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EVALUATION OF GROWTH, CARBAZOLE BIODEGRADATION AND ANTHRANILIC ACID PRODUCTION BY *Pseudomonas stutzeri*

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Abstract - The proportion of nitrogenated compounds such as carbazole in heavy fractions of crude oil is higher in Brazil than in other parts of the world. The degradation of this compound by microorganisms has already been described for bacteria such as *Pseudomonas stutzeri* ATCC 31258. Assays were undertaken to assess the influence of different carbazole concentrations on cell growth, carbazole degradation and the formation of anthranilic acid (an intermediate in the carbazole degradation pathway). The results indicated that there was an accumulation of anthranilic acid in the medium with the higher concentration of substrate (10 g/L), which could be related to the inhibition of *Pseudomonas stutzeri* growth in an excess of carbazole. With 1 g/L of carbazole, growth was found to be ten times greater (0.37 g dry cell weight/L) and there was no accumulation of anthranilic acid (formation of around 7 mg/L), with complete carbazole degradation after three days.
Keywords: Carbazole; Anthranilic acid; Biodegradation; Biodenitrogenation; BDN.

INTRODUCTION

Carbazole and dibenzopyrroles are nitrogenated aromatic heterocyclic compounds that are commonly found in crude oil, as is the case of Brazilian crude (Leite et al., 2005), which are recalcitrant to removal. The environmental problems associated with the presence of these compounds in oil and other fuels include the generation and emission of oxides of nitrogen (NO_x), which are active in the formation of acid rain and the destruction of the ozone layer. Research into their degradation has been intensified in the last decade as the increasingly strict environmental regulations have forced countries to reduce their emission levels. Also, nitrogen compounds have an economic impact on oil refining processes, because they poison the catalysts used for cracking, inhibit hydrodesulfurization (HDS), and alter the quality of the products derived from them (Benedik et al., 1998; Kilbane II, 2006). Currently, hydroprocessing is used to remove nitrogen and sulfur heteroatoms (HDN and HDS, respectively). These processes require high temperatures and pressures and affect the other constituent parts of oil, which could be overcome by coupling this with biodegradation pathways, due to the selectivity and mild conditions required for biorefining (Bressler et al., 2003; Larentis, 2005; Kilbane II, 2006).

There are several microorganisms described in the literature that are capable of degrading carbazole (many of them using it as the sole source of nitrogen, carbon and energy) and that have been isolated from soils, contaminated waters and activated sludges (Nojiri and Omori, 2007). These strains are generally

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described as Gram-negative rod bacteria, such as those presented in Table 1, and there are still others that are being isolated and studied. The literature also contains descriptions of Gram-positive strains capable of degrading carbazole (Table 1).

In the study by Hisatsuka and Sato (1994), a Gram-negative strain was isolated and identified as *Pseudomonas stutzeri* (deposited under code ATCC 31258), which grows well aerobically with carbazole as a sole source of carbon and nitrogen. Anthranilic acid, an intermediate in the biosynthesis of L-tryptophan, was identified as a metabolite in the degradation of carbazole by the bacteria and its accumulation in large quantities was observed in the culture medium. After four days' growth at 30°C with 10 g/L carbazole in a culture medium containing non-ionic surfactants, around 4 g/L of anthranilic acid was produced.

With a view to undertaking biodegradation assays using *Pseudomonas stutzeri* ATCC 31258, growth curves were obtained for two initial carbazole concentrations: 10 g/L (10.000 ppm) and 1 g/L (1000 ppm). The assays were undertaken in order to measure carbazole degradation and anthranilic acid formation over time. The purpose of the higher concentration was to compare with the biodegradation test described by Hisatsuka and Sato (1994), while the second concentration was chosen to see how well the strain would grow in a culture medium with a ten times lower concentration of carbazole.

Table 1: Carbazole-degrading bacteria.

