

Studies on Decolourisation of Acid Blue 113 Using *Staphylococcus Aureus* and *Escherichia Coli* Isolated From Tannery Wastewater

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ABSTRACT: Biodegradation of acid blue 113 has been studied using two strains-*Staphylococcus aureus* and *Escherichia coli*. The effect of pH, time, initial dye concentration, the inoculum size, the co-substrate, static and shaking on decolourisation was studied. The optimum condition for decolourisation was found to be at pH 7 for an initial dye concentration of 200 mg/L at 37 °C under static condition. The removal of dye by *S.aureus* and *E.coli* at optimum condition was 90% and 88% within 30 h and 42 h respectively. Addition of fructose and starch as carbon source increased the removal of colour by *S.aureus* and *E.coli* respectively. Similarly addition of ammonium formate and glycine as nitrogen source under static condition increased the colour removal by *S.aureus* and *E. coli*. The enzyme activity of azoreductase for *S. aureus* and *E. coli* was found to be 7.33 U/ml and 7.24 U/ml respectively. The COD reduction, FTIR and TLC analysis of the sample before and after decolourisation confirmed the biodegradation of dye and the formation of new metabolite. Phytotoxicity studies indicated that the microbial treated effluent is safe for disposal into the environment.

KEYWORDS: Biodegradation, Colour removal, *E. coli*, *S.aureus*, Wastewater.

I. INTRODUCTION

The wastewater discharged from industries such as textile, leather and dye manufacturing units have been of a major environmental concern for many years, due to its refractory nature, colour, toxicity and high levels of chemical oxygen demand (COD) and biological oxygen demand (BOD)[1,2]. Azo dyes are molecules with one or more double bonded azo (N=N) groups, which are bound to substituted aromatic and heterocyclic groups such as aromatic amine and phenyl. The substituted rings of these dyes are responsible for the intense colour, the water solubility and the resistance to degradation under conventional wastewater treatment [3].

As dyes are designed to be chemically and photolytically stable, they are highly persistent in natural environment and may be inhibitory towards biological wastewater treatment systems due to its complex chemical structure. 10-15% of these dyes are usually lost in the effluent during synthesis and dyeing process [4]. The discharge of the effluent containing dyes and their derived product like benzidine and other aromatic compounds may cause potential toxicity to animals and human [5, 6]. Several physico-chemical processes such as adsorption, chemical oxidation, filtration and incineration are used to treat wastewater containing dyes and dye products. These methods are rather expensive and produce hazardous byproducts [7]

Biological decolourisation by fungi, algae, actinomycetes and higher plants has been tried and seems to be an attractive method, because of their metabolic pathways and versatility of the microorganism [8]. The possibility of using bacteria to decolourise wastewater containing dyes is more advantageous, due to the presence of azoreductase enzyme which has the ability to degrade azo compounds. Moreover, such decolourisation and degradation is an

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environmentally friendly and cost competitive alternative to chemical decomposition process [9]. Studies have reported that some of the aerobic microorganisms, that have the ability to reductively cleave azo bonds under aerobic condition [10, 11]. According to recent studies, bacterial decolourisation showed good colour removal effects, under both static and anaerobic conditions [12,13,14,15]. Some strains of aerobic bacteria have the ability to use dye as the sole carbon and nitrogen sources and reductively cleave azo bonds under aerobic condition [11,16,17,18]. The aim of the present study was to investigate the decolourisation efficiency of the two bacterial strains (*S.aureus* and *E.coli*) at various operational conditions such as pH, initial dye concentration, and temperature and also evaluate the toxicity of treated effluent by plant.

II EXPERIMENTAL

II.1. MATERIALS

II.1.1. DYE AND CHEMICAL

Acid blue 113 Dye (CAS No.3351-05-1) used in this study was purchased from Sigma Aldrich India. All microbiological media and medium ingredients purchased from Hi-media Laboratories, Mumbai, India. Structure and characteristics of the dye is given in the Table 1

