# EVALUATION OF THE BIOSTIMULATION AND BIOAUGMENTATION TECHNIQUES IN THE BIOREMEDIATION PROCESS OF PETROLEUM HYDROCARBONS CONTAMINATED SOIL

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# ABSTRACT

Frequent oil spills in Brazilian's clay-bearing soils are stimulating researches in the bioremediation area. However, there is a great difficulty of cleanning up those contaminated soils due to the strong soil/contaminant interactions and the low permeability, usually making impracticable the application of traditional *in-situ* bioremediation techniques. Biodegradation experiments were carried out in order to evaluate the efficiency of pollutant removal by the addition of native oil-degrading microorganisms (M4 – *Nocardia nova*, M29 – *Nocardia nova*, M31 - *Pandoraea sp*., M36 - *Rhodotorula glutinis*) and to define the best microbial pool to be used in the biotreatment of an oil-contaminated soil for three years. Moreover, for this pool, the influence of four nutrients ratio (C:N:P) of 100:1.25:1, 100:5:1, 100:1.25:5 and 100:5:5 and two inoculum size ($10^6$ and $10^8$ CFU/g of soil) on the biodegradation efficiencies were investigated. The experiments were performed and analysed through the experimental design by the *Statistica*™'99 software version 5.1 for *Windows*®. The results showed that best biodegradation efficiency (7.39%) was gotten by the addition of the pool constituted by M29 and M36 strains in an inoculum size of $10^8$ CFU/g of soil and correction of soil C:P ratio to 100:1. The highest C:N and C:P ratios used presented inhibitory effects in the biodegradation of the oil-contaminated soil and, more specifically, in the oil-degrading microorganisms. The M4 and M31 strains were harmful to the biodegradation possibly due to the competition for nutrients in the soil.

# INTRODUCTION

The risk of oil spillage, involved in many activities of petroleum industry, poses a serious environmental problem, due to the possibility of air, water and soil contamination. Recent oil spill occurrences in clay-bearing soils are motivating studies in the soil bioremediation field, especially due to the lack of scientific knowledge in the bio-treatment of this type of soil. Concerns about this subject led to the establishment of a partnership between The Center for Mineral Technology (CETEM/MCT), Petrobras Research Centre (CENPES) and The School of Chemistry of the Federal University of Rio de Janeiro (EQ/UFRJ), which has, as the main objective, the proposal of a new bioremediation technology to be applied to clay bearing soils. It has been shown that the presence of clay minerals in soil impairs the application of *in-situ* remediation techniques, frequently leading to the adoption of *ex-situ* alternatives. Recalcitrance of petroleum hydrocarbons in the type of soil can be ascribe to a high interaction between the soil matrix and those pollutants, associated to low oxygen and nutrients availability, reinforcing the importance of more efficient systems as aeration and stirring.

The maximum benefit of bioremediation process is the mineralization in which the pollutant is degraded to $CO_2$ and $H_2O$ by the aerobic metabolic way (1). There are two bioremediation techniques which can be used in all the available technologies of treatment in order to try to maximize its efficiencies (2): the biostimulation, in which there is the increase of the indigenous populations activity by adding nutrients and/or a terminal electron acceptor, and the bio-augmentation, in which there is the increase of the pollutant degradation potential by adding exogenous degrading microbial strains.

The present work was carried out and evaluated using as tool the experimental design and its strategy was divided in two stages described as follow: evaluation of the efficiency of pollutant removal by the addition of native oil-degrading microorganisms (M4 – *Nocardia nova*, M29 – *Nocardia nova*, M31 - *Pandoraea sp*., M36 - *Rhodotorula glutinis*) in an oil-contaminated soil for three years and the definition of the best microbial pool and determination of a better nutrients ratio (C:N:P) and inoculum size for the selected pool.

# MATERIALS AND METHODS

## Soil

The present work used a contaminated soil with crude oil for three years approximately. Some characterization data of that soil (3) are: 26.88% of water holding capacity (WHC), 5.38% of total petroleum hydrocarbons (TPH), 2.0 mg P / Kg of soil, 4.79% of $C_{organic}$ and 0.6g N / Kg of soil.

The contaminated soil was sampled by a specialized team from Petrobras in order to guarantee a representative sample which was stored at 5°C.

## Experimental Design

The generation of the experimental design matrix and the analysis of the results gotten at the end of the experiments had been carried through in Statistica$^{TM.99}$ software for Windows version 5.1. The results had been analysed statistically in a 95% confidence level, using only a dependent variable (biodegradation efficiency).

## Biodegradation Experiments

### Description and Details of the Experimental System

The biodegradation experiments were carried out in 250 ml conical flasks (kitazatos), containing 50g of contaminated soil and others additives such as nutrients and inoculum. The moisture content and pH were adjusted to 50% of WHC and 7.0, respectively. The nitrogen and phosphorus correction was performed using $NH_4NO_3$ and $K_2HPO_4$ solutions, respectively. The conditions were incubated at 30$^oC$ for 41 days and, during the experiments, they were aerated in 48 hours intervals for 2 minutes.

