the survival values stay low. The lyophilization conditions used did not yield good survival. Although some strains (T902 and TF1) belong to the same genus (*Rhodococcus* and *Acinetobacter*, respectively) as collection strains, they do not have the same behavior during the drying process.

To improve the yield, and to verify whether the difference between the two categories stayed the same, other drying techniques were tested.

Table 1
Collection Strains Description and Their Survival Ratio After Standardized Lyophilization

| Strains | Genera and species | Hydrocarbon used | Other characteristics | Survival ratio (%) |
|---|---|---|---|---|
| LB208 | *Rhodococcus* sp | Pyrene, fluoranthene, phenanthrene | | <0.01 |
| SK15 | *Arthrobacter* sp | Biphenyl | | 0.36 |
| LB126 | *Sphingomonas* sp | Fluorene | | 0.05 |
| AEX5 | *Alcaligenes eutrophus* | 3-CBA, 4-CBA, 3CBP, 4-CBP, 2-CBP, BP[a] | Cd, Ni, Zn resistant | 0.11 |
| LH240 | *Pseudomonas* sp | Oil | Lux marked[b] | <0.01 |
| PaW1 | *P. putida* | Biphenyl | | 0.09 |
| GpO1 | *P. oleovorans* | Oil | | 0.01 |
| LH168 | *Acinetobacter calcoaceticus* | Oil | | 0.16 |
| LB400 | *Pseudomonas* sp | Biphenyl | | 0.11 |
| A5.1. | *Alcaligenes eutrophus* | Biphenyl, 4-CBP | pSS50[c] | –[d] |

[a] 3-chloro-benzoate, 4-chloro-benzoate, 3-chloro-biphenyl, 4-chloro-biphenyl, 2-chloro-biphenyl, biphenyl, respectively.
[b] Ref. *16*.
[c] Catabolic plasmid, Springael, D. personal communication.
[d] Not determined.

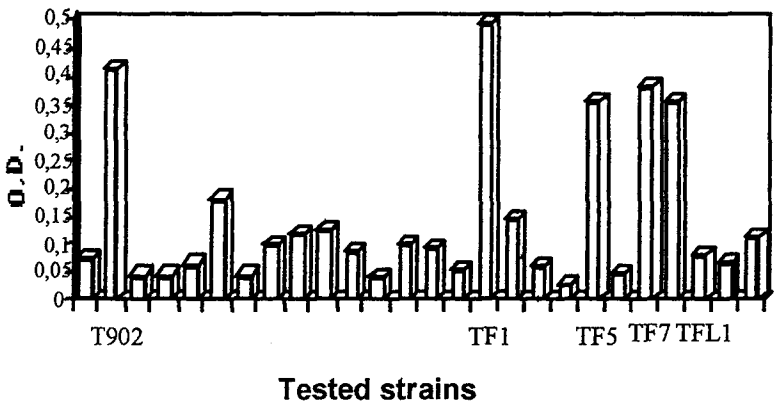


Fig. 1. Growth level (OD, 600 nm) of the collected soil strains in mineral liquid medium with diesel as sole source of carbon, measured after 4 d.

## Improvement of Survival Ratio

The technique adapted from Mattimore *(15)* consists of a slow extraction of moisture from the cells. It affords higher survival yields than the standardized lyophilization used so far. The results are given in Table 3.

Table 2
Identification and Survival Ratio After Standardized Lyophilization of Some Strains Collected from Dried Soil

| Strains | Genera and species | Survival ratio (%) |
|---|---|---|
| T902 | *Rhodococcus erythropolis* | 1.54 |
| TF1 | *Acinetobacter johnsonii* | 1.89 |
| TF7 | *Micrococcus luteus* | 2.55 |
| TFL1 | *Methylobacterium extorquens* | 1.03 |

Table 3
Survival Ratio of Different Selected Strains After Slow Drying

| Strain | 48 hr (%) | 15 d (%) | Fluidized bed (%) |
|---|---|---|---|
| T902 | 68 | 14.84 | 15.5 |
| TF1 | 6.61 | 1.36 | – |
| TF7 | 2.98 | 2.73 | 12.5 |
| TFL1 | 23.38 | 17.50 | – |
| LB208 | 0.56 | 0.15 | – |
| SK15 | –[a] | – | – |
| LB126 | 0.21 | <0.01 | – |
| AEX5 | 0.90 | <0.01 | – |
| LH240 | 0.79 | 0.77 | – |
| PaW1 | 0.17 | 0.10 | – |
| GpO1 | 1.16 | 0.01 | – |
| LH168 | 0.27 | 0.03 | 2 |
| LB400 | 0.35 | 0.03 | – |

[a] Not determined.