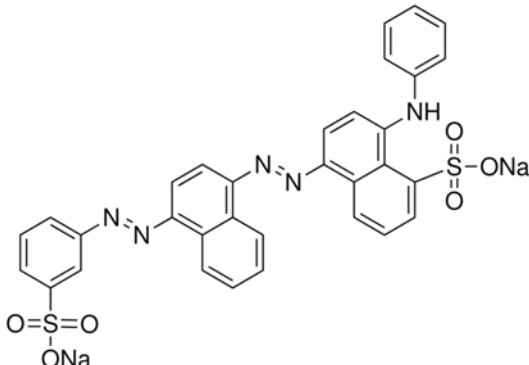
| Dye Name | Structure | Properties |
|---------------|--|--|
| Acid Blue 113 |  | Molecular formula- $C_{32}H_{21}N_5Na_2O_6S_2$ Molecular weight-681.648 Composition C (56.38%) H (3.11%) N (10.27%) O (14.08%) Na (6.74%) S (9.40%) λ max 566 |

Table 1 : Properties of the Dye used in the study

II.1.2. ORGANISM AND CULTURE CONDITIONS

The two strains *S. aureus* and *E. coli* were isolated from tannery wastewater and pure culture was obtained by culture enrichment technique. The pure cultures were grown in 250 mL Erlenmeyer flask containing 100 mL of nutrient broth [(g L^{-1}): peptic digest of animal tissue 5, sodium chloride 5, beef extract 1.5] at 30°C for 24h incubation under static condition.

II.2. DECOLOURISATION STUDIES

The decolourisation study was performed in 100 mL Erlenmeyer flask containing 50 mL nutrient broth. Acid blue 113 was added to the nutrient medium, inoculated with the microorganism and incubated at room temperature under static as well as shaking condition. To study the effect of parameters like dye concentration (50 to 300 mg L^{-1}), temperature (22°C to 42°C), pH (5-9) and inoculum size (200-1000 $\mu L L^{-1}$) were varied individually for the decolourisation of acid blue 113. Also, different carbon sources, (0.5 mg L^{-1} of each) such as fructose, starch, dextrose, and mannitol were used as a co-substrate to study the decolourisation. Similarly, in order to observe the effect of different nitrogen sources, ammonium formate, glycine, and melamine (1 g L^{-1} each) were added to the medium. All experiments were done in duplicate.

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II.3. MEASUREMENT OF DYE CONCENTRATION

The dye concentration was measured with a UV/VIS spectrophotometer (Hitachi U-2000 Spectrophotometer) at regular intervals during the decolourisation process at the wavelength of maximum absorbance of the dye solution (566nm). The percentage of decolourisation was calculated as follows

$$\text{Decolourisation (\%)} = \frac{\text{Initial Absorbance} - \text{Final Absorbance}}{\text{Initial Absorbance}} \times 100$$

II.4. TLC AND FT-IR ANALYSIS FOR THE DEGRADATION PRODUCT OF ACID BLUE113

Biodegradation of acid blue 113 was further confirmed by TLC and FT-IR. The decolourised medium after incubation was centrifuged at 8000 rpm for 15min. The supernatant obtained was added with equal volume of ethyl acetate to extract the metabolite and then evaporated under vacuum. The residue was dissolved in methanol and TLC analysis has been done. The developing solvent system used was isopropanol: acidic acid: water, in the ratio of 19:9:1 respectively. The dry samples were used for FTIR spectral analysis (FTIR spectrum 2000 Perkin Elmer spectrophotometer).

II.5. AZOREDUCTASE ASSAY AND SDS-PAGE

The *S.aureus* and *E.coli* in nutrient broth medium at 37°C were harvested by centrifugation at 17,000 rpm (4°C) for 15 min. The cell pellet was re-suspended into 10 ml acetate buffer (0.1 M, pH 7) and subjected to ultrasonication. Ultrasonication was carried out using sonicator for 8 cycles, each cycle lasting 20 second and 1 minute interval was given between each cycle at 4°C. The crude extract was then subjected to ammonium sulphate precipitation at 80% saturation at 4°C by adding 50.51 g of ammonium sulphate to 90 mL of crude extract. The precipitated enzyme was collected by centrifugation (17,000 g, X 15 min and dissolved in 0.1 M acetate buffer at pH 7. The solution was then dialyzed against 0.01 M acetate buffer (50 mM) overnight. Further purification was carried out by anion exchange chromatography using DEAE anion exchanger. The dialysed enzyme was then used for azoreductase assay [19]. In order to find the molecular weight of azoreductase enzyme, involved in the decolourisation, the purified enzyme was subjected to SDS -PAGE. Molecular weight was compared with standard molecular weight markers ranging from 15-100 kDa.