### Microorganisms

Four crude oil-degrading microorganisms, previously isolated and selected from the contaminated soil (4), were used in the biodegradation experiments, entitled as M4, M29, M31 and M36. These strains had been identified by the Tropical Foundation of Research and Technology "André Tosello" as: M4 - *Nocardia nova*, M29- *Nocardia nova*, M31 - *Pandoraea sp*. e M36 – *Rhodotorula glutinis var. dairenesis*. Two inoculum sizes ($10^6$ e $10^8$ CFU/ g of soil) were used in the experiments and gotten in the exponential growth phase of these strains determined by previous growth kinetics studies (4).

### Development of the Experiments

#### *Definition of the Best Microbial Pool:*

This stage had the objective to define the microbial pool that better stimulated the oil biodegradation efficiency, based in all possible combinations of the four strains in the presence and absence of nutrients (N and P) using as tool the fractionary factorial experimental design $2^{(6-2)}$, resulting in 16 experimental conditions. The inoculum size used was $10^6$ CFU/g of soil and the nutrients ratio (C:N:P) was 100:10:1. Therefore, the following variables had been investigated: M4, M29, M31 and M36 strains and C:N and C:P ratios. All the conditions were performed in duplicates. The following conditions had been investigated (Table 1).

#### *Evaluation of the Best Nutrients Ratio and the Best Inoculum Size:*

This stage aimed at defining a better nutrients ratio (C:N:P) and a better inoculum size for the best pool defined in the previous stage. For this purpose, a full factorial experimental design $2^3$ was carried out, resulting in 8 experimental conditions. The following variables and its respective levels had been studied: C:N (100:1.25 and 100:5), C:P (100:1 and 100:5) and inoculum size ($10^6$ and $10^8$ CFU/g of soil). All the conditions were performed in triplicates and, for each condition, two additional sacrifice flasks to

initial and intermediate quantitative determination of microorganisms were used. The following conditions had been investigated (Table 2).

## Quantitative Determination

A Hewlett-Packard 5890A II gas chromatograph with a thermal conductivity detector (TDC), set at 220° C, and a CHROMOSORB 102 column were used for the determination of the $CO_2$ content in the headspaces from conical flasks containing soil samples. The carrier gas used was ultra-high purity helium. Gas samples (0.5 ml) from all flasks were taken daily during the fist week of the test and three times a week after the first week.

The carbon dioxide results were used to estimate the total amount of contaminant consumed during biodegradation tests. Results were expressed by biodegradation efficiency (BE), calculated as follows:

Totally Biodegraded Carbon = 2 x Carbon from $CO_2$ Evolved

BE% = (Totally Biodegraded Carbon) x 100 / Soil Total Organic Carbon

The quantification of total heterotrophic microorganisms was made by pour plate technique (triplicates) in a solid organic medium (g/L): glucose, 10.0; peptone, 5.0; yeast extract, 2.0; NaCl, 5.0, Agar-Agar, 20.0. The Petri dishes had been incubated at 30°C for 48 hours and the results had been expressed in CFU/g of soil. The quantification of oil-degrading microorganisms was made by the Most Probable Number (MPN) technique (5). The liquid mineral medium (6), the crude oil (0.5 % v/v) and the microorganisms inoculum were added to the NMP tubes which were incubated at 30°C for 7 days. The results had been expressed in cells/g of soil.

# RESULTS AND DISCUSSION

## Definition of the Best Microbial Pool

Based in the total amount of the $CO_2$ evolved during the tests, the values of the biodegradation efficiencies (BE) had been determined (Table 3).

In order to select the microbial pool that more influenced positively the biodegradation efficiency, the Response Surface Graphics (Figures 1 and 2), generated by Statistica™.99 software, after the analysis of the matrix of the fractionary factorial experimental design (Table 3), were analysed. The influence of the strains used in the presence of nitrogen adjustment on the biodegradation efficiencies is observed as follow.

Nitrogen presented an inhibitory effect to the oil biodegradation when adjusted to the C:N ratio of 100:10 for all strains tested. In addition, it is possible to conclude that only the M29 strain (*Nocardia nova*) (Figure 1b) and the M36 strain (*Rhodotorula glutinis var. dairenesis*) (Figure 1d) had enhanced the biodegradation efficiencies, in the absence of nitrogen addition (C:N = 100:1.25).

These two microbial genera have been usually reported in the literature as oil degrading (7). It had been proved that *Rhodotorula* species has the ability of degrading

anthracene in soil which presented a biodegradation efficiency of 81% (8). Additionally, *Nocardia* species are found in soils and in aquatic environments and are able to degrade hydrocarbons and other recalcitrant compounds (9).

The M4 (*Nocardia nova*) and M31 (*Pandoraea sp*.) strains had presented a negative effect in the biodegradation efficiency (Figure 1). It suggests that these strains, when added to the contaminated soil, had not been able to mineralize the pollutant and had competed with the other strains for the nutrients in order to surviving or carrying through another metabolic route. This fact can have led to non ideal or harmful nutrients conditions to the strains capable to mineralise the crude oil (10, 11, 12).