Strain	Gram	References
Pseudomonas resinovorans CA06 and CA10	negative	Ouchiyama et al., 1993; Habe et al., 2001
Pseudomonas stutzeri ATCC 31258 / INCQS 00520	negative	Hisatsuka and Sato, 1994; Larentis, 2005
Pseudomonas sp. KUKK-1,2,3,8; *Escherichia coli* KUKK-6; *Serratia* sp. KUKK-7	negative	Kobayashi et al., 1995
Pseudomonas cepacia F297	negative	Grifoll et al., 1995
Pseudomonas sp. LD2	negative	Gieg et al., 1996
Burkholderia cepacia CB1; *Xanthamonas* sp. CB2	negative	Shotbolt-Brown et al., 1996
Sphingomonas CB3, formerly *Pseudomonas*	negative	Shotbolt-Brown et al., 1996; Shepherd and Lloyd-Jones, 1998
Pseudomonas stutzeri OM1	negative	Ouchiyama et al., 1998
Sphingomonas sp. CDH-7	negative	Kirimura et al., 1999
Ralstonia sp. RJGII.123, formerly *Xanthomonas ampelina*	negative	Grosser et al., 1991; Schneider et al., 2000
Pseudomonas putida ATCC 17484	negative	Loh and Yu, 2000
Novosphingobium sp. KA1, formerly *Sphingomonas* sp. KA1	negative	Habe et al., 2002; Inoue et al., 2004; Gai et al, 2010
Pseudomonas rhodesiae KK1	negative	Yoon et al., 2002
Sphingomonas sp. GTIN11	negative	Kilbane II et al., 2002
Pseudomonas sp. C3211	negative	Jensen et al., 2003
Neptuniibacter sp. CAR-SF	negative	Fuse et al., 2003; Nagashima et al., 2010
Sphingomonas sp. CP19	negative	Bressler et al., 2003
Pseudomonas sp. XLDN4-9	negative	Li et al., 2004; Li et al., 2006
Pseudomonas sp. K23, K22, K15 and J11; *Janthinobacterium* sp. J3 and J4; *Pantoea* sp. J14; *Novosphingobium* sp. J30; *Sphingomonas* sp. J40 and M2	negative	Inoue et al., 2004
Acinetobacter sp. IC001; *Pseudomonas* sp. IC017; *Sphingomonas* sp. IC033, IC075, IC081, IC097 and IC145; *Burkholderia* sp. IC049, IC129 and IC138; *Achromobacte*r sp. IC074; *Erythrobacter* sp. IC114; *Janthinobacterium* sp. IC161; *Stenotrophomonas* sp. IC193; *Marinobacterium* sp. IC961 and IC977	negative	Inoue et al., 2005
Burkholderia sp. IMP5G	negative	Castorena et al., 2006
Sphingomonas sp. XLDN2-5	negative	Gai et al., 2007; Gai et al., 2010
Novosphingobium sp. NIY3	negative	Ishihara et al., 2008
Sphingomonas sp. VKM B-2434	negative	Baboshin et al., 2008
Klebsiella sp. LSSE-H2	negative	Li et al., 2008
Kordiimonas sp. OC3, OC6S, OC9 and OC11S; *Erythrobacter* sp. OC4 and OC8S; *Hyphomonas* sp. OC5; *Sphingosinicella* sp. OC5S; *Caulobacter* sp. OC6 and OC10; *Lysobacter* sp. OC7	negative	Maeda et al., 2009a; Maeda et al., 2009b; Maeda et al., 2010
Sphingomonas sp. JS1	negative	Yang et al., 2009
CBZ-21	unidentified	Baboshin and Golovleva, 2010
Bacillus sp. KUKK-4,5	positive	Kobayashi et al., 1995
Janibacter sp. YY-1	positive	Yamazoe et al., 2004a; Yamazoe et al., 2004b
Nocardioides aromaticivorans IC177	positive	Inoue et al., 2005; Inoue et al., 2006
Gordonia sp. F.5.25.8	positive	Santos et al., 2006
Arthrobacter sp. P1-1	positive	Seo et al., 2006
Bacillus sp. T2.3 to T2.6, T3.1 and T3.3, T4.1 to T4.3, T6.1 to T6.6 and T7.0	positive	Cunha et al., 2006
Dietzia cinnamea P4	positive	Von der Weid et al., 2007
Chryseobacterium sp. NCY; *Achromobacter* sp. NCW	positive	Guo et al., 2008

MATERIALS AND METHODS

The strain *Pseudomonas stutzeri* ATCC 31258 was deposited at the Laboratório de Materiais de Referência/Departamento de Microbiologia/ INCQS/ Fiocruz under code INCQS 00520.

