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BIODEGRADATION OF 4-AMINOBENZENE SULPHONIC ACID BY A LOCAL TEXTILE MILL *ASPERGILLUS NIGER* ISOLATE

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Abstract

4-aminobenzene sulphonic acid (4-ABS) is an intermediate formed during the degradation of azo dye loaded effluent containing the unutilized azo dyes used by the textile industry during dyeing and printing processes. Released in the environment, it is a recalcitrant and mutagen. Degradation of 4ABS by fungal strain *Aspergillus niger* RH19 was investigated in batch shake flask-cultures. *Aspergillus niger* RH19 showed more than 40% degradation of 4ABS. Experiments were carried out at initial concentrations between 50-500mg L^{-1}. With increasing initial 4ABS concentrations in shake-flask cultures, percentage removal of 4ABS decreased and residual 4ABS concentrations increased proportionally. Temperature, pH and inoculum size for degradation were optimized. Optimum temperature and pH for degradation was 34^{o}C and 5.0 respectively. Degradation in shaking condition at various concentrations of 4ABS (50-500 mg L^{-1}) was 30-60% whereas in static condition degradation was <20%. Degradation of 4ABS was not affected with addition of glucose as secondary source of carbon at 250mg L^{-1}, however at 500mg L^{-1}, addition of glucose resulted in decreased degradation.

Introduction

Pakistan is a developing country and its major cash crop is cotton. There are more than 200 textile units producing finished products. Textile manufacture consists of a series of processes starting from raw cotton to finished products, comprising pretreatment, dyeing, printing, finishing and treatment of fibers. All these processes require large amounts of water and a variety of chemical and metal complexed dyes (Marks & DeLeo, 1997). Due to unplanned industrial growth, much of the land and nearby water bodies are polluted by indiscriminate dumping of solid and liquid wastes generated by these units (Khan, 2001).

During the dyeing process, azo dyes are used for colouring in textile industry and are among the largely used dyes (Chen, 2006). All azo dyes contain one or more azo (-N=N-) linkages. Degradative product of azo dyes are sulphonated aromatic amines which can be divided into two main groups, linear alkyl benzene sulphonic acid (Jimenez *et al.*, 1991; Mumpel *et al*., 1998) and the sulphonated aromatic compounds, comprising of the aromatic sulphonates, which include 2-aminobenzene sulphonic acid, 3-aminobenzene sulphonic acid, 4-aminobenzene sulphonic acid (4-ABS or sulphanilate) and 4-toluene sulphonic acid (Tan & Field, 2000). 4-ABS is a typical representative of aromatic amines, and is an intermediate in degradation of azo dyes. Its degradation is difficult, because the sulphonyl group is a xenobiotic structural element. Microbes are capable to degrade azo dyes to 4-ABS, which is excreted into the environment, where it accumulates and remains resistant to further microbial degradation (Kulla *et al*., 1983). Thurnheer *et al*., (1986) grew and selected bacteria capable of degrading 4-ABS, sulphonamide, 4-sulphobenzoic acid and phenol sulphonic acid. Four different bacterial strains were able to use aminobenzene sulphonic acids, as sole energy and carbon source.

Feigel & Knackmuss (1988) isolated two cultures strain S1 *Hydrogenophaga palleronii* and strain S2 *Agrobacterium radiobacter*, which were capable of growth with 4-ABS as a sole source of carbon, nitrogen, sulphur and energy. Only mixed cultures degraded 4ABS, whereas, monoculture of strain S2 did not convert 4-ABS (Feigel & Knackmuss, 1993). A bacterial strain (strain S5), was obtained by continuous adaptation of *Hydrogenophaga palleronii* S1, from the mixed coculture isolated by Feigel & Knackmuss (1988). This strain acquired the ability to grow aerobically with 4-ABS (Blümel *et al.*, 1998).