Inhibitory effects to the biodegradation process caused by the excessive nitrogen addition have been vastly reported in literature (13, 14, 15). The nitrogen, when added through the ammonium salts, can be toxic to the microorganisms due to the possibility of ammonia generation in the soil, which can be lethal in high concentrations. Moreover, the ammonium ion promotes the increase of the oxygen demand which can cause problems to the ecosystem (13).

The influence of the four strains on the biodegradation efficiencies in the presence of phosphorus adjustment is observed in Figure 2.

The phosphorus addition stimulated the biodegradation when it was adjusted to C:P ratio of 100:1 (Figure 2). In these conditions, the M29 and M36 strains had also presented a beneficial effect on the biodegradation efficiencies which can be evidenced by the Figures 2b and 2d, respectively. The phosphorus addition can stimulate the biodegradation of petroleum hydrocarbons, however some sources (phosphate and ortho-phosphate) can have diverse effect on the biodegradation, depending on its toxicity and solubility (16).

## Evaluation of the Best Nutrients Ratio and the Best Inoculum Size

The $CO_2$ analysis during the biodegradation experiment allowed to evaluate the $CO_2$ evolution profile of each condition, as presented in the Figure 3.

It could be verified in Figure 3 that there wasn't a lag phase for all conditions tested, probably due to the previous exposition of the pollutant to the native microorganisms leading to a selection in favour of the oil-degrading ones. Moreover, exogenous oil-degrading microorganisms, isolated from the contaminated soil, were added in the same soil which also eliminate the adaptation phase (2, 17, 18).

Based in the total amount of generated $CO_2$, the values of the biodegradation efficiencies (BE) had been determined for the tested conditions (1 to 8) and for the control (without nutrients adjustment and without inoculum addition) (Table 4).

In order to verify the influence of the variables tested on the biodegradation efficiency, the Response Surface Graphics (Figures 4, 5 and 6), generated by Statistica$^{TM.99}$ software, after analysing the matrix of the full factorial experimental design (Table 4), were analysed. The influence of the nutrients adjustment on the biodegradation could be observed in Figure 4.

Both nutrients were very harmful to the biodegradation efficiency when adjusted to the highest nutrients ratio (Figure 4). The biodegradation of the pollutant was higher in the C:N:P of 100:1.25:1 as the biodegradation efficiency for this ratio was at least 81%, 81% and 54% higher than the biodegradation efficiencies for the C:N:P of 100:5:5, 100:5:1 and 100:1.25:5, respectively.

The influence of the inoculum sizes on the biodegradation in the presence of nitrogen (Figure 5) and phosphorus (Figure 6) adjustments could be verified as follow.

The inoculum size of $10^8$ CFU/g of soil, in the C:N:P of 100:1.25:1 (Figure 5), led to a biodegradation efficiency increase of 37% when compared to the inoculum size of $10^6$ CFU/g of soil, in the same nutrients ratio. In the C:N of 100:5 (Figure 5), the highest inoculum size did not stimulate the biodegradation when compared to the minor inoculum size used and, in the C:P of 100:5 (Figure 6), this increase was 10%. This fact evidences that the nutrients had been only beneficial in the lower used nutrients ratio (C:N:P of 100:1.25:1).

It is well known that each system has its proper optimal C:N:P ratio as it depends on the contaminant type, concentration and bioavailability and, in addition, the ability of the microorganisms in degrading it. The C:N:P ratio of 100:10:1 is extensively used, but under certain conditions, the carbon of the oil is not completely assimilated to the biomass because some components are recalcitrant or metabolised by long periods (19). In this case, it can lead to harmful effect to the microorganisms due to this nutrients ratio to be higher than the amounts required. It was noticed in the conditions which also contained the nutrients ratio of 100:10:1 in the previous stage and in the conditions which contained nutrients ratios 100:5:1, 100:1.25:5 and 100:5:5 in this stage, suggesting that only a small amount of carbon proceeding from the crude oil is being assimilated to the biomass.

The oil biodegradation inhibition caused by the nitrogen adjustment is not uncommon. Inhibitory levels of nitrogen range from 100 to 4000 mgN/Kg of soil (15). In the experiments carried out in the first stage, the nitrogen concentration of the C:N of 100:10 was higher than 4000mgN/Kg of soil (4573mgN/Kg of soil) and in the second stage, the nitrogen concentration of the C:N of 100:5 was within the inhibitory range (2286.5mgN/Kg of soil).

