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# Biodegradation of aniline by a consortium of *Bacillus* species isolated from oil contaminated soil in Mexico

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**A B S T R A C T**

We assessed the potentials of a bacterial consortium isolated from oil contaminated soil for the biodegradation of aniline. The consortium (CAHY2) was found to be composed of *Bacillus subtilis*, *Bacillus lentimorbus* and *Bacillus mycoides* and was able to utilize aniline and two of its chlorinated derivatives, 4-chloroaniline (4-CA) and 2,4-dichloroaniline (2,4-DCA), as sole nitrogen and carbon sources in the preferential order 2,4-dichloroaniline >4-chloroaniline >aniline and at the rates of 0.78 to 3.82 µmoles $L^{-1}$ $h^{-1}$. The acclimatized bacterial consortium metabolized 260 $mgL^{-1}$ each of 4-CD and 2,4-DCA by as much as 9 and 40% respectively. The additional nitrogen sources such as $KNO_2$ and $NH_4Cl$ enhanced the biodegradation of aniline 6 and 10 times, respectively. Although various intermediate products were formed during the biodegradation of aniline, only 1,4-benzenediol was identified, but it disappeared after 21 days. When $KNO_2$ was used as an additional nitrogen source, two intermediates (biphenyl-amine and 4-phenyl-aniline) were detected, while three (3-formylaniline, 2-acetylaniline and 1,4-benzenediol) were detected when $NH_4Cl$ was used as an additional nitrogen source. We conclude that the consortium of *Bacillus* species could biodegrade aniline and its chlorinated derivatives to less toxic intermediates and that additional nitrogen sources could enhance their metabolism.

## Introduction

Anilines are aromatic compounds used for the manufacture of pesticides, dyes, plastics and pharmaceutical products(ATSDR 1999). These aromatic amines are also formed during the biotransformation of nitroaromatics such as explosives and dinitroaniline herbicides (de Souza *et al.,* 1998; Cullington and Walker, 1999; el-Deeb *et al.,* 2000; El-Fantroussi *et al.,* 2000; Tixier *et al.,* 2001; Tixier *et al.,* 2002). As a consequence of their wide distribution in the environment, they are frequently found in sediments, soils and residual industrial water (Kosson and Byrne 1995). In principle, a great number of physicochemical processes can affect the destiny of aniline in the environment, including evaporation, autoxidation, photoxidation, chemical oxidation, chemical transformation and biodegradation. In soils, aniline binds to humic materials as a result of a chemical transformation, and thus, their mineralization is very slow. In aquatic media, biodegradation is an important mechanism for its removal. However, some media rich in organic matter could cause some chemical transformations that are not conducive for its removal. These transformations also occur in the process of disinfection of residual waters, where the action of oxidizing agents, such as hypochlorous acid (in the presence of organic matter and nitrogenous sources such as amines or ammonia) results in the formation of chloride aryl amines in the biological treatments. Free chloride or bromide can also bind with organic compounds such as aryl amines or phenols through microbial routes by means of halogenation reactions, resulting in the formation of chloramines (Tachikawa *et al.,* 2005) or bromophenols during drinking water chlorination (Acero *et al.,* 2005). On the other hand, various microorganisms that degrade aniline and chloride anilines have been reported, among them *Pseudomonas putida* UCC22 (pTDN1)(Konopka et al 1989; Paris and Wolfe 1987; Fujii *et al.,* 1997; Boon *et al.,* 2000, 2001; Dejonghe *et al.,* 2002), *Acinetobacter* sp. YAA (Chaudhry and Chapalamadugu 1991; Fetzner and Lingens 1994; Fukumori and Saint 1997), *Rhodococcus* sp. AN-22(Matsumura *et al.,* 2006), and *Delftia tsuruhatensis* H1 (Zhang *et al.,* 2010); and *Erwinia* sp (Li *et al.,* 2010). However, there is a dearth of literature implicating *Bacillus* species in aniline biodegradation; but even then, such reports are mostly on axenic cultures (Yao *et al.,* 2011; Sewarde and Gawai, 2014; Liu *et al.,* 2014). There is paucity of information on aniline biodegradation by *Bacillus* consortia and the influence of additional nitrogen sources on the biodegradation of the pollutant. In this paper, we report on the biodegradation of aniline by a consortium of three *Bacillus* species in an effort to develop an active microbial consortium of relevance in the bioremediation of aniline compounds polluted environments.

