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ECOFRIENDLY TREATMENT OF REACTIVE YELLOW 15 : BIODEGRADATION USING *BACILLUS LICHENIFORMIS*

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ABSTRACT

The dye reactive yellow 15was degraded by *Bacillus licheniformis* bacterium optimally at pH 9, temperature at 37°C, 50 mg/l of dye concentration at 20% inoculums size. Glucose, NH_4NO_3 were found to be the best carbon and nitrogen sources. The extracellular enzyme from *Bacillus licheniformis* was studied for dye decolourization potential. Biodegradation was confirmed by analyzing the product using thin layer chromatography (TLC) and gas chromotograpy - mass spectrometry (GC-MS). GC-MS analysis revealed the formation of uric acid, 2-Methyl sulfonyl-5-dimethylaminomethyleneamino pyrimidine, diethyl phthalate and phenol as final metabolites formed by *Bacillus licheniformis*. Phytotoxicity test was accomplished in pearl millet to assess the toxicity of the untreated and microbially treated textile dye effluents. These results indicated that the high potential of *Bacillus licheniformis* to serve as an excellent biomass for the use in dye removal.

Key words: Reactive yellow 15, *Bacillus licheniformis*, lignin peroxidase, Biodegradation and Gas chromotograpy- Mass spectrometry- Phytotoxicity.

Introduction

Industrialization is the main constraint for the economic growth of a nation and modernization of life. Thus, different large and medium scale industries play a role in nation's economic development both in developed and developing countries. The textile industry takes the biggest share due to its capacity to create a variety of job opportunities and its incorporation with different economic divisions both globally and locally. It is the most expanding and evolving sector. Cotton, woolen, synthetic fibers, synthetic dyes, chemical, and process water are amongst the major attribute raw materials in this industry and all these inputs spread the economic importance of the textile industry. Based on fibers production, the textile industries can be categorized into two major processes such as dry and wet fabric processes. In dry processing, solid wastes are mainly generated, while liquid wastes are mainly generated in wet processing steps (Teshale adane *et al*., 2021).

As per environmental laws, it is the responsibility of the textile industries to remove the wastes before discharge into water in the same industry (Ahluwalia Sunita Malhotra, 2009). Along with the reductive enzymes, some investigators have demonstrated the oxidative enzymes such as lignin peroxidase, laccase and tyrosinase, in the decolourization and degradation of azo dyes (Bhatia, 2008). Extracellular enzymes break down the large, complex molecules and enable their entry into the cell. The genes of certain enzymes have inducible operons while others are constitutive (Rajendran and Gunasekaran, 2006). Globally, the Environmental Protection Agency has cited global warming, ozone depletion, and increasing species extinction are the greatest environmental threats to future generations (Michael McKinney *et al.,* 2007). Biological treatment of textile effluents may be either aerobic, anaerobic or a combination of both, depending on the type of microbe being employed (Rajendran and Gunasekaran, 2006). microbial metheds have received attention outstanding to its ease of application, low cost and envirnmental benigity (Shailesh Dave and Riddhi Dave, 2012).

Materials and Methods

Site Description and Sample Collection

The effluent and soil sample was collected from Infra Tex textile industry in Perundurai (lat: 11°13'18.6"N long: 77°39'18.5"E) Erode district for decolourization studies.

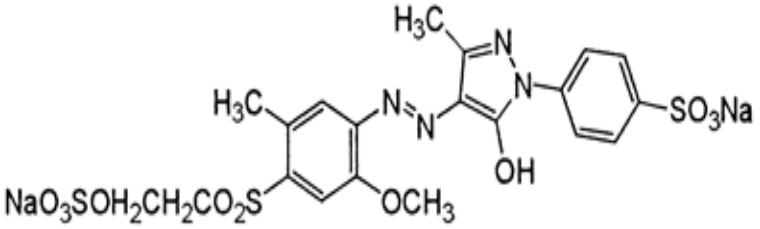
Dye and Chemicals

Reactive yellow 15 was obtained from Infra Tex textile industry. Chemicals used in this study were also of analytical grade.

Reactive Yellow 15

Reactive Yellow 15 is also known as Reactive Yellow GR, Reactive yellow KN-GR. It comes with the molecular structure of single azo class with a molecular formula of $C_{20}H_{20}N_4Na_2O_{11}S_3$ and molecular weight of 634.34 g/Mol.