The inorganic nitrogen can be related better with the soil moisture ($N_{H2O}$) than with the substrate level (C:N) or dry soil mass (Ns) in order to supply more adequately this nutrient for the bioremediation avoiding the inhibition. A super-fertilization is dependent on the soil water content which can decrease the activity of oil-degrading microorganisms that are sensible to the soil water potential. Soils with a high water content better dilute the nitrogen than soils with a low water content. However, it must bear in mind that the increase of water content in soils leads to a reduction of the $O_2$ transfer rate. The optimal $N_{H2O}$ concentration is approximately 2000mgN/Kg of $H_2O$ and the threshold concentration is 2500mgN/Kg of $H_2O$ (15). The C:N ratios of 100:1.25 and 100:5 used in the biodegradation experiments resulted in the $N_{H2O}$ values of 5004 and 20015 mgN/ Kg of $H_2O$, respectively. The lowest C:N ratio used of 100:1.25 (value found in the soil) resulted in a $N_{H2O}$ value higher than the threshold concentration proposed.

The phosphorus, when adjusted to the C:P of 100:5, also presented a inhibitory effect to the biodegradation efficiency. Probably, there was a inhibition of the microbial growth and/or the oil degradation metabolism caused by the phosphorus toxicity in this concentration to the soil microbial population.

Another way to verify the influence of the nutrients ratio on the biodegradation is to evaluate the percentage (logarithmic base) of the degrading microorganisms in the total heterotrophic population during the experiments (Table 5).

It is possible to verify that there was a reduction in the degrading microorganisms percentage to the end of the experiment in the conditions with different nutrients ratios of 100:1.25:1 (conditions 2, 3, 4, 6, 7 and 8) when compared with the initial percentage. Only the conditions 1 and 5 kept the percentage above 90% during all the period of experiment execution. Both the nitrogen and phosphorus, in the highest nutrients ratios, reached inhibitory levels to the degrading population what can be observed by the lower percentage in these conditions. Moreover, for these nutrient ratios, the degradation and/or consumption of other organic compounds in the soil can have been favoured (17, 20).

# CONCLUSIONS

Among all the possible combinations of the four degrading microbial strains, isolated from the oil contaminated soil, the pool that more stimulated the biodegradation process was constituted by the M29 (*Nocardia nova*) and M36 (*Rhodotorula glutinis var. dairenesis*) strains. When added to the contaminated soil, the M4 (*Nocardia nova*) and M31 (*Pandoraea sp.*) strains had presented a negative effect on the biodegradation efficiency, suggesting that these strains had not been capable to mineralise the pollutant and had competed for the nutrients leading to non ideal nutrients conditions to the strains capable to mineralise the crude oil.

The nitrogen adjustments to the C:N ratios of 100:10 and 100:5 caused an inhibitory effect to the degrading microbial population, harming extensively the biodegradation. The phosphorus only presented an inhibitory effect when it was adjusted to the C:P ratio of 100:5. It is possible to conclude that the best C:N:P ratio investigated was 100:1.25:1, being only necessary to adjust the phosphorus content in the soil samples. The best inoculum size tested was $10^8$ CFU/g of soil.

Despite the biodegradation efficiencies had still been low, it must be considered that the contaminated soil is aged and contains a recalcitrant fraction to the microbial degradation. It can be noted that a pollutant fraction seems to be inaccessible to the biodegradation in contaminated soils for a long time due to the weathering. The decrease of the bioavailability may result from (21): incorporation of the pollutant to the soil natural organic material due to the chemical oxidation reactions, slow diffusion into very small pores, sorption into organic matter and formation of semi-rigid films around non-aqueous-phase liquids (NAPL), causing a high resistance to the mass transfer. Moreover, this soil contains a high contamination level (5.38% of TPH) as compared with values mentioned in the literature. Under certain conditions, the threshold TPH concentration in soil is approximately 2.8% (22). In higher concentrations, the biodegradation does not occur or occurs in very low rate (23).

However, even with a high oil concentration, a biodegradation efficiency of 7.39% was reached by the addition of two degrading microorganisms (M4 - *Nocardia nova* and M36 - *Rhodotorula glutinis var. dairenesis*) in the inoculum size of $10^8$ CFU/g of soil and adjustment of the C:N:P ratio to 100:1.25:1. This efficiency was approximately 99.7% higher than the efficiency reached for the control without inoculum addition and nutrients adjustment.

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# Tables:

**Table 1.** Tested conditions in the Fractionary Factorial Experimental Design – $2^{(6-2)}$.

| CONDITIONS | VARIABLES | | | | | |
|---|---|---|---|---|---|---|
| | M4 | M 29 | M 31 | M 36 | C:N | C:P |
| 1 (Control) | -1 | -1 | -1 | -1 | -1 | -1 |
| 2 | 1 | -1 | -1 | -1 | 1 | -1 |
| 3 | -1 | 1 | -1 | -1 | 1 | 1 |
| 4 | 1 | 1 | -1 | -1 | -1 | 1 |
| 5 | -1 | -1 | 1 | -1 | 1 | 1 |
| 6 | 1 | -1 | 1 | -1 | -1 | 1 |
| 7 | -1 | 1 | 1 | -1 | -1 | -1 |
| 8 | 1 | 1 | 1 | -1 | 1 | -1 |
| 9 | -1 | -1 | -1 | 1 | -1 | 1 |
| 10 | 1 | -1 | -1 | 1 | 1 | 1 |
| 11 | -1 | 1 | -1 | 1 | 1 | -1 |
| 12 | 1 | 1 | -1 | 1 | -1 | -1 |
| 13 | -1 | -1 | 1 | 1 | 1 | -1 |
| 14 | 1 | -1 | 1 | 1 | -1 | -1 |
| 15 | -1 | 1 | 1 | 1 | -1 | 1 |
| 16 | 1 | 1 | 1 | 1 | 1 | 1 |

-1: C:N= 100:1.25 (without adjustment) or C:P =100:0.004 (without adjustment) or absence of inoculum.
1: C:N= 100:10 or C:P= 100:1 or inoculum of $10^6$ CFU/g of soil.

