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Biodegradation of *p*-aminoazobenzene by *Bacillus subtilis* under aerobic conditions

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Biological oxidation of organic dyes is important for textile industry wastewater treatment. The aim of this work was to assess the biodegradation kinetics of a specific azo-dye, *p*-aminoazobenzene. The degradation of *p*-aminoazobenzene by *Bacillus subtilis* was examined through batch experiments in order to investigate the effect of *p*-aminoazobenzene on the bacterial growth rate and elucidate the mechanism of dye degradation. The results proved that *B. subtilis* cometabolizes *p*-aminoazobenzene in the presence of glucose as carbon source, producing aniline and *p*-phenylenediamine as the nitrogen–nitrogen double bond is broken. The azo-dye was found to act as an inhibitor to microbial growth. A mathematical model was developed that describes cellular growth, glucose utilization, *p*-aminoazobenzene degradation and product formation.

Keywords: *Bacillus subtilis*; *p*-aminoazobenzene; dye removal; modelling; azo-dyes

Nomenclature

C_p = *p*-aminoazobenzene concentration (mg L^{-1})
K_I = *p*-aminoazobenzene inhibition constant (mg pAAB L^{-1})
K_p = constant (mg pAAB L^{-1})
K_s = glucose half saturation constant (mg glucose L^{-1})
P_1 = aniline concentration (mg L^{-1})
P_2 = *p*-phenylenediamine concentration (mg L^{-1})
S_g = glucose concentration (mg L^{-1})
t = reaction time (h^{-1})
X = cell mass concentration (mg L^{-1})
$Y_{x/s}$ = yield coefficient for cells on glucose (mg cells mg glucose^{-1})
$Y_{c_p/x}$ = yield coefficient for *p*-aminoazobenzene on cell mass (mg *p*-aminoazobenzene mg cell mass^{-1})
$Y_{p_1/x}$ = yield coefficient for aniline on cell mass (mg aniline mg cell mass^{-1})
$Y_{p_2/x}$ = yield coefficient for *p*-phenylenediamine on cell mass (mg *p*-phenylenediamine mg cell mass^{-1})

Greek letters

β_1 = non-growth-associated pAAB degradation coefficient (mg pAAB mg cell mass^{-1} h^{-1})
β_2 = non-growth-associated aniline formation coefficient (mg aniline mg cell mass^{-1} h^{-1})
β_3 = non-growth-associated *p*-phenylenediamine formation coefficient (mg *p*-phenylenediamine mg cell mass^{-1} h^{-1})
μ_{max} = maximum specific growth rate (h^{-1})

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Introduction

The removal of colour from textile industry and dyestuff manufacturing industry wastewaters represents a major environmental concern. Organic dyes cause both organic pollution and a higher colouration of the effluents. Dye compounds are difficult to treat because of their ability to resist light and oxidizing agents [12]. They are not readily degradable and are typically not removed from water by conventional wastewater treatment systems [1,15]

Azo-dyes represent the largest group of industrial dyes both in number and amount produced. They are characterized by the presence of nitrogen to nitrogen double bonds (—N═N—). The color of azo-dyes is attributed to the azo-bond, the associated auxochromes and a system of conjugated double bonds (aromatic hydrocarbons).

Only a limited amount of research on the aerobic treatment of specific dye compounds in industrial wastewaters has been done in the past. Some researchers have shown azo-dyes to be slowly biodegraded under aerobic conditions [9]. Shaul *et al* [17] found that among 18 azo-dyes tested in activated sludge, only three were degraded to a significant extent. Doyanyos *et al* [3] studied the degradation of 20 azo-dyes under aerobic conditions and observed little or no biodegradation. The results proved that the dye removal is based on absorption on activated sludge. This view was supported by Pagga and Brown [15].

Although bacterial azo-reduction normally requires anaerobic environment, there are few studies reporting that azo-dyes could be degraded by bacteria under aerobic conditions. Reduction of simple azo-compounds by *Aeromonas hydrophila* in the presence of oxygen has been observed [7]. *Pseudomonas cepacia* was also shown to reduce azo-dyes both aerobically and anaerobically [6]. Zimmermann *et al* [18] reported that azoreductase, the enzyme responsible for the initiation of degradation (azo-bond cleavage), from *Pseudomonas* KF46 can degrade azo-dyes aerobically. However, structural variation of an azo-dye influ-

ences the enzyme activity substantially. Mechsner and Wuhrmann [13] noted that the reduction rate should be influenced by the electron density of the azo-group.

