



Ecological engineering: from concepts to applications.

Feasibility of mixed enzymatic complexes to enhanced soil bioremediation processes

R. Gómez Jiménez-T^a, E. Moliterni^b, L. Rodríguez^b, F.J. Fernández^c, J. Villaseñor^{c, *}

^aChemical Engineering Department, Technical College of Civil Engineers, University of Castilla-La Mancha, 13071, Ciudad Real, Spain

^bAlquimia Soluciones Ambientales, S.L. C/ de la Calidad, 3, 13250 Daimiel (Ciudad Real), Spain

^cChemical Engineering Department, Institute for Chemical and Environmental Technology (ITQUIMA), University of Castilla-La Mancha, 13071, Ciudad Real, Spain

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Abstract

This work deals about the feasibility of using different enzymatic complexes to enhance bioremediation of hydrocarbon polluted soils. These complexes were generated by non defined mixed microbial consortia that were isolated from three different oil contaminated sites at an oil refinery, and enriched through weekly aerobic cultivations using diesel hydrocarbons as the sole carbon source. Extracellular proteins mixtures were produced during laboratory batch diesel biodegradation experiments using the three microbial consortia, and after separation from the biomass and remanent substrate, the proteins production and the enzymatic activity were measured in every cases. The trend of the enzymatic activity of these consortia was similar qualitatively, achieving the maximum at the exponential phase of the standard bacterial growth curve; but one of the consortia (C) reached the highest activity (275.316 U/mg). In a second step of this work, batch bioremediation experiments were done to evaluate the feasibility of such enzymatic complexes using the the one that provided the best results (C) to enhance the hydrocarbon removal from a polluted soil carried out by the microbial consortia.

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1. Introduction

The contamination of soil and groundwater with petroleum hydrocarbons is unfortunately a common phenomenon which causes serious environmental problems. This contamination can be caused by accidents, such as breaks in pipelines or transport accidents, or by poor waste-treatment processes and the subsequent discharge of pollution. Bioremediation is considered to be one of the best approaches for restoring oil-contaminated soils because the technology is cost-effective and environmentally benign [1].

The enzymatic degradation can be defined as the use of the enzymes in the contaminated site to degrade the pollutants; these enzymes could be previously produced by the microorganisms that degrade the pollutant. It is an

* Corresponding author. Tel.: +34-902-20-4100; fax: +34-926-29-5242.

E-mail address: jose.villaseñor@uclm.es (J. Villaseñor).

innovative technique that combines methods already common in the traditional decontamination, such as pumping, separation or injection of air, with enzymatic processes.

Enzymes have the ability to breakdown the bonds of organic compounds and/or catalyze its transformation into less toxic compounds and more biodegradable forms. Therefore, they reduce the time required for the process or provide significant advantages over traditional chemical or physical methods.

Some other advantages of the enzymatic bioremediation processes are that the enzymes are products with biological specificity for one type of pollutant, allowing them to remove selectively the contaminants and preclude the undesirable or unnecessary reactions. The enzymes mixtures are not toxic and they are derived from natural organism, what ensures the maximum mixing efficiency. The main disadvantage is that the enzymatic complexes may lose activity over time, i.e., they can be inactivated due to various factors such as thermal denaturation or loss of the prosthetic group [2].

Since there was already extensive experience in the Department of Chemical Engineering (University of Castilla-La Mancha) in the study of the bioremediation of soils contaminated with diesel hydrocarbons [3,4], it was considered interesting to meet a specific study on the enzymatic bioremediation of these soils using the extracellular enzymes secreted by the microorganisms.

Therefore, the bioremediation process of a diesel-contaminated soil, improved through the use of enzymatic complexes, was carried out using three different microbial consortia obtained from oil-contaminated soils and adapted to the degradation of diesel oil. First, during previous batch diesel-biodegradation, it was studied the generation of extracellular enzymatic complexes by microorganisms. In a second step, it was studied the feasibility of improving the process of microbial bioremediation of soils contaminated with diesel oil by the addition of those enzymatic complexes.

2. Materials and methods

2.1. Microorganisms and culture conditions

Three diesel-oil contaminated soils (named as SA, SB and SC) were collected from three different sites around an oil refinery in central Spain. These soils were characterised upon arrival at the laboratory and the different consortia of unidentified microorganisms contained in the soils (XA, XB and XC respectively) were isolated from these soils, maintained and enriched over several weeks, as described below.