**Table 2.** Tested conditions in the Full Factorial Experimental Design - $2^3$.

| CONDITIONS | VARIABLES | | |
|---|---|---|---|
| | C:N | C:P | Inoculum Size |
| 1 | -1 | -1 | -1 |
| 2 | 1 | -1 | -1 |
| 3 | -1 | 1 | -1 |
| 4 | 1 | 1 | -1 |
| 5 | -1 | -1 | 1 |
| 6 | 1 | -1 | 1 |
| 7 | -1 | 1 | 1 |
| 8 | 1 | 1 | 1 |

-1: C:N= 100:1.25 or C:P = 100:1 or inoculum size of $10^6$ CFU/g of soil.
1: C:N= 100:5 or C:P = 100:5 or inoculum size of $10^8$ CFU/g of soil.

**Table 3.** Matrix of the Fractionary Factorial Experimental Design – $2^{(6-2)}$.

| CONDITIONS | VARIABLES | | | | | | BE (%) * |
|---|---|---|---|---|---|---|---|
| | M4 | M 29 | M 31 | M 36 | C:N | C:P | |
| 1 (Control) | -1 | -1 | -1 | -1 | -1 | -1 | 3.70 ± 0.56 |
| 2 | 1 | -1 | -1 | -1 | 1 | -1 | 1.75 ± 0.06 |
| 3 | -1 | 1 | -1 | -1 | 1 | 1 | 2.14 ± 0.26 |
| 4 | 1 | 1 | -1 | -1 | -1 | 1 | 4.62 ± 0.55 |
| 5 | -1 | -1 | 1 | -1 | 1 | 1 | 1.38 ± 0.44 |
| 6 | 1 | -1 | 1 | -1 | -1 | 1 | 2.49 ± 0.37 |
| 7 | -1 | 1 | 1 | -1 | -1 | -1 | 3.55 ± 0.53 |
| 8 | 1 | 1 | 1 | -1 | 1 | -1 | 1.79 ± 0.20 |
| 9 | -1 | -1 | -1 | 1 | -1 | 1 | 4.04 ± 0.44 |
| 10 | 1 | -1 | -1 | 1 | 1 | 1 | 2.11 ± 0.38 |
| 11 | -1 | 1 | -1 | 1 | 1 | -1 | 1.84 ± 0.30 |
| 12 | 1 | 1 | -1 | 1 | -1 | -1 | 5.76 ± 0.54 |
| 13 | -1 | -1 | 1 | 1 | 1 | -1 | 1.01 ± 0.60 |
| 14 | 1 | -1 | 1 | 1 | -1 | -1 | 3.15 ± 0.77 |
| 15 | -1 | 1 | 1 | 1 | -1 | 1 | 7.56 ± 0.49 |
| 16 | 1 | 1 | 1 | 1 | 1 | 1 | 2.73 ± 0.13 |

-1: C:N= 100:1.25 (without adjustment) or C:P =100:0.004 (without adjustment) or absence of inoculum.
1: C:N= 100:10 or C:P= 100:1 or inoculum of $10^6$ CFU/g of soil.
* means and standard deviation gotten by duplicates.

**Table 4.** Expanded Matrix of Full Factorial Experimental Design - $2^3$.

| CONDITIONS | VARIABLES | | | BE(%) * |
|---|---|---|---|---|
| | C:N | C:P | Inoculum Size | |
| 1 | -1 | -1 | -1 | 5.39 ± 0.66 |
| 2 | 1 | -1 | -1 | 2.82 ± 0.09 |
| 3 | -1 | 1 | -1 | 3.35 ± 0.18 |
| 4 | 1 | 1 | -1 | 2.75 ± 0.21 |
| 5 | -1 | -1 | 1 | 7.39 ± 0.03 |
| 6 | 1 | -1 | 1 | 2.87 ± 0.26 |
| 7 | -1 | 1 | 1 | 3.69 ± 0.11 |
| 8 | 1 | 1 | 1 | 3.06 ± 0.15 |
| Control | indigenous microorganisms + soil nutrients relation | | | 3.70 ± 0.56 |