4-ABS is a recalcitrant compound with xenobiotic characters and requires unusual catabolic activities. Upto now, limited data is available on biodegradation of sulphanilate. The aim of this study was to assess 4-ABS degradation capability of a fungal strain, *Aspergillus niger* RH19, at various conditions to develop a low cost biotechnological solution for local small textile units.

Materials and Methods

Aspergillus niger RH19 was isolated from Koh-i-Noor Textile Mills, located in a thickly populated peri-urban suburb of Rawalpindi, Pakistan as described earlier (Faryal & Hameed, 2005). This strain was isolated and maintained on sabouraud dextrose agar slants at 4^oC.

A carbon free mineral salt media (MSM) was to used to check growth and degradation as described by DeFrank & Robbins (1976). Composition of the media was $KH_2PO_4$13.6%, $(NH_4)_2SO_4$ 2.4% and NaOH 2.5% for solution A and $MgSO_4.7H_2O$ 8.0%, $FeSO_4.H_2O$ 0.2% and 4% HCl for solution B. Mineral salt media was prepared by mixing 50 mL solution A and 7 mL solution B in 1 litre of distilled water. Mineral salt medium with glucose was prepared by adding 5 mM glucose (MSM-G).

Analytical methods: 4-ABS was analyzed by high performance liquid chromatography (HPLC) on a C-18 cartridge column (3.9 x 150 nm), having a mobile phase of methanol/distilled deionized water (70:30) at 30ºC, with a flow rate of 0.5 mL min^{-1} and retention time of 10 minutes. Peaks were detected by a UV detector at 257 nm and identified by comparing UV spectra and retention times with those of reference compounds. Samples were prepared by filtration through 0.45µm millipore syringe filter, followed by centrifugation at a relative centrifugal force of 10,000 *g* thrice, for 3 minutes each.

The methods proposed by Clausen *et al.*, (2000) and Price *et al.*, (2001) for determination of metal ions resistivity, were applied to determine maximum resistivity level of 4-ABS. Spore suspensions of $1x10^5$ spores mL^{-1} of inoculum were added to mineral salt media (pH 7.0), containing 50 mg L^{-1} 4-ABS at 34ºC in an orbital shaker (100 rpm). All experiments were performed in duplicate to minimize errors in analysis. Two flasks were taken at 0, 3, 6, 9, 12, 15 and 50^{th} days, the contents filtered through pre-weighed Whatmann filter paper no.1, and the filtrate was analyzed for degradation of 4-ABS.

Degradation at various temperatures, pH and inoculum sizes: In order to find out conditions where there is maximum 4-ABS degradation, mineral salt media containing 50 mg L^{-1} 4-ABS was assessed at various temperatures (28°C, 34°C, 37°C and 40°C), pH (4.0 to 8.0) and spore inoculum sizes ($1x10^3$, $1.2x10^5$ and $1.8x10^8$).

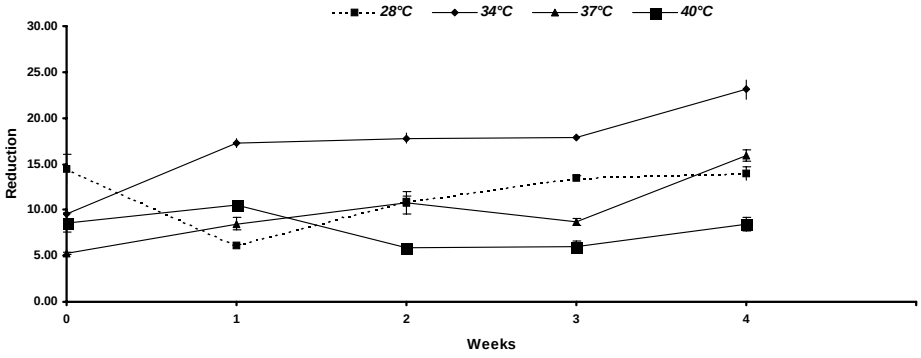

Fig. 1. Reduction of 4-ABS (mg L^{-1}) by *Aspergillus niger* RH19 at various temperatures.