## Materials and Methods

### Enrichment cultures

A mixed culture of bacteria was isolated from waste sludge from an oil refinery plant by enrichment, using a mixture of aniline and chloroaniline as the sole source of carbon and nitrogen in a mineral saline medium (MSM) (Kästner *et al.,* 1994). The composition of MSM was ($L^{-1}$): $Na_2HPO_4$, (2.1 g); $KH_2PO_4$ (1.3 g) and then 2.5 ml each of 1 $molL^{-1}$ $MgSO_4$ and 36 $mmolL^{-1}$ $FeSO_4{\cdot}7H_2O$ and a trace elements solution were added. The pH of the MSM was adjusted to 6.5, autoclaved and allowed to cool. Three sets of media were used; one MSM containing $KNO_2$ (0.8 g) as nitrogen

source, the second MSM with $NH_4Cl$ (0.5 g) as nitrogen source, both with a C/N ratio of 2, corresponding to modified MSM (McCarthy 1987; Alaoui *et al.,* 2001), and the third without an additional nitrogen source (C/N ratio of 6). Biodegradation studies were carried out with aniline or chloroanilines added to the three mineral media at the required concentration from a stock solution (5% w/w of each compound). The cultures were incubated aerobically for eight weeks at 28°C on a horizontal shaker at 200 rpm.

**Isolation and maintenance of chlorophenol-degrading microorganism CAHY2**

From the enrichment culture, several bacterial isolates were recovered in the presence of 1.6, 2.0 and 2.7mM of a mixture of aniline and chloroaniline in accordance with the description of Pries *et al.* (1994). The isolates were maintained through subsequent transfers in MSM with 1.6, 2.0 and 2.7 mM of 2,4-dichloroaniline (2,4-DC), 4-chloroaniline (4-CA) and aniline, respectively, and incubated as described earlier (Pries *et al.,* 1994). A sample of the 8-week old culture was spread onto the surface of Nutrient agar (Bioxon, Mexico) and incubated at 28°C. Three morphologically distinct colony types were isolated, purified and identified.

**Identification of bacterial isolates**

The three purified bacterial isolates were identified by 16S rDNA sequencing as described by Estrada-de los Santos *et al.* (2001). The nucleotide sequences of the amplicons were analyzed by the Basic Local Assignment Search Tool (BLAST) to confirm the identities of the different bacterial isolates.

**Aniline and chloroanilines biodegradation experiments**

Batch culture experiments for the degradation of aniline and chloroanilines, using the bacterial consortium were carried out individually in Erlenmeyer flasks (250 mL) containing 2.7 mM of aniline, 2 mM of 4-chloroaniline, and 1.6 mM of 2,4-dichloroaniline in 100 mL of previously steam sterilized (110°C for 30 min) MSM without an additional nitrogen source. All experiments were carried out in triplicate. The flasks were inoculated with 10% v/v of the bacterial consortia culture (CAHY2) to yield an initial biomass concentration of140 mg $L^{-1}$, and incubated aerobically at 28°C with agitation at 200 rpm. Culture samples were taken at regular intervals over a period of 7 days and analyzed for biomass, aniline and chloroanilines. Control experiments were setup in parallel to estimate the levels of abiotic losses. All experiments and controls were carried out in triplicate.

**Kinetics of aniline biodegradation**

The kinetics of aniline biodegradation was assessed under the same conditions as described above. Three sets of media were used, one containing MSM without an additional nitrogen source, and two others with an additional nitrogen source ($NH_4Cl$)**,** corresponding to the MSM described above, and another with $KNO_2$ (McCarthy 1987; El Alaoui *et al.,* 2001) termed MSM modified. Triplicates of these experiments (using 5.3 mM of aniline) were incubated at 28°C and 200 rpm. Kinetic samples were taken at 0, 7, 14 and 21 days, and analyzed for biomass, aniline and metabolites, prior to acid (HCl1M) extraction with ethyl acetate, followed by GC analysis. Control experiments were used with non-inoculated flasks under the same conditions.