Chemical structure of reactive yellow 15



Isolation and Screening of Bacteria

Bacteria were isolated from the soil sample and effluent by preparing aliquot (10 ml) dilutions. Nine millilitre of sterile water was taken in test tubes and labelled as 10^{-1} to 10^{-6}, and one ml of effluent sample was transferred into each tube using a sterile pipette. Then 1ml was taken from both soil and effluent aliquots and plated on dye fermentation agar medium containing reactive yellow 15 and incubated at 37ºC for 72 hrs. Totally fifty isolates (bacteria) were isolated and further tested in agar plates with uniform cell densities. The cultures capable of growth in this medium were isolated and purified by sub-culturing on dye fermentation agar medium (Ola *et al.,* 2010). Based on the preliminary screen results, one isolates were taken for further studies.

Identification of Bacteria

Morphological, Gram staining, motility and endospore staining were performed. Biochemical tests were performed for identification. Biochemical tests like IMVIC, catalase, oxidase, gelatin liquefaction test, nitrate reduction test, triple sugar iron test, H_2S production and urease test were performed (Paul *et al*., 2009).

Enzyme Activity Assay

The numerous isolated strains tested, one strain showed good probable and selected for further work. *Bacillus* species was selected and grown in nutrient broth at 30°C for 24 hours and centrifuged at 6000 rpm for 20 minutes. These cells were suspended in 50 $mmol^{-1}$ potassium phosphate buffer (pH 7.4) and temperature at 4° C. This extract was used as an enzyme source without centrifugation (Dawker, 2008). The supernatent fraction was filtered through a sterile syringe filter (Millipore) and filtrate was selected as the extracellular fraction for the assay of extracellular enzymatic activity.

Oxidative enzymes

Lignin Peroxidase enzyme Activity Assay

Lignin peroxidase activity has been performed at 40°C using $CaCO_3$ as a substrate. The assay mixture contains 0.1M citrate/phosphate buffer pH 4.0. The oxidation of $CaCO_3$ led to an absorbance increase at 420 nano meters (Dawker *et al*., 2008). Lignin peroxidase was determined by monitoring the formation of propanaldehyde. 2, 4-dinitrophenylhydrazine reagent and Schiff reagent were used as a chemical reagents for propanaldehyde estimation.

2, 4 - Dinitrophenyl Hydrazine Test

Cell supernatant was collected into reaction tube and 2, 4-dinitrophenyl hydrazine (20 drops) was added. Then precipitate formation was noted, if the precipitation was not occurring, the next step was carried out. The reaction mixture was rinsed, it indicates the removal of acid and recrystallized the product using ethanol (5 ml) and it was allowed to dry. Then to heat for a few minutes and again the reagent 2, 4-dinitrophenyl hydrazine was added. Finally colour change was noted.

Schiff Test

Cell supernatent was separated and Schiff reagent were added and the colour change was noted after two minutes (Bansal Raj, 2009).

Decolourization Studies

Decolourization studies were followed in static condition by bacterial isolate. Biodegradation of pollutant is highly affected by various environmental factors in any ecosystems. Such as pH, temperature, dye concentrations and inoculum size, etc. (Fulekar, 2010). Various carbon sources, nitrogen sources, and combination of Carbon / Nitrogen sources at different pH were followed. The reactive azo dye was selected for decolourization purpose that is reactive yellow 15. Carbon source such as sucrose, glucose, lactose, maltose, and nitrogen source such as ammonium sulphate, ammonium nitrate, effect of pH at 7, 8 and 9, temperature at 28°C, 37°C and 45°C and, the effect of inoculums concentrations of 5%, 10%, 15% and 20% and effect of dye concentrations of 50, 100, 150 and 200 mg / l were carried out for decolourization studies. It was taken into a conical flask and microbial inoculums was added to each and incubated for 11 days, by using mineral salt medium (, KH_2PO_4 - 0.2 g, K_2HPO_4 - 1.6 g $(NH_4)SO_4$ - 1.0 g, , $FeSO_4.7H_2O$ - 0.01 g, $MgSO_4.7H_2O$ - 0.2 g, NaCl - 0.1, $COCl_2.2H_2O$ - 0.002 g, glucose - 3 g, yeast extract - 1.0 g, pH .7) (Moosvi *et al.*, 2007).

Dye removal was calculated according to the equation (Olukanni *et al*., 2009).

$$\text{Decolourization (\%)} = \frac{A_o - A_t}{A_o} \times 100$$

Where:

A_o =Absorbance of the dye solution

A_t = Absorbance of the treated dyes solution at specific time, t.