Effect of shaking on degradation: To assess degradation effect of shaking, $1.2x10^5$ spores m L^{-1} of the fungus were inoculated in mineral salt media (pH 5.0) in duplicate series of conical flasks, having 4-ABS in the range of 50-500 mg L^{-1}. These flasks were kept for 5 weeks at 34ºC, one set in an orbital shaker (100 rpm) and the other in a static incubator.

Effect of secondary carbon source on degradation: Glucose (5mM), was added to mineral salt media (pH 5.0) and incubated at 34ºC in an orbital shaker having 4-ABS concentrations 250- 500 mg L^{-1}.

Statistical analysis: Data are represented as Mean ± Standard Error of Mean. Data were subjected to statistical analysis through Student's 't'-test, as described by Steel and Torrie (1960).

Results

In order to screen fungal isolate's ability to degrade 4-ABS, maximum resistivity level was determined on solid mineral salt media supplemented with various concentrations of sulphanilate (50-2000 mg L^{-1}). *Aspergillus niger* RH19 could resist high levels of 4-ABS, upto1500 mg L^{-1} at 28°C and pH 7.0. In shake flask experiments, initially no degradation of 4-ABS was observed at pH 7.0. Upto 15 days of the incubation, minimal degradation of 4-ABS was seen, however, after 50 days, a good degradation was detected. Degradation of 4-ABS was observed at four different temperatures of 28°C, 34°C, 37°C and 40°C. The optimum temperature for degradation was found to be 34°C, however, at all temperatures, degradation of 4-ABS did occur. After 4 weeks incubation, optimal degradation (46.25 ± 1.056%) was observed at 34°C. When growth of *Aspergillus niger* RH19 was monitored at different temperatures, the best growth was seen at 37°C and 40°C, however, percentage degradation of 4-ABS by *Aspergillus niger* RH19 was maximum at 34°C (Fig. 1).

Optimum pH for degradation was 5.0, however, *Aspergillus niger* RH19 exhibited good growth in the pH range of 4.0 to 6.0. The best degradation (65.00 ± 0.25%) was observed at 5.0 pH followed by 36.09 ± 0.12% at pH 4.5 (Fig. 2). With incubation at various pH levels there was a significant increase ($p< 0.05$) in degradation over the time period, however, maximum biomass of *Aspergillus niger* RH19 was produced after 4 weeks (0.0289 g/100 mL) at pH 5.0.

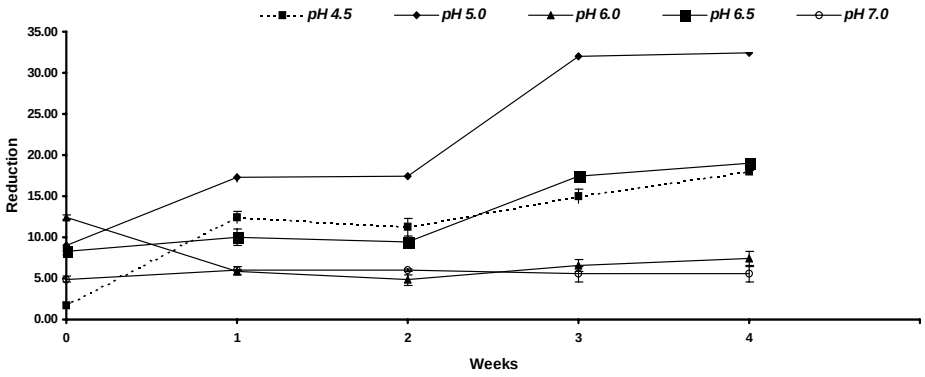

Fig. 2. Reduction of 4-ABS (mg L^{-1}) by *Aspergillus niger* RH19 at various pH.

