Enzyme Mediated Amido Black Decolourization by Soil borne RS-II Strain Isolated from an Industrial Town.

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Abstract: Total 10 strains of microorganisms were isolated from the soils exposed to dyeing industry effluent, in and around Baddi. (HP). The isolate, RS-II, tentatively identified as *Galactomyces* sp. showed maximum amido black (azo dye) decolourization activity (72.08%), on primary screening. However, this isolate exhibited 81.43% decolourization of amido black, under optimal conditions of pH (8.0) and temperature (37^0C). The decolourization activity was found to be pH and temperature dependant, and mediated by enzymatic step. The SDS-PAGE gel electrophoresis results spotted a 66 Kd band. The purification of crude enzyme was carried out by Ion Exchange Chromatography. The activity of the pure fraction (eluted CM sepharose) was recorded as 5.5 moles/min/ml. The study highlights that RS-II has an adequate potential to decolourize the amido black dye, and the pure fraction of enzyme has even higher potential to do so. The findings could be a safe and viable solution for bioremediation of azo dye containing effluents, and could be an effective gateway to evolve more advanced and effective strategies based on the use of pure or immobilized enzymes.
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1. Introduction

Synthetic dyes offer a vast range of new colours to food and textile industry. Many synthetic dyes are used in textile, paper, leather, ceramics, cosmetics, foods processing and ink industries (Buitron *et al*., 2004). The majority of these dyes are azo dyes, that are characterized by the presence of -N = N group. Azo dyes account for approximately 60-70% of all dyes, used in food and textile manufacture. The worldwide production of these dyes has been estimated at 4,50,000 tons/year, and almost 50,000 tons/year, are lost in effluent during application and manufacture. About 2-50% of these dyes are lost as waste effluents, at the time of production and application (Sokman *et al*., 2001; Olukanni *et al*., 2009). It has been reported that 90% of the reactive textile dyes, entering activated sludge sewage treatment plants, pass through unchanged, and are discharged into rivers (Pierce, 1994). Some of these dyes are toxic and may be hazardous to human health (Saue *et al*., 2002). Dyeing factory effluent, that alters the colour and quality of the water bodies, has also proved to be hazardous to aquatic organisms. The effluents containing azo dyes, adversely affect water resources, soil fertility, plant growth, aquatic organisms and ecosystem integrity. They can be lethal, genotoxic, mutagenic and carcinogenic to the aquatic organisms and animals. The toxic compounds, present in dye effluents, enter the aquatic organisms, and reach man through the food chain. Thus, causing various physiological disorders like hypertension, sporadic fever, renal damage, cramps, etc. The bioaccumulation of these toxicants depends on availability and persistence in water and food, and physiochemical properties of the toxicants (Jalandoni-Buan *et al*., 2010).

The dyes are not readily degradable under natural conditions. Moreover, the conventional waste water treatment systems are not effective for their removal. These dyes are mainly metabolized by bacteria to colorless aromatic amines by azoreductases. These amines are further degraded aerobically by bacteria. Some bacteria can degrade azo dyes both aerobically and anaerobically. Extensive work has been carried out on the problems associated with the discharge of dye effluent from industries. It has been documented that the safe method for azo dye biodegradation is combined aerobic treatment (Mabrouk and Yusef, 2008; Olukanni *et al*., 2009). Microorganisms use their enzymatic machinery to decolourize synthetic dyes. Different enzymes such as azoreductases, lignin and manganese peroxidase and laccases are involved in microbial decolourization of dyes (Naidu *et al*., 2003; Ghasemi *et al*., 2010; Suwannawong *et al*., 2010).

Evaluation of optimal enzyme activity, time profile and expression profile are useful to target the enzyme mediated biotransformation process of dyes. Thus,

screening of microflora with effective decolourization, and know how about the enzyme involved, could evolve plausible bioremediation strategies for removal of azo dyes from effluents.

The present work focuses on isolation and screening of indigenous microbial strains for their ability to decolorize azo dyes aerobically, and optimizes the pH and temperature, required for efficient decolourization. Further, an effort has also been made to purify and characterize the enzyme to ascertain its the role in decolourization process.