Identification of Metabolic Intermediates

Thin Layer Chromatography (TLC)

Thin layer chromatography (TLC) is useful for qualitative analysis screening and can also be used for quantitative analysis (Christian, 2004). After complete decolourization by bacterial isolate at 7 days of incubation period, the decolourization medium was centrifuged at 7500 rpm for 4 minutes and the supernatant extracted with ethyl acetate (Asad *et al*., 2007).

The extract and the aqueous phases were evaporated in a rotary evaporator. The concentrated extracts were dissolved in 1ml methanol and used for thin layer chromatography

(TLC). A sample was spotted with a micropipette on the plate, and the chromatogram was developed by placing the bottom of the plate in a solvent. The mobile phase is methanol: hexane (3:1). The compounds were observed under UV light and spotted. Then it was scrapped and mixed with methanol, for further GC - MS analysis.

Gas Chromatography - Mass Spectrometry (GC - MS) Analysis

Biodegradation analysis via GC-MS is one of the most objectives of the present research work to study the metabolites formed as a result of these reactive dye with the selected bacterial isolate. For this purpose GC-MS analysis was performed.

In this study, 100 ml of decolourized samples was taken and centrifuged at 10,000 rpm. Extraction of metabolites was carried from the supernatant using equal volumes of ethyl acetate. The Rotary evaporated sample was dissolved in methanol and GC-MS analyses of metabolites were carried out (Dawker *et al.,* 2008) using a Thermo DSQ II Mass spectrometer fitted with GC Trace Ultra Version 5.0 at temperature programming mode with DB 35-MS capillary non polar column. The initial column temperature was at 50^{o}C then increased linearly to 260^{o}C at 10^{o}C/min. The carrier gas helium was used with a flow rate of 1.0 ml min^{-1}. Identification of degradative products was made by comparison of retention time and fragmentation pattern with known reference compounds as well as mass spectra in the NIST spectral library.

Molecular characterization of bacteria

16S rRNA sequencing of possible bacterial isolate was carried out at Sri Ramachandra Medical University, Porur, Chennai, as recommended by Farrell Robert (2005). Amplification of 16S rRNA fragment was performed using 16SF (AGAGTTTGATCMTGGCTCAG) and 16SR (TACGGYTACCTTGTTACGACTT). The thermo cycler programme included an initial denaturation of 96°C for 10 seconds and at 94°C for 30 seconds, while it was annealed at 50°C for 5 seconds and extended at 60°C for 4 minutes, these steps cycled a total of 30, while the program was finally extended at 72°C for 10 minutes. The purified products were sequenced by Run 3730, Applied Biosystem 3.0 version.

Phylogenetic Analysis

Phylogeny is the name given to a systematic ordering of the species into larger grouping based on inheritable genetic traits. All cells must have rRNA to complete translation, and the structure of the rRNA is remarkably consistent for all types of cells. Therefore, the rRNA has proven an excellent molecule for doing phylogeny (Bruce Rittmann and Perry McCarty, 2001).

The completed 16S rRNA sequence was aligned and their closest relative sequence was determined using BLAST search and multiple sequence alignment with CLUSTAL X. The method of Jukes and Cantor (1969) was used to calculate evolutionary distances. Phylogenetic dendogram was constructed by the neighbour-joining method and tree topologies were evaluated by performing bootstrap analysis of 1000 data sets using MEGA 4 (Molecular Evolutionary Genetic Analysis).

Phytotoxicity Test

It is very important to know whether biodegradation of a dye leads to detoxification of the dye or not. This can be done by performing a phytotoxicity test of the original dye and its

biodegradative products. Phytotoxicity test was performed in pearl millet to measure the toxicity of the untreated and microbially treated textile dye effluents. Pearl millets are belonging to,

Family - Poaceae
Order - Poales
Genus - *Pennisetum*
Species - *glaucum*.

Millets are a group of highly variable small - seeded grasses widely grown around the world as cereal crops in the tropics of Asia and (especially in India, Nigeria and Niger) with 97% of millet production in developing countries. This crop is favoured due to its productivity and short growing under dry, high temperature conditions. The most widely grown millet is pearl millet which is an important crop in India.