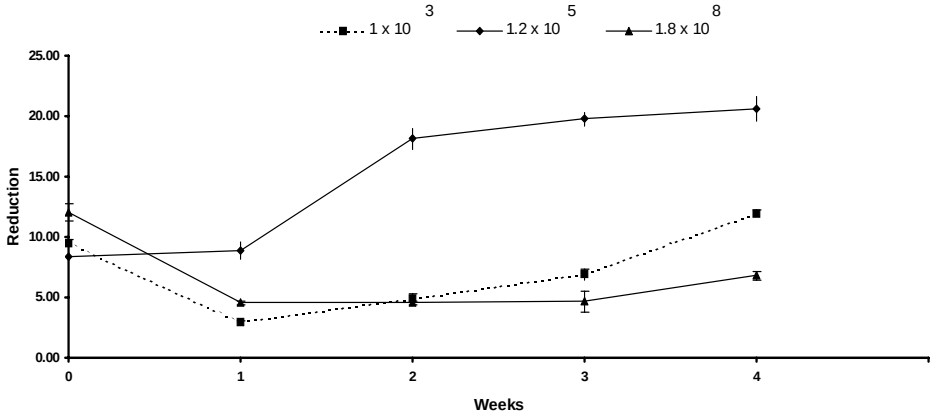

Fig. 3. Reduction of 4-ABS (mg L^{-1}) by *Aspergillus niger* RH19 with different inoculum sizes (spores/mL).

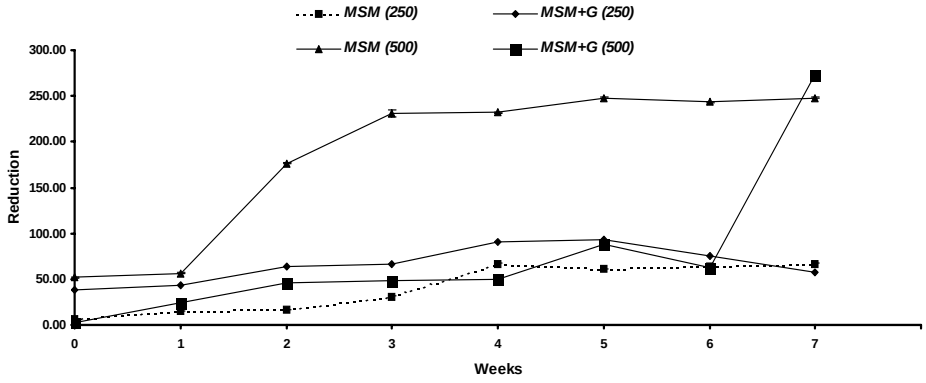

Fig. 4. Reduction of 4-ABS by *Aspergillus niger* RH19 at various concentrations (mg L^{-1}) in Mineral Salt Medium (MSM) and with Glucose (MSM+G).

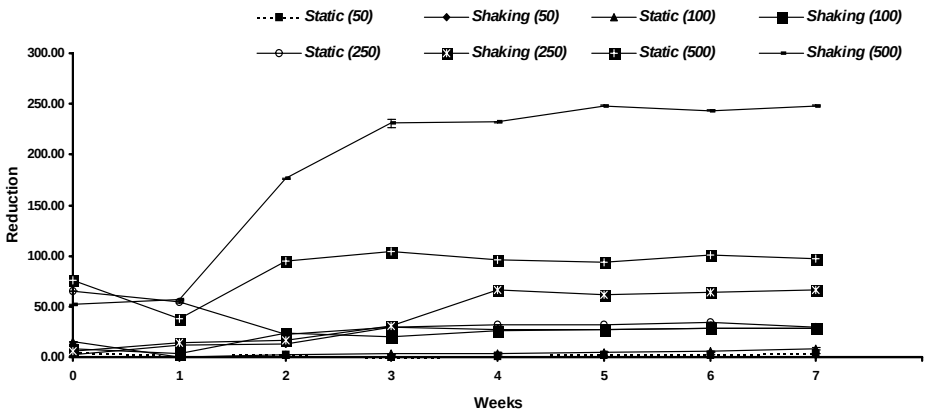

Fig. 5. Reduction of 4-ABS by *Aspergillus niger* RH19 at various concentrations (mg L^{-1}) in static and shaking conditions.