II.6. COD AND PHYTOTOXICITY

COD was determined using colorimetric method after the sample was digested by closed reflux method [20]. The by- products formed after dye degradation are concern due to for the safe disposal of effluent in the environment, phytotoxicity studies were carried out using 200 mgL⁻¹ of acid blue 113 and its extracted metabolite using seeds *Trigonella foenumgraecum* with water as control at room temperature (34°C ± 2).

III.1. DECOLOURISATION UNDER STATIC AND SHAKING CONDITION

Bacterial species *S.aureus* and *E.coli* exhibited better decolourisation of acid blue 113 under static condition than under shaking condition. *S. aureus* exhibited 90% decolourisation within 30 h and *E. coli* exhibited 88% decolourisation within 42h of incubation (Fig. 1). Similar studies on decolourisation of acid Maroon under static and shaking condition have been reported by Moosvi et al. 2005; Yogesh et al.2012 [14,21]. This may be attributed to 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 [21, 22].

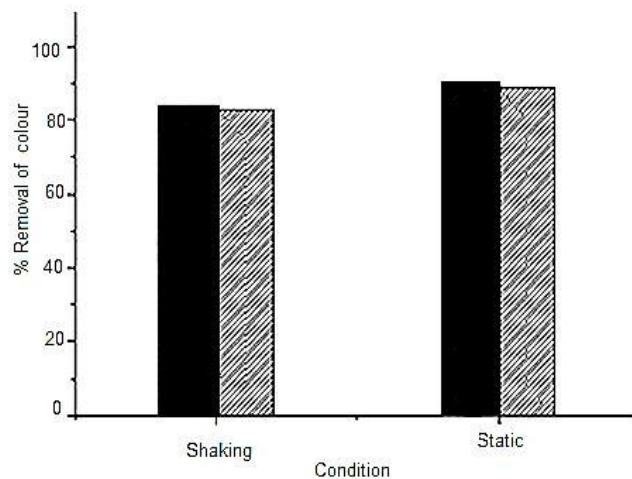


Fig 1. Effect of static and shaking on decolourisation by *S. aureus* (■) and *E. coli* (▨) at pH= 7, Dye concentration = 200 mgL⁻¹, T= 37°C.

III.2. EFFECT OF TEMPERATURE AND pH

Fig. 2 shows effect of different temperature (22-45°C) on removal of colour by *S.aureus* and *E.coli* static condition. The percentage removal of colour by *S.aureus* was 90.02% and that of *E.coli* was 88.7% at 37°C. Further increase in temperature resulted in the marginal reduction of decolourisation by *S.aureus* and *E.coli*. This is due to the loss of cell viability or to the denaturation of the azoreductase enzyme [8].

Since pH plays an important role in the decolourisation of dye, the experiments were conducted at varying pH 5-9. Fig. 3 shows the effect of pH on removal of colour by *S.aureus* and *E.coli* at 37°C under static condition. The percentage removal of colour by *S.aureus* and *E.coli* was 90.2% and 88.6% respectively at pH 7. Similar studies have been reported for decolourisation of azo dyes by *E.coli* [23, 24]. In general, decolourisation by bacteria occurs at nearly pH 7. The major effect of pH may be attributed to the transport of dye molecule across the cell membrane which can be treated as the rate limiting step for decolourisation [15,25].

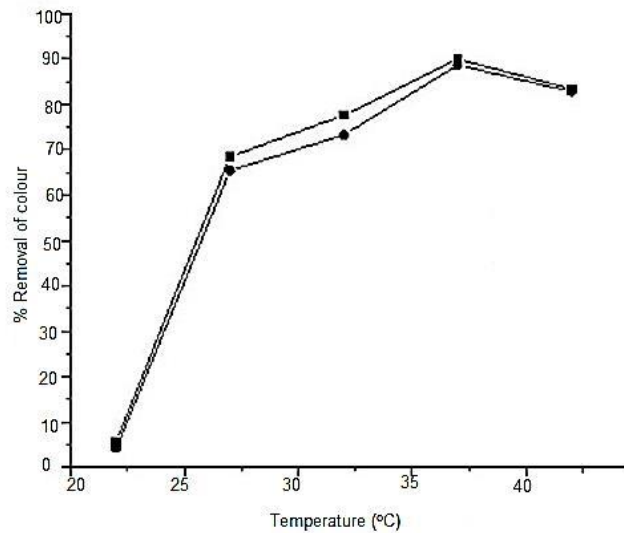


Fig. 2. Effect of Temperature on decolourisation of dye *S. aureus* (■) and *E. coli* (●) at pH=7, Dye concentration =200 mgL⁻¹.

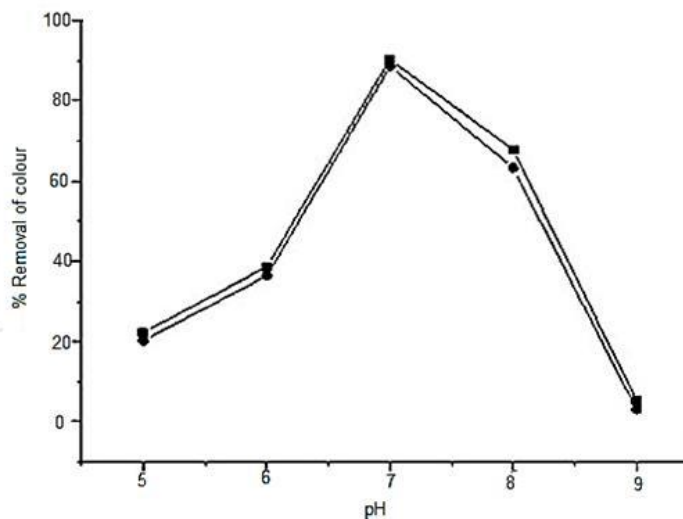


Fig 3. Effect of pH decolourisation of dye by *S. aureus* (■) and *E. coli* (●), T = 37°C, Initial dye concentration= 200 mgL⁻¹.

III.3. EFFECT OF INITIAL BACTERIAL BIOMASS

Decolourisation activity of *S.aureus* and *E.coli* was studied using Acid blue 113 at different initial biomass concentrations varying from 200-1000l/50 ml (7.7×10^{-7} CFU/mL). It is clear from the fig. 4 that the percentage removal of colour increased with an increase in the biomass concentration which is due to the excess available adsorption.

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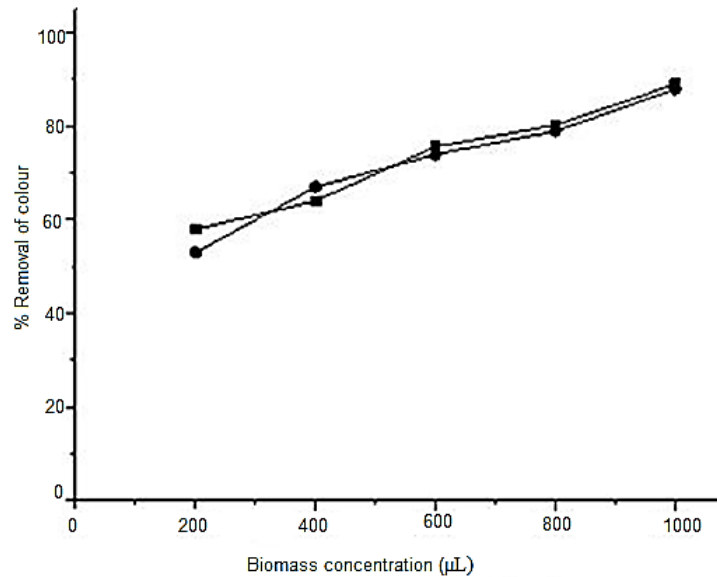


Fig 4. Effect of biomass concentration on decolourisation of dye *S. aureus* (■) and *E. Coli* (●), T= 37°C, Dye concentration =200 mgL⁻¹, pH= 7.

III.4. EFFECT OF INITIAL DYE CONCENTRATION ON BACTERIAL CULTURE ON DECOLOURISATION

The decolourisation of acid blue was studied at various increasing initial concentration such as 50 – 250 mg/L at pH 7, temperature 37°C. Fig. 5 depicts that decolourisation decreased with increase in initial dye concentration. Under static condition both *E.coli* and *S. aureus* could decolourise more than 95% acid blue dye within 32 h. Further increase in the initial dye concentration from 150-250 mg/l resulted in decrease of colour removal up to 73%. The decrease in decolourisation efficiency was due to toxicity of the dye to bacterial cells through inhibition of metabolic activity or because the available bacterial cells would not have been sufficient to degrade the dye [9, 26].