Recently the white rot fungus *Phanerochaete chrysosporium* was reported to aerobically degrade some azo-dyes [2], under limited nutrient nitrogen (NH_3) conditions, which provides for ligninolytic activity. Later, Paszczynski *et al* [16] showed that *Phanerochaete chrysosporium* and *Streptomyces chromofuscus* mineralized, aerobically, sulfonated azo-dyes. Peroxidases seem to perform an essential role in azo-dye transformations.

Degradation of azo-dyes, by bacteria, can occur in both aerobic and anaerobic environments. Under anaerobic conditions, no further degradation has been observed [14], whereas permeability through the cell wall has often been found to be the rate-limiting step in the reduction process [13]. Under aerobic conditions, the initial step of azo-bond cleavage is typically followed by hydroxylation and ring opening of the aromatic intermediates. Kulla *et al* [10] however, investigating the aerobic degradation of two sulfonated azo-dyes, did not observe complete mineralization. Zimmerman *et al* [18] demonstrated that the azoreductase under aerobic conditions is highly specific and this specificity plays an important role in determining which azo-dyes are susceptible to bacterial attack.

Horitzu *et al* [5] have demonstrated that *Bacillus subtilis* is capable of *p*-aminoazobenzene reduction under anaerobic conditions. In the present study, we focus on the aerobic biodegradation of this dispersed azo-dye, *p*-aminoazobenzene (pAAB), by *B. subtilis*, growing on a synthetic medium. Such a well-defined system secures reproducibility of the results and allows a clear accounting of all experimental observations. The objectives of this study were to investigate the way of dye transformation, the effect of dye concentration on the bacterial growth and the products of biodegradation under aerobic conditions. On the basis of the experimental data, a mathematical model describing the kinetics of pAAB degradation was developed and the kinetic parameters of the process were estimated.

Materials and methods

Microorganism

B. subtilis was purchased from the American Type Culture Collection (ATCC 6051). The organism was maintained on nutrient agar slants at 4°C.

Culture medium and growth conditions

The nutrient agar (Difco, Detroit, MI, USA) contained (g L^{-1}): Bacto-beef extract 3, Bacto-peptone 5, Bacto-agar 15. The growth medium used consisted of (g L^{-1}): glucose 3, NH_4Cl 1, NaCl 1, $MgSO_4 \cdot 7H_2O$ 0.2, $CaCl_2$ 0.0264, KH_2PO_4 15, $KHPO_4$ 50, yeast 0.05 and peptone 0.05. The medium was also supplemented with 1 drop L^{-1} trace elements (0.5% w/v $MnCl_2$ $CuSO_4$, $FeCl_3$, $Na_2MoO_4 \cdot 2H_2O$). Glucose (Merck, Hohenbrunn, Germany) was used as carbon and energy source and ammonium chloride as nitrogen source. *p*-Aminoazobenzene (Merck) was added in the medium at a concentration of up to 20 mg L^{-1}, the solubility limit of the dye in water. All the experiments were carried out under sterile conditions. The pH was adjusted to 7.1. Three clusters of cells maintained on agar were precultured on a rotary shaker at 25°C in 1-L Erlenmeyer flasks containing 500 ml of the above medium. The conditions were aerobic.

Experimental system

The precultured cells (150 ml) were inoculated in a F-2000 Multigen Bench Fermentor (New Brunswick Scientific Co, Edison, NJ, USA) with 1500-ml working volume. The experiments were carried out under aerobic conditions at 25°C and pH 7.1. Air was supplied to the fermentor, by an internal air pump through an air filter, at a constant flow rate of 3 L min^{-1}. Temperature was controlled by a Solid State Temperature Controller with a fast response Thermowell Thermistor Sensor. Agitation was provided by means of a drive motor magnetically coupled to a magnet assembly located inside the vessel. The suspended medium was continuously stirred at a constant rate of 500 rpm during all the runs. The DO was monitored via a steam sterilized galvanic (Johnson & Borkowski) type oxygen electrode (Virtis Co, Gardiner, NY, USA).