Optimum inoculum size of *Aspergillus niger* RH19 was found to be 1.2 x 10^5 spores mL^{-1}, where degradation rate was 41.15 ± 1.011% at pH 5.0 and 34°C. Increasing the inoculum size reduced the degradation rate, which decreased to 13.58 ± 0.384%, when 1.8 x 10^8 spores/mL were used (Fig. 3).

In these degradation studies of 4-ABS, an additional carbon source, besides 4-ABS, was given in the form of glucose. Degradation in presence of second source of carbon was not affected with addition of glucose as secondary source of carbon at 250mg L^{-1}, however at 500mg L^{-1} addition of glucose decreased degradation (Fig. 4).

On comparison of 4-ABS degradation in static and shaking conditions, *Aspergillus niger* RH19 showed more degradation in shaking condition at all the concentrations (mgL^{-1}) of 4-ABS studied (Fig. 5). Under static conditions, *Aspergillus niger* RH19 degraded 4-ABS at 50, 100, 250 and 500 mg/L concentrations. At all concentrations, significant increase ($p< 0.05$) in degradation was observed during the first 3 weeks of incubation. After 3rd week, a nonsignificant increase in degradation of 4-ABS occurred. Initially, soon after inoculation of *Aspergillus niger* RH19 in MSM containing 4-ABS, there was a rapid adsorption, which was later released into the medium. Maximum degradation was seen at a concentration 250 and 500 mg/L 4-ABS, which was 11.75±0.305 and 19.36±0.695% respectively (Fig. 5). With regard to biomass production, maximum biomass (0.88 g/100 mL) was produced in media containing 50 mg/L 4-ABS, after 3 weeks. After the 4th week, there was a gradual decrease in the growth of *Aspergillus niger* RH19.

Under shaking conditions, maximum degradation (57.38±0.640%) was observed with 50 mg/L 4-ABS at 34°C and pH 5.0. Significant increase ($p<0.05$) in the degradation of 4-ABS, over the time period of 4 weeks was observed. After 4 weeks, degradation rate did not increase significantly. At a higher concentration (500 mg/L 4-ABS), *Aspergillus niger* RH19 degraded 49.50±0.530% 4-ABS after 4 weeks. Trend analysis of biomass produced at various concentrations of 4-ABS, showed an increase in biomass upto the 7th week. Maximum biomass was found after the 4th week of incubation.

Discussion

Pakistan has large number of small textile mills, and these units are considered to discharge effluent having dyes and their degradation products within the permissible limits. Contamination by industrial effluents discharged by various industries in the industrial area of Rawalpindi, Lahore, Sheikhupura & Kala Shah Kaku is well documented (Lone *et al*., 1999; Faryal & Hameed, 2005). The study under discussion was initiated to develop a low cost biosorbent to remediate textile dyes and their degradative product 4-ABS loaded effluent.

Initially, no degradation of 4-ABS was observed, but, after a prolonged incubation of 50 days, degradation was recorded. Adaptation of the fungal strain to this compound enabled it to use 4-ABS as the carbon and energy source. Similarly, Brown & Hamburger (1987) found no degradation of 4-ABS under anaerobic conditions by activated sludge, however, after 28 days under aerobic conditions, upto 99% degradation was observed. Tan *et al*., (1999), observed no degradation of 4-ABS aerobically, even after extended period of operation of the bioreactor, as well as batch experiments upto 245 days. However, 4-ABS degradation was found after bioaugmentation with Rhine river sediment, which was exposed to sulphonated aromatic amines, as it received waste water from a plant treating domestic textile waste water. In the present study, fungal strains from textile mill effluent contaminated soil were used, where *Aspergillus niger* RH19 degraded 4-ABS accompanied by change in pH from 7.0 to 5.0, after 50 days of incubation. Perei *et al*., (2001), also observed a similar effect, change in pH of *Pseudomonas paucimobilis* over time as well as with the consumption of 4-ABS. It can thus be suggested that 4-ABS degradation is correlated with pH, which in turn, affects growth and biomass formation.

Fungal strain *Aspergillus niger* RH19 exhibited degradation of 4-ABS over a wide range of temperature. At even 40°C, *Aspergillus niger* RH19 still degraded 4-ABS, only the percentage degradation was reduced. This strain could be used for removal of 4-ABS from textile wastewater, which have normally temperatures above 40°C. Degradation of 4-ABS has been reported earlier in several studies at 30°C and 37°C (Thurnheer *et al.*, 1990; Feigel and Knackmus, 1993; Perei *et al*., 2001). Feigel and Knackmus (1988), reported 30°C the optimum temperature for the degradation of 4-ABS at pH 7.2, using 25 mM 4-ABS. Maximum degradation was obtained at pH 5.0, which was optimal growth pH as well. However, increasing the pH afterwards led to a decrease in 4-ABS degradation by *Aspergillus niger* RH19. It is a well documented fact that fungal strains grow well over acidic pH.

Aspergillus niger RH19 effectively grew in 4-ABS and utilized the products as the sole carbon source for growth, within a concentration range of 50-500 mg L^{-1}, since mineral salt media was a carbon limiting medium. The decrease in degradation of 4-ABS with increasing concentrations of 4-ABS is in line with the work of Perei *et al*., (2001), who observed that a 10 mM 4-ABS concentration yielded 98% degradation at pH 7.0 and 30°C, which was reduced to 20% by increasing the concentration to 100 mM.

In order to develop a viable option to design tanks at industrial sites, degradation was also assessed at static conditions. Degradation of 4-ABS by *Aspergillus niger* RH19 took place not only under shaking, but also in static conditions. However, percentage degradation was much higher in agitated liquid cultures, as compared to the static cultures. This is indicative of aerobic biodegradation pathway for this compound. This

finding is in agreement with an earlier study on PCP degradation by *Phanerocheate chrysosporium*, using both modes of culture (Laugero *et al*., 1997). In the study under discussion, effect of glucose as a secondary carbon and energy source was also analyzed. Addition of glucose as secondary carbon and energy source did not alter preference of fungus for this complex sulphonated aromatic amine, which is contrary to the findings of Peréz *et al*., (1997), who recorded an increase in chlorophenol degradation by *Phanerocheate chrysosporium*, with an increase in glucose concentration.

In the literature, only one study on 4-ABS degradation by a fungus *Phanerocheate chrysosporium* is reported, in which using U-ring- ^{14}C labeled 4-ABS at an initial concentration of 200 mg L^{-1} was mineralized 40% at 37°C in a shaking culture (Paszezynski *et al*., 1992). In our study, for the first time *Aspergillus niger* has been used for degradation of the recalcitrant 4-ABS, a product from the incomplete degradation of textile dyes, and which has demonstrated the ability to degrade 4-ABS. Absence of peaks other than those of 4-ABS in the HPLC chromatogram suggest that there was a complete mineralization of 4-ABS by *Aspergillus niger* RH19, as degradation was over an extended period of one month. Complete mineralization of azo dyes by fungal strains, as compared to bacterial strains, is possible as there is no limitation of uptake of such compounds into fungal cells. Fungal transformations are mediated by exoenzymes and, therefore, the rate limiting membrane permeation of the substrate is not necessary. However, further studies are needed to enhance, and achieve complete mineralization of 4-ABS by *Aspergillus niger* RH19. Textile mills are an important part of Pakistan's growing economy and efforts need to be concentrated in bringing them among industrial units whose effluent can be transformed from eco-toxic to eco-friendly through bioremediation.

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