# Green bean coffee as nutrient source for pesticide degrading-bacteria

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Glucose and peptone are generally used as primary substrates in the study of pollutant cometabolism. However, they sometimes fail because the presence of more favorable carbon sources can inhibit the degradation of xenobiotics. The use of natural waste rich in nutrients, as defective green coffee beans, may be used as co-substrate to remove toxic recalcitrant pollutants. Coffee, like many biological products, has a great chemical and biological complexity. In this work, four bacterial strains were isolated from green coffee beans and identified for their biochemical characteristics. They were also examined for their capacity to grow and to remove 1,1,1-trichloro-2,2-bis (4-chlorophenyl) ethane (DDT) and 1,2,5,6,7,7-hexachloro-5-norbornene-2,3-dimethanol cyclic sulfite (endosulfan) in a liquid medium using defective green coffee beans, glucose or peptone as co-substrates. Results showed that coffee bean was an adequate nutrient source for bacterial growth and it significantly enhanced DDT and endosulfan biodegradation in comparison with glucose and peptone.

**Keywords** coffee; pesticide: biodegradation; *Pseudomonas; Flavimonas*

## 1. Introduction

The nutritional requirements that must be considered for microbial growth are the sources of carbon, energy and nitrogen, other major mineral nutrients such as sulfur, phosphate, potassium, magnesium, calcium, and trace metal requirements [1].

Glucose and peptone are generally used as primary substrates in the study of pollutant cometabolism. However, they sometimes fail because the presence of more favorable carbon sources can affect the xenobiotics degradation [2]. Alternatively, the use of structural non-chlorinated analogues of pesticides, for example diphenylmethane, diphenylethanes or biphenyl has been proposed for degradation of DDT and their metabolites [3-6]. However these compounds are in the Environmental Protection Agency's priority pollutant list and their application as co-substrate in bioremediation processes is obviously not permitted.

The use of natural waste to remove toxic recalcitrant pollutant from polluted places is a recent environmentally sustainable strategy [7-9] and it could also solve disposal problems of these wastes.

Due to oversupply of coffee on world markets coffee producers have committed to remove the lowest quality coffee, i.e., "defective" coffee or triage from the market to stabilize prices. In Brazil, defective beans represent about 20% of the total coffee production [10] and in México, 5% of total coffee exportation (about 12 000 tons annually). It is therefore necessary to look for alternative uses for defective green bean coffee.

Coffee, like many biological products, has a great chemical and biological complexity; more than 1,000 substances have been identified and their biological functions defined. Among them are vitamins, minerals (3%) caffeine (1%), trigonelline (1%), lipids (12%), chlorogenic acids (5.5%), aliphatic acids (1.5%), amino acids (2%), proteins (11%), oligosaccharides (6%), and polysaccharides (50%) [11].

This study shows the application of defective green bean coffee to support growth and enhance organochlorine pesticide removal by bacteria in liquid media, comparatively with two traditional carbon sources.

## 2. Materials and Methods

### 2.1 Reagents and chemical

Technical grade DDT and endosulfan were supplied by Teckchem and Velsimex Company (Mexico). All chemicals used were reagent-grade and solvents were HPLC-grade (Merck).

### 2.2 Green coffee bean (*Coffea arabica*)

Coffee beans were supplied by Consejo Mexicano del Café. Beans were air-dried, ground and sieved (10-20 Mesh). Coffee sterilization was done by irradiation with $^{60}$Co at 28 kGy. Carbon, nitrogen and phosphorus composition was determined by standards methods**.**

 

2.3 Culture medium.

The composition of the mineral salt medium (MSM) used was ($gL^{-1}$): $Na_2HPO_4$, 2.4; $KH_2PO_4$, 2.0; $NH_4NO_3$, 0.1; $MgSO_4.7H_2O$, 0.01; $CaCl_2$, 0.01. The pH was adjusted to 6.5. The medium was sterilized by autoclaving at 121°C for 20 min [12]

2.4 Isolation of organochlorine pesticide degrading bacteria from coffee beans

The procedure to isolate bacteria consisted in the addition of 0.1 g of ground coffee beans (not sterilized), to a flask containing 50 mL of MSM medium, with 200 $mgL^{-1}$ of pentachloronitrobenzene (PCNB) as the organochlorine pesticide model and as a fungal inhibitor.
Cultures were incubated at 30 °C and shaken at 100 rpm for 7 days. 2 mL were then transferred to a fresh MSM medium containing 200 $mgL^{-1}$ of PCNB and incubated under the same conditions. Cultures from the fifth transfer were plated on nutritive agar medium and incubated for 24 h at 30°C. Colonies were isolated on the basis of their morphology and identified for their biochemical characteristics. Strains capable to grow on agar with DDT, endosulfan and PCNB were selected for further use in in liquid media.