**Isolation and characterization of the aniline metabolites in batch experiments**

The *Bacillus* consortium was incubated at 28°C in Erlenmeyer flasks containing MSM or MSM modified medium with aniline at 5.3 mM was added. After the different incubation times, the contents of the flasks were collected and centrifuged at 10000*g*. The supernatant fluid was acidified with HCl 1 M and then extracted with ethyl acetate. The organic layers were dried over anhydrous sodium sulphate, evaporated to dryness in a rotary evaporator (Schlosser *et al.*, 2000) and dissolved in 1ml of ethyl acetate for gas chromatography-mass spectrometry (GC-MS) analysis.

**Analytical procedures**

The quantification of aniline, chloroanilines and their biodegradation products was done by means of a gas chromatograph, using HP 6890 adapted to a mass spectrometer detector model 5972 (GC-MS) and injector 7973. The GC-MS was equipped with an HP5MS and HP1 column of 30 m length, 0.25 mm internal diameter and 0.25 µm of film thickness. Helium was used as the carrier gas at a flow rate of 2.9 ml $min^{-1}$. The conditions for analysis were: split 1:20; range of mass scan 50–500; with temperatures as follows: injector 260°C, detector 280°C; gradient program: 65°C to 96°C (4°C $min^{-1}$), 96°C to 160°C (8°C $min^{-1}$) and up to 230°C (12°C $min^{-1}$). The MS spectra of the metabolites were compared to those of the standards and previously published spectra (Environmental Protection Agency/National Institute of Health 1980).Biomass was monitored as cell dry weight by filtering 5 mL of culture effluent onto pre-weighed polycarbonate filters (pore size, 0.4 mm), drying the preparation at 60°C for 24 h, and weighing the filter with the cells.

**Chemicals**

Aniline, 4-chloroaniline (4-CA), 2,4-dichloroaniline (2,4-DCA), 1,4-benzenediol, and ethyl acetate (analytical grade) were purchased from the Sigma-Aldrich Chemical Company Co., Inc.

**Results and Discussion**

**Isolation of aniline and chloroanilines degrading CAHY2-bacteria**

The bacterial consortium obtained after the enrichment process was capable of growing in a mineral medium containing a mixture of aniline and chloroanilines as the carbon and nitrogen sources. Members of the bacterial consortium were identified as *Bacillus subtilis*, *Bacillus lentimorbus*, and *Bacillus mycoides*. This consortium was subsequently used to study the biodegradability of aniline, 4-CA, and 2,4-DCA.

**Use of aniline and chloroanilines**

The ability of the CAHY2 consortium to use aniline or chloroanilines as sole sources of carbon and nitrogen were assessed. The results of the biodegradation are as presented in table 1. The CAHY2 consortium utilized the aniline and two of its chlorinated derivatives (4-CA, and 2,4-DCA) for growth and preferentially metabolized them in the following order: 2,4-CA>4-CA>aniline. Aniline and 4-CA were biodegraded to a lesser extent. The explanation for the low biodegradation of 4-CA could be the position of the halogen in the molecule, that could determine its biodegradation. Given that the biodegradation of aniline was very low (0.78 µmoles $L^{-1}$ $h^{-1}$), it was necessary to acclimatize the consortium to this compound, up to 3.0 mM. Preliminary

results revealed that under these conditions, the cell biomass doubled within seven days from an initial biomass of approximately140 mg $L^{-1}$ and also that KNO2 as a source of nitrogen was able to enhance aniline biodegradability. The acclimatized *Bacillus* consortium was able to metabolize4-CA and 2,4-DCA by9% and 40%, respectively.