Growth Conditions and Culture Medium Composition

Pseudomonas stutzeri ATCC 31258 / INCQS 00520 was grown in 100 mL at 30ºC and with rotation of 200 rpm for three days. A minimal growth medium was used, which comprised: 10 g carbazole, 10 g $Na_2HPO_4.12H_2O$, 5.5 g KH_2PO_4, 0.25 g $MgSO_4.7H_2O$ and 0.01 g $FeSO_4.7H_2O$ in 1 L distilled water, plus 200 μL Tween 20, according to the description in Hisatsuka and Sato (1994). The surfactant was added to increase the dispersion of carbazole in water, to improve accessibility to this compound by the strain. Another substrate concentration was tested, using 1 g carbazole with the same composition for 1 L of culture medium.

Cell Growth Measurements

Cell growth was measured every 12 hours in the experiments with 10 g/L and 1 g/L of carbazole by absorbance at 600nm (Abs_{600nm}) and by counting colony-forming units (CFU) on LB agar plates [1% (m/v) NaCl, 1% (m/v) bacto-tryptone and 0.5% (m/v) yeast extract, pH 7.5, adjusted with NaOH, and 1.5% (m/v) agar]. After plating 10 μL of the culture medium diluted 10^{10}-fold, the plates were incubated for around 18h at 37°C to obtain isolated colonies.

The conversion from absorbance measured at 600 nm (Abs_{600nm}) to the dry cell weight of *Pseudomonas stutzeri* ATCC 31258 was obtained for the points after three days of cell growth and samples were taken in duplicate. For each 30 mL of 3-day culture medium, 0.0112 g was obtained, giving a concentration of 0.37 g dry cell weight /L.

Carbazole Determination by Gas Chromatography After Extraction with Ethyl Acetate

Carbazole was extracted from the culture medium in two stages, using 4 mL ethyl acetate in an acidic medium for each 2 mL of culture medium at each stage. It was detected by gas chromatography (Varian 3380 with an FID detector and CP-SIL5CB capillary column measuring 15 m in length, 0.25 mm external diameter and 0.25 μm internal diameter), using the following temperatures: 250°C at the injector, 300°C at the detector, column heated to 150-250°C / 8 min and a 1:8 split (volume in the column:volume discharged), with nitrogen as the carrier gas at 60 kPa. Areas detected in FID-GC from known carbazole concentrations were used as standard for substrate determination. The surfactant addition minimizes sampling errors inherent to irregular dispersion of the insoluble substrate in the medium.

Determination of Anthranilic Acid

Anthranilic acid was determined using Ehrlich's reagent, which consists of a solution of 1 g *p*-dimethyl-aminobenzaldehyde, 50 mL of 25% HCl and 5 mL ethanol, and analyzed by absorbance at 450 nm, as described in Hisatsuka and Sato (1994); 100 μL of Ehrlich's reagent was used in 1 mL. A molar absorption coefficient was obtained for determining anthranilic acid in an aqueous medium (minimal growth medium for *Pseudomonas stutzeri*) by the linear correlation (R^2=0.99): $Abs_{450nm} = 0.0011\ AA\ (\mu M)$.

RESULTS AND DISCUSSION

The results for cell growth, carbazole degradation and anthranilic acid formation over three days' growth of *Pseudomonas stutzeri* ATCC 31258 at two different carbazole concentrations (10 g/L and 1 g/L) are discussed below. Results for 10 g/L carbazole are presented in Table 2 and Figure 1.