2.5 Effect of carbon source on bacterial growth and pesticide biodegradation

Erlenmeyer flasks containing 50 mL of MSM medium with 10 $gL^{-1}$ glucose, 2 $gL^{-1}$ peptone or 2 $gL^{-1}$ sterilized ground coffee beans amended with 50 $mgL^{-1}$ DDT or 50 $mgL^{-1}$ endosulfan were inoculated with 5mL of biomass (0.3 $gL^{-1}$ dry biomass) and incubated in the dark at 100 rpm, 30 °C for 7 days. Addition of 17 g NaCl to the medium was used to stop bacterial growth. Two experimental sets (for cell growth and degradation tests), were conducted in duplicate.
A biomass ($gL^{-1}$) vs time curve was performed using 5 mL aliquot of culture broth. A control for the determination of the pesticide recovery efficiency was performed under the same culture conditions, but pesticides were added after culture inactivation. Dichloromethane extracts were dehydrated and concentrated.
The residue was dissolved in 5 mL of methanol and 2 µL were injected into the GC/MS. *p'p*-DDT and endosulfan (I+II) were quantified using the external standards method**.** Metabolites were identified by comparison with MS spectra library records [13]. A DB-5 fused silica capillary column (30m x 0.25 mm) was used. The column temperature program was, 140 °C for 2 min followed by ramping at 5 °C $min^{-1}$ to 240 °C and then maintaining 240 °C for 27 min. The temperatures in the injector and the transfer line were 250 °C and 280 °C**.** The ionization was carried out at 70Ev. Each experiment was performed in duplicate and data were analized through analysis of variance ($p<0.05$ (SAS, v 6.08 (8), SAS Institute S.A de C.V, Mexico)

## 3. Results and discusion

3.1 Isolation of organochlorine pesticide degrading bacteria from coffee beans

The magnitude and diversity of the microbial populations associated with the natural processing of coffee (*C. arabica*) have previously been assessed and members of the genera *Aeromonas, Pseudomonas, Enterobacter, Serratia, Cellulomonas, Arthobacter, Microbacterium, Dermabacter and Lactobacillus* [14] have been identified. The fungi which have been isolated include *Cladosporium, Fusarium, Penicillium* and *Aspergillus* [14].

The most used method for isolating microorganism with the ability to degrade toxic compounds is by enrichement culture, using the pollutant target as inductor [2]. For example, phenanthrene is a common polynuclear aromatic hydrocarbon (PAH), used as a model for more toxic structures of PAH's [15]. A novel approach considers degradative capabilities of the strains developed on natural substances [16]. For example,microbial communities isolated from *Eucalyptus* and *Pinus radiata* mulch were able to grow on aromatic hydrocarbons, polychlorinated biphenyls, heterocyclic aromatic compounds and organochlorine pesticides.as DDT. [17].

There is not a compound used as a model to isolate organochlorine pesticide degrading bacteria, for this reason in this study it was used pentachloronitrobenze as inductor, because it does not show acutely toxicity, it is not carcinogenic or mutagenic to humans and it inhibits fungal growth.

In this study, four glucose nonfermenting gram-negative bacteria, capable to degradate organochlorine pesticides, were isolated from coffee beans and identified according with the biochemical characteristics showed in Table 1 as *Pseudomonas aeruginosa, P. putida, Flavimonas oryzihabitans,* and *Stenotrophomonas malthophilia.* Degradation of DDT metabolites by *P. aeruginosa*, *P. putida* and *S. malthophilia* has been previously reported [2][17-18]

**Table 1**. Biochemical profiles of strains isolated from green bean coffee. (+) Positive test; (-) Negative test; (d) weak

| Test | *P. aeruginosa* | *P. putida* | *S. malthophilia* | *F. oryzihabitans* |
|---|---|---|---|---|
| Catalase | + | + | + | + |
| Oxidase | + | + | - | + d |
| Glucose (96 h) | + | + | + | + |
| Urease | + | - | - | + |
| Casein hydrolysis | + | - | + | - |
| Tween 80 | + | - | + | - |
| L-arginine | + | + | - | - |
| Lysine decarboxylase | - | - | + | - |
| Deoxyribonuclease production | + | - | + | - |
| Growth at 4°C | - | + | - | - |
| 41°C | + | - | + | - |
| Growth in Mac Conkey Agar | + | + | + | + |
| Simmons' citrato medium | + | + | - | + |
| Nitrate reduced to nitrite | + | - | - | - |
| Phenylalanine | - | - | - | - |
| Gelatinase hydrolysis | + | - | + | + |
| Aesculin hydrolysis | + | - | - | - |

Among the four bacteria isolated from green bean coffee, only *F. oryzihabitants and P. aeruginosa* were able to grow on the plates with DDT or endosulfan or PCNB as the sole carbon source as it is seen in Table 2. Therefore, they were selected to further experiments. The evaluation of growth on the the plate with organochlorine pesticides by isolated bacteria to coffee bean was carried out by visual monitoring.