**Effect of the addition of an additional nitrogen source on aniline biodegradation**

The effect of additional nitrogen sources on aniline biodegradation was assessed and the results are a presented in figure 1. The kinetic of growth revealed a slow lag phase during the first 7 days of reaction, with a subsequent exponential growth, reaching a maximum biomass of 0.7g $L^{-1}$. The aniline biodegradation followed a rapid pattern for the first 14 days, and after 21 days of reaction approximately 60% of the aniline had been biodegraded at 6.6 ± 0.1 µmoles $L^{-1}h^{-1}$. The pH remained constant during the process of biodegradation. The biodegradation of aniline by the consortium in the presence of $KNO_2$as an additional nitrogen source permitted the detection of only two metabolites by GC analysis (Table 2). After 7 days of culture, metabolites I and II, with retention times of 10.02 and 17.88 min, began to appear and their concentration slightly diminished and finally disappeared by day 21. Intermediate I was identified as biphenylamine with the molecular ion peak as the base peak at m/z 169. The loss of a phenyl group (m/z 77) afforded the peak at m/z 91 (Budzikiewicz *et al.,* 1991). Intermediate II was identified as 4-phenylaniline with molecular ion peak at m/z 169, and major peak fragments at m/z 154 due to the loss of $NH_2$ from the molecular ion, and m/z 77 corresponding to a phenyl group (Budzikiewicz, 1988).

Additionally, kinetic studies of the aniline biodegradation were carried out using an MSM medium containing ammonium chloride ($NH_4Cl$) as the nitrogen source. The growth kinetic of the consortium showed a slow lag phase during the first 7 days, with a subsequent exponential growth, reaching a maximum biomass of 0.8 g $L^{-1}$. Aniline biodegradation increased steadily attaining about 95% degradation in 14 days and complete biodegradation in 21 days (Figure 2). The biodegradation rate was 10.56 ± 0.35 µmoles $L^{-1}$ $h^{-1}$.

The products of aniline biodegradation were extracted with ethyl acetate from the culture at different incubation times. Three intermediates of aniline biodegradation in the cultures with $NH_4Cl$ as an additional nitrogen source were identified by GC-MS (Table 3). The intermediate I was identified as 3-formylaniline with the molecular ion peak at *m/z* 121 (100) plus 93 (88), 77 (10) and 66 (76) mass fragments. The intermediate II was identified as 2-acetylaniline with a molecular ion peak at *m/z* 135 (28) and 93 (100), 77 (6), and 65 (24) as major fragments. The *m/z* pattern of metabolite III with the molecular ion at *m/z* 110 as the base peak, and ion fragments at *m/z* 81 (71), 63 (15), and 53 (60) was identified as 1,4-benzenediol.

The presence of an additional nitrogen source also affected the biodegradation level (Table 4). A C/N ratio favoured the aniline biodegradation compared with the control where only aniline was used as nitrogen and carbon source.

CAHY2 was capable of degrading aniline, mono and dichloroanilines as the only source of carbon and nitrogen. It would appear that the presence of the halogens moiety and its position was a determining factor in the biodegradability of chlorinated compounds, but in this case, the biodegradation level was low for aniline. A comparison of the biodegradability of

aniline compounds was undertaken by Paris and Wolfe (1987), demonstrating that the presence of derivatives diminishes the level of biodegradability of the compounds.

The capacity of *Bacillus* species to biodegrade different halogenated compounds was demonstrated in our previous report(Herrera *et al.,* 2008). In the present study, the consortium tolerated high concentrations of aniline and chloroanilines. Also, this study demonstrated that the inhibitory effect of the substrates can be reduced and the degrading capacity of the consortium increased by means of the previous acclimatization of the microbial consortia to the different compounds. It has been reported that the mineralization of many organic compounds in different environments is preceded by a period of adaptation that can vary depending on the compound (Radianingtyas *et al.,* 2003; Vangnai and Petchkroh, 2007).

The mineralization and biotransformation of toxic compounds have been widely studied by various authors (Spain *et al.,* 1980; Wiggins *et al.,* 1987; Berg and Nyholm, 1996; Nyholm *et al.,* 1996; Raymond *et al.,* 2001; Ahtiainen *et al.,* 2003; Torang and Nyholm, 2005). These studies demonstrated that the degrading capacity of the microorganisms used strongly depends on the environmental conditions as well as the microbial interactions involved when natural or mixed consortia are used (Campo *et al.,* 2011). Biotransformation can produce more soluble and less toxic compounds by means of detoxifying processes as well as more toxic intermediaries through processes of metabolic activation (Alexander, 1999).