Surface soil was collected from agricultural fields and thirty plastic bags were filled with soil for three divisions of experimental work. Tap water was added to the soil. Then the seeds of pearl millets were sown. One to two seeds were sown in each bag at a depth of 5mm - 10 mm and after sowing water were irrigated daily. The effect of microbially treated textile dyes and also untreated textile dyes at the dye concentration of 300 ppm were used to study on the growth of millet. Seeds germinated in water irrigated bags were used as control. All bags were kept at room temperature for 9 days. Seeds were considered germinated when the radical and hypocotyls appeared. The factors like germination, shoot length, root length (cm) early seedling of the millet crop were evaluated. Germination percentage, root, shoots lengths of seedling was measured after 3rd, 6th and 9th day of experiment (Barakat *et al.*, 2009).

Results

Isolation and Screening of Bacterial Isolate

The bacterial strain was selected based on formation of a clear zone on nutrient agar plates. Among the potential seven bacterial strains, one strain was selected based on its ability to form a high dye decolourization zone on agar plates.

Enzyme Activity

Estimation of Lignin Peroxidase Enzyme

The lignin peroxidase enzyme was observed in bacterial isolate, qualitatively during propanaldehyde estimation. The Schiff test and 2, 4-Dinitrophenylhydrazine test gave qualitative information of enzyme activity.

Schiff Test

Formation of magenta colour in the reaction it indicates presence of propanaldehyde.

2, 4-Dinitrophenylhydrazine Test

Brown colour precipitate was formed during the reaction. It indicates presence of Propanaldehyde. So these two methods denote the presence of lignin peroxidase enzyme.

Quantification of Lignin Peroxidase Enzyme

Bacillus strain grown on solid media with and without substrate excreted extracellular lignin peroxidase enzyme. The culture was tested for the lignin peroxidase enzyme production at

3rd, 5th, and 7th day of incubation. There was no enzyme production up to 6 days, after the 6th day, it showed enzyme production to a certain level (Sudha and Balagurunathan, 2013).

Biodegradation Analysis

Thin Layer Chromatography (TLC)

The comparison of TLC chromatogram of the decolourized sample of *Bacillus* Sp. UV light showed that the decolourized sample of reactive yellow 15 had additional bands, which might originate from the dye by-products. In reactive dye Rf value was noted as 0.9 by *Bacillus licheniformis* for reactive yellow 15.

Biodegradation of reactive yellow 15 by *Bacillus* species

The untreated textile dye reactive yellow 15 showed ten peaks in its chromatogram. The compounds analyzed for these peaks were found to be toxic products present in the untreated raw dye sample (**Figure 1**). Biodegradation analysis showed a major reduction in the entire organic compound and the peaks that were observed was reduced five to six to a significant extent for reactive yellow 15 (**Figure 2)**.

Based on our proposed pathway, the peroxidase catalyzed initially the asymmetric cleavage, resulted in the intermediate product which was identified as uric acid with retention time 27.29 minutes and a mass peak of 168.1, (**Figure 3**) 2-Methyl sulfonyl-5-dimethylaminomethyleneamino pyrimidine with retention time 27.56 minutes and a mass peak 228.2, (**Figure 4**) supports the oxidative asymmetric cleavage of reactive yellow 15. The reduction of uric acid giving rise to the intermediate product in this reaction as diethyl phthalate at retention time 21.23 minutes and a mass peak 222.2 (**Figure 5**). Diethyl phthalate giving rise the intermediate phenol at retention time 16.64 minutes and a mass peak 206 (**Figure 6**) in this pathway *by Bacillus* species. The pathway is proposed in degradation of reactive yellow 15, **Figure 7** showing various steps involved in reactive yellow 15 dye degradation.

