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RESEARCH ARTICLE

BIODECOLORIZATION OF ANTHRAQUINONE TEXTILE (ACID BLUE 25) DYE BY *KLEBSIELLA SP*

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ABSTRACT

Acid Blue 25 is an Anthraquinone based dye extensively used in textile industries**,** many bacterial isolates obtained from the textile effluents showed the decolorization activity, among all isolates, *Klebsiella sp.* showed maximum decolorization potential. In the present study different tests have been conducted to optimize the decolorization efficiency, such as enrichment of carbon & Nitrogen source with different concentrations, P^H, Temperature, size of inoculums etc., 70% to 90% of decolorization activity was recorded within 48 hrs of incubation at static conditions by *Klebsiella sp*. The degradation was confirmed by UV-Visible spectrum and TLC analysis. Microbial Toxicity study revealed the degradation of Acid blue 25 in to non- toxic products by *klebsiella sp.* High efficiency of dye decolorization is a key for the degradation of biological treatment of polluted effluents. Therefore *Klebsiella sp.* appears to be the promising organism for acid blue 25 dye degradation.

INTRODUCTION

Large number of chemicals including dyes was manufactured and used day to day life. Approximately 10,000 different dyes and pigments are used industrially and over 7 million tons of synthetic dyes are produced annually worldwide. The discharge of pollutants from various industries poses threat to the biodiversity of the earth. The textile finishing generates a large amount of dyes containing wastewater from dyeing industries and subsequently that forms one of the largest contributions to water pollution.

The removal of the dye amended pollutants is a difficult task, particularly for small scale textile industries before disposal they have to treat the discharged water from the industry. Microbial decolorization and degradation is an environmental friendly and cost competitive to chemical decomposition (Verma & Madamwar, 2003). Bioremediation depends on the pollution removal process with microorganisms has much attention as microbial decolorization (Kalyani *et al.*, 2008) The general approach of bioremediation is to improve natural degradation capacity of the native organisms.

Anthraquinone dyes are persistent due to their fused aromatic structures and thus remained colour for a longer time in waste water. Microbial degradation of Anthraquinone dyes produces aromatic amines which are highly carcinogenic and mutagenic, so microbial population has an amazing capacity to degrade various organic compounds. Several physicochemical techniques have been proposed for the treatment of colored effluents. These include adsorption on different materials, oxidation and precipitation by Fenton's reagent, bleaching with chloride or ozone, photo degradation or membrane filtration. The economic and safe removal of the polluting dyes is still an important issue.

Bioremediation is becoming important, because it is cost effective and environmentally friendly and produce less sludge. Anaerobic degradation of dyes also leads to the formation of highly carcinogenic and mutagenic. This study represents the isolation of aerobic bacteria from soil and identified the bacterial strain that were shown to be highly effective for decolorization of Anthraquinone dyes (Acid blue 25) and evaluate their performance under various conditions of carbon, nitrogen, temperature, pH etc.

Table 1 Identification of dye decolorizing bacteria from textile effluents

Test	Isolate
Gram's nature	-
Shape	Rod
Motility	Non-Motile
Methyl red	-
Indole production	-
Voges-Prausker	-
Catalase	+
Oxidase	-
Citrate utilization	-
Identity of the isolate	Klebsiella sp.

+ = Positive - = Negative

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MATERIALS AND METHODS

Sample Collection

Waste water effluents and soil samples contaminated with dyes of textile industry were collected from the discharges of Siera Silk Mills Ltd., Bangalore. The effluent samples were collected under aseptic conditions in sterilized screw cap bottles and soil samples collected by polythene bags.

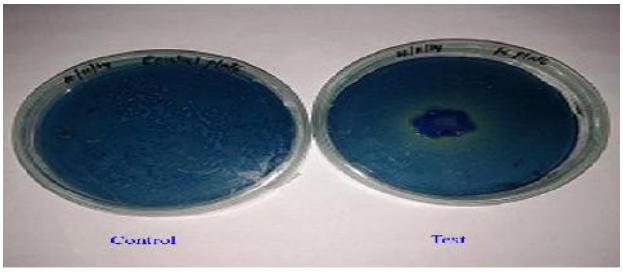


Fig 1 Decolorization activity of *klebsiella sp.* was observed by plate assay. Decolorization zone was observed.