Five grams of each soil sample were added to sterile flasks containing 50 mL of BH (Bushnell-Haass) basal-enrichment medium broth (BD, Franklin Lakes, NJ USA, ref. 257820), and the bottles were placed in an Ecotron incubator-shaker overnight at 50 rpm and 26°C. Each water-soil suspension was centrifuged at $1,016\times g$ for 15 min to obtain the supernatants containing the unidentified cultures. These supernatants were inoculated at a ratio of 1% (v/v) into three flask bottles containing sterile BH broth, a defined mineral-salts medium, with 1% (v/v) diesel oil as the sole carbon source. These three consortia were maintained for several months by weekly subcultivation and enrichment under aerobic conditions in the Ecotron incubator-shaker (50 rpm and 26°C). The BH broth composition was (g L^{-1}): MgSO_4 (0.2), CaCl_2 (0.02), KH_2PO_4 (1), $(\text{NH}_4)_2\text{HPO}_4$ (1), $(\text{NH}_4)\text{NO}_3$ (1) and FeCl_3 (0.05). The media was prepared with pure water, adjusted to a final pH of 7 ± 0.2 and autoclaved; the diesel oil was sterilised by filtration (0.2 μm) prior to addition. Previous studies conducted by the authors in the same laboratory have demonstrated the feasibility of obtaining stable consortia by this method [3,4].

2.2. Hydrocarbon characterization

The hydrocarbons used in this work were those contained in a conventional petroleum-derived diesel fuel from a petrol station in Ciudad Real, Spain. The density of the diesel oil was 832 g L^{-1} (Method: EN-ISO 3675:1998, crude petroleum and liquid petroleum products. Laboratory determination of density: hydrometer method) and its composition, estimated through a distillation curve at atmospheric pressure (ASTM D86, Standard Test Method for Distillation of Petroleum Products at Atmospheric Pressure) with the help of the Hysys® Oil-Program Manager (Aspen Technology, Inc. Burlington, Massachusetts, USA), was about 75% saturated hydrocarbons (primarily paraffin including *n*-, *iso*- and cycloparaffin), and 25% aromatic hydrocarbons (including naphthalene and alkylbenzene). Chain length of the *n*-alkanes was also identified using a diesel standard pattern (Absolute Standards,

Inc. Hamden, Connecticut, USA) by comparing the retention times with the chromatographic profile of the diesel oil used. The results indicated the presence of n-alkanes with chain lengths ranging from 10 to 26 carbon atoms and other typical compounds such as pristane and phytane.

2.3. Production and analysis of the enzymatic complexes

The experiments were performed in 1-L Erlenmeyer flasks with rubber stoppers in an orbital shaker bath at 25°C and 130 rpm to ensure oxygen and substrate availability.

Each flask contained 300 mL of BH broth enriched with 1% diesel and they were inoculated with 1% (v/v) of a microbial consortium. The samples (by triplicate) were taken at different times and centrifuged to obtain a cell-free supernatant with the enzymatic complexes. This procedure allowed obtaining the extracellular enzymes secreted by microorganisms, which were supposed to be involved in the first step of bioremediation. Samples were taken and analyzed over 2 weeks to measure the evolution of the total enzymatic activity and the concentration of proteins, and to calculate the specific enzymatic activity.

Total enzymatic activity (U/ml) was determined treating the sample with Sulphanilamide – azocasein and with trichloroacetic acid (ATC). Then, the optical density (OD) of the culture medium was measured at 379 nm using a Shimadzu UV-1700 spectrophotometer and, with the equation above, was related with the total enzymatic activity.

$$\text{Total Activity (U/mL)} = \frac{\text{OD} \cdot 1000}{16.67} \quad (1)$$

Total extracellular proteins were measured by the Lowry Method [5] as described by Verdin et al. [6]. The total concentration was obtained from the OD at 450 nm as:

$$\text{Protein Concentration (mg/ml)} = \frac{35.7 \cdot \text{OD} \cdot 5}{0,1} \quad (2)$$

The specific enzymatic activity (U/mg protein) was calculated with the following mathematical equation:

$$\text{Specific Activity (U/mg)} = \frac{\text{Total Activity (U/ml)}}{\text{Protein Concentration (mg/ml)}} \quad (3)$$

2.4. Soil bioremediation experiment with enzyme addition

A laboratory batch experiment was carried out with diesel polluted soil in a water suspension (slurry system). An inoculum of microbial consortia was added to produce the diesel biodegradation, together with an extra amount of the extracellular enzymatic complex previously produced by the same consortia. To observe the possible improvement of the diesel degradation caused by the enzyme complex, it was simultaneously carried out a control experiment using the same operating conditions, but with no enzyme addition.