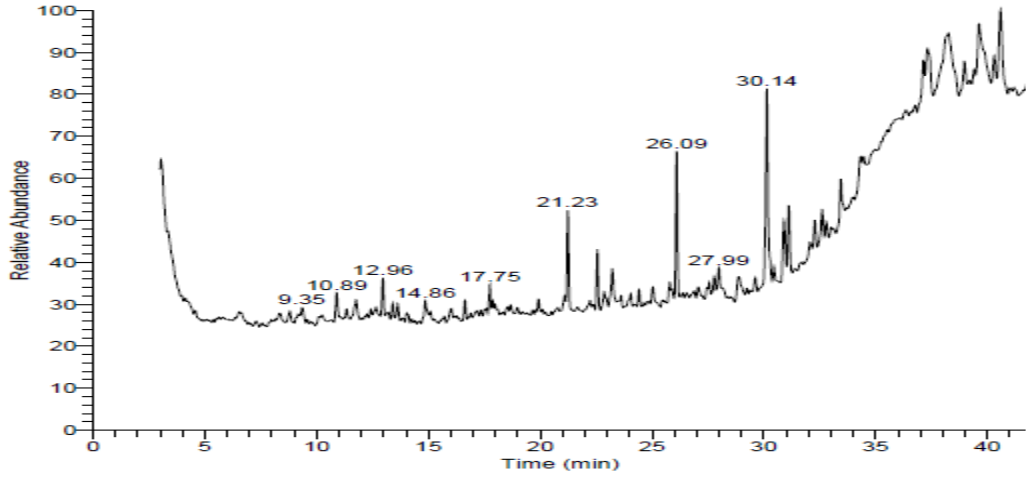


Figure 1. Gas chromatogram of untreated Reactive Yellow 15 dye sample.

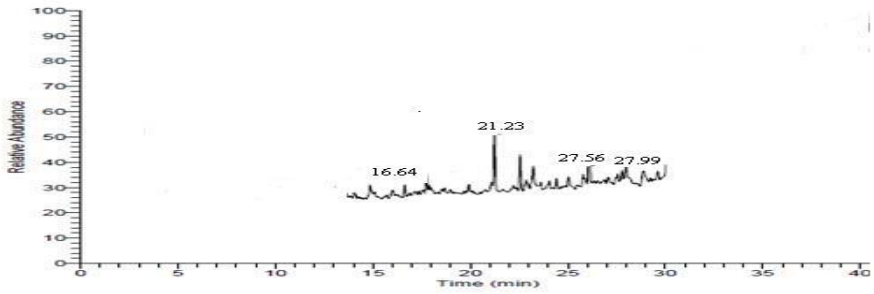


Figure 2. Gas chromatogram of biodegraded reactive yellow 15 dye

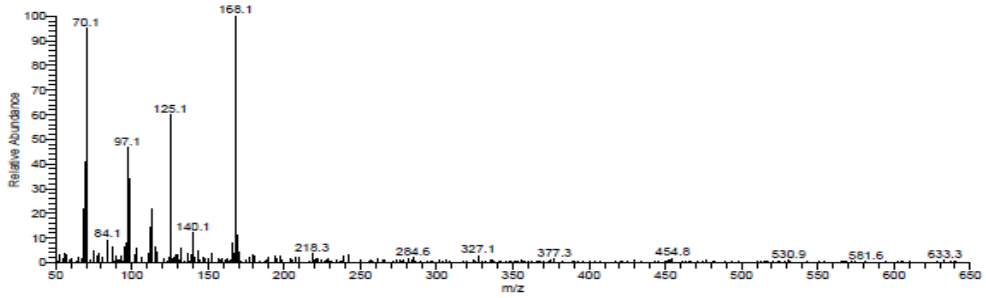


Figure 3. Mass spectral data of the reactive yellow 15 degradation product, uric acid

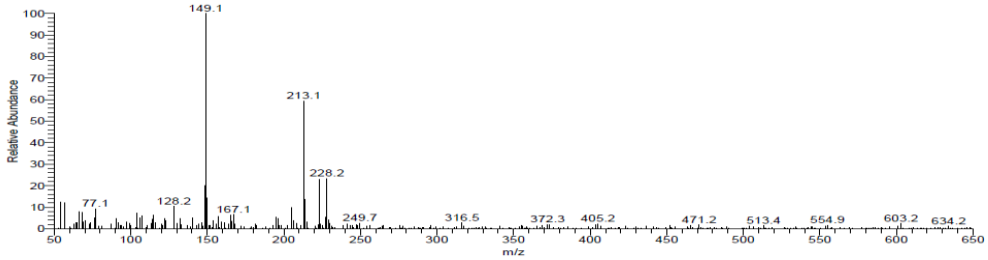


Figure 4. Mass spectral data of the reactive yellow 15 degradation product, 2-Methyl sulfonyl-5-dimethylaminomethyleneaminopyrimidine