2. Materials and Methods

2.1 Collection of Samples

The soil samples were collected in sterile plastic bottles under aseptic conditions, in duplicate, at random, from three different sites, in and around Baddi, Distt. Solan (H.P), India. Baddi is an industrial town located in Himachal Pradesh, India ($30^0$57' 31.08" N, 76^0 47' 17.87"E) at 1375 ft. above sea level.

2.2 Enrichment and Isolation of Amido Black Decolourizing Microorganism

10 different isolates of microorganisms were obtained using standard methods of cultivation.

Mineral salts medium with 1% amido black was used for the enrichment of Amido Black decolourizing bacteria (Cohen-bazire, 1954). The inoculated culture flasks were incubated at 37^0C in shaker (REMI-CIS-24BL) at 120 rpm. The decolorizing cultures were further enriched by transferring aliquots of enriched cultures into fresh media, and processed as done earlier. This step was repeated once more.

The organisms were isolated from the final enriched culture, on nutrient agar, using streak plate method. The isolates were purified and maintained for further investigations. The pure cultures were identified on the basis of morphological and biochemical characteristics.

2.3 Screening for Decolourization Activity

10 ppm amido black dye solution in distilled water was scanned spectrophotometrically (Systronics 2202) to find out maximum absorbance (λ_{max}) for amido black dye. Decolourization experiments were performed in 100 ml conical flasks, containing 50 ml nutrient broth with 50 ppm amido black. Three flasks, each for an isolate, were inoculated with approximately 20 mg dry cell mass (Biomass measured using OD 600), and were incubated at 37^0C. The decolorization was measured spectrophotometrically at λ_{max} (617.60 nm). Three ml sample was collected at 72 h incubation, and centrifuged (4000 x g for 15 min) to exclude biomass. Percentage decolourization was calculated as per method documented (Olukanni *et al.*, 2006).

$$\text{Decolourization (\%)} = \frac{A_0 - At}{A_0} \times 100$$

Where,

A_0 = Absorbance of the blank (dye solution)

At = Absorbance of the treated dyes solution at specific time.

2.4 Percentage Decolourization at Different pH and Temperature

Nutrient broth supplemented with 50 ppm amido black was prepared, and was dispensed in different conical flasks, each containing 50 ml. The pH of the broth in different flasks, was adjusted from 2.0 to 11.0 using NaOH or HCl. Then, added approximately 20 mg dry cell mass of RS-II to each flask. The experiments were performed in triplicate. The flasks were incubated at 37^0C for 24 hrs. The absorbance was recorded to calculate percentage decolourization. The percentage decolourization by RS-II was also determined at different temperatures (20^0C, 25^0C, 30^0C, 37^0C, 40^0C, 45^0C and 50^0C) to find out optimum temperature required for maximum decolorization.

2.5 Protein Isolation and Precipitation

For the isolation of proteins, 24 h old culture broth (with 50 ppm amido black) was centrifuged (8000 x g at 4^0C for 10 min). The pellet was re-dissolved in 50 ml. sonication buffer (10 mM Tris HCl, 5 mM EDTA, 5% (v/v) ethanol, 1mM sodium azide, 1mM DTT). Then, vortexed it for 10 minutes, and sonicated at 10% maximum amplitude, 5 sec pulse interval for 5 minutes at 4^0C in sonicator (Misonix, New York). The sonicated products were then centrifuged at 8000 x g for 10 minute at 4^0C, to remove the cell debris. Equal volumes of 70% ammonium sulphate (ice cold) were added to the supernatant. Then, centrifuged the contents at 12000 x g for 20 minutes at 4^0C. The supernatant was discarded and the pellet was washed twice with PBS (pH 7.0) by centrifugation at similar conditions. Protein concentration was determined by Biuret method.

The protein sample pellet obtained by ammonium sulphate precipitation method was mixed with 30 ul of sample loading buffer. Samples were loaded in the wells of the gel. The samples were subjected to SDS-PAGE (Laemmli, 1970). Then, the protein bands were visualized using silver stain.