The soil used was a sterile clay soil, sterilized at 121°C during 15 minutes. The microbial consortium used was the one that provided the best results on the previous enzymatic activity experiments, that is the XC consortium supplemented with its own enzymatic complex. The experiment consisted on the addition of BH broth on different Erlenmeyer flask that were previously filled with 15 g of the sterile soil and inoculated with the microorganisms. Two groups of flasks must be distinguished: (a) the “control flasks” that would only serve to evaluate the bioremediation with no enzyme addition, and (b) the “experiment flasks” with addition of enzymatic complex. The first ones (a) were filled with 60 ml of Bushnell Broth and inoculated with 300 µl of the XC consortia suspension. The other flasks (b) were identical, but also 60 ml of BHB Broth, enriched with the enzymatic complex, were added. To obtain previously this enriched broth, 300 ml of BHB were poured in a 1 L Erlenmeyer flask supplemented with 3 ml of diesel and 3 ml of XC microorganism suspension, and then incubated during 24 hours. The cell-free supernatant after centrifugation containing the enzymatic complex was poured in the “experiment flasks”. The specific activity of the enzyme complexes was measured before they were added to the experiment, and the result obtained was 147.029 U/mg. Finally, all flasks were incubated at 25°C and shaken at 130 rpm.

Sampling was made by taking complete flasks at different times. Diesel oil had to be extracted from the aqueous and solid phase, so the sample was shaken at 11000 rpm during 10 minutes and at 4°C. Then, the two phases were separated and treated individually. On one hand, 5 g of sodium sulfate anhydrous was added to the solid phase and then, the diesel was extracted adding 3 ml of n-hexane and shaken at 11000 rpm during 10 minutes at 4°C. On the other hand, the liquid phase only needed the addition of the extraction agent (2 ml n-hexane), and then the sample was shaken to extract the diesel.

The diesel extracted was analyzed by Gas chromatography to determine the concentration of total petroleum hydrocarbon (TPH). The equipment used was a Thermo-Fischer Trace GC Ultra gas chromatograph equipped with a flame-ionization detector, where the hydrocarbons of the samples were separated in a micro Ultra Fast capillary column (5 m × 0.1 mm id × 0.4 μm). The carrier gas used was helium, the injection volume was 1 μL and a split injection mode was employed. Qualitative analysis was performed using an *n*-alkane reference-calibration mixture (Absolute Standards Inc - Hamden, CT, USA) and calibration curves were prepared from serial dilutions for quantitative analysis.

3. Results and Discussion

3.1. Enzymatic activity

Fig. 1 shows the results of the experiments described in section 2.3 (production and analysis of the enzymatic complexes). The figure plots the average concentration of proteins and the total enzymatic activity obtained from the three consortia studied. Vertical bars indicate the standard deviation.