After 48 h of drying, the first survival value is measured when the $a_w$ of the bacteria is in equilibrium with the environment water activity, equaled to 0.49. The second value, measured after 15 d, shows the evolution of the survival ratio over time, i.e., the stability of the dry product.

Some samples were also dried in a fluidized bed. This technique allows a slow water activity depletion as well.

The strains selected for their drought-resistance characteristics exhibit a better survival level than the bioremediation collection strains, when dried with these techniques as well. Furthermore, most of the collection strains undergo a rapid decay of their surviving population with time. The dry products are not stable at 25°C. The bacteria collected from the dried soil yield much better drying resistance, and are more stable in time.

## Characterization of Carbon Source Pattern of the Strains

Some soil bacteria were streaked on minimal medium Petri dishes with different C sources, in order to determine their pattern of degradation

Table 4
Description of Hydrocarbons Metabolized by Strains Collected from Dried Soil

| Strains | General and species | Hydrocarbons degraded |
|---|---|---|
| T902 | *Rhodococcus erythropolis* | Alkanes ($C_{10}$–$C_{15}$), branched alkanes, diesel |
| TF1 | *Acinetobacter johnsonii* | Branched alkanes, diesel |
| TF7 | *Micrococcus luteus* | Benzene, phenol, m-xylene, diesel |
| TFL1 | *Methylobacterium extorquens* | Diesel |
| T901 | –[a] | Diesel |
| T981 | – | Xylenes, p-cymene, biphenyl, diesel |
| T982 | – | Diesel |
| T986 | – | Diesel, benzene |
| T987 | – | Alkanes ($C_{10}$–$C_{11}$), diesel |
| TFL3 | – | Alkanes ($C_8$–$C_{20}$), diesel |

[a] Not determined.

potentialities. Their growth was evaluated after 4 d on 40 different compounds, ranging from simple light alkanes to the heaviest polyaromatic hydrocarbons. A summary of the results is given in Table 4.

Since the first screenings were done with diesel as C source, not surprisingly, the strains selected are only able to grow on the most easily degradable molecules: n-alkane, and the simplest aromatic molecules. The more recalcitrant compounds are not decomposed. The selection should, therefore, be made on the recalcitrant compounds, if strains are to be isolated with other degradation properties.

The screening test with the dried soil samples was carried out again with a mixture of PAH (naphtalene, phenanthrene, anthracene) as sole source of C. No strain was isolated from this soil in these conditions.

## Maintenance of Biodegradation Properties After Drying

The influence of the drying process on the maintenance of the degradation properties of four microorganisms selected from dried soil (*Rhodococcus erythropolis, Acinetobacter johnsonii, Micrococcus luteus, Methylobacterium extorquens)* and of two of the collection strains *(Acinetobacter calcoaceticus,* LH168, and *Alcaligenes eutrophus,* A5.1.) was investigated. The effect of the different drying procedures (lyophilization and Mattimore's slow drying) on the bioremediation properties of the cells was measured by comparing their ability to grow on rich medium and on mineral medium, with a xenobiotic compound as sole source of C and energy.

The direct measurement of the degradation abilities of the bacteria after lyophilization is equal to the measurement made before drying, even for *A. eutrophus* A5.1, known to be a plasmid bearer. One hundred % of the survivors are still able to grow on decane, chosen as the xenobiotic C