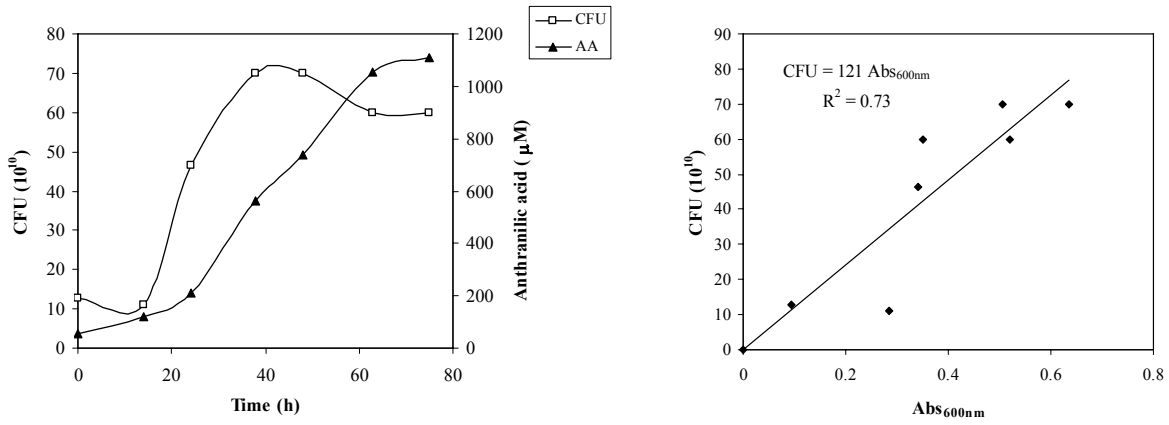
The results obtained for 10 g/L carbazole were similar to those obtained by Hisatsuka and Sato (1994), with an accumulation of anthranilic acid as the strain grew, although at a lower concentration than identified by these authors (after three days, around 1 mM or 140 mg/L anthranilic acid (MW_{AA} = 136 g/gmol) was measured). The carbazole data under these assay conditions were not deemed satisfactory because of the inefficient extraction using ethyl acetate caused by the excess substrate. The growth curve and product formation are shown in Figure 1 (a).

It was found that, under these conditions, the absorbance measurements at 600 nm (Abs_{600nm}) suffered interference from the excess carbazole in the culture medium, and it correlated poorly with the count of CFUs, as can be see in Figure 1 (b).

The results obtained for growth in 1 g/L carbazole differed significantly from the results in the culture medium with a higher concentration of carbazole described in the literature (Hisatsuka and Sato, 1994), as shown in Table 3 and Figure 2.

Table 2: Growth of *Pseudomonas stutzeri* ATCC 31258 in 10g/L carbazole in a minimal medium.

Points	Time (h)	Abs_{600nm}	CFU (10^{10})	AA (μM)
0	0	0.093	13	54.5
1	14	0.285	11	120.5
2	24	0.342	46	212.7
3	38	0.635	70	561.4
4	48	0.505	70	738.6
5	63	0.520	60	1056.8
6	75	0.350	60	1109.1

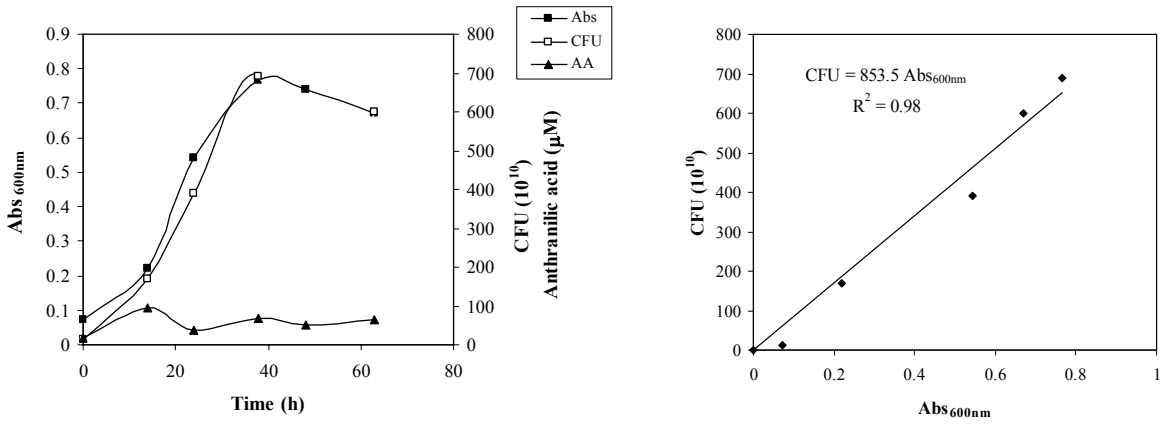


(a) (b)

Figure 1: (a) Growth (CFU) and anthranilic acid formation for *Pseudomonas stutzeri* ATCC 31258 in 10 g/L carbazole in a minimal growth medium. (b) Correlation between Abs_{600nm} and count of CFUs on LB agar plates for *Pseudomonas stutzeri* ATCC 31258 growth in 10 g/L carbazole.