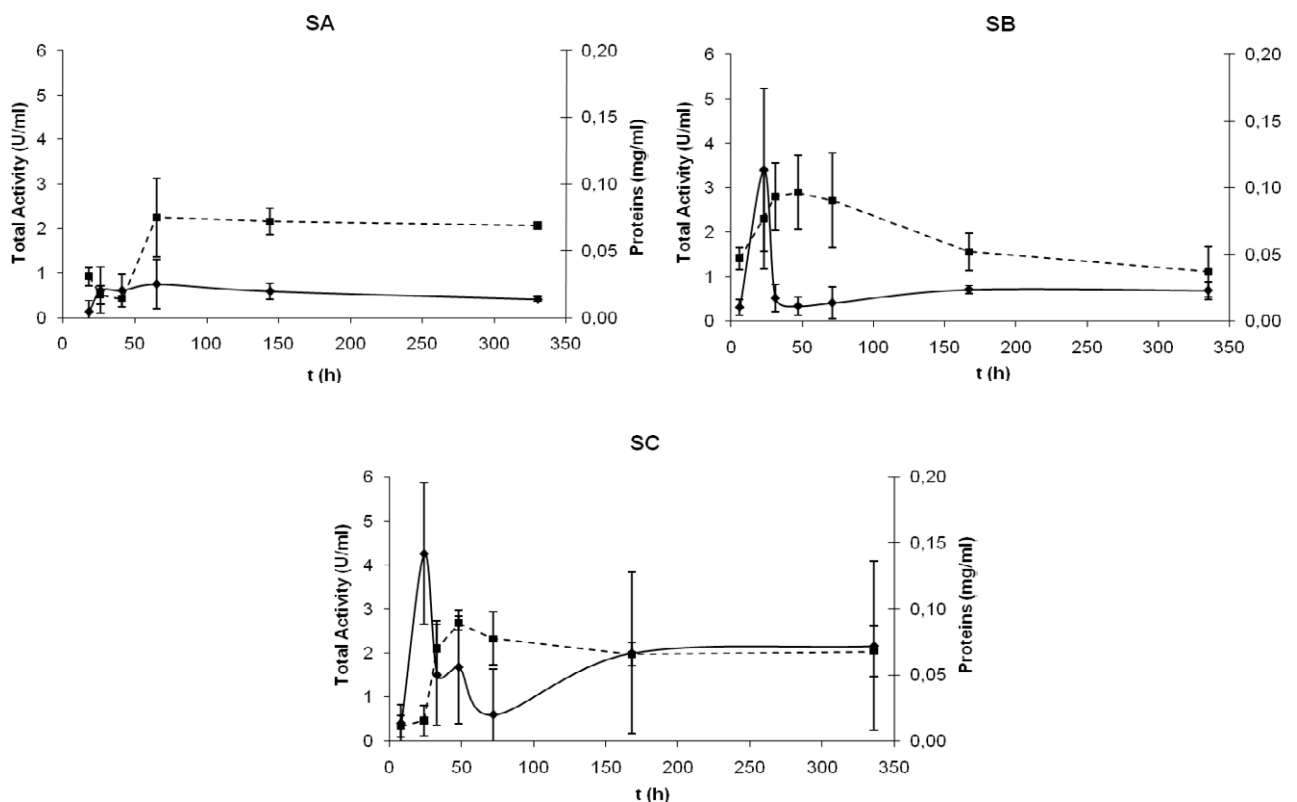


Fig. 1. Evolution with time of the total enzymatic activity and proteins' concentration (—●— Total Activity, - -■- Proteins)

The total activity increased during the first fifty hours in all experiments, and then decreased to the same range until it was obtained a constant value. Concerning the concentration of proteins, it also increased at the beginning of the experiments, then decreased a bit and finally remained constant at this value [7,8].

Comparing the results of the three consortia, it can be said that the consortium that provided the highest total activity was XC, with 4.26 U/ml at 24 hours. The other two consortia provided 3.40 U/ml at 24 hours (XB) and 0.76 U/ml (XA). The low value of XA could be due to the bad state of the consortium at the moment of the experiments. According to the concentration of proteins, it can be observed something analogous, obtaining similar values for the B and C consortia, 0.0964 and 0.0893 mg/ml respectively, and 0.0750 mg/ml for the consortium A.

Fig. 2 shows the specific enzymatic activity of the three consortia used in the experiments. The trend of the three curves is similar to the one observed in the total activity results. It can be observed that the three lines are qualitatively similar, showing an initial rise between 0 and 50 hours (corresponding with the exponential phase of the microbial growth) and then decreasing sharply until a final value similar to the one of the beginning.

Making a deep analysis, it is possible to say that on the one hand the consortia coming from the A and B soils have a similar behaviour, reaching a maximum of the activity of 43.41 U/mg at 41 hours, and 44.29 at 23 hours respectively. On the other hand, the XC consortium reaches the maximum of the specific enzymatic activity at 24 hours, with a value of 275.32 U/mg. It must be also said that the final value of the specific activity of XC is higher than that of the other two consortia.