Fig 2 Decolorization of *klebsiella sp.* was observed by broth assay.

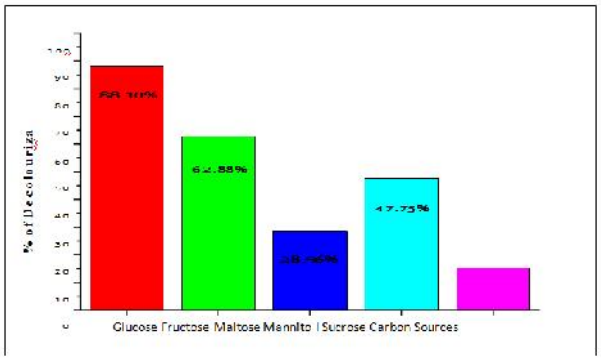
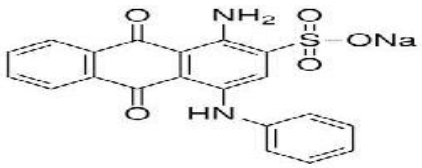


Fig 3 Effect of carbon sources on dye decolorization by *Klebsiella sp.*

Dye

The Acid blue 25 is an Anthraquinone based dye was used for the present study and it was supplied by sigma Aldrich chemicals limited (CHEMIE GmbH). Acid blue is a water soluble textile dye and widely used in several textile industries. Its maximum absorption was 600-630 nm with the following chemical structure



Chemical structure of Acid blue 25

Medium

The bacterial culture *klebsiella sp.* was grown at 37^{0}C in the basal culture medium, Zhou and Zimmermann medium (ZZ) containing the following in g/l, Glucose 0.5%,Yeast extract 0.5%, $(NH_4)_2SO_4$- 0.5g/l, KH_2PO_4 - 2.66g/l, Na_2HPO_4 - 4.32 g/l, dye - 100 ppm.

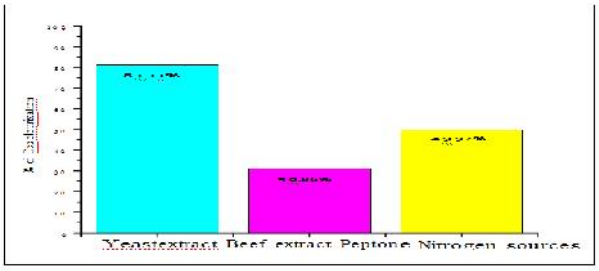


Fig 4 Effect of Nitrogen sources on dye decolorization by *Klebsiella sp.*

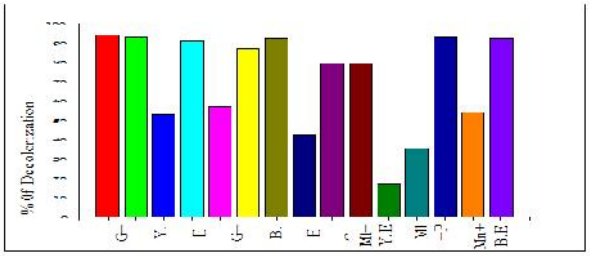


Fig 5 Effect of different combinations of Carbon and Nitrogen sources on dye decolourization

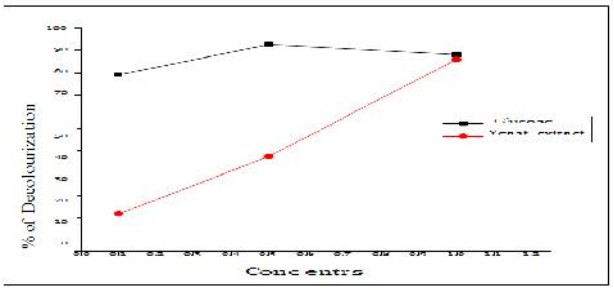


Fig 6 Effect of different concentrations of carbon and Nitrogen sources on dye decolourization