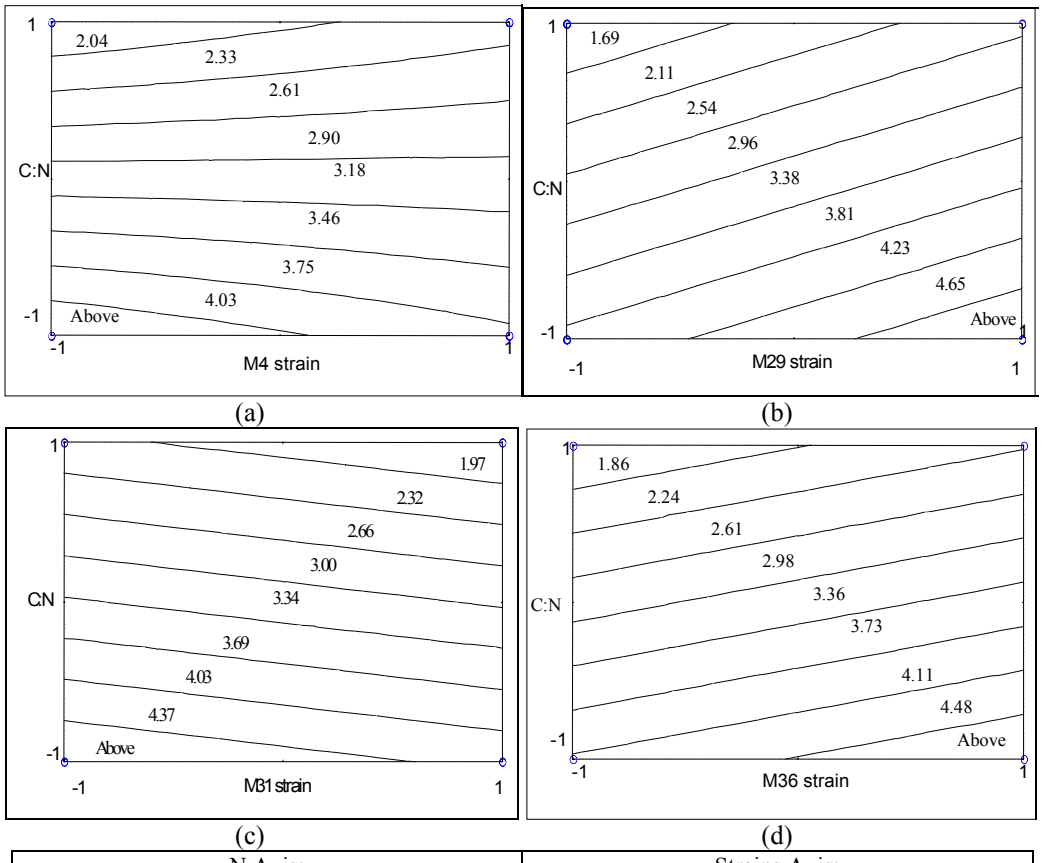
-1: C:N= 100:1.25 or C:P = 100:1 or inoculum size of $10^6$ CFU/g of soil.
1: C:N= 100:5 or C:P = 100:5 or inoculum size of $10^8$ CFU/g of soil.
* means and standard deviation gotten by triplicates.

**Table 5.** Degrading Microorganisms Percentage (logarithmic base).

| Conditions | C:N | C:P | Initial Degrading Microorg. (cells/g of soil) | Final Degrading Microorg. (cells/g of soil) | Initial Degrading Microorg. (%) | Final Degrading Microorg. (%) |
|---|---|---|---|---|---|---|
| 1 | LR | LR | $2.38x10^6$ | $4.28x10^6$ | 98.20 | 90.34 |
| 2 | HR | LR | $2.38x10^5$ | $1.90x10^5$ | 85.40 | 70.71 |
| 3 | LR | HR | $1.43x10^6$ | $1.43x10^5$ | 97.64 | 63.86 |
| 4 | HR | HR | $2.38x10^5$ | $1.52x10^5$ | 88.19 | 70.54 |
| 5 | LR | LR | $1.05x10^8$ | $9.03x10^7$ | 90.97 | 93.34 |
| 6 | HR | LR | $6.18x10^6$ | $1.05x10^6$ | 80.19 | 74.45 |
| 7 | LR | HR | $4.28x10^6$ | $7.13x10^4$ | 77.08 | 58.93 |
| 8 | HR | HR | $2.38x10^6$ | $1.43x10^5$ | 76.07 | 63.81 |