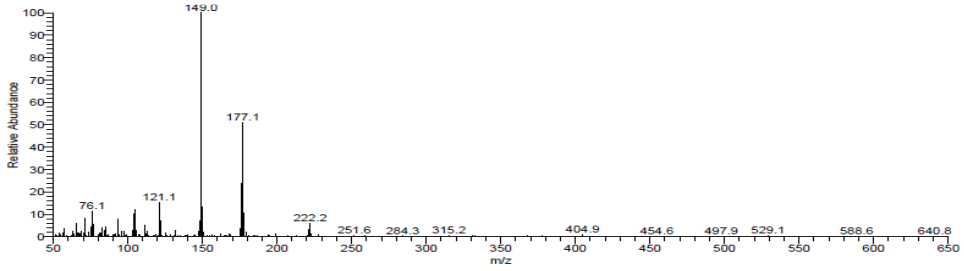


Figure 5. Mass spectral data of the reactive yellow 15 degradation product, Diethyl phthalate

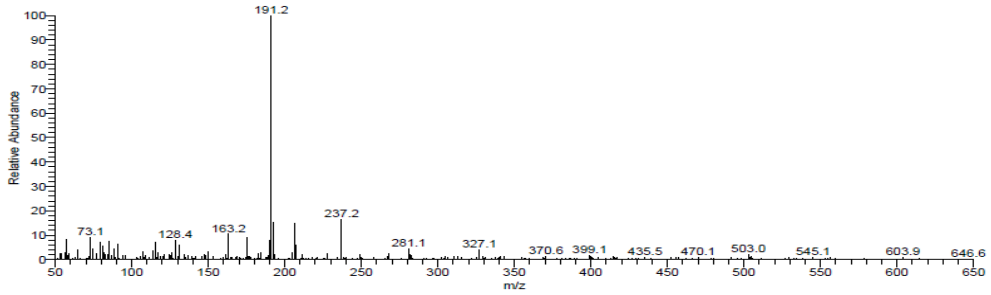


Figure 6. Mass spectral data of the reactive yellow 15 degradation product, Phenol

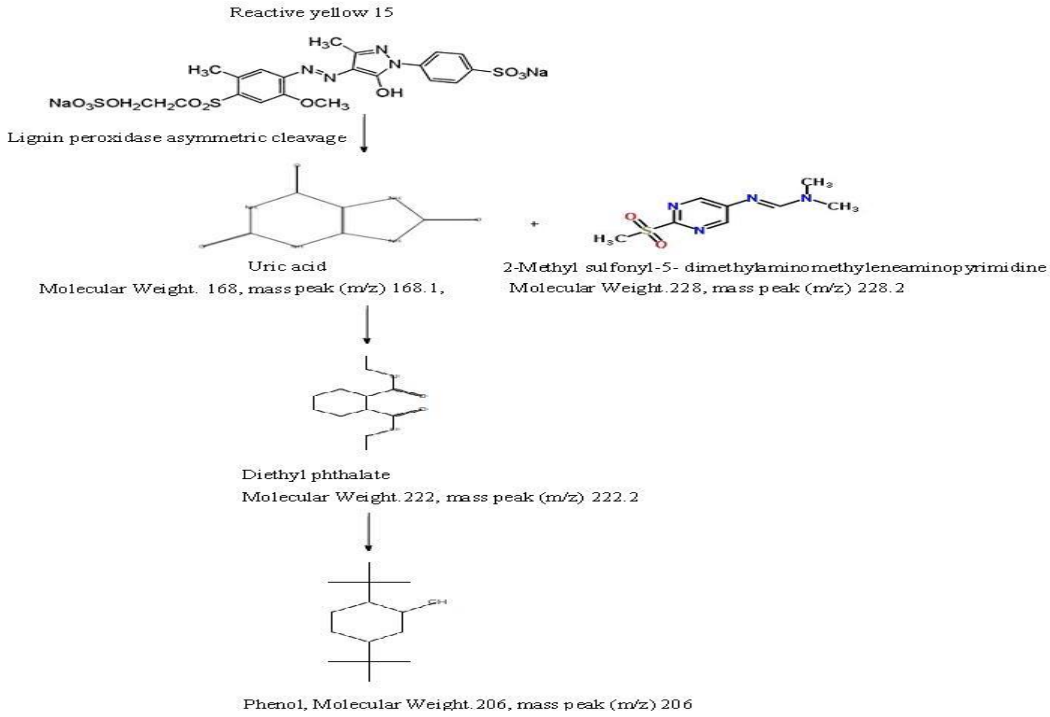


Figure 7. Possible pathway for degradation of reactive yellow 15 by *Bacillus* species

Identification of bacterial isolate

Bacterial isolate was primarily identified by doing morphological and biochemical test. The results of the morphological and biochemical test were presented in **Table 1**.