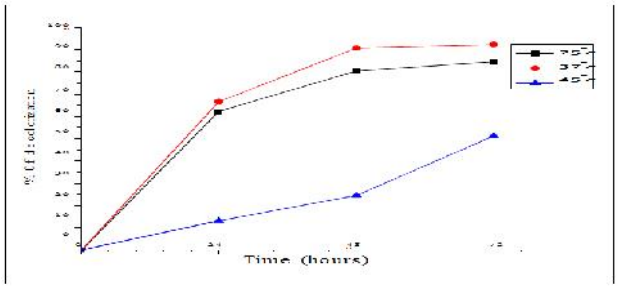


Fig 7 Effect of Temperature on decolorization of Acid blue 25 by *Klebsiella sp.*

Isolation of pure cultures and Screening of dye degrading bacteria

Textile effluents & contaminated soil were collected then inoculated on to the nutrient agar and incubated at 37^{o}C. The organisms isolated on nutrient agar was further sub cultured on modified ZZ medium containing 100ppm of acid blue 25 dye and incubated at 37^{o}C under static conditions. After six days of incubation the decolourization potential was calculated. The bacterial isolates utilizing the dyes as a source of nutrient, showing high efficiency of decolourization were selected for the study. The maximum decolorization potential of the selected bacterial culture showed with in 48 hrs of incubation.

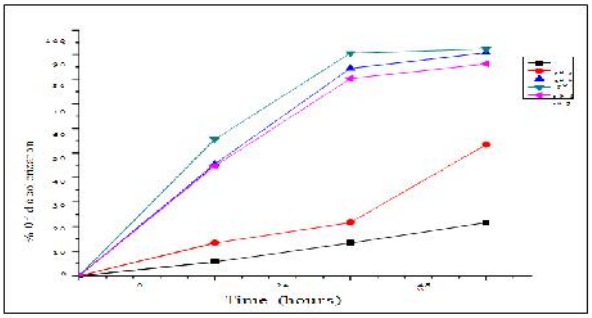


Fig 8 Effect of pH on decolourization of Acid blue 25 by *Klebsiella sp.*

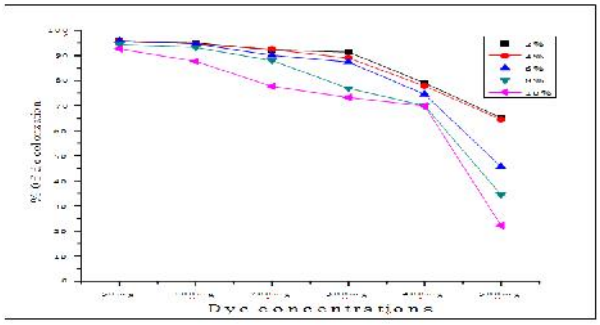


Fig 9 Effect of inoculums size at different dye concentrations by *Klebsiella sp.*

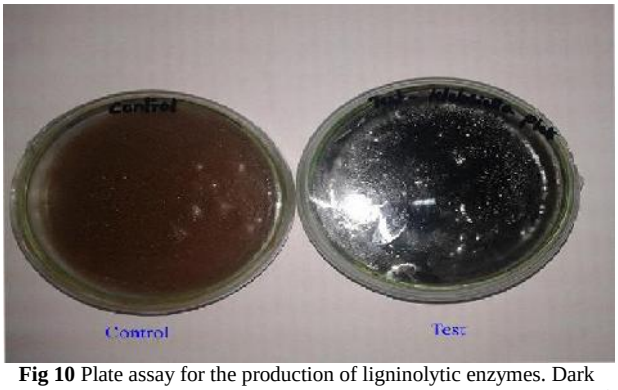


Fig 10 Plate assay for the production of ligninolytic enzymes. Dark brown color was observed in the Test plate and concluded that some of the enzymes are responsible in dye decolorization.

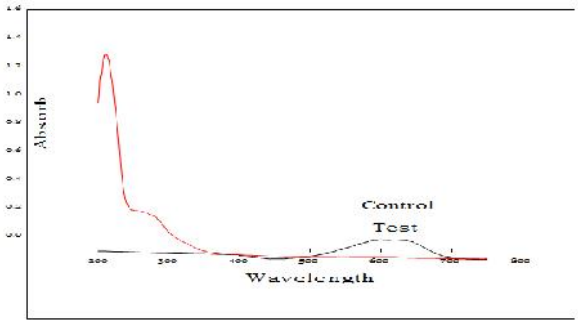


Fig 11 UV-visible scan of Acid Blue 25 decolorized by *Klebsiella* sp. In control dye peak was obtained at 600nm and in decolorized broth medium peak was disappeared.

Identification of dye degrading bacteria

The selected bacterial pure cultures were maintained on nutrient agar plates and also based on growth, morphology, microscopic observation, staining and biochemical tests, the isolates were identified by using Bergey's manual of Systematic bacteriology.

Detection of Decolorization activity

Both nutrient broths (Himedia) and slightly modified Zhou and Zimmerman medium containing 100 ppm of Acid blue 25 dye in the medium used for the detection of decolorization activity by plating and broth dilution method respectively:

Fig 12 Thin Layer Chromatography analysis of Acid blue 25 by klebsiella sp.. C, in control spot was not observed and T, test or treated sample, spot was observed and it was confirmed degradative products were present.

Plate method

The Nutrient agar and ZZ agar plates were prepared under aseptic conditions with required amount of dye content and bacterial culture was added at the centre of the plates then incubated at 37˚c for decolourization activity.

Broth method

100 ml of each Nutrient broth and ZZ media (broth) was used for the present study. And 100 ppm of dye content was added with 1% (v/v) of culture inoculated into the broth. The flasks are kept for incubation at 37^{o}C under static conditions and without inoculums served as control.

Dye Decolorization

Decolorization experiment was conducted using 250ml conical flask containing 100 ml of ZZ medium with 100 ppm of dye and 1 ml of culture and incubated at 37˚C. After 2 days of incubation decolorized medium was centrifuged at 10000 rpm for 20 minutes to separate the cell lysate and supernatant.

Decolourization potential was measured by absorbance at 600 nm using UV-Vis spectrophotometer. Percentage of decolourization calculated by the following formula

$$\text{Decolourization (\%)} = \frac{\text{Initial absorbance} - \text{observed absorbance}}{\text{Initial absorbance}} \times 100$$

Bio adsorption assay

After the decolourization the medium subjected to centrifugation and the pellet was suspended in distilled water. Then the suspension was vortexes and filtered by using What Mann number filter paper and the supernatant was measured at 600 nm for the bio adsorption study. The decolourization efficiency due to bio adsorption was expressed by the following Equation.

Decolourization efficiency by bio adsorption (%) = L/I × 100

Where I = initial absorbance and L = absorbance of filtrate (liquid phase)

Optimization of dye decolourization by Nutrients

Effect of carbon sources on dye decollourization

Optimization of the decolourization activity conducted by using various carbon sources i.e. Glucose, Sucrose, Fructose, Maltose and Mannito l of 1% with 1 % (v/v) of inoculums. The decolourization of Acid blue 25 in modified ZZ medium and without inoculums was served as control. All the flasks kept

for incubation at 37˚c under static conditions for six days then the results were recorded**.**

Effect of different nitrogen sources on dye decolourization

Optimization of decolorization activity by different Nitrogen sources of 1 % (w/v) Yeast extract, Beef extract, Peptone with 1 % (w/v) of inoculum for the decolourization of Acid blue 25 in modified ZZ medium and without inoculum was served as control. All the flasks incubated at 37˚ c under static conditions for six days then the results were recorded**.**

Effect of different carbon & Nitrogen sources as co-substrates on dye decolourization

Different combinations of carbon & nitrogen sources have been incorporated individually to ZZ medium for about 0.5%, this test is a key for both carbon and nitrogen sources acts as co-substrates which influences the decolourization activity**.**

Effect of different concentrations of carbon source on dye decolourization

Decolourization activity may also measured by using different concentrations of carbon sources i.e. 0.1%, 0.5%, & 1 % (w/v) with 1% inoculum for the decolourization of Acid blue 25 in modified ZZ medium and without inoculum was served as control. All the flasks incubated at 37˚C under static conditions for six days then the results were recorded**.**

Effect of different concentrations of Nitrogen source on dye decolourization

Decolourization activity may also measured by using different concentrations of nitrogen sources i.e. 0.1%, 0.5%, & 1 % (w/v) with 1% inoculums for the decolourization of Acid blue 25 in modified ZZ medium and without inoculums was served as control. All the flasks incubated at 37˚C under static conditions for six days then the results were recorded**.**