Table 3: Growth of *Pseudomonas stutzeri* ATCC 31258 in 1g/L carbazole in minimal medium.

Points	Time (h)	Abs_{600nm}	CFU (10^{10})	AA (μM)
0	0	0.072	13	18.2
1	14	0.220	170	95.5
2	24	0.543	390	38.2
3	38	0.765	690	66.4
4	48	0.740	-	52.3
5	63	0.670	600	65.9



(a) (b)

Figure 2: (a) Growth (Abs_{600nm} and CFU) and anthranilic acid formation for *Pseudomonas stutzeri* ATCC 31258 in 1 g/L carbazole in minimal growth medium. (b) Correlation between Abs_{600nm} and count of colony formation units (CFU) on LB agar plates for *Pseudomonas stutzeri* ATCC 31258 growth in 1 g/L carbazole.

The carbazole analysis by gas chromatography indicated that, for the lower initial carbazole concentration (1 g/L), there was significant substrate uptake, and around 60% of the carbazole was degraded in 48h, 75% at 63h and complete degradation was observed after three days. This is a very promising biodegradation assay and is comparable with the best results for other carbazole-degrading strains described in the Introduction section. Under these assay conditions, no accumulation of anthranilic acid was found in the culture medium, as can be seen in Figure 2 (a); its levels remained very low (around 50 μM or 7 mg/L) throughout the entire growth period.

According to the data presented in Table 3, in the culture medium with the lower carbazole concentration, growth of around 600 CFUs (Abs_{600nm} ~ 0.7) was observed after three days of cell growth, corresponding to 0.37 g/L dry cell weight. At this concentration, a high correlation was identified between the colony count (CFU) and the absorbance at 600 nm (Abs_{600nm}), indicating that the latter measurement can be reliably used (Figure 2b).

A comparison of the results in Figures 1 and 2 shows that growth in the culture medium with the lower carbazole concentration (1 g/L) was around ten times greater than in the medium with the higher concentration (10 g/L). The production of anthranilic acid was assessed for both initial carbazole concentrations and it was found that, in the lower concentration, around 7 mg/L was obtained, while in the culture medium with a high carbazole concentration there was an accumulation of anthranilic acid (nearly 140 mg/L after three days). These results indicate that the accumulation of anthranilic acid in the culture medium may be related to the inhibition of the growth of *Pseudomonas stutzeri* in a medium with excess amounts of carbazole.

CONCLUSIONS

With a view to undertaking biodegradation assays, curves for *Pseudomonas stutzeri* ATCC 31258 growth, carbazole degradation and anthranilic acid formation were assessed for two different carbazole concentrations (10 g/L and 1 g/L). After three days, 0.37 g/L cells (dry weight) were grown in the 1 g/L culture medium, complete degradation of the initial carbazole was observed, and 7 mg/L of anthranilic acid were formed, confirming carbazole as a sole source of carbon and energy for the bacteria. When the carbazole concentration was higher, the growth was ten times lower and the excess carbazole led to an accumulation of 140 mg/L anthranilic acid, which inhibited the growth of the bacteria.

The 1 g/L (1000 ppm) assay results for *Pseudomonas stutzeri* ATCC 31258 carbazole biodegradation are very promising for the application of this strain in biorefining of Brazilian crudes, which contain more nitrogenated compounds than in other parts of the world (Leite et al., 2005).

NOMENCLATURE

AA	anthranilic acid concentration
Abs_{450nm}	absorbance measured at 450 nm
Abs_{600nm}	absorbance measured at 600 nm
ATCC	American Type Culture Collection
BDN	biodenitrogenation
CFU	colony-forming unit
HDN	hydrodenitrogenation
HDS	hydrodesulfurization
INCQS	Instituto Nacional de Controle de Qualidade em Saúde / Fiocruz
LB	Luria Bertani
MW	molecular weight
ppm	part per million

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