Table 5
Maximal Growth Rate, Generation Time, Hydrocarbon Uptake Rate, and Lag Phase Duration of *Rhodococcus erythropolis* (T902) and *Micrococcus luteus* (TF7)

| Strain | Xenobiotic compound | | $\mu_{max}$ ($h^{-1}$) | g (h) | $-dS/dt$ (ppm/h) | Lag time |
|---|---|---|---|---|---|---|
| T902 | Decane | Before drying | 0.0129 | 54 | 88 | 0 |
| | | After drying | 0.0114 | 60 | 85 | 60 hr |
| T902 | Diesel | Before drying | 0.0210 | 33 | 44 | 0 |
| | | After drying | 0.0180 | 38 | 34 | 48 hr |
| TF7 | Decane | Before drying | 0.0115 | 60 | 92 | 0 |
| | | After drying | 0.0130 | 54 | 108 | 48–72 hr |
| TF7 | Diesel | Before drying | 0.0195 | 35 | 59 | 0 |
| | | After drying | 0.0168 | 41 | 52 | 24–48 hr |

All parameters were measured before and after lyophilization.

source. On the other hand, when dried with the slow technique, activity is lost during conservation. A part of the survivors have lost their ability to grow on the xenobiotic compound after a conservation of 15 d at 25°C of the dried powders *(see* Fig. 2). Loss of activity does not come from the drying process itself, but rather from the storage period. The phenomenon is common to both strain categories, but a difference appears between the drought-resistant bacteria and the collection ones. Eighty % of the survivors of the A5.1. strain already lost their degradation properties after 48 h and 75% of LH168 after 15 d, but only 10% of T902, 18% of TF7, 30% of TF1, and 43% of TFL1 lost their properties after 15 d.

In a second phase, two strains, *R. erythropolis* (T902) and *M. luteus* (TF7), showing the best rate of growth with hydrocarbons as sole source of C, were further studied. The maximum rate of growth ($\mu_{max}$), and the rate of hydrocarbon uptake ($-dS/dt$), with decane and diesel, were measured from the growth curve and the hydrocarbon degradation curve *(see* Fig. 3). Their values were compared before and after drying. The lag phase before growth start was estimated. The values are given in Table 5.

*R. erythropolis* (T902) and *M. luteus* (TF7) both grow faster on diesel than on decane alone, but they retain comparable generation time, before and after lyophilization. However, the lag time before the exponential growth starts gets longer after drying, especially on decane alone as C source. The lyophilization process does not have any effect on the growth rate of the strains, as measured by this experiment.

The degradation rate of decane is higher than that of diesel, although the growth rates are in the reversed order, for both strains. Since diesel is a complex mixture of compounds, some of them might not be decomposed at all, which makes the overall degradation rate slower, but, in diesel, the bacteria find other compounds that allow them to grow faster than on decane alone. The degradation rate values, measured before and after ly-