Table 1 Primary Identification of Bacterial Isolates

Results	
Characteristics	**Strain. DC6**
Colony morphology	Bright yellow colour colonies with cream pigmentation
Microscopic observations	
Gram'staining	Gram positive rod in chains
Spore staining	Ellipsoidal spores
Motility test	Motile
Biochemical test	

Indole	Negative
Methyl red	Negative
Voges Proskauer test	Positive
Citrate utilization	Positive
Catalase	Positive
Oxidase	Positive
Triple sugar iron test	Negative
Nitrate reduction test	Positive
Starch hydrolysis	Positive
Gelatin liquefaction	Positive
Urease	Negative

Molecular Characterization

Further, the sequence analysis of the 16S rRNA study of *Bacillus licheniformis* was performed, followed by phylogenetic analysis. The sequence was submitted to NCBI and received the Gen bank accession number. The accession number is KC866382 for *Bacillus licheniformis*. The BLAST search was analyzed to search for their closest identity in the Genbank database (Sudha and Balagurunathan, 2013).

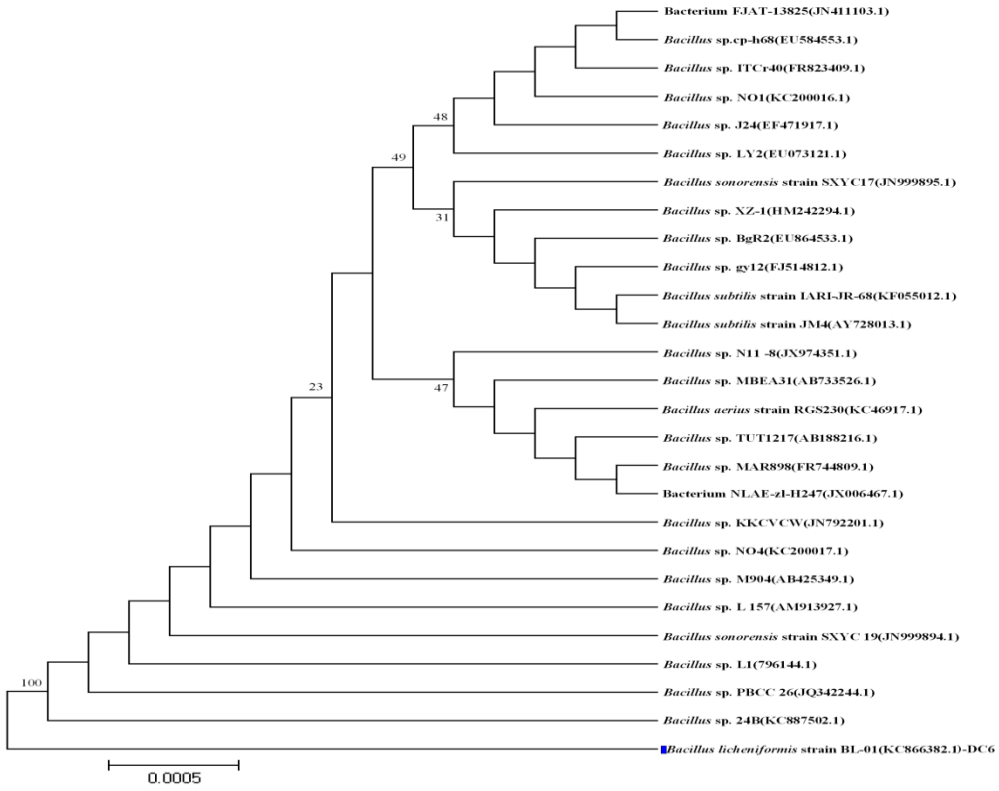


Figure 8. Phylogenetic tree of *Bacillus licheniformis*

Phytotoxicity Analysis

The effect of bacterially treated (decolourized) and untreated dyes solutions and control (water) on seed germination, shoot and root elongation was noted through this experiment. The seed germination percentage (Figure 9) of treated and control was slightly differ, when compared to untreated dye solutions. There was 90% of germination in control (water), 81% of germination in treating dye samples, 61% of germination in treating dye solutions and 300 ppm untreated dye solutions showed very less with 31% of germination. There was 5.2 cm root length was observed when seeds were irrigated with water. