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

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(Submitted: April 10, 2010 ; Revised: August 8, 2010 ; Accepted: September 27, 2010)

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 (NOₓ), 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

*To whom correspondence should be addressed
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.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Gram</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Pseudomonas resinosovorans</em> CA06 and CA10</td>
<td>negative</td>
<td>Ouchiyama et al., 1993; Habe et al., 2001</td>
</tr>
<tr>
<td><em>Pseudomonas stutzeri</em> ATCC 31258 / INCQS 00520</td>
<td>negative</td>
<td>Hisatsuka and Sato, 1994; Larentis, 2005</td>
</tr>
<tr>
<td><em>Pseudomonas sp. KUKK-1,2,3,8, Escherichia coli KUKK-6; Serratia sp. KUKK-7</em></td>
<td>negative</td>
<td>Kobayashi et al., 1995</td>
</tr>
<tr>
<td><em>Pseudomonas cepacia</em> F297</td>
<td>negative</td>
<td>Grifoll et al., 1995</td>
</tr>
<tr>
<td><em>Pseudomonas sp. LD2</em></td>
<td>negative</td>
<td>Gieg et al., 1996</td>
</tr>
<tr>
<td><em>Burkholderia cepacia</em> CB1; <em>Xanthomonas sp. CB2</em></td>
<td>negative</td>
<td>Shotbolt-Brown et al., 1996</td>
</tr>
<tr>
<td><em>Sphingomonas CB3, formerly Pseudomonas</em></td>
<td>negative</td>
<td>Shotbolt-Brown et al., 1996; Shepherd and Lloyd-Jones, 1998</td>
</tr>
<tr>
<td><em>Pseudomonas stutzeri</em> OM1</td>
<td>negative</td>
<td>Ouchiyama et al., 1998</td>
</tr>
<tr>
<td><em>Sphingomonas sp. CDH-7</em></td>
<td>negative</td>
<td>Krimura et al., 1999</td>
</tr>
<tr>
<td><em>Ralstonia sp. RCGIL123, formerly Xanthomonas amgelina</em></td>
<td>negative</td>
<td>Grosser et al., 1991; Schneider et al., 2000</td>
</tr>
<tr>
<td><em>Pseudomonas putida</em> ATCC 17484</td>
<td>negative</td>
<td>Loh and Yu, 2000</td>
</tr>
<tr>
<td><em>Novosphingobium sp. KA1, formerly Sphingomonas sp. KA1</em></td>
<td>negative</td>
<td>Habe et al., 2002; Inoue et al., 2004; Gai et al, 2010</td>
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<tr>
<td><em>Pseudomonas rhodesiae</em> KK1</td>
<td>negative</td>
<td>Yoon et al., 2002</td>
</tr>
<tr>
<td><em>Sphingomonas sp. GTIN11</em></td>
<td>negative</td>
<td>Kilbane II et al., 2002</td>
</tr>
<tr>
<td><em>Pseudomonas sp. C3211</em></td>
<td>negative</td>
<td>Jensen et al., 2003</td>
</tr>
<tr>
<td><em>Neptunibacter sp. CAR-SF</em></td>
<td>negative</td>
<td>Fuse et al., 2003; Nagashima et al., 2010</td>
</tr>
<tr>
<td><em>Sphingomonas sp. CP19</em></td>
<td>negative</td>
<td>Bressler et al., 2003</td>
</tr>
<tr>
<td><em>Pseudomonas sp. XLDN4-9</em></td>
<td>negative</td>
<td>Li et al., 2004; Li et al., 2006</td>
</tr>
<tr>
<td><em>Acinetobacter sp. IC001; Pseudomonas sp. IC017; Sphingomonas sp. IC033, IC075, IC081, IC097 and IC145; Burkholderia sp. IC049, IC129 and IC138; Achromobacter sp. IC074; Erythrobacter sp. IC114; Janthinobacterium sp. IC161; Stenotrophomonas sp. IC193; Marinobacterium sp. IC961 and IC977</em></td>
<td>negative</td>
<td>Inoue et al., 2004</td>
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<td><em>Burkholderia sp. IMPSG</em></td>
<td>negative</td>
<td>Castorena et al., 2006</td>
</tr>
<tr>
<td><em>Sphingomonas sp. XLDN2-5</em></td>
<td>negative</td>
<td>Gai et al., 2007; Gai et al., 2010</td>
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<tr>
<td><em>Novosphingobium sp. Niy3</em></td>
<td>negative</td>
<td>Ishihara et al., 2008</td>
</tr>
<tr>
<td><em>Sphingomonas sp. VKM B-2434</em></td>
<td>negative</td>
<td>Baboshin et al., 2008</td>
</tr>
<tr>
<td><em>Klebsiella sp. LSSE-H2</em></td>
<td>negative</td>
<td>Li et al., 2008</td>
</tr>
<tr>
<td><em>Kordiimonas sp. OC3, OC6S, OC9 and OC11S; Erythrobacter sp. OC4 and OC8S; Hyphomicrobium sp. OC5; Sphingosinicella sp. OC5S; Caulobacter sp. OC6 and OC10; Lysobacter sp. OC7</em></td>
<td>negative</td>
<td>Maeda et al., 2009a; Maeda et al., 2009b; Maeda et al., 2010</td>
</tr>
<tr>
<td><em>Sphingomonas sp. JS1</em></td>
<td>negative</td>
<td>Yang et al., 2009</td>
</tr>
<tr>
<td><em>CBZ-21</em></td>
<td>unidentified</td>
<td>Baboshin and Golovleva, 2010</td>
</tr>
<tr>
<td><em>Bacillus sp. KUKK-4,5</em></td>
<td>positive</td>
<td>Kobayashi et al., 1995</td>
</tr>
<tr>
<td><em>Janibacter sp. YY-1</em></td>
<td>positive</td>
<td>Yamazoe et al., 2004a; Yamazoe et al., 2004b</td>
</tr>
<tr>
<td><em>Nocardioides aromaticivorans</em> IC177</td>
<td>positive</td>
<td>Inoue et al., 2005; Inoue et al., 2006</td>
</tr>
<tr>
<td><em>Gordonia sp. F5-25</em></td>
<td>positive</td>
<td>Santos et al., 2006</td>
</tr>
<tr>
<td><em>Arthrobacter sp. PI-1</em></td>
<td>positive</td>
<td>Seo et al., 2006</td>
</tr>
<tr>
<td><em>Burkholderia sp. T2.3 to T2.6, T3.1 and T3.3, T4.1 to T4.3, T6.1 to T6.6 and T7.0</em></td>
<td>positive</td>
<td>Cunha et al., 2006</td>
</tr>
<tr>
<td><em>Dietzia cyanina</em> P4</td>
<td>positive</td>
<td>Von der Weid et al., 2007</td>
</tr>
<tr>
<td><em>Chryseobacterium sp. NCY; Achromobacter sp. NCW</em></td>
<td>positive</td>
<td>Guo et al., 2008</td>
</tr>
</tbody>
</table>
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 30°C and with rotation of 200 rpm for three days. A minimal growth medium was used, which comprised: 10 g carbazole, 10 g Na₂HPO₄·12H₂O, 5.5 g KH₂PO₄, 0.25 g MgSO₄·7H₂O and 0.01 g FeSO₄·7H₂O 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 600 nm (Abs₆₀₀nm) 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 10¹⁰-fold, the plates were incubated for around 18h at 37°C to obtain isolated colonies.