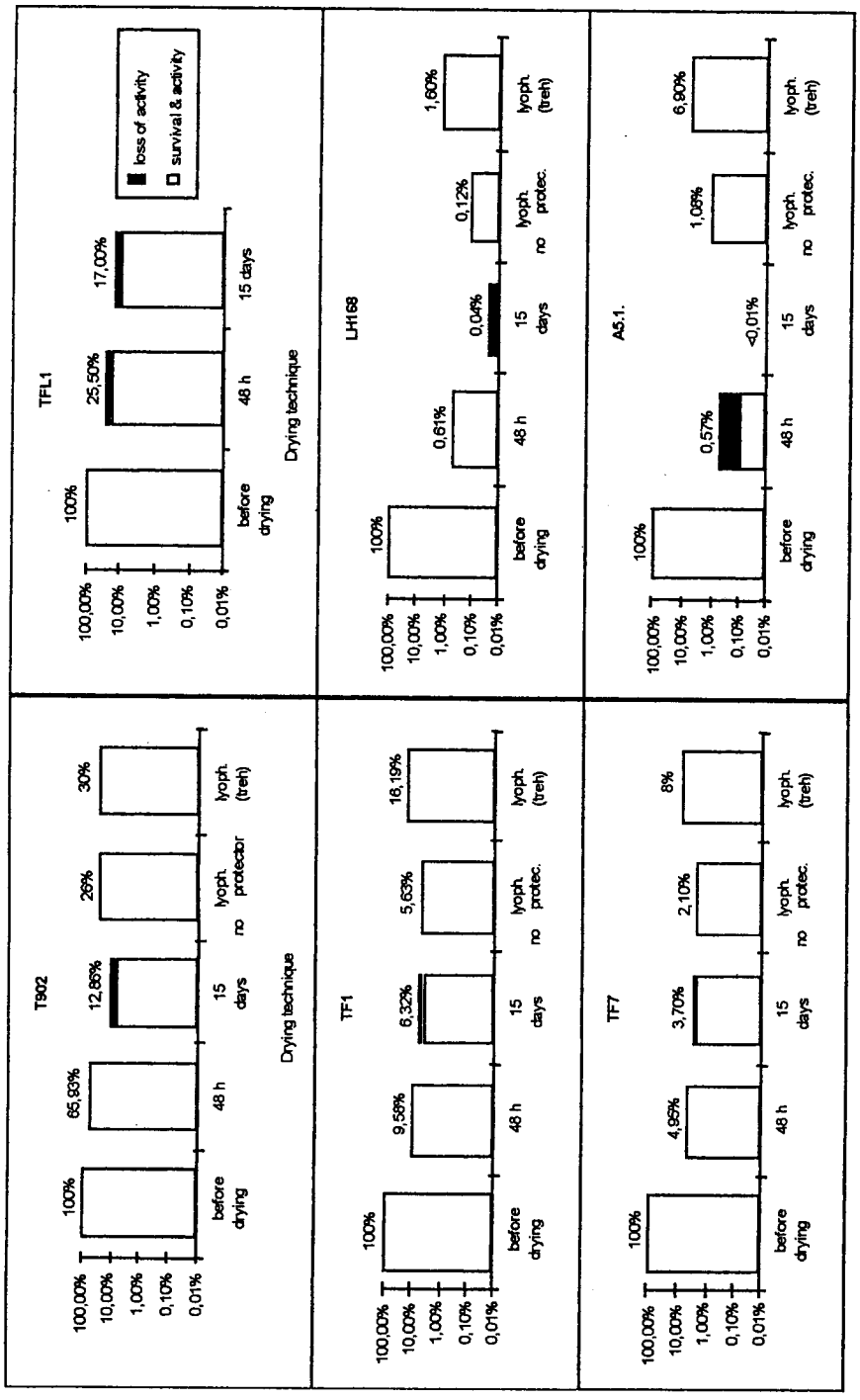


Fig. 2. Survival and maintenance of the biodegradation properties of soil strains and of two collection strains after 48 h of slow drying, 15 d of storage, lyophilization without protector, and lyophilization with 0.5% (w/v) trehalose as protector.

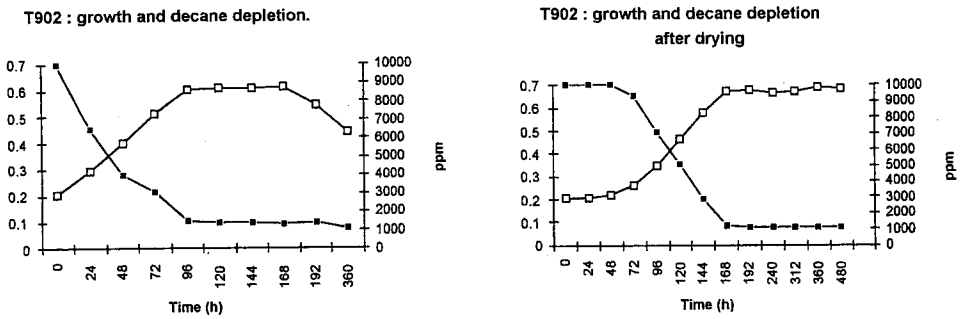


Fig. 3. Growth (O.D.) and decane consumption before and after drying of the strain T902.

ophilization, are essentially the same. The lyophilization process does not seem to have any effect on the rate of biodegradation of decane or diesel, once the lag phase is finished. However, because of the longer lag phase of the lyophilized starter, the overall speed of hydrocarbon uptake is slowed down compared to the use of fresh inoculum. This is true for both strains.

## CONCLUSIONS

This new approach to the selection of potential bioremediation strains, which are suitable for the conditioning process, yields good results. The strains selected from dried soil have a survival ratio two orders of magnitude higher than the collection strains. However, since they grow only on the compound on which they were selected, their biodegradation potentialities could be improved.

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