In the current study, it was demonstrated that the presence of an additional nitrogen source in the culture medium, could increase the biodegradation level 6 and 10 times with $KNO_2$ and $NH_4Cl$, respectively. The biodegradation level and the intermediate products formed were also different in both conditions.

With the identities of these intermediates, it was possible to propose the principal metabolic reactions which occur during the biodegradation of aniline. These reactions consisted of acylation, oxidation, and condensation. The products formed can also be classified into two categories according to their structure: 1) products derived from aniline and 2) by-products of oxidation. Beside the oxidative desamination reactions caused by dioxygenase attacks, intermediary products were produced by formylation and acylation reactions. The formation of these products has been documented by other authors (Russel and Bollag, 1977; Lyons *et al.,* 1984; Zeyer *et al.,* 1985; Chaudhry and Chapalamadugu, 1991; Fetzner and Lingens, 1994; Takenaka *et al.,* 2003) and has been considered as a by-product of detoxification reactions.

Results of this study suggest that the principal biodegradation pathway for aniline by the *Bacillus* consortium in the medium with ($NH_4Cl$) appears to involve formylation and acylation reactions, resulting in the formation of 3-formylaniline, 2-acetylaniline. The biodegradation of aniline and its metabolites was complete by day 21. However, the formation of 1,4-benzenediol, the final by-product of the biodegradation of aniline, permits the assumption that the most important route was oxidative desamination (Lyons *et al.,* 1984). In other studies with p-chloroaniline, this compound was metabolized completely by *Chlorella fuscarubra* mostly to water-soluble products. After 4 weeks of degradation, 28% of the radioactivity was recovered from algae and 35% from the aqueous medium.

**Table.1** Biodegradation rates of aniline compounds by *Bacillus* consortium (CAYH2) after 7 days of incubation

| Compound | Biodegradation rate (µmoles $L^{-1}$ $h^{-1}$) ± SE |
|---|---|
| Aniline | 0.78 ± 0.03 |
| 4-chloroaniline | 1.46 ± 0.04 |
| 2,4-dichloroaniline | 3.82 ± 0.02 |

Values are means of three replicates. SE represents standard error

**Table.2** Intermediates from aniline biodegradation by CAHY2,cultures with an additional nitrogen source (KNO2) and their fragmentation ion (m/z) peaks

| Intermediate* | Ions (m/z) [$M^{+\cdot}$] Molecular Ion |
|---|---|
| **I**Biphenyl amine | 169 [$M^{+\cdot}$](100), 154(2), 141(5), 128(1), 115(3), 104(1), 91(2), 84(18), 77(18), 65(10), 51(21), 39(9), 28(1). |
| **II**4-phenyl aniline | 170 (2), 169 [$M^{+\cdot}$](100), 154(2), 141(6), 128(2), 115(4), 104(1), 91(1), 83(16), 76(10), 66(6), 59(1), 51(10), 39(4), 28(2). |

*Ethyl acetate extraction 3 times from growth medium from three replicates.

**Table.3** Intermediate from aniline biodegradation by CAHY2 cultures with an additional nitrogen source (NH4Cl), and their fragmentation ion (m/z) peaks

| Intermediate* | Ions (m/z). [$M^{+\cdot}$] Molecular Ion |
|---|---|
| **I**3-formylaniline | 121[$M^{+\cdot}$](100), 93(88), 87(2),77(10), 66 (76), 61 (10), 51 (18) |
| **II**2-acetylaniline | 135 [$M^{+\cdot}$](28), 119(1), 106(1), 93(100), 77(6), 65(24), 51(8) |
| **III**1,4-benzenediol | 110 [$M^{+\cdot}$](100), 81(71), 69(3), 63(15), 53(60). |

*Ethyl acetate extraction 3 times from growth medium from three replicates.