Analytical methods

Bacterial growth was determined by measuring the absorbance at 660 nm in a Milton Roy Spectronic 601 Spectrophotometer (Rochester, NY, USA). A calibration curve relating cell concentration and absorbance was constructed. The concentration of *p*-aminoazobenzene was measured spectrophotometrically, at the dye's absorption maximum of 370 nm according to the following procedure: the total absorbance at 370 nm during the culture was the sum of the dye and biomass absorbance. A calibration curve at 370 nm has been constructed for the biomass in the absence of the dye as well as for the dye in the absence of biomass. The dye concentration in experiments was calculated from an equation which correlated the total absorbance at 370 nm and the concentrations of biomass and dye. The concentration of biomass was known from the absorbance at 660 nm (since the dye did not absorb at this wavelength). The presence of the products of the reaction did not interfere at 370 nm. Since the products did not absorb at this wavelength this method yielded reliable values for dye concentrations. Glucose was determined using the rapid spectrophotometric procedure for determination of total carbohydrates [4]. The determination of glucose is based on the formation of highly coloured materials from the reaction of acid degradation products with polyaromatic compounds.

Aniline and *p*-phenylenediamine were determined by high pressure liquid chromatography on a model 9010 Solvent Delivery System (Varian Analytical Instruments, Sunnyvale, CA, USA) and a model 9050 UV-VIS Detector (Varian). The column used was a Nucleosil C18 (Hichrom Ltd, Berkshire, UK) (reversed phase 25 cm, 4.6 mm). The elution was isocratic and the mobile phase consisted of 60% methanol and 40% water (HPLC grade, Merck). At a flow rate of 1 ml min^{-1} the retention time was 4.1 min for aniline and 3.2 min for *p*-phenylenediamine. The UV-Vis detector was set at 238 nm and 254 nm for aniline and *p*-phenylenediamine respectively.

Experimental results

Since the *B. subtilis* did not grow with *p*-aminoazobenzene as a carbon source, it was assumed that the dye was cometabolically degraded in the presence of glucose. A set of three experiments was performed in order to assess the influence of glucose on *p*-aminoazobenzene removal. We observed an increase of *p*-aminoazobenzene removal with an increase in initial glucose concentration (Figure 1), since growth ceased after glucose was consumed. The extent of removal was 30%, 60% and 90% for glucose concentrations 1, 3 and 6 g L^{-1} respectively. The enhancement of the extent of degradation by increasing a utilizable energy source (primary substrate) implicates the cometabolic process in the system.

In order to investigate the effect of pAAB on the growth of *B. subtilis*, a set of batch cultures was carried out with different initial concentrations of *p*-aminoazobenzene. A preculture of *B. subtilis* was used to inoculate three cultures with 0, 10 and 20 mg L^{-1} of *p*-aminoazobenzene, with identical inocula. The initial concentration of glucose was 3070 mg L^{-1}. We observed that the specific growth rate decreases as *p*-aminoazobenzene increases (Figure 2). So we conclude that pAAB acts as a growth inhibitor. The inhibition is slight for a pAAB concentration of 10 mg L^{-1} but quite pronounced for 20 mg L^{-1}.

A batch experiment was carried out to assess the biodegradation kinetics of *p*-aminoazobenzene. Figure 3 shows the aerobic growth of *B. subtilis* and glucose utilization in a batch culture containing 10 mg L^{-1} *p*-aminoazobenzene initially. The initial glucose concentration was 3070 mg L^{-1} and after its exhaustion no further cell growth took place, confirming that glucose was the growth-limiting substrate. In this experiment the oxygen was monitored continuously to ensure that the conditions were truly aerobic. The oxygen concentration was maintained above 4 mg L^{-1} throughout

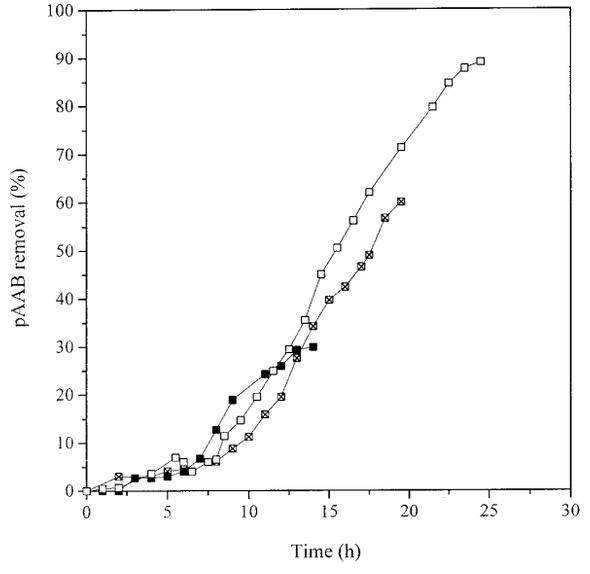


Figure 1 Experimental profiles of pAAB removal during batch cultures of *B. subtilis* with three different initial glucose concentrations in the culture medium. –■– 1 g L^{-1} glucose; –⊠– 3 g L^{-1} glucose; –□– 6 g L^{-1} glucose.

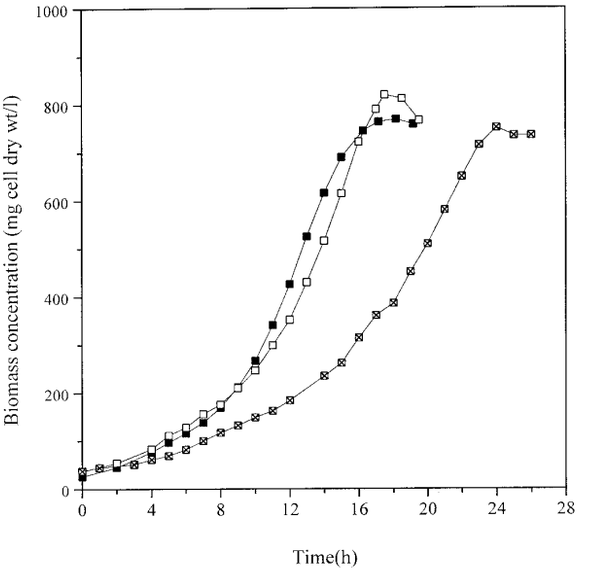


Figure 2 Experimental profiles of biomass concentration during aerobic growth of *B. subtilis* with three different initial pAAB concentrations in the culture medium. –■– Without PAAB; –□– with 10 mg L^{-1} PAAB; –⊠– with 20 mg L^{-1} PAAB.

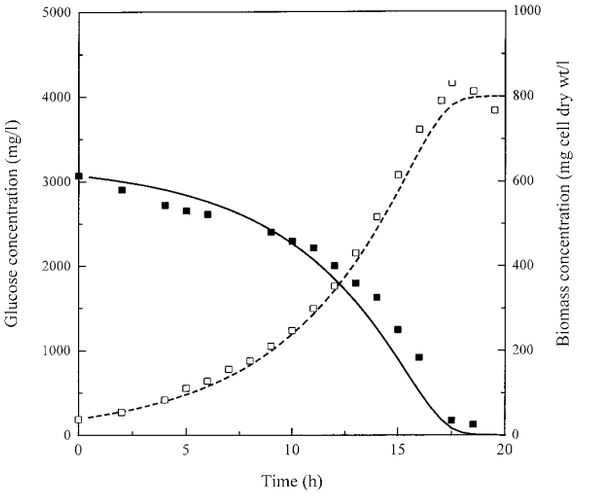


Figure 3 Predicted and experimental biomass and glucose concentration profiles for a glucose-limited batch culture of *B. subtilis*. Initial pAAB concentration 10 mg L^{-1} (C_{gluc_0} = 3070 g L^{-1}, C_{paab_0} = 10 mg L^{-1}, T = 25°C, pH = 7.1). ---- Biomass model; □ biomass experiment; —— glucose model; ■ glucose experiment.

the experiment. Aniline and *p*-phenylenediamine were obtained as the products of reduction cleavage of the azo-bond. Figures 4 and 5 show the degradation of *p*-aminoazobenzene and the formation of products during cultivation. According to the stoichiometry of the reaction, reduction of one mole of pAAB should yield one mole aniline and one mole *p*-phenylenediamine. The experimental results of Figures 4 and 5 are in complete agreement with this expected stoichiometry. Consequently the reaction for the degradation of *p*-aminoazobenzene by *B. subtilis* is shown in Scheme 1.

The next experiment was similar to the previous one with the exception that the initial concentration of *p*-aminoazo-

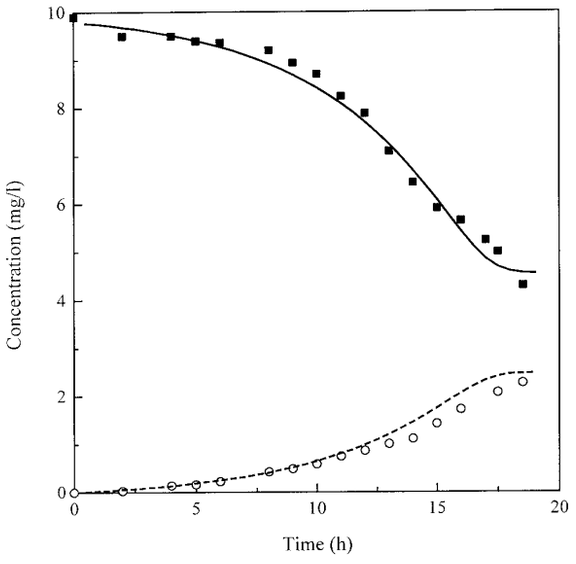


Figure 4 Predicted and experimental concentration profiles of pAAB and aniline during cometabolism of pAAB by *B. subtilis*. —— PAAB model; ■ PAAB experiment; ---- aniline model; ○ aniline experiment.

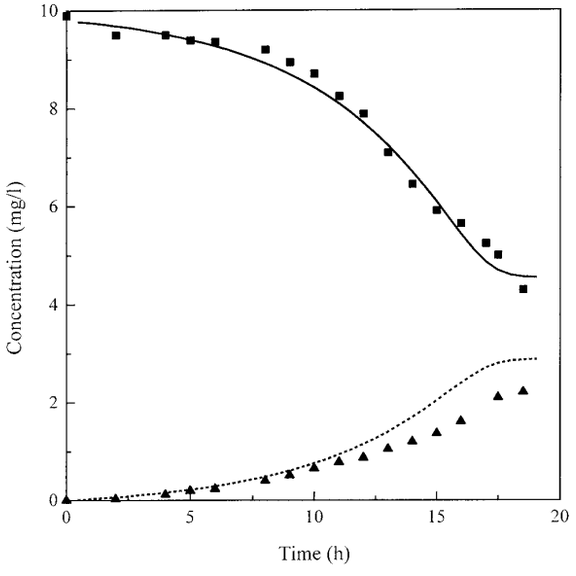
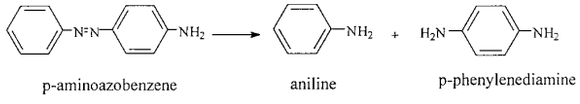


Figure 5 Predicted and experimental concentration profiles of pAAB and *p*-phenylenediamine during cometabolism of pAAB by *B. subtilis*. —— PAAB model; ■ PAAB experiment; ---- *p*-phenylenediamine model; ▲ *p*-phenylenediamine experiment.



Scheme 1

benzene was 19 mg L^{-1}. Figure 6 shows the decrease of pAAB concentration and the increase of the products. From these experimental results we observe that the ratio of moles aniline per mole *p*-phenylenediamine was unity but the ratio of moles of the products per mole of pAAB consumed was less than unity.

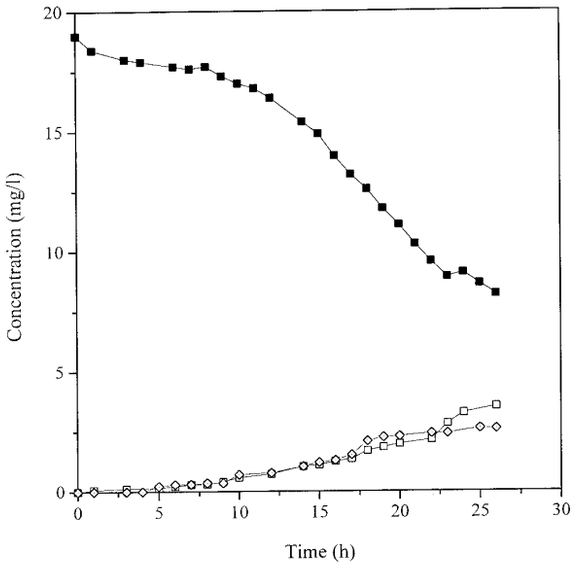


Figure 6 Experimental concentration profiles of pAAB –■–, aniline –□–, and *p*-phenylenediamine –◇– during cometabolism of pAAB by *B. subtilis*. Initial pAAB concentration 19 mg L^{-1} (C_{gluc_o} = 3070 mg L^{-1}, C_{paab_o} = 19 mg L^{-1}, T = 25°C, pH = 7.1).

The ability of *B. subtilis* to degrade pAAB under aerobic conditions may be attributed to an oxygen-insensitive azoreductase, found to be present in the soluble fraction of biomass [5]. Horitzu [5] *et al* have studied the enzyme activity only under anaerobic conditions. Idaka *et al* [8] reported that the azoreductase activity from *P. cepacia* was also observed by incubation under air. Liu and Yang [11] pointed out that oxygen inhibited azoreduction only by purified azo-reductase but not by whole suspensions of *Pseudomonas* S-42. Zimmermann *et al* [18] also reported that azoreductase from *Pseudomonas* KF46 can degrade azo-dyes aerobically.

In order to investigate if this is a constitutive or inducible enzyme, another set of experiments was carried out. Two batch cultures were grown initially on 0 and 10 mg L^{-1} *p*-aminoazobenzene respectively under glucose-limited conditions. An equal amount of *p*-aminoazobenzene was added, as the only organic compound, when the growth ceased after glucose exhaustion. There was very slow but little degradation of pAAB past the stationary phase (probably due to remaining activity). It was observed that pAAB was degraded slowly by both cultures without any lag phase. This observation suggests that *p*-aminoazobenzene is not an inducer of the bacterial enzyme.

From the above results one can conclude that aniline and *p*-phenylenediamine are the products of *p*-aminoazobenzene degradation. Experiments were carried out, with these compounds in the starting medium, in order to examine whether further microbial degradation of aniline and *p*-phenylenediamine is possible. No biodegradation of either compound by *B. subtilis* was observed under aerobic conditions. We conclude therefore that this organism is incapable of further degradation of these compounds.

Kinetic modelling

In this section a model that describes the system under batch aerobic conditions during exponential growth is developed. Assuming Monod kinetics for carbon limitation, the rate equations associated with cell growth are expressed as:

- *cell growth*

$$\frac{dX}{dt} = \frac{\mu_{max} \cdot S_g}{K_s + S_g} \cdot X \tag{1}$$

- *carbon utilization*

$$\frac{dS_g}{dt} = -\frac{1}{Y_{x/s}} \cdot \frac{\mu_{max} \cdot S_g}{K_s + S_g} \cdot X \tag{2}$$

where S_g is the glucose concentration and X is the biomass concentration in mg L^{-1}. A batch experiment was carried out in the absence of *p*-aminoazobenzene to evaluate the maximum specific growth rate, μ_{max}, the half saturation coefficient, K_s and the yield coefficient based on carbon substrate, $Y_{x/s}$. The $Y_{x/s}$ was determined from the slope of the straight line that relates growth rate (dX/dt) and glucose utilization rate (dS_g/dt) (Figure 7). It was found equal to 0.248 mg cells mg glucose^{-1}. The kinetic parameters K_s, μ_{max} were determined from Equations (1), (2) using a least-squares fit. Figure 8 shows the experimental data and the model prediction for this experiment.

In the presence of *p*-aminoazobenzene the kinetics of aerobic growth of *B. subtilis* are assumed to be subject to carbon limitation and dye inhibition. Thus our model equations become:

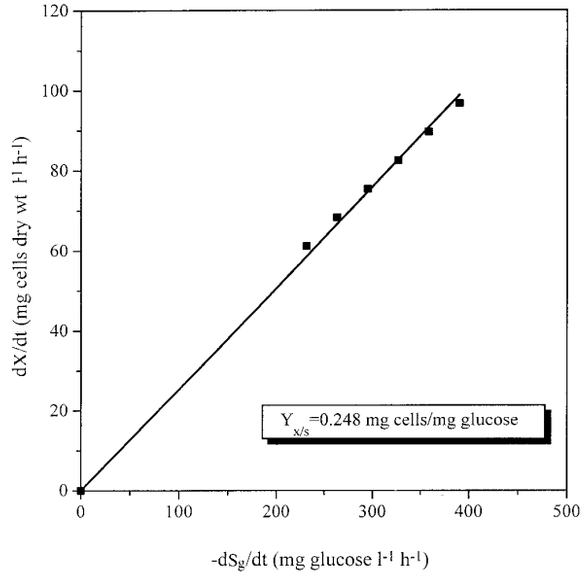


Figure 7 Relationship between bacterial growth rate and glucose utilization rate. Data obtained from the batch culture without pAAB in the culture medium.

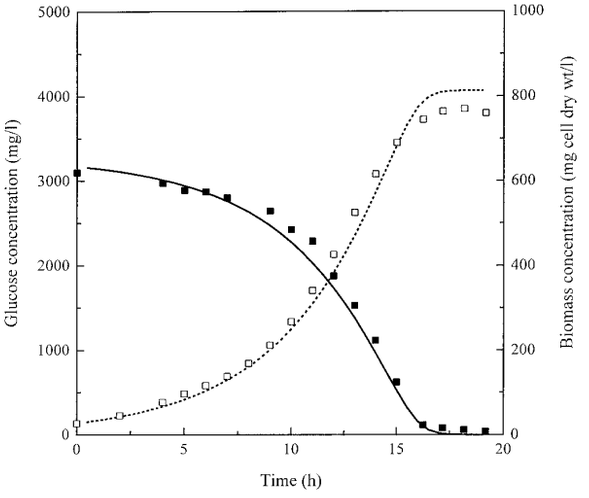


Figure 8 Predicted and experimental biomass and glucose concentration profiles for a batch culture of *B. subtilis* in the absence of pAAB (C_{gluc_o} = 3070 g L^{-1}, T = 25°C, pH = 7.1). —— Glucose model; ■ glucose experiment; ---- biomass model; □ biomass experiment.

- *biomass*:

$$\frac{dX}{dt} = \mu(S_g, C_p) \cdot X \tag{3}$$

- *glucose*:

$$\frac{dS_g}{dt} = -\frac{1}{Y_{x/s}} \cdot \mu(S_g, C_p) \cdot X \tag{4}$$

- *p-aminoazobenzene*:

$$\frac{dC_p}{dt} = -Y^{appar}_{c_p/x} \cdot \mu(S_g, C_p) \cdot X - \beta_1(C_p) \cdot X \tag{5}$$

- *aniline*:

$$\frac{dP_1}{dt} = Y^{appar}_{p_1/x} \cdot \mu(S_g, C_p) \cdot X + \beta_2(C_p) \cdot X \tag{6}$$

- *p-phenylenediamine:*

$$\frac{dP_2}{dt} = Y^{appar}_{p_2/x} \cdot \mu(S_g, C_p) \cdot X + \beta_3(C_p) \cdot X \tag{7}$$

where

$$\mu(S_g, C_p) = \mu_{max} \frac{S_g}{K_s + S_g} \cdot \frac{K_I}{K_1 + C_p} \tag{8}$$

$$Y^{appar}_{1/x} = \frac{C_p}{K_p + C_p} \cdot Y^{true}_{1/x} \; I = C_p, P_1, P_2 \tag{9}$$

Equations (5), (6) and (7) are based on the assumption of combined growth associated and non-growth-associated contributions. Since only minor and very slow degradation of pAAB was observed past the stationary phase, the non-growth associated conversion of pAAB was neglected (all

