

Microbial degradation of Amido Black by *Pseudomonas* sp. and its kinetics

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ABSTRACT

Azo dyes constitute the largest and most versatile class of synthetic dyes used in the textile, pharmaceutical, food and cosmetics industries and represent major components in wastewater from the industrial dyeing processes. Biological degradation of Amido Black (AB), an azo dye, by a lab isolate of *Pseudomonas* sp. was studied by varying the culture conditions. About 90% of AB (100ppm) degradation was observed within 45 h of incubation in nutrient broth medium at $28\pm 2^\circ\text{C}$ under static conditions. The effect of inoculum size, dye concentration and initial pH of the solution on AB degradation was analyzed. UV-Visible spectra and FTIR spectra of the dye samples before and after decolorization confirmed the ability of the *Pseudomonas* sp. to degrade AB. The kinetic parameters for biodegradation of AB were estimated using three kinetic models available in the literature. AB biodegradation was found to follow the pseudo-second order kinetic model and their correlation coefficients were found to be around 0.995–0.998. In addition, the phytotoxicity study revealed the degradation of AB into a non-toxic product by *Pseudomonas* sp.

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Introduction:

Synthetic dyes have complex aromatic molecular structures, making them very stable and difficult to biodegrade. Such dyes are used extensively in textile dyeing, paper printing, color photography, and in the pharmaceutical, food, cosmetic and other industries [1]. The environmental problems created by the textile industry have received increased attention for several decades because this industry is one of the largest generators of contaminated effluents [2], which mainly arise from dyeing and finishing processes and is associated with the water pollution caused by the discharge of untreated or poorly treated effluents [3]. Based on the chemical structure of the chromophoric group, dyes are classified as azo, anthraquinone, triphenylmethane, heterocyclic, and polymeric dyes; among these, the versatile azo and triphenylmethane dyes account for most textile dyestuffs produced [4].

Azo dyes are the most widely used dyes and represent over 60% of the total dyes used in industries [5]. They are complex aromatic compounds with significant structural diversity. Their properties are enhanced to provide a high degree of chemical, biological and photocatalytic stability and resist

breakdown due to time, exposure to sunlight, microorganisms, water and soap. Many of them are resistant to degradation [3]. Some of the azo dyes are degraded by reducing agents but the reductive cleavage of azo linkages resulted in the formation of amines, which are classified as toxic and carcinogenic [6]. Many physical, chemical and photochemical methods, including adsorption, coagulation, precipitation, filtration and oxidation, have been used for the treatment of dye-containing effluents [7].

Although effective in dye removal, particularly for nonionic dyes, these methods are expensive, add operational complexity to the process and can generate large amounts of dye contaminated sludge that must be disposed of [8]. Due to the inherent drawbacks of physical, chemical and photochemical approaches to dye removal, the effectiveness of biological methods for the treatment of textile wastewaters has been studied by several researches [9].

Biological treatment of azo dye wastewater has attracted more attention due to its cost effectiveness, lower sludge production, and environmental friendliness [10]. In the natural environment azo dye can be transformed or degraded by a variety of

microorganisms, including aerobic and anaerobic bacteria and fungi [11]. However, the long growth cycle and moderate decolorization rate limit the performance of the fungal system [12]. In contrast, the bacterial reduction of the azo bond is usually nonspecific and bacterial decolorization is normally faster. Microbial decolorization and degradation has been reported to be an eco-friendly and cost-competitive alternative to chemical decomposition processes [13].

The present work studied the efficiency of biodegradation of Amido Black by *Pseudomonas* sp. The effect of process variables on the rate of dye degradation was studied for optimizing the condition. In addition, the Amido Black biodegradation kinetics of *Pseudomonas* sp. was also examined using different models. The phytotoxicity of the product formed after decolorization was studied, to assess the toxicity of the degradation product.

Materials and methods:

1. Dye and Chemicals:

The textile reactive azo dye (Amido black) was purchased from the local market and was used without any further purification. All chemicals used were of the highest purity available and of an analytical grade.

2. Bacterial strain and culture conditions:

The soil samples were collected from three different sites of dye effluent discharge, placed in sterile bags and brought to the laboratory within 24hrs to isolate potent dye decolorizing bacteria. The isolation of bacterial strain was carried out by serially diluting the soil samples and subsequently plating on Nutrient Agar medium amended with 100ppm Amido black dye. After one day of incubation at $28 \pm 2^\circ\text{C}$, the colonies were screened for their ability to form a clear zone around them. A bacterial isolate which showed significant amido black dye degradation was identified as *Pseudomonas* sp. based on its microscopic, morphological, and biochemical characters [14].

3. Effects of different parameters on dye decolorization:

Pseudomonas sp. was grown for 24 h at $28 \pm 2^\circ\text{C}$ in Erlenmeyer flasks (250ml) containing 100ml of nutrient broth. Effects of various parameters including initial dye concentration (20, 40, 60, 80 and 100 mg l⁻¹), inoculum size (1%, 2%, 3%, 4% and 5%) and initial pH (5, 6, 7, 8 and 9) and mechanical shaking of cultures on dye decolorization were investigated in triplicate sets of experiments.

Nutrient broth containing dye solution without bacteria served as control. Aliquots of nutrient broth (3ml) were taken at periodic intervals and centrifuged at 5000 rpm for 5 min and the absorbance of supernatant was spectrophotometrically determined using spectrophotometer (620nm). The rate of decolorization was calculated using the following equation:

$$(\text{OD}_i - \text{OD}_t)$$

$$\text{Decolorization (\%)} = \frac{(\text{OD}_i - \text{OD}_t)}{\text{OD}_i} \times 100$$

Where OD_i refers to the initial absorbance, OD_t refers to the absorbance after incubation; and t refers to the incubation time.

4. FTIR spectral analyses:

After complete decolorization, culture filtrate was centrifuged at 13,000 rpm for 30 min. The samples were mixed with spectroscopically pure KBr in the ratio of 2:200, pellets were fixed in a sample holder and the analyses were carried out in a FTIR instrument (Perkin-Elmer, Spectrum one) [15]. The FTIR analysis was done in the mid-IR region of 400–4000 cm⁻¹ with 16 scan speed. The spectra obtained for the biodegraded dye samples were compared with that of control dye

5. Biodegradation kinetics:

The experimental biodegradation data were modeled using zero order, pseudo-first and pseudo-second order equations. The linearized form of the zero order model [16], pseudo-first order model [17] and pseudo-second order model [18] are shown below as Eqs. 1-3, respectively:

$$\text{Log } S = \text{Log } S_0 - K_0 \cdot t/2,303 \quad (1)$$

$$\text{Log } (q_e - qt) = \text{Log } (q_e) - K_1 \cdot t \quad (2)$$

$$t/q_t = 1/K_2 \cdot q_e^2 + 1/q_e \quad (3)$$

6. Phytotoxicity studies:

Phytotoxicity tests were performed to assess the toxicity of the untreated and treated dye [19]. The phytotoxicity of Amido Black (100ppm) and its degradation product was studied as seedling growth response of *Vigna radiata* and *Sorghum vulgare* (10 seeds each) observed in Petri plates lined with coarse filter paper which were wetted separately with aliquots (5 ml) of aqueous solutions of Amido Black (100 ppm) and its degradation product obtained after its decolorization. Simultaneously control sets were carried out using distilled water. Rate of germination (%), shoot and root length of the test plants' seedlings were recorded after 5 days.

Results and Discussion:

1. Effect of mechanical shaking of cultures:

Shake – flask cultures of *Pseudomonas* sp. showed better growth than that under static conditions but higher dye decolorizing activity (90%) was exhibited by the static cultures than that of shake – flask cultures (37 %). The maximum rate of decolorization was reached within 45 hrs of incubation and further incubation did not improve decolorization. Static conditions were reported to be necessary for bacterial decolorization though the cell growth was poorer than that under higher aeration obtained under mechanical shaking conditions [20]. Azo dyes are reportedly resistant to attack by bacteria under shake – flask conditions [21]. Azo dye decolorization by bacterial

species is often initiated by enzymatic reduction of azo bonds. The presence of oxygen normally inhibits the azo bond reduction activity since aerobic respiration may dominate utilization of NADH, thus impeding the electron transfer from NADH to azo bonds [22]. The results were similar to the studies on *E. coli* and *Pseudomonas luteola* [23].

2. Effect of initial bacterial biomass:

To estimate the optimum biomass of *Pseudomonas* sp., required for the removal of 100 mg/l AB, different inoculum size (1 % - 5%) was used during various contact times (Figure 1). Result showed that the rate of dye removal increased significantly along with an increase in the biomass concentration until it reached the value of 82.49% for the biomass at 5%. There was no proportionate increase in the percentage of decolorization with increase in the inoculum size. Similar observation was reported during decolorization by static cultures of *Kocuria rosea* at $28 \pm 2^\circ\text{C}$ and at an inoculum size of 10% [24]. Junnarker et al. [25] reported that the Direct Red 81 decolorization rate was increased to the maximum rate of 2.53 mg/l/h when the bacterial consortium size was increased to 20% (v/v).

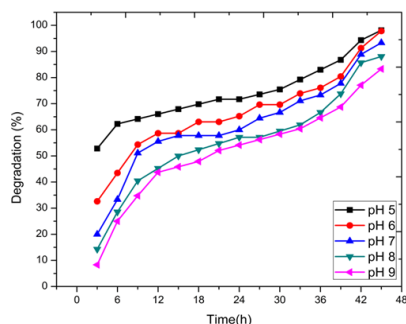


Figure 1. Effect of different inoculum size on decolorization of Amido black by *Pseudomonas* sp.

3. Effects of initial dye concentration on degradation:

In order to study the effect of the initial concentration of AB, the experiments were carried out at a fixed inoculum size (5%) and at different initial dye concentrations of AB (20, 40, 60, 80 and 100 ppm) for different time intervals (3, 6, 9, 12 and 45 h) at $28 \pm 2^\circ\text{C}$. Figure: 2 shows the effect of the initial dye concentration on the decolorization. As the initial AB concentration increased from 20 to 100 ppm, the percentage of AB degradation decreased from 98.97% to 89.93% after 45 h. Khehra et al. [26] suggested that the decrease in decolorization efficiency might be due to the toxic effect of dyes on the cultures. Parshetti et al. [24] reported that only 6% of decolorization of Malachite Green by *Kocuria rosea* MTCC 1532 was observed at 100 mg/l dye concentration. Similarly no decolorization was observed by the *Kurthia* sp. strain

when the concentration of MG was higher than 70 mg/l [27].

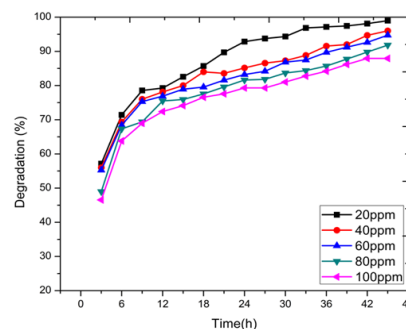


Figure 2. Effect of different initial dye concentration on decolorization of Amido black by *Pseudomonas* sp.

4. Effect of medium pH on degradation:

The effect of initial pH (5.0– 9.0) of nutrient broth on the biodegradation efficiency AB as represented in figure 3 indicate that the rate of dye degradation decreased with a higher initial pH of the medium as the highest rate of AB degradation (98.11 %) was observed at the initial medium pH 5. Similar to our results, Ayed et al. [28] has reported that the optimum pH for Methyl red degradation by *Sphingomonas paucimobilis* was in the range between pH 3.0 and 5.0. Daneshvar et al. [29] reported that the biodegradation of Malachite Green dye by *Cosmarium* sp. was higher in the acidic pH of the medium. The observations indicated that *Pseudomonas* sp. could treat practically wastewaters containing acidic dyes.

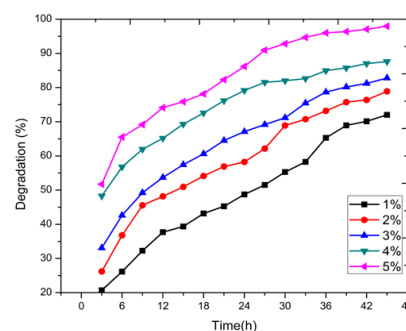


Figure 3. Effect of medium pH on decolorization of Amido black by *Pseudomonas* sp.

5. Dye degradation analysis:

FTIR spectrum of control Amido Black dye was shown in figure 4a. Original absorption at 3495.81cm^{-1} (associated hydroxyl group) was overlapped by the strong stretching mode of N-H. The peaks at 1640.83 and 674.64cm^{-1} indicate N-H bending primary amines. The absorption bands at 2095.23 and 1543.83cm^{-1} were due to C=C stretching of the benzene ring. The band appeared at 1358.80cm^{-1} was assigned to -N=O stretching of -C-NO₂ vibrations.

The FTIR spectrum of partially purified degradation product of Amido Black dye is shown in figure 4b. The peaks at 2744.65 and 2606.50 cm^{-1} were due to C-H and S-H stretching vibrations. The peaks for N-H stretching and CH=CH₂ stretching modes were noticed at 2458.25 and 960.11 cm^{-1} , respectively. The band at 1354.09 cm^{-1} was due to the stretching mode of C-N stretching vibrations. On comparing figure 4a and 4b it could be seen that certain additional peaks had appeared and some had shifted to higher frequency region, providing information that dye degradation had taken place.

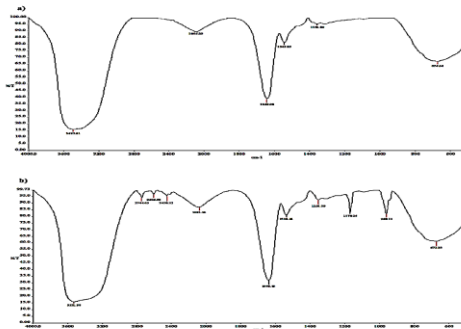


Figure: 4. FTIR spectra of Amido black (a) and its metabolites degraded by *Pseudomonas* sp. (b)

6. Biodegradation kinetics:

The degradation kinetics is very important for the process design and operation control of an industrial degradation process. In biodegradation processes, several kinetic approaches describing the transformation of organic compounds by suspended microorganisms have been evaluated [30]. Zero order, pseudo-first order and pseudo-second order rate kinetics are some of them. The constants for all models were evaluated for the biodegradation of AB by *Pseudomonas* sp. They are listed in table 1 and the corresponding plots are shown in figure 5.

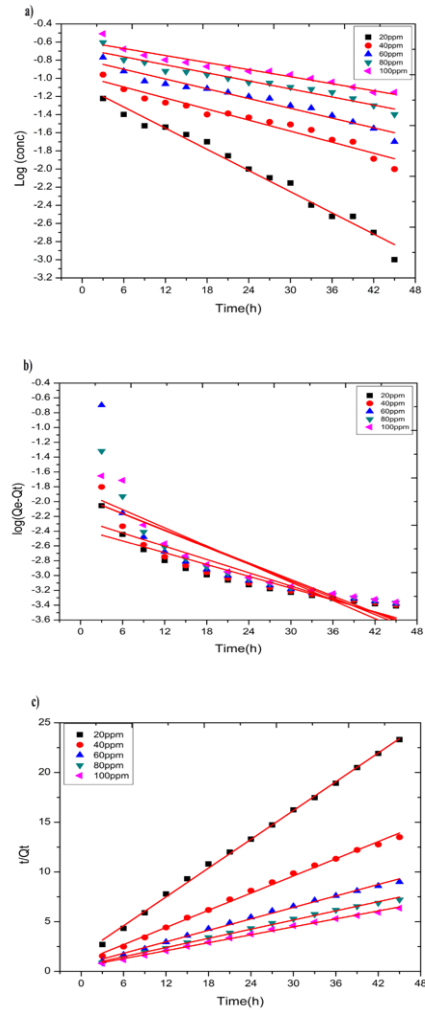


Figure: 5. Plot of zero order (a), pseudo-first order (b) and pseudo-second order (c) kinetics for the removal of Amido black

Table: 1. Constant parameters of zero-order, pseudo-first and pseudo-second order kinetic models for different initial Amido black concentration

S (g/l)	Zero order kinetics			First order kinetics		Second order kinetics	
	K ₀ (g/(1h))	S ₀ (g/l)	R ²	K ₁ (h)	R ²	K ₂ (g/(mg h))	R ²
0.02	0.0006	12.078	0.981	0.0004	0.856	3.78	0.998
0.04	0.0003	9.397	0.952	0.0005	0.818	2.405	0.996
0.06	0.0003	6.194	0.958	0.0007	0.638	1.546	0.995
0.08	0.0002	4.709	0.946	0.0006	0.774	1.233	0.996
0.10	0.0002	3.917	0.943	0.0006	0.825	1.012	0.997

K₀ and S₀ are zero order rate constants and can be obtained by the zero-order kinetic model, Eq. 5. The correlation coefficient obtained for zero order kinetics was found in the range of 0.943–0.981 for various initial Amido Black concentration values. The value of S₀ and K₀ was found to decrease with the increment in the AB concentration. In the zero order kinetics, the reaction rate is independent of the concentration of the

reacting substance [31]. But our results showed that the reaction rate was dependent on AB concentration.

Annur *et al.* [32] hypothesized that the degradation of Trypan Blue followed the first-order reaction kinetics model in which the rate of decolorization was directly proportional to the initial concentration of the dye. The correlation coefficients obtained for the pseudo first order kinetics model for

AB degradation by *Pseudomonas* sp. was comparatively lower than for the other two models and it varied between 0.638 and 0.856. The constant K_1 was found to be influenced by the initial concentration only between 80 ppm and 100 ppm AB dye.

In the case of the pseudo-second order model, the correlation coefficients were found to be around 0.995–0.998, which indicated a good fit of the experimental data. The constant K_2 gradually decreased with an increase in Amido Black concentration. Data obtained in the present study fitted the pseudo-second order model than the Zero order and pseudo-first order kinetics models. Similar results were reported by Filomena *et al.* [30] who observed that pseudo-second order kinetic model suited the biodegradation of diethylketone by *Arthrobacter viscosus*.

7. Phytotoxic effect of AB dye and its degradation product:

The use of untreated and treated dyeing effluents in agriculture has a direct impact on the fertility of the soil. The relative sensitivity of the two plants *Vigna radiata* and *Sorghum vulgare* to AB dye and its degradation products is represented in table 2. The nutrient broth medium allowed 100% of germination of both the plants studied. Amido black dye solution (100ppm) inhibited germination of *Vigna radiata* and *Sorghum vulgare* by 20 % and 30% respectively. However, in the presence of the biodegradation products of AB, *Vigna radiata* and *Sorghum vulgare* showed 100% and 90% germination respectively indicating the non toxic nature of the biodegradation products of AB.

Table: 2. Phytotoxicity of Amido black dye and its metabolites formed after degradation by *Pseudomonas* sp.

Parameter studied	<i>Vigna radiata</i>			<i>Sorghum vulgare</i>		
	NB	AB (100ppm)	degradation product	NB	AB (100ppm)	degradation product
Germination (%)	100	80	100	100	70	90
Shoot length (cm)	3.92±0.27	0.57±0.21	3.61±0.14	3.12±0.31	0.79±0.20	2.68±0.29
Root length (cm)	4.18±0.15	0.72±0.18	3.88±0.19	4.02±0.19	0.96±0.32	3.11±0.25

The shoot length and root length of the two plants were strongly inhibited by the untreated AB dye (0.57 cm and 0.72 cm respectively for *Vigna radiata*; 0.79 cm and 0.96 cm respectively for *Sorghum vulgare*). But the AB dye degradation products induced better growth rates which reached the control values (3.61cm and 3.88cm respectively for *Vigna radiata*; 2.68cm and 3.11cm respectively for *Sorghum vulgare*). Thus the phytotoxicity study revealed that microbial biodegradation of the AB by *Pseudomonas* sp. has resulted in non-toxic break down products.

Conclusion:

The present study revealed the biodegradation potential of a soil isolate of *Pseudomonas* sp. in degrading Amido black. Biodegradation was carried out as a function of shaking/ stationary mode, bacterial inoculum, dye concentration and pH. The experimental results indicated that stationary mode of degradation was efficient when compared to shaking mode of experiment in inducing higher rate of dye degradation. Amido black removal increased significantly along with an increase in the inoculum size up to 5% which resulted in 82.49% color removal in 45 h of incubation. Initial pH of the medium at 5 brought about 98.11% amido black degradation at 45 h of incubation under dark condition. The degradation data were found to follow the pseudo second- order kinetic model. Degradation products were nontoxic to seedlings of *Vigna radiata* and *Sorghum vulgare* as against the effect of the dye. Overall findings ensured the ability of *Pseudomonas* sp. for the complete degradation of Amido black dye within 45 hours of incubation at pH 5

and initial dye concentration of 100 ppm by a static culture of *Pseudomonas* sp. at an inoculum size of 5%.

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