The conversion from absorbance measured at 600 nm (Abs₆₀₀nm) 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-dimethylaminobenzaldehyde, 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 (R²=0.99): Abs₄₅₀nm = 0.0011 AA (μM).

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 (MWAA = 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 (Abs₆₀₀nm) 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.

<table>
<thead>
<tr>
<th>Points</th>
<th>Time (h)</th>
<th>Abs600nm</th>
<th>CFU (10^10)</th>
<th>AA (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>0.093</td>
<td>13</td>
<td>54.5</td>
</tr>
<tr>
<td>1</td>
<td>14</td>
<td>0.285</td>
<td>11</td>
<td>120.5</td>
</tr>
<tr>
<td>2</td>
<td>24</td>
<td>0.342</td>
<td>46</td>
<td>212.7</td>
</tr>
<tr>
<td>3</td>
<td>38</td>
<td>0.635</td>
<td>70</td>
<td>561.4</td>
</tr>
<tr>
<td>4</td>
<td>48</td>
<td>0.505</td>
<td>70</td>
<td>738.6</td>
</tr>
<tr>
<td>5</td>
<td>63</td>
<td>0.520</td>
<td>60</td>
<td>1056.8</td>
</tr>
<tr>
<td>6</td>
<td>75</td>
<td>0.350</td>
<td>60</td>
<td>1109.1</td>
</tr>
</tbody>
</table>

**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 Abs600nm 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.

<table>
<thead>
<tr>
<th>Points</th>
<th>Time (h)</th>
<th>Abs600nm</th>
<th>CFU (10^10)</th>
<th>AA (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>0.072</td>
<td>13</td>
<td>18.2</td>
</tr>
<tr>
<td>1</td>
<td>14</td>
<td>0.220</td>
<td>170</td>
<td>95.5</td>
</tr>
<tr>
<td>2</td>
<td>24</td>
<td>0.543</td>
<td>390</td>
<td>38.2</td>
</tr>
<tr>
<td>3</td>
<td>38</td>
<td>0.765</td>
<td>690</td>
<td>66.4</td>
</tr>
<tr>
<td>4</td>
<td>48</td>
<td>0.740</td>
<td>-</td>
<td>52.3</td>
</tr>
<tr>
<td>5</td>
<td>63</td>
<td>0.670</td>
<td>600</td>
<td>65.9</td>
</tr>
</tbody>
</table>

**Figure 2:** (a) Growth (Abs600nm and CFU) and anthranilic acid formation for *Pseudomonas stutzeri* ATCC 31258 in 1 g/L carbazole in minimal growth medium. (b) Correlation between Abs600nm 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 (Abs600 nm ~ 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 (Abs600 nm), 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
Abs400 nm absorbance measured at 450 nm
Abs600 nm 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

ACKNOWLEDGMENTS

This study was supported by the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) and PETROBRAS.

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