**Table 2.** Bacterial growth on plate with pesticides as sole carbon source

| Strain | Pentachloronitrobenzene | DDT | Endosulfan |
|---|---|---|---|
| *P. aeruginosa* | + | + | + |
| *S. malthophilia* | + | + | - |
| *P. putida* | - | + | - |
| (*F. oryzihabitans* | + | + | + |

(+) With bacterial growth (-) Without bacterial growth

3.2. Effect of carbon source on bacterial growth and pesticide biodegradation.

Figure 1 and Figure 2 show the time course for maximum biomass production for *F. oryzihabitants* and *P. aeruginosa* in liquid media with coffee, glucose and peptone suplemented with endosulfan and DDT from 24 to 48 h, at 30 °C and 100 rpm. There were significant differences between bacterial growth in the three media ($p<0.05$).

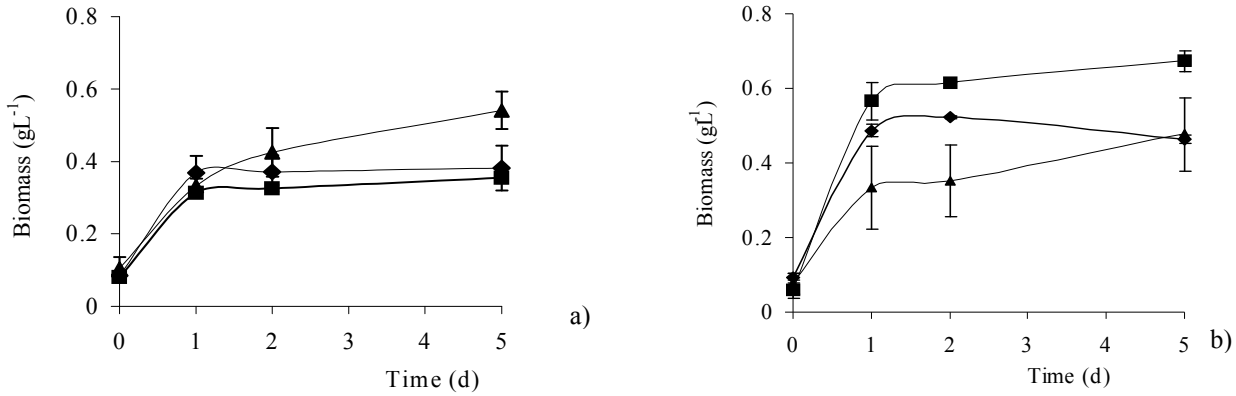


**Fig. 1.** Time course of cell growth of *F. oryzihabitants* (a) and *P. aeruginosa* (b) with different carbon sources: 2 g peptone $L^{-1}$ (◆ ), 10 g glucose $L^{-1}$ (■ ), and 2 g ground coffee $L^{-1}$ (▲) supplemented with 50 mg endosulfan $L^{-1}$.

 

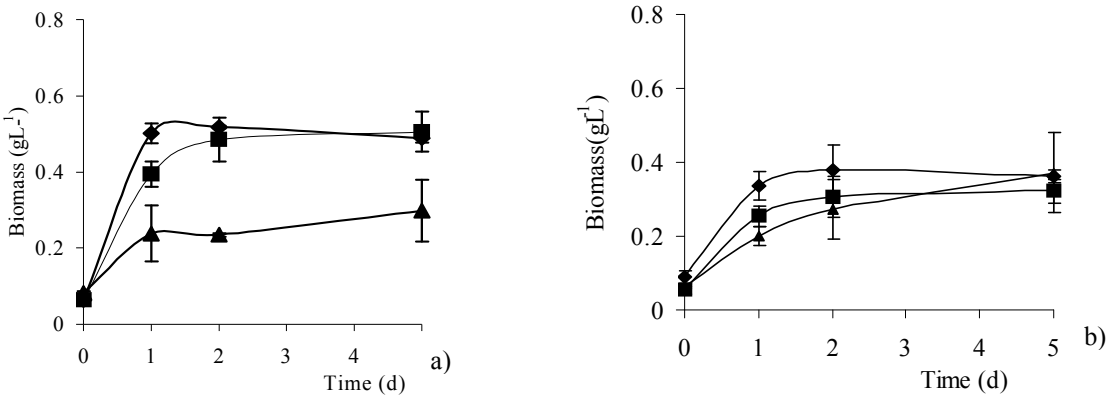


**Fig 2.** Time course of cell growth of *F. oryzihabitants* (a) and *P. aeruginosa* (b) with different carbon sources: 2 g peptone $L^{-1}$ (◆ ), 10 g glucose $L^{-1}$ (■ ), and 2 g ground coffee $L^{-1}$ (▲), supplemented with 50 mg DDT $L^{-1}$.