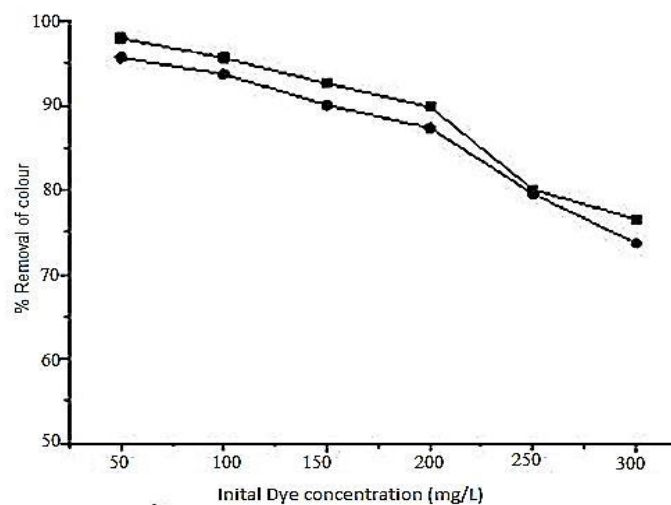


Fig 5. Effect of initial dye concentration on decolourisation of dye *S. aureus* (■) and *E. Coli* (●), T= 37°C, Dye concentration= 200 mgL⁻¹, pH=7.

III.5. EFFECT OF CARBON AND NITROGEN SOURCE ON DECOLOURISATION

Fig. 6 depicts the effect of different carbon sources (fructose, starch, dextrose and mannitol- 500 mg/L of each) on decolourisation of acid blue 113 by *Saureus* and *Ecoli* at pH 7 for an initial dye concentration of 200 mg/L. It was found that maximum colour removal was 96.9% with fructose and 94.02 % with starch for *S.aureus* and *E.coli* respectively. This research shows that addition of various carbon and nitrogen sources improved decolourisation of acid blue 113. Fig. 7 shows the effect of different nitrogen sources such as ammonium chloride, ammonium formate, glycine and melamine. Among the organic nitrogen sources, addition of glycine causes maximum colour removal up to 94.3% for *E. coli*. Among all nitrogen sources, addition of ammonium formate shows the maximum colour removal up to 95% for *S.aureus*. Similar studies have reported that organic nitrogen sources are considered as an essential medium supplement for the generation of NADH that act as an electron donor for the reduction of azo dyes by microorganism [27,28,29] Levin et al. 2010;Nachiyar and Rajkumar 2003; Bibi et al. 2012).

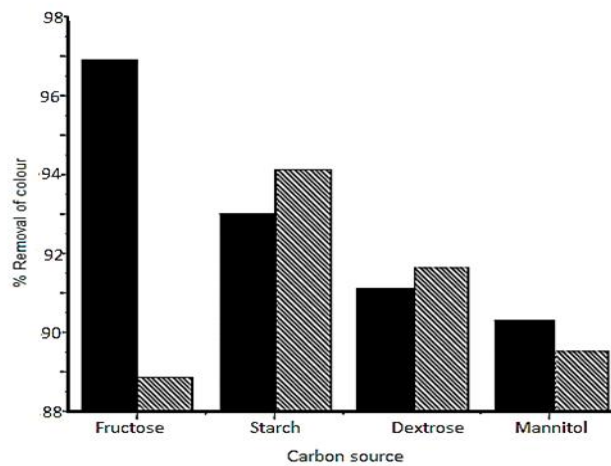


Fig 6. Effect of different carbon sources on decolourisation of dye by *S.aureus* and *E.coli*, *S. aureus* (■) and *E. coli* (▨), pH =7, T= 37⁰ C, Dye concentration=200 mgL⁻¹.

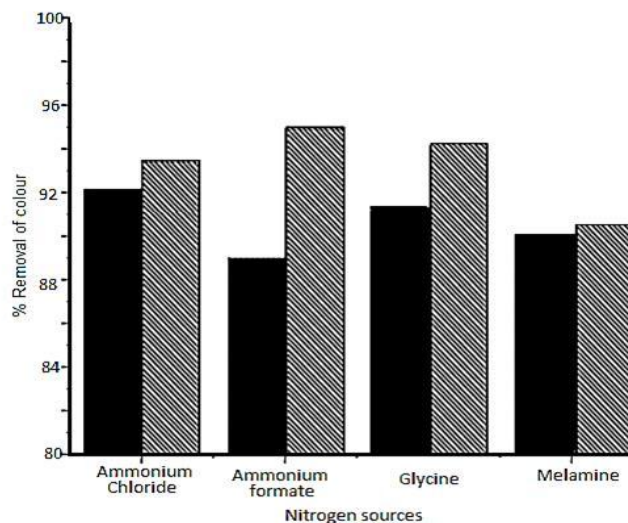


Fig 7. The effect of different nitrogen sources on decolourisation of dye *S. aureus* (■) and *E. coli* (▨), pH =7, T= 37⁰ C, Dye concentration=200 mgL⁻¹.