The purification of the crude protein was carried out by Ion Exchange chromatography. The activity of all fractions of ion exchange chromatography was measured at 617.60 nm.

$$\text{Enzyme activity} = \frac{\mu\text{mol of substrate hydrolysed}}{\text{Minutes X ml. of sample}}$$

The biochemical and physical characteristics of solid wastes (e.g., constituents, pH, and moisture) and operating conditions of solid waste composting (e. g., carbon to nitrogen ratio, aeration rate, reaction temperature and pressure) impose significant effects on an ecological succession of microorganisms (Vallini, 1993; Huang, 2000). Although relationships between these factors have been stressed, it is often difficult to synthesize such a large volume of materials. Generally, the factors that affect composting processes, such as temperature and oxygen availability, are controlled to maintain a relatively better growth environment for microorganisms during the process of composting. Analytical and numerical modeling of the composting process could be used as a tool to analyze composting system performance under different operating scenarios.

3. Results

10 different microbial isolates were screened for amido black (azo) dye decolorization. Among all isolates, the maximum decolourisation was observed in the flask inoculated with RS-II; followed by that of RS-V. The isolates viz. RS-I, III, IV, VI showed almost same results. 90% of all the isolates were found to decolorize azo dyes.

Table 1. Percentage decolourization of Azo dye by RS-II at different pH

pH				**Absorbance at 617.60 nm**		**Decolorization (%)**
Initial		**Final**				
Control	Test	Control	(Test pH ± S.D.)	Control	(Test ±S.D)	
2.00	2.00	2.00	1.76 ± 0.2828	0.114	0.084 ± 0.0021	26.31
3.00	3.00	3.00	2.94 ± 0.1273	0.436	0.245 ± 0.0041	43.80
4.00	4.00	4.00	3.91 ± 0.0424	0.557	0.214 ± 0.0035	61.57
5.00	5.00	5.00	5.09 ± 0.0848	1.232	0.428 ± 0.0318	65.21
6.00	6.00	6.00	7.72 ± 0.1273	1.469	0.449 ± 0.0883	69.43
7.00	7.00	7.00	7.98± 0.0070	1.519	0.394 ± 0.0487	74.06
8.00	8.00	8.00	8.19 ± 0.0848	1.814	0.336 ±0.0487	81.43
9.00	9.00	9.00	8.36 ± 0.2404	1.735	0.330 ± 0.0367	80.97
10.00	10.00	10.00	9.67 ± 0.0141	0.587	0.490 ± 0.1605	16.52
11.00	11.00	11.00	9.99 ± 0.0212	1.159	1.045 ± 0.0049	9.83

Secondary screening of isolates for percentage azo dye decolourization was also carried out. (Table.1). It was observed that RS-II had maximum decolourization (72.08%) of amido black followed by RS-V (69.49%) at λ_{max} (617.60 nm) (Figure. 1).

The influence of different pH on percentage decolorization of amido black was also recorded. Maximum decolourization activity (81.43%) was observed at pH 8.0. (Table.1). It was also observed that the decolorization increased from 26.31% to 81.43% with increasing pH (from 2.0 to 8.0). Further increase in pH of the media led to decline in decolourization percentage with minimum activity (9.83%) at pH. 11. (Figure. 2).

The influence of different temperatures on percentage decolourization of amido black was studied at optimized pH 8.0. (Table 2).

Maximum activity (81.43%) was recorded at 37°C. This value was almost similar to data obtained on effect of pH earlier. The percentage decolourization showed increasing trend with rise in temperatures till 37^0C. Further, rise in temperature, beyond 37^0C, led to decline in decolourization ability of the strain.(Figure. 3).