**Table.4** Aniline biodegradation by CAHY2 using an additional nitrogen source

| Nitrogensource | C/N ratio | Biodegradation rate (µmoles $L^{-1}$ $h^{-1}$) ± SE |
|---|---|---|
| Without | 6 | 0.8 ± 0.05 |
| $KNO_2$ | 2 | 6.6 ± 0.17 |
| $NH_4Cl$ | 2 | 10.5 ± 0.22 |

Values are means of three replicates. SE represents standard error

**Figure.1** Kinetic profile of aniline biodegradation and biomass production of CAHY2 in MSM with KNO2 as an additional nitrogen source. (▲) aniline a (◆) pH and (●) Biomass

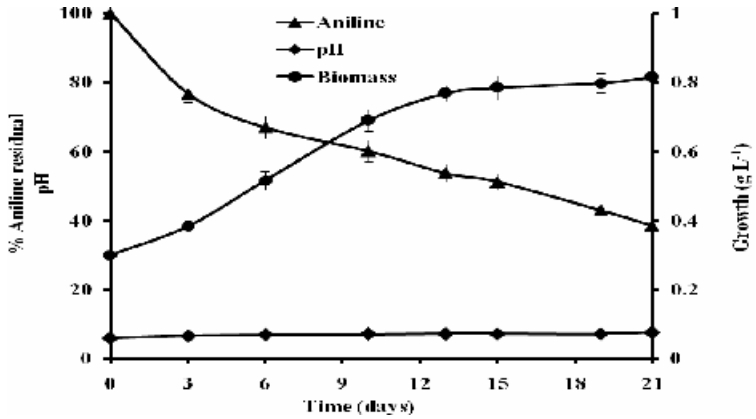
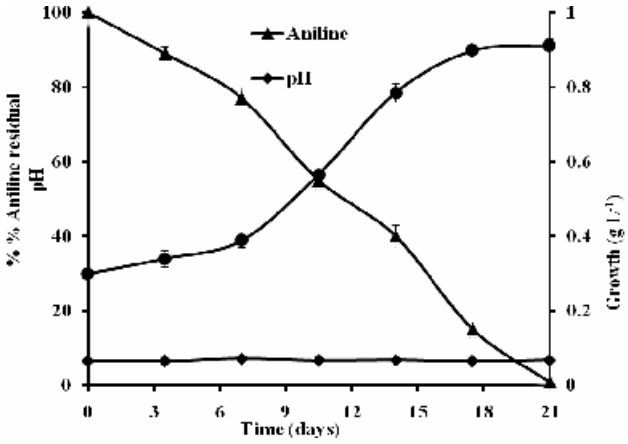



**Figure.2** Kinetic profile of aniline biodegradation and intermediate product formation and depletion during degradation by CAHY2 in MSM with NH4Cl as an additional nitrogen source (▲) Aniline (◆) pH and (●) Biomass




Metabolites isolated were p,p'-dichloroazoxybenzene and p,p'-chloroazobenzene from algae and p-chloro-formanilide and p-chloroanilide from the nutrient medium (Anagnostopoulos *et al.*, 1978). Future studies should be designed to evaluate the potential toxicity of these intermediates and also determine the biodegradability of aniline monocultures of the *Bacillus* species.

The synthesis of the enzymes for the catabolism of an aromatic substrate generally requires the presence of an inducer, which is either the aromatic substrate itself or an intermediate in the degradation pathway. In most aniline-degrading microbes, aniline degradation has been reported to be inducible. Kaminski *et al.* (1983) found that aniline metabolism in *Rhodococcus* strain An117 was induced by

chloroanilines, even though the organism could not grow on them. In *Moraxella* strain G, aniline oxidation was induced by a wide variety of halogenated anilines, some of which supported the growth of the organism (Zeyer *et al.*, 1985). After the investigation of the biodegradability of aniline under aerobic conditions with an additional nitrogen source, differences between the biodegradation level and intermediates formation were observed. These results are very important for the development of strategies for the biodegradation of some aniline based pesticides in natural environments.

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## Compliance with Ethical Standards

The authors declare that they have no conflict of interest. This article does not contain any studies with human participants or animals performed by any of the authors. Ethical clearance is not applicable to this study.

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