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III.6. DETERMINATION OF AZOREDUCTASE ACTIVITY BY ENZYME ASSAY AND SDS PAGE

The enzyme obtained from the bacterial cells was found to decolourise acid blue 113 dye using NADH as electron donor. The absorbance decreased which showed that NADH acted as a coenzyme for azoreductase. The enzyme activity of azoreductase for *S. aureus* and *E. coli* was found to be 7.33 U/ml and 7.24U/ml respectively. The molecular weight of purified azoreductase determined by gel electrophoresis using a standard marker is 22kDa.

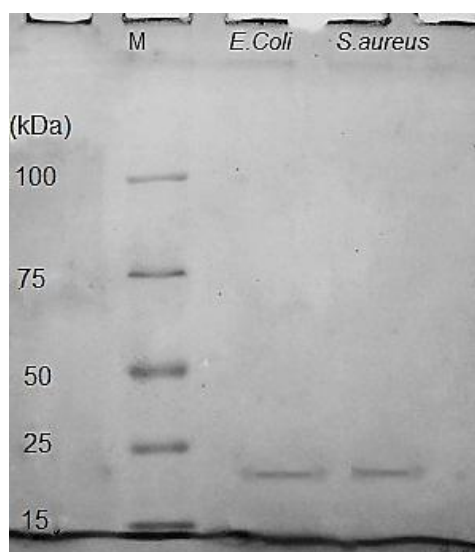


Fig 8. SDS-PAGE of purified Azoreductase by *E. coli* and *S. aureus*. It shows the molecular weight of purified azoreductase to be approximately 22 kDa.

III.7. ANALYSIS OF DEGRADATION PRODUCT BY TLC AND FTIR

The dye decolourisation was further confirmed by TLC analysis. The spot observed in the initial dye solution (Rf value of Acid blue 113 =0.76) was different from the spot obtained after decolourisation by *S. aureus* (Rf value; 0.65) and *E. coli* (Rf value; 0.68). This indicated that the decolourisation was due to its degradation into unidentified intermediates. FTIR spectral analysis of acid blue 113 before and after decolourisation by *S. aureus* and *E. coli* showed various peaks. Fig. 9a shows peaks in the control dye which represents N-H stretching of amine or O-H and aromatic =C-H stretching at 3440 cm^{-1} . The peaks at 1598-1566 cm^{-1} may be attributed to C=C bending, N=N stretching due to the azo bond was observed at peaks 1495 cm^{-1} and 1455 cm^{-1} . The peak at 1190 cm^{-1} and 1102 cm^{-1} indicates SO_2 symmetric and asymmetric stretching. Aromatic C-H out-of-plane bending vibrations at 816-627 cm^{-1} and in-plane bending vibrations at 1036 cm^{-1} were observed.

The spectrum of the dye degraded by *E. coli* (Fig. 9b) showed N-H amine stretch at 3382 cm^{-1} , stretching of C-H bond at 2196 cm^{-1} . The peak at 1642 cm^{-1} may be attributed to aryl carboxylic acid or quinone formed during decolourisation. N-H stretching of aliphatic amines showed the peak at 1110 cm^{-1} . The spectrum of dye degraded by *S. aureus*.

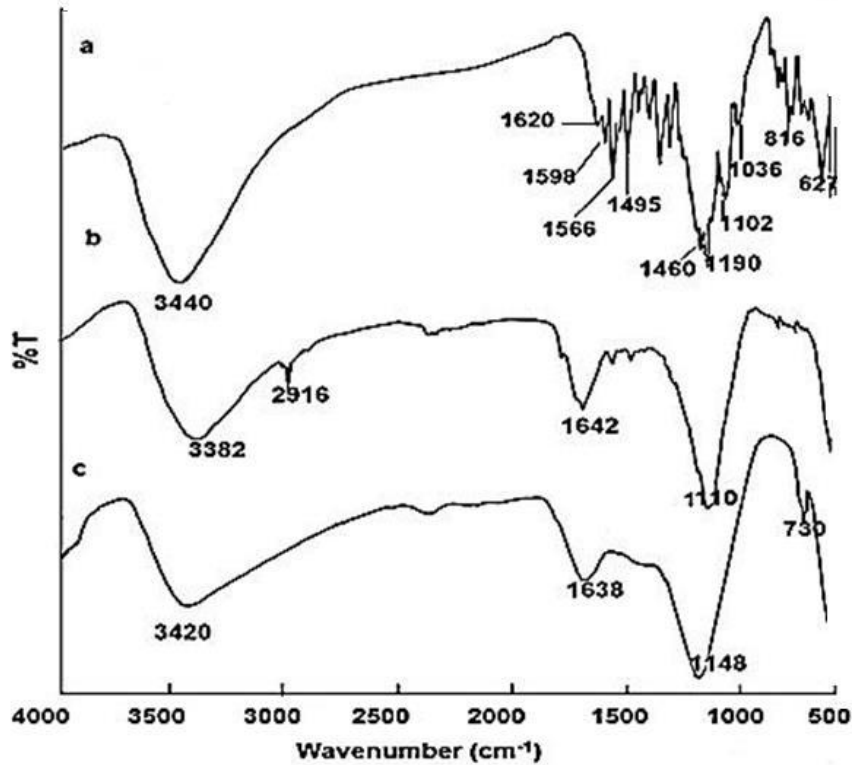


Fig 9. FTIR spectra of Acid blue before decolourisation and after decolourisation a: control, b: after degradation by *E. coli*, c: After degradation by *S. aureus*. The changes in peaks prove the degradation of the dye by the two micro organisms.

III.8. COD AND PHYTOTOXICITY

Initial and final COD was estimated in order to confirm the biodegradation of acid blue dye by *S. aureus* and *E. coli*. The percentage removal of COD by *S. aureus* and *E. coli* was 58.2% and 50.7% respectively at pH 7. The initial COD was 2045 mgL⁻¹. This showed the presence of organic matter in the decolourised medium, and there is no exact correlation between the percentage removal of colour and COD as reported by Mohana et al.2007; Yogesh et al.2012 [15, 30]. The phytotoxicity study shows seed germination rate as well as growth in Root length and Shoot length for the plant grown in the treated effluent after decolourisation as compared with initial dye effluent. Table 2 shows the growth of shoot and root length in the *Trigonella foenumgraecum* seeds grown in *S. aureus* and *E. coli* treated dye effluent after 4th day. Phytotoxic studies indicates reduced toxicity of treated effluent formed after biodegradation of acid blue by *S. aureus* and *E. coli* ensuring a safe disposal of effluent in the environment.

Table 2: Phytotoxicity studies of Acid blue and its by-products formed after biodegradation.

| Parameters Studied | Distilled water | Acid blue 113 | <i>S. aureus</i> | <i>E. coli</i> |
|------------------------|-----------------|---------------|------------------|----------------|
| | | | Metabolite | Metabolite |
| Germination (%) | 100 | 85 | 93 | 89 |
| Shoot length (cm ± SD) | 5.8± 0.56 | 2.1±0.043 | 5.1 ± 0.23 | 4.7± 0.54 |
| Root length (cm ± SD) | 4.3±0.34 | 1.8± 0.211 | 4.8± 0.13 | 4.1± 0.042 |

Values are mean of germinated seeds in duplicate. SD: Standard deviation

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IV. CONCLUSION

The present study, decolorization potential of isolated bacteria *S.aureus* and *E. coli* was studied with a focus on the process variables such as pH, temperature, initial dye concentration, carbon and nitrogen sources. The optimum condition for decolourisation was found to be at pH 7, 37°C, for 200 mgL⁻¹ dye concentration showing a maximum colour removal of 90% for *S. aureus* and 88% for *E. coli*. Further addition of fructose as carbon source and ammonium formate as nitrogen source showed a colour removal of 95% for *S.aureus*. In the case of *E. coli*, starch as carbon source and glycine as nitrogen source showed a colour removal of 94%. FTIR, TLC and SDS PAGE analysis confirmed that decolourisation was due to the enzyme azoreductase. *S.aureus* and *E. coli* has the capability to decolourise and detoxify the azo dye acid blue 113. The study has confirmed the potential application of bacterial cultures *S.aureus* and *E.coli* for the decolourisation of tannery effluent.

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