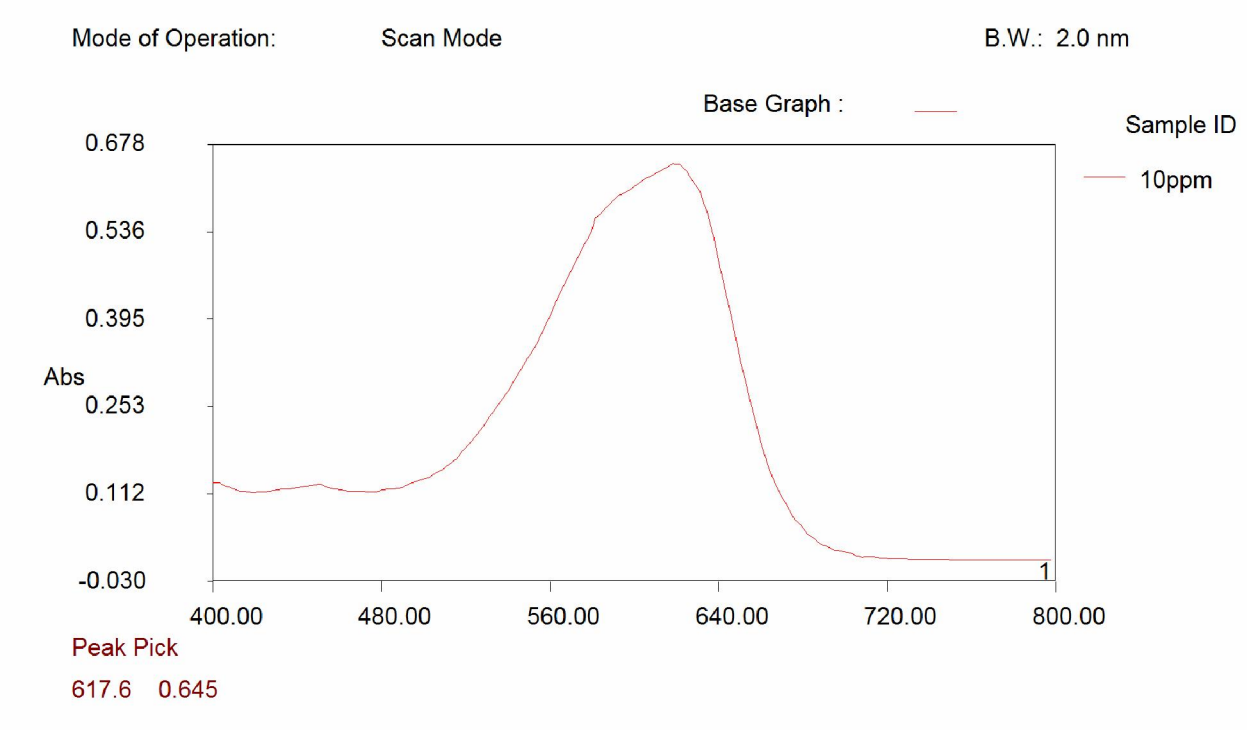

Figure 1. λmax for Amido Black Azo dye (400-800 nm on UV-VIS spectrophotometer)