It has been reported that green coffee bean is a material rich in nutrients that promotes the growth of microorganisms [11][19]. Coffee beans composition (dry matter) used in this research was carbon 46.86 %, nitrogen 5.03 %, phosphorus 0.056 %, and ash 3.97 %, therefore a high biomass production is expected.
Table 3 shows the highest biomass production ($P<0.05$) was obtained for *F. oryzihabitans* in glucose when the pesticide was endosulfan and with glucose and peptone for DDT. Endosulfan removal by *F. oryzihabitans* in peptone was 16.3 % but in glucose and green bean coffee no removal was observed, in spite of having the highest biomass production in glucose. In contrast, this bacterium removed DDT, where the highest value with coffee was obtained, even though it had the least biomass production

**Table 3.** Growth and pesticide degradation by *F. oryzihabitans*

| Carbon source | DDT | | Endosulfan | |
|---|---|---|---|---|
| | Biomass ($gL^{-1}$) | Degradation (%) | Biomass ($gL^{-1}$) | Degradation (%) |
| Glucose ($10gL^{-1}$) | 0.489 ± 0.040 | 39.5± 1.5 | 0.660± 0.009 | 0± 2.0 |
| Peptone ($2gL^{-1}$) | 0.436± 0.036 | 26.7± 7.3 | 0.428± 0.074 | 16.3±3.2 |
| Coffee ($2gL^{-1}$) | 0.296± 0.084 | 63.2 ± 1.6 | 0.407± 0.102 | 0 ± 1.8 |

There were no significant differences ($P>0.05$) of *P. aeruginosa* growth on media with DDT as it is showed in Table 4. However, the highest biomass production in medium with coffee and endosulfan was obtained. On the other hand, endosulfan removal by *P. aeruginosa* was observed in all media but the highest endosulfan removal (51.2%), was obtained in medium with coffee beans. Only 32.6 % DDT concentration remained in the medium after 7 days of incubation. The best DDT removal efficiencies with both bacteria were obtained with coffee bean; this may be explained by its complex chemical composition, including aromatic compounds as lignin [20]. Lignin-degrading microorganisms have been associated with the degradation of environmental pollutants [21].

**Table 4.** Growth and pesticide degradation by *P. aeruginosa*

| Carbon source | DDT | | Endosulfan | |
|---|---|---|---|---|
| | Biomass ($gL^{-1}$) | Degradation (%) | Biomass ($gL^{-1}$) | Degradation (%) |
| Glucose ($10gL^{-1}$) | 0.264 ± 0.046 | 32.5 ± 5.4 | 0.342 ± 0.023 | 30 ± 11 |
| Peptone ($2gL^{-1}$) | 0.337 ± 0.012 | 16.1 ± 0.1 | 0.345 ± 0.079 | 30 ± 12.2 |
| Coffee ($2gL^{-1}$) | 0.334 ± 0.010 | 67.4 ± 0.2 | 0.531 ± 0.071 | 51.2 ± 10.6 |

It has been showed that carbon sources other than target chemicals may influence the degradation rates. Addition of glucose to the culture medium increased bacterial mass of *Alcaligens denitrificans* (From 0.78 mg to 8.4mg), but DDT metabolism was only 3% increased [22]. Also, the presence of favorable substrates as sodium succinate, sodium acetate, sodium citrate, glucose or sucrose inhibited DDT biodegradation by *Serratia marcensces* [2]. Other sources such as sodium acetate and sodium succinate have been reported to inhibit endosulfan degradation [23]. Thus, a convenient nutrient source should promote microorganism growth and pollutant degradation. The results in this research show that green bean coffee support growth and pesticide degradation for *P. aeruginosa* and *F. oryzihabitans.*

Additionally, green coffee bean structure can promote microniches and biofilm formation on its surface to enhance pesticide biodegradation [24].