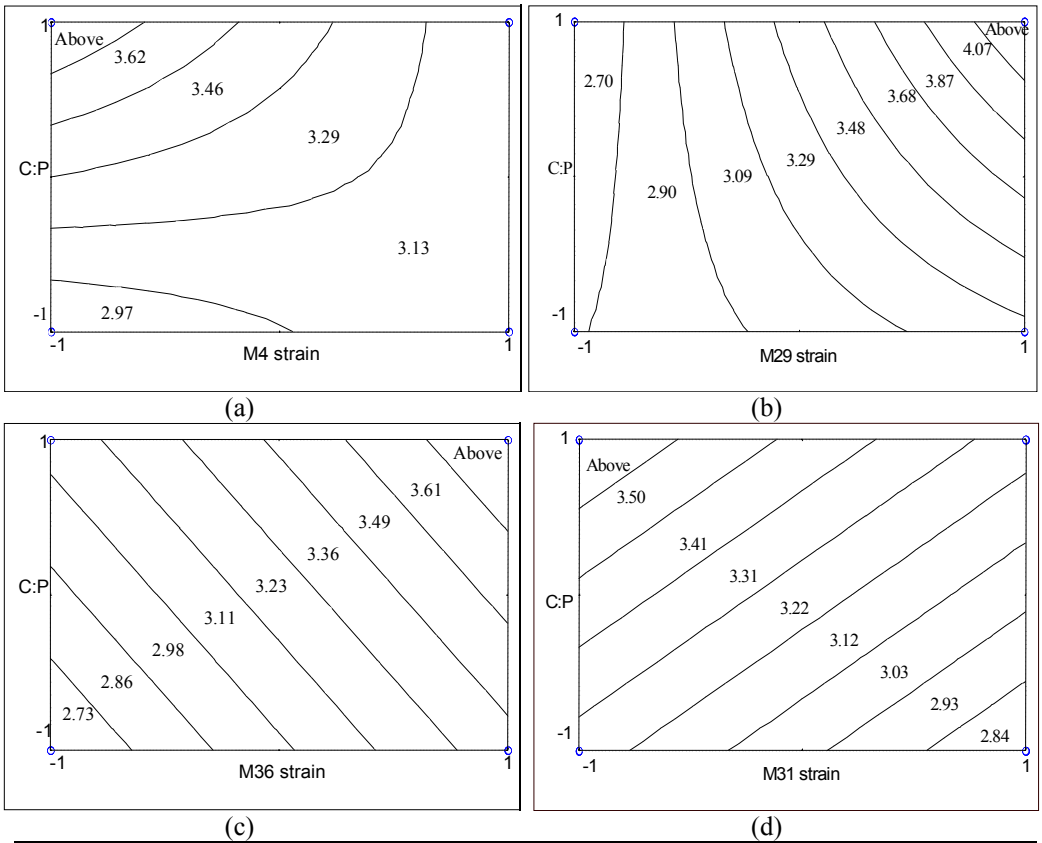
LR – low nutrient ratio used. HR – high nutrient ratio used.

# Figures:




| N Axis: | Strains Axis: |
|---|---|
| -1: C:N= 100:1.25 (without adjustment).<br>1: C:N= 100:10 (adjustment). | -1: absence of inoculum.<br>1: inoculum of $10^6$ CFU/g of soil. |
| shown values correspond to the biodegradation efficiencies. | |

**Figure 1.** Influence of the (a) M4 (b) M29 (c) M31 e (d) M36 strains in the presence of nitrogen adjustment.




| P Axis: | Strain Axis: |
|---|---|
| -1: C:P= 100:0,004 (without adjustment).<br>1: C:P= 100:1 (adjustment). | -1: absence of inoculum.<br>1: inoculum of $10^6$CFU/g of soil. |
| values in the graphics correspond to the biodegradation efficiencies. | |