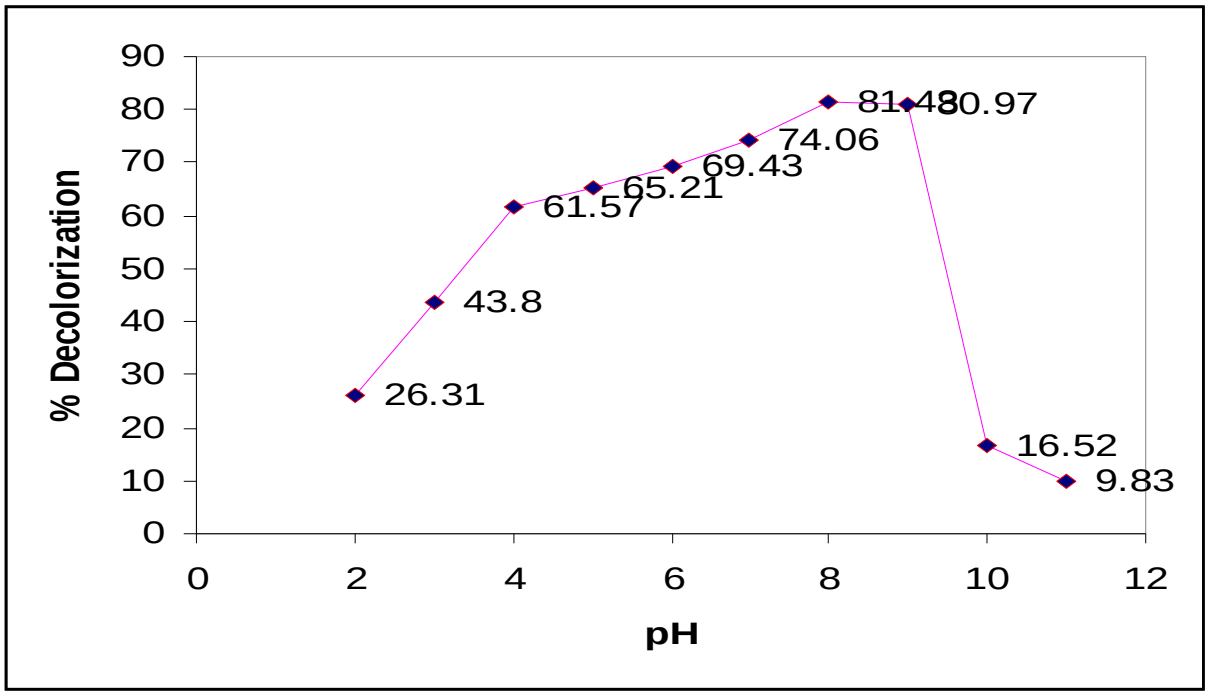

Figure 2. Plot of pH Vs Decolourization % of Azo Dye by RS-II

Table 2. Percentage Decolourisation of Azo Dye by RS-II at Different Temperatures

Tempera -ture (° C)	pH				Absorbance (617.60 nm)		Decolourization (%)
	Initial		Final				
	Control	Test	Control	Test pH±S.D.	Control	TEST± S.D.	
20° C	8.00	8.00	8.00	8.31 ± 0.0212	1.706	1.211±0.3471	8.70
25° C	8.00	8.00	8.00	8.35 ± 0.0777	1.471	1.343±0.0841	17.52
30° C	8.00	8.00	8.00	8.37 ± 0.9899	1.883	1.553±0.0452	29.01
37° C	8.00	8.00	8.00	8.30 ± 0.0707	2.160	0.401±0.0339	81.43
40° C	8.00	8.00	8.00	8.52±0.0707	1.721	1.013±0.2814	41.13
45° C	8.00	8.00	8.00	7.70±0.1414	1.976	1.831±0.3316	18.45
50° C	8.00	8.00	8.00	7.45±0.0494	1.897	1.547±0.875	7.33