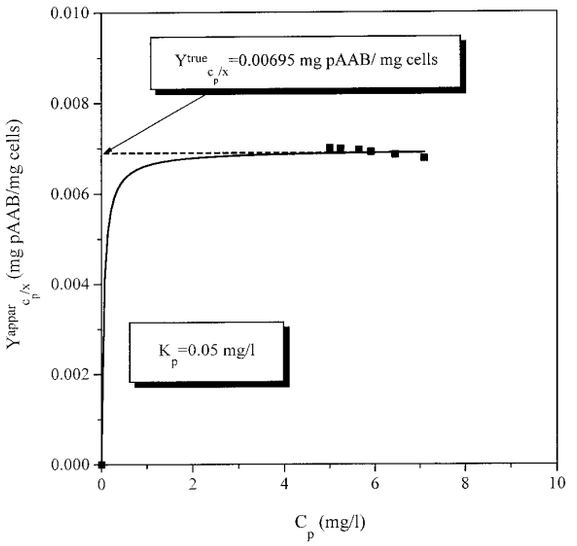


Figure 9 Relationship between apparent yield coefficient and pAAB concentration. Data obtained from the batch culture with 10 mg L^{-1} pAAB in the culture medium.

β_i's were set equal to zero). The inhibition coefficient K_1 was determined from Equations (3), (4), (5) using a least-squares method. Expression (9) is used in order to secure non-negative dye concentration, in the event the dye is exhausted before total consumption of glucose. $Y^{true}_{c_p/x}$ is the maximum yield coefficient attainable for $C_p >> K_p$. The values of the parameters $Y^{true}_{c_p/x}$ and K_p, were determined by plotting $Y^{appar}_{c_p/x}$ $((-dc/dt)(dx/dt)^{-1})$ *vs* C_p (Figure 9). The theoretical best fit was obtained for $Y^{true}_{c_p/x} = 0.00695$ mg pAAB mg $cells^{-1}$ and $K_p = 0.05$ mg L^{-1}. The values of $Y^{true}_{p_1/x}$ and $Y^{true}_{p_2/x}$ were determined from the reaction stoichiometry. All the values of the kinetic parameters obtained from the batch experiments are summarized in Table 1.

The system of Equations (3)–(7) was integrated numerically with the determined values of K_s, K_I, μ_{max}, $Y_{x/s}$, $Y_{c_p/x}$ $Y_{p_1/x}$ and $Y_{p_2/x}$, in order to predict cellular growth, glucose utilization, *p*-aminoazobenzene degradation, aniline and *p*-phenylenediamine production. Model productions are compared with the experimental results in Figures 3–5. It is observed that agreement between the experimental observations and the predictions of the model is very good for all experiments. There is only a slight deviation between the experimental data and the model prediction for *p*-phenylenediamine concentration towards the end of the experiment. This is probably due to the instability of *p*-phenylenediamine during measurements.

Table 1 Kinetic parameters obtained from aerobic experiments

Parameter	Value
μ_{max}	0.245 h^{-1}
K_s	290 mg glucose L^{-1}
K_I	43 mg *p*-aminoazobenzene L^{-1}
K_p	0.05 mg *p*-aminoazobenzene L^{-1}
$Y_{x/s}$	0.248 mg cells mg $glucose^{-1}$
$Y^{true}_{c_p/x}$	0.00695 mg *p*-aminoazobenzene mg $cells^{-1}$
$Y^{true}_{p_1/x}$	0.00328 mg aniline mg $cells^{-1}$
$Y^{true}_{p_2/x}$	0.00381 mg *p*-phenylenediamine mg $cells^{-1}$

Conclusions

Kinetic experiments were performed in a batch reactor containing a pure culture of *B. subtilis* and *p*-aminoazobenzene as a cometabolite. *p*-Aminoazobenzene was partially degraded during growth of *B. subtilis*. Experiments were carried out in order to assess the influence of *p*-aminoazobenzene on bacterial growth rate. It was found that cell growth is inhibited by the presence of pAAB.

The results presented in this study demonstrate that aniline and *p*-phenylenediamine are the stoichiometric products of *p*-aminoazobenzene indicating that azo-linkage is reduced yielding the corresponding amines. The enzyme responsible for azo-dye degradation, was found to be synthesized independently of the presence of the dye.

Based on the experimental observations, a kinetic model was developed, that describes well the aerobic growth of *B. subtilis*, the carbon utilization, the *p*-aminoazobenzene degradation and the product formation.

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