**Figure 2.** Influence of the (a) M4 (b) M29 (c) M31 e (d) M36 strains in the presence of phosphorus adjustment.

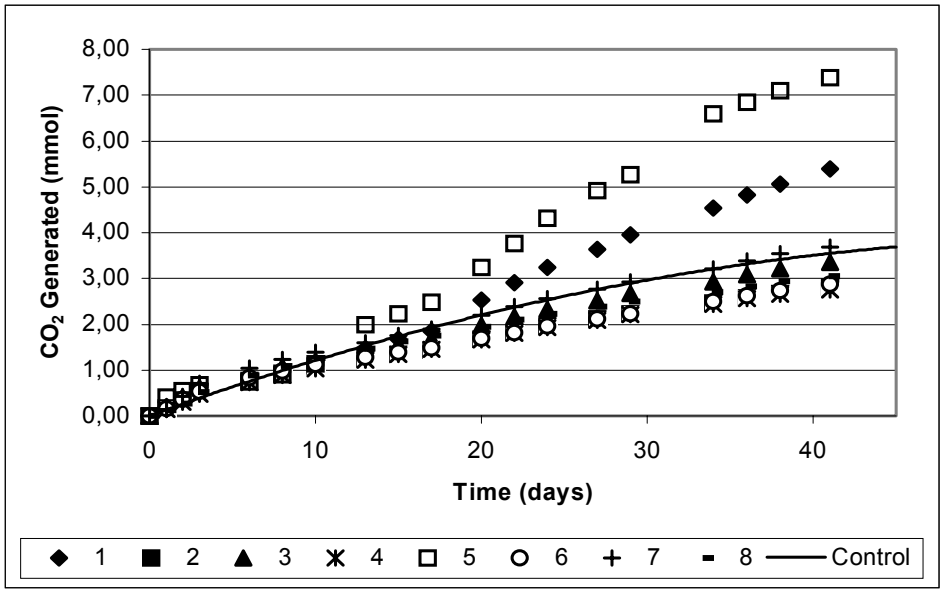
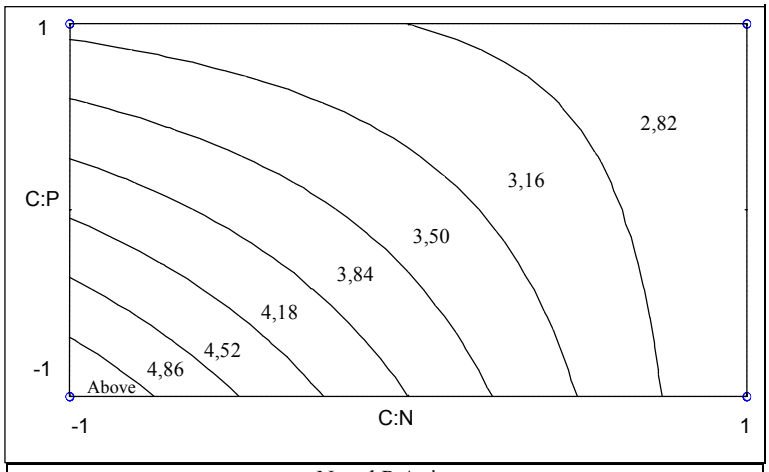
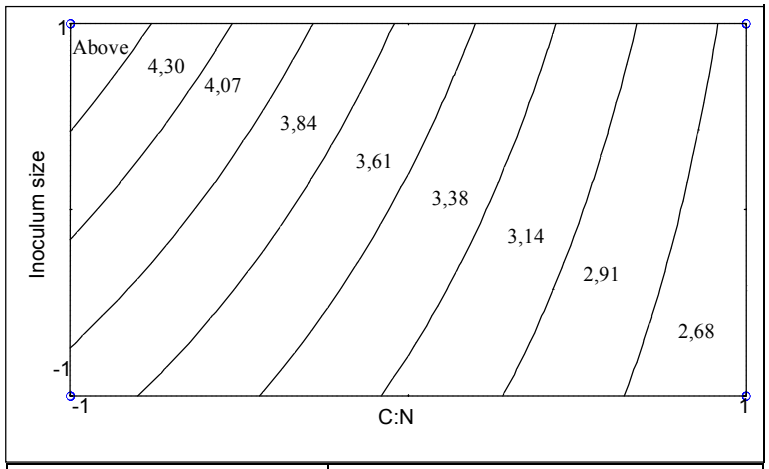



**Figure 3.** Profiles of $CO_2$ Generation.



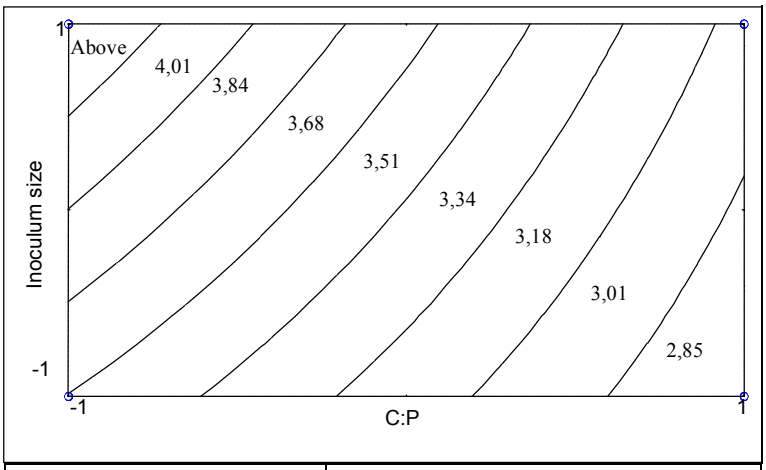

| N and P Axis: | |
|---|---|
| -1: C:N=100:1.25 or C:P = 100:1. | 1: C:N or C:P = 100:5. |
| Values in the graphic correspond to the biodegradation efficiencies. | |

**Figure 4.** Influence of the Nutrients Adjustment in the Biodegradation.




| N Axis: | Pool Axis: |
|---|---|
| -1: C:N= 100:1.25.<br>1: C:N= 100:5. | -1: inoculum size of $10^6$ CFU/g of soil.<br>1: inoculum size of $10^8$ CFU/g of soil. |
| Values in the graphic correspond to the biodegradation efficiencies. | |

**Figure 5.** Influence of Inoculum Sizes and the N Adjustment on the Biodegradation.




| P Axis: | Pool Axis: |
|---|---|
| -1: C:P= 100:1.<br>1: C:P= 100:5. | -1: inoculum size of $10^6$ CFU/g of soil.<br>1: inoculum size of $10^8$ CFU/g of soil. |
| Values in the graphic correspond to the biodegradation efficiencies. | |

**Figure 6.** Influence of Inoculum Sizes and the P Adjustment on the Biodegradation.