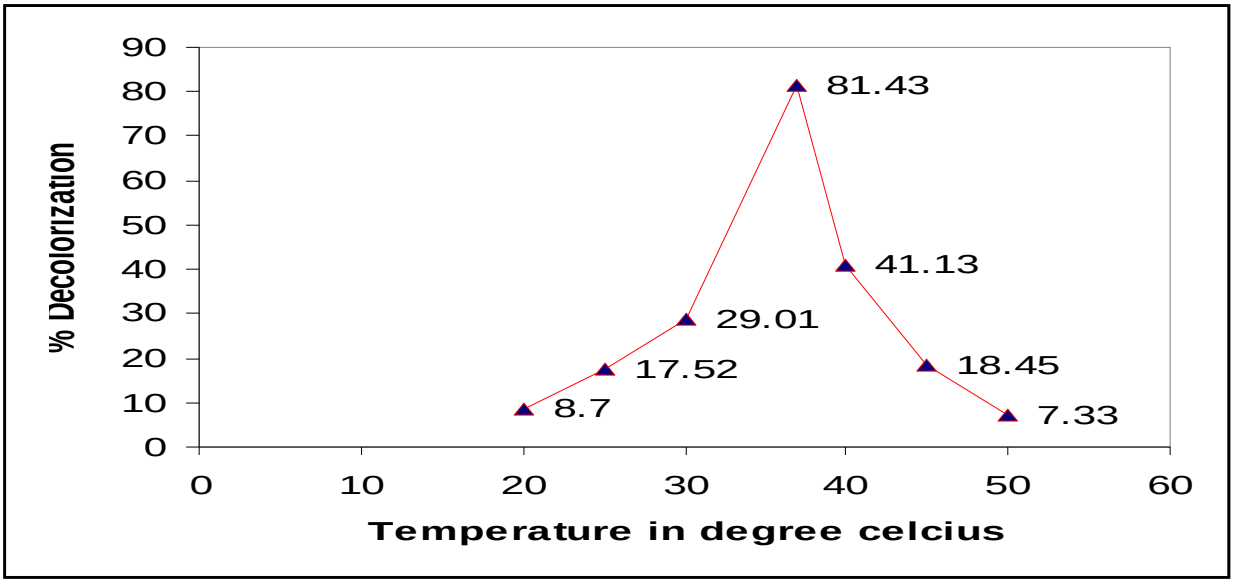

Figure 3. Temperature Vs Decolourization % of Azo dye by RS-II

The protein estimation using SDS PAGE showed two bands (25Kd and 66 Kd). The crude protein was purified using Ion Exchange chromatography. The activity of all the fractions of ion exchange chromatography was determined. The enzyme responsible for decolourization of amido black azo dye was found to effectively bound to cationic exchanger i.e. CM Sepherose. Thus, indicating that the enzyme responsible for amido black decolourisation is a positively charged protein. The enzyme activity of the eluted CM fraction (66Kd) was found out to be 5.5 moles/min/ ml.

4. Discussion

All the isolates showed different decolourization ability for amido black. RS-II strain exhibited highest decolourization at 37^0C. The optimal conditions for decolourization of amido black by this isolate were found to be, pH 8.0 and temperature 37^0C. Since, each microbial strain and its enzymes are highly specific to

pH and temperature, so decrease or increase in decolourization extent of dye might be due to variation in pH and temperature. A large number of workers have investigated the effect of pH and temperature on decolourization of dyes by microorganisms (Raghukumar, 2008; Jadhav *et al*., 2008; Shedbalkar *et al*., 2008; Saratale *et al*., 2009). It is documented that behaviour of each strain varies for dye decolourization with variation in pH (Nosheen *et al*., 2010). The work on azo dye and bacterial combinations to determine optimal conditions for maximum decolourisation efficiency has been documented (Maier *et al*., 2004; Olukanni *et al*., 2009). The authors have concluded that the significant suppression of decolourizing activity at different pH and temperatures, might be due to loss of cell viability or deactivation of enzymes responsible for decolourization. The favourable pH and temperature for dye decolourization by different strains has been reported as 7.0-8.0, and 30-37^{0}C respectively. These findings are similar to our observations.

The decolourizing process of microorganisms is reported to be mediated by different enzymes viz. azoreductases, laccases, peroxidases (Morozova *et al*., 2007; Suwannwong *et al*., 2010). During our studies, two protein bands (25 Kd and 66 Kd) were noticed on SDS PAGE. Earlier, a 66.2 Kd spot on SDS PAGE gel electrophoresis has been observed on azo dye decolourization by *Phanerochaete chrysosporium* (Ghasemi *et al*., 2010). It has been reported that most commonly fungal laccases lie between 50-130 Kd, and that type of dye has no significant effect on extracellular enzyme production (Morozova *et al*., 2010).

Further purification of the protein fraction by Ion Exchange Chromatography and determination of enzymatic activity exhibited significant activity for azo dye decolourization. Thus, it is obvious that the isolate RS-II harbors active enzymatic machinery for amido black decolourization and could be a useful bioremediation tool for treatment of dyeing industry effluent.

5. Conclusion

The study led us to conclude that the isolate has adequate potential to decolourize the azo dye, and that the pure fraction of enzyme has even higher potential to do so. This finding could be a safe and viable solution to bioremediation of azo dye containing effluents and can be an effective gateway to evolve more advanced and effective strategies based on use of pure or immobilized enzymes.

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