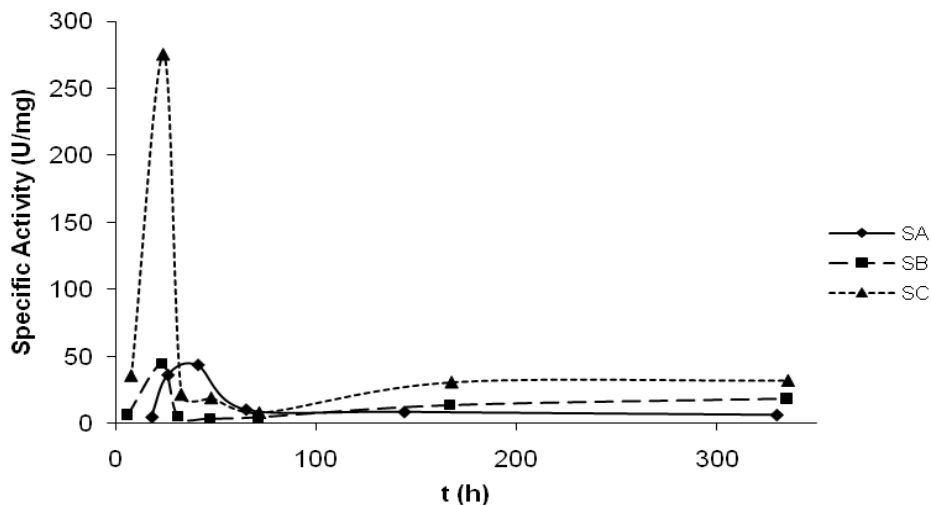


Fig. 2. Evolution of the specific enzymatic activity along time

3.2. Enzymatic bioremediation viability

Fig. 3 shows the results of the diesel biodegradation in the soil bioremediation experiments (section 2.4) with and without addition of enzymatic complexes. It can be observed the improvement caused by the addition of the enzymatic complexes: the decrease of the substrate concentration in the experiment with enzymes (discontinuous line) was higher than without enzymes (continuous line), observing a sharp decline during the first hours, and later reaching a diesel concentration that remains nearly constant over time. According to the bioremediation experiment with no enzymatic complex, the decrease of the TPH occurs more gradually, reaching a final value higher than the one for the other experiment.

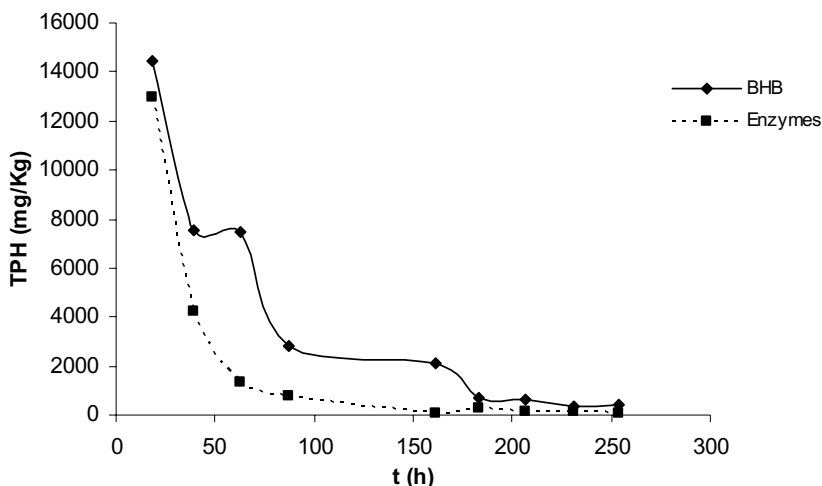


Fig. 3. Removal of diesel (total petroleum hydrocarbon, TPH) along time during the bioremediation and the enzymatic-enhanced bioremediation experiments (BHB: bioremediation experiment using only BHB medium with no enzymes addition)

The principal difference between the bioremediation process and the enzymatic-enhanced bioremediation process was that the last one produced higher diesel degradation than the first one: a degradation of approximately 90% was reached at 60 hours; while in the first one, about 175 hours were needed to reach that level of diesel degradation.

4. Conclusion

It can be concluded that the addition of extracellular enzymatic complexes, generated by the microorganisms that degrade the diesel, provides better results than the process of bioremediation without these enzymes, reaching a higher degradation of diesel. The time to reach the end of the process was also lower than in the diesel bioremediation only using microorganisms. Enzymes act as catalysts for the reaction. Therefore in the enzymatic bioremediation process, as the broth is enriched with these enzymes, the microorganisms could begin the reaction before and achieve a higher degradation yield. The application of this technique would depend of the final economical balance, comparing the costs of enzyme production to the improvement of the process.

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References

- [1] F. Menezes, F.A. de Oliveira, B.C. Okeke, and W.T. Frankenberger, *Microbiol. Res.*, 160(2005)249.
- [2] L. Gianfreda, and M. Rao, *Enzyme Microb. Technol.*, 35(2004)339.
- [3] E. Moliterni, F.J. Fernández, L. Rodríguez, M. Villar, and J. Villaseñor, *Proc. 4th European Bioremediation Conference*, Crete, Greece, 2008.
- [4] E. Moliterni, F.J. Fernández, L. Rodríguez, M. Villar, and J. Villaseñor, *Proc. 18th International Congress of Chemical and Process Engineering CHISA*, Prague, Czech Republic, 2008.
- [5] O.H. Lowry, N. J. Rosebrough, A.L. Farr, and R.J. Randall, *J. Biol. Chem.*, 193(1951)265.
- [6] A. Verdin, A. Lounès-Hadj, and R. Duran, *Int. Biodet. Biodeg.*, 53(2004)65.
- [7] K. Das, and S.K. Mukherjee, *Appl. Microbiol. Biot.*, 69(2005)192.
- [8] O. Segev, A. Abeliovich, and A. Kushmaro, *Chemosphere*, 68(2007)958.