Detoxification of endosulfan by aerobic microorganisms often results in the formation of a toxic endosulfan sulfate [25]. Also, other metabolites, such as endosulfan diol or endosulfan ether can be produced by microbial metabolism but neither of these were identified by GC/MS in the culture extracts

Figure 3 shows GC-MS chromatogram of culture broth extracts for different treatments of DDT. Changes in the DDE concentration indicate its production during DDT biodegradation as well as its degradation, in the medium with coffee bean addition. DDMU and DDOH metabolites were also identified by GC/MS in the culture broth extract (Fig 3c).

The conversion of DDE to DDMU through the reductive dechlorination by bacteria and marine sediments has been reported [26, 27] The DDOH peak in the chromatogram indicates further degradation of DDMU [28].

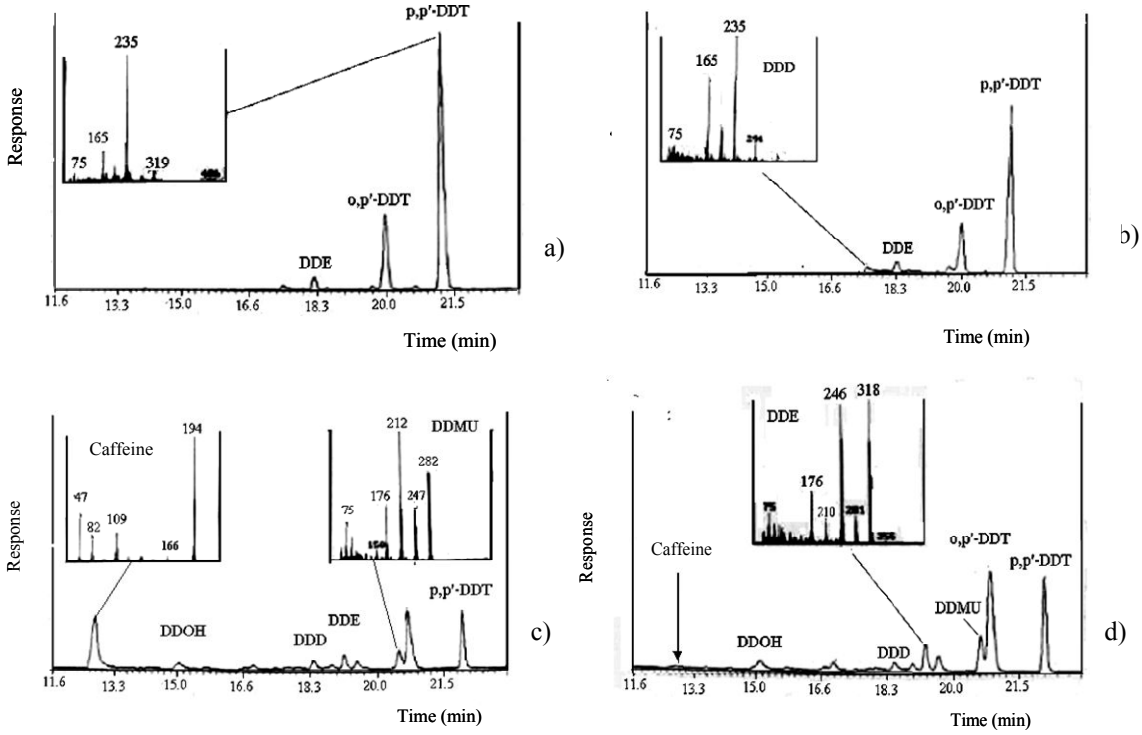


**Fig. 3** GC/MS chromatograms of dichloromethane extracts for different treatments: a) DDT with inactivated bacteria (control); b) *F. oryzihabitants* with glucose; c) *P. aeruginosa* with coffee; d) *F. oryzihabitants* with coffee. All chromatograms are at the same scale.

## 4. Conclusions

*Flavimonas oryzihabitans* and *Pseudomonas aeruginosa* were isolated from defective green beans coffee with traditional enrichment techniques using PCNB as an organochlorine pesticide model. These microorganisms were able to grow and use green coffee bean as source of nutrients. In all cases, free biomass production was higher than 3 g $L^{-1}$, getting the maximum growing to 24-48 h in incubation at 30°C and 100 rpm. There was not endosulfan degradation by *F. oryzihabitans* with neither glucose nor green coffee bean, only in peptone where 16.3 % of endosulfan degradation was obtained. In contrast, a 63.2% DDT removal was observed, with coffee beans at 7 days of incubation. *P. aeruginosa* was able to remove both endosulfan and DDT (51.2%) and 67.4%, respectively) when coffee bean was used as nutrient source. Thus, defective green coffee bean is a readily available and relatively inexpensive material, which could be used as nutrient source to enhance organochlorine pesticide removal.

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