Optimization of dye decolourization

Temperature

Optimization studies were conducted by different Temperatures ranged from 25˚C, 37˚C, & 45˚C with 1% inoculums, the known concentration of dye Acid blue 25 incorporated into the modified ZZ medium for decolourization and without inoculums was served as control. All the flasks were incubated at 37˚C under static conditions for six days and the results recorded**.**

***P*^{H}:** For the optimizations different pH ranges i.e. 5, 6,7,8,9 and 1% of bacterial culture, known concentrations of dye (Acid blue 25) into the modified ZZ medium for decolourization and without inoculum as control. After the incubation at 37˚ c under static conditions for six days results were noted and recorded**.**

Dye concentrations

Optimizations studies carried out by using different concentrations of dye i.e. 50 ppm to 500 ppm with 2% inoculum on dye decolourization of Acid blue 25 in modified ZZ medium, without inoculum was served as control. The flasks kept for incubation at 37˚C under static conditions for six days. Further the results of decolourization activity were recorded**.**

Inoculums size

Optimizations decolorization activity was carried out by using different concentrations of inoculums i.e. 2%,4%,6%,8%,10% for the decolourization of Acid blue 25 in modified ZZ medium containing 100 ppm dye and without inoculum was the control. All the flasks were incubated at 37˚C under static conditions for six days and results were recorded**.**

Microbial toxicity test

Microbial toxicity test conducted in order to measure the impact of dye toxicity after decolourization of *klebsiella sp.* by acid blue 25. This test widely used for agricultural fields to check out the antagonistic activity. For the microbial toxicity test, Bacillus cereus was used as a test organism. Bacillus cereus was inoculated and spreaded on ZZ Agar plate. Wells were prepared on agar plate, to this decolorized broth medium was poured in the well and incubated the plate at 37˚C for 2 days. Zone of inhibition around the well shows the toxicity effect of the dye and no zone of inhibition around the well shows non toxic effect.

Factors determining the Enzyme analysis

Decolorization is mainly a biological process; in decolorization activity some enzymes were present. To study the enzymes involved in decolorization, a plate screening method was used.0.1ml decolorized broth is poured in sterilized petriplate and then molten agar medium with separately sterile 0.5% tannic acid is added and agar is allowed to solidify and plate is incubated at 37ºC for 4 days.

Biodegradation analysis of *klebsiella sps* by UV spectrum

Decolorized medium was analyzed by spectrophotometer and further the decolorization potential was calculated**.**

Thin layer chromatography

After complete degradation the medium subjected to centrifuge at 10,000rpm for 20 minutes and supernatant obtained was used to extract metabolites with equal volume of ethyl acetate and extracts were dried over anhydrous sodium sulphate and evaporated for dryness by rotary evaporator. The residue dissolved in small amount of methanol analyzed by TLC by using mobile solvent -ethyl acetate: hexane (2:3v/v).The observations were recorded**.**

RESULTS

Bacteria isolated from the dye amended soils collected from the dye amended sites with waste water contamination from the dyeing industrial effluents. A bacterial strain *klebsiella sp.*with high decolourization activity against Acid blue 25 was isolated by using nutrient broth and ZZ medium with glucose and yeast extract as co substrates. Bacterial isolate was identified by biochemical tests and was shown in Table 1and identified as *klebsiella sp.* (M.Ponraj *et al*., 2011). Among all the isolates, more frequently encountered organism was identified as *Klebsiella sp.* Further pure culture of *Klebsiella sp.* was however used to detect the decolourization activity (Fig-1) a clear zone observed around the site of inoculum on agar plate.

In broth medium, the pure culture of *klebsiella sp.* showed maximum decolourization activity with initial dye concentration (100 ppm) within 48 hrs under static condition. The percentage of decolorization was more than 92% (Fig-2).

Optimizations of different parameters were carried out in ZZ medium for the decolourization potential. Decolorization potential of *klebsiella sp.* was enhanced by supplementing different carbon sources (1% of Glucose, Sucrose, Fructose, Maltose, Mannitol) in modified Zimmermann's medium. Glucose supplement showed more than 85 % decolourization of Acid Blue 25 (100 mg/l) by *Klebsiella sp.* within 48 hrs under static conditions. No decolourization activity by sucrose, maltose and mannitol. But in the presence of fructose 62.8 % of decolorization activity under static conditions was recorded. The range of decolourization activity of Acid blue 25 with Mannitol was about 47.7%, Sucrose 5.4%, Maltose 28.5% under partial static conditions. Glucose alone showed 85 % decolourization and was shown in (Fig-3).with the combination of Glucose and nitrogen sources more than 92% of decolourization was observed (Fig-5). From these findings *klebsiella sp.* was found to be most effective and efficient decolorizable among all isolates.

The Nitrogen sources such as 1% each of Beef extract, Peptone and Yeast extract were used for decolourization and showed that yeast extract 85%, peptone 49.9% and beef extract 45.4% respectively (Fig-4). Yeast extract supplement showed maximum decolourization of Acid blue 25 by klebsiella sp.under static conditions.

Decolourization was studied by different combinations of carbon and nitrogen sources as co-substrates into the dye enriched medium by adding 0.5% carbon source and 0.5% nitrogen source. The results was recorded that Glucose and Yeast extract showed decolourization activity was 94%, Glucose & Beef extract showed 92%, Glucose & Peptone showed 53%. Sucrose & Yeast extract showed decolourization activity was 93%, Sucrose & Beef Extract was 57% and Sucrose & Peptone was 87%. Fructose & Yeast extract showed 92%, Fructose & Beef Extract showed 42%, Fructose & Peptone 79%. Maltose & Yeast extract (79%), Maltose & Beef Extract (17%), Maltose & Peptone (85%). Mannitol & yeast extract (91%) Mannitol & Beef extract (54%), Mannitol & peptone (92%).The results showed that in combination of Glucose and Yeast extract had showed high decolourization potential activity compared to other co-substrates. Sucrose and Yeast extract, Fructose and Yeast extract Mannitol and Yeast extract, Glucose and Beef extract and Mannitol and Peptone also acts as good co-substrates for decolorization of Acid blue 25 dye by *Klebsiella sp.* (Fig-5).

Different concentrations of glucose (0.1%, 0.5% & 1 %,) used to analyse the decolourization potentiality of *Klebsiella sp.*, carbon source alone supplemented in to the medium 90 % decolourization efficiency was observed and also high decolorization potential observed in 0.5 % glucose rather 1% & 0.1% and shown in (Fig-6).
Decolourization efficiency of *Klebsiella sps* was observed with different concentrations of Yeast extract (0.1%, 0.5% & 1 %,) as Nitrogen source supplemented in to the medium the efficiency of decolourization on dye Acid blue 25. More than 80 % decolourization was observed in 1 % yeast extract compare to 0.5% & 0.1 % (Fig-6).

Temperature is one of the most important parameter in Biodecolorization. It plays important role in microbial growth and enzyme activity. The dye decolourization activity of culture was found to increase with increase in incubation temperature at 25 to 45˚ C and maximum activity was observed at 37˚C and decrease in decolourization was observed with increase in temperature (Fig-7).

The results of the effect of pH on dye decolourization was found in increase of pH at 5 to 9 and maximum decolourization was observed at pH 8 and decrease in decolourization was observed pH 5.High efficacy of decolourization was observed at 7 to 8 pH within 48 hrs of incubation (Fig-8).

Decolourization activity of bacterial culture of *Klebsiella sp.* was studied using Acid blue 25 at different concentrations varying from 50 mg to 500 mg. Rate of decolourization increased with increase in initial dye concentration 200mg (92 % decolourization) . If further dye concentration increases resulted decrease in decolourization activity. (Fig-9)

The percentage of decolourization increased as inoculum size was increased. Maximum rate of decolourization (400mg/l) was observed at a 4 % inoculum concentration. Maximum decolourization was in the range of 85 to 95 % (Fig-9).

In microbial toxicity test, No zone of inhibition observed surrounding the wells containing decolorized medium, indicating that the biodegradation or decolourization product was non- toxic to the soil bacterium.

It should be noted that decolourization process might occur by bioadsorption or/and biodegradation. Thus, it was interesting to determine the percentage of colour removal that contributed by bioadsorption and biodegradation. Results indicated that the decolourization due to bioadsorption and biodegradation were 20.46 % and 92.04 %, respectively. Here clearly observed that decolourization was by degradation of *klebsiella sps.*

In plate screening method the results showed (Fig-10) that the plate was completely changed with dark brown colour producing ligninolytic enzymes responsible for dye degradation (Bhoosreddy *et al*.,2014)

Inoculation of *Klebsiella sps* to media containing Anthraquinone dyes resulted in the decolourization of the dye Acid blue 25. The Biodecolorization was confirmed by UV-VIS spectrum corresponding to control and sample of decolourization experiments. The absorbance analyzed from 200 nm to 800 nm. The control sample showed the highest peak at the wave length of 600 nm and the decolorized sample showed lowest peak to a minimal absorbance. This result (Fig-11) indicates that decolourization is due to dye degradation (Hui Wang *et al*., 2009).

TLC analysis (Ganesh Parshetti *et al*., 2006) of degraded product showed RF value of 0.55 conforming the biodegradation of dye.The results suggested that *klebsiella sps* was able to degrade Acid blue 25 dye and most of the metabolite products are involved in the dye degradation (Fig-12).

DISCUSSION

Bacteria are frequently found everywhere and can be collected from water,waste water and soil. The textile industries releases the effluents and causes serious pollution. In this study, isolation of bacteria was carried out from dye contaminated waste water of textile industry by using Acid blue 25 as the sole source of carbon and nitrogen. The results showed that the bacterial strain isolated through enrichment of liquid media were capable of decolorizing Acid blue 25 dye. *Klebsiella sp.*

completely decolorized anthraquinone dye (Acid blue 25) under static condition with in 48 hours.Similar reports (Deepti Gulati *et al*., 2014; and Abdulraheema Giwa *et al*., 2012) decolorized anthraquinone dye (Reactive blue 19) by klebsiella sp., enterococcus sp. and Bacillus cereus within 72 hrs. Carbon sources such as Glucose, Sucrose, Fructose, Maltose and Mannitol were used and the decolorization activity was high in Glucose. Nitrogen sources like Yeast extract, Beef Extract and Peptone were used and the decolorizationwas high in Yeast extract (M.Ponraj *et al*., 2011) reported a Bacillus sp. decolorized by 87% Orange-3R dye of sucrose and Beef extract as carbon and Nitrogen sources.

Combinations of different co-subtrates Glucose and yeast extract,Sucrose and yeast extract showed maximum decolorization activity.Similar work was reported (Gurulakshmi *et al*.,2008 and Madhuri M.sahasrabudhe *et al*.,2011).(Maulin P Shah *et al*.,2014) describe the use of Bacillus spp.ETL-1979 for sequential decolorization and detoxication.Optimizations of temperatures by *klebsiella sp*.showed decolorization at 37^0C , no decolorization was observed at 47^0C.similar report was observed (Joshi *et al*.,2013 and Praveen Kumar *et al*., 2012) at Bacillus megaterium and Bacillus cereus.In the present study, results was observed at pH 7-8 *klebsiella sp*. showed maximum decolorization compared to acidic pH. Similar results (MP.Shah *et al*.,2013) reported in pseudomonas stutzeri ETL-79 at pH 7.

The decolorized products were tested for their toxic effect on the agriculture importances soil bacterium Bacillus cereus.It was reported by (Usman Aftab *et al*., 2011).(Mohandass Ramya *et al*.,2007) reported at pH 3 decolorization was effective and few chemically different dyes were decolorize moderately by Aspergillus sp..In another study (Sonia seth *et al*.,2012,Hui wang *et al*.,2008, Poonam Dayaram *et al*.2008, Ganesh Parshetti *et al*.,2006, Olaganathan Rajee *et al*.,2012 and Singh RP *et al*., 2014) reported that the synthetic and reactive dyes showed decolorization of textile effluents of dye contaminated soil.

In plate screening method, (Bhoosreddy *et al*., 2014) production of ligninolytic enzymes was responsible for the degradation of Acid blue 25 dye. The enzymes involved were lignin peroxidase, Manganese peroxidase, Laccase were involved in the degradation process.

CONCLUSION

The nutritional and environmental factors needs to be optimized to enhanced the decolourization efficiency of strain. The observed results showed that in presence of different nitrogen sources and carbon sources decolourization efficiency was enhanced. The results obtained have characterized and identified dye degrading efficacy *of klebsiella sps*. The ability of the strain to decolorize and degrade the Anthraquinone dyes at high concentrations has advantage to the treatment of textile effluents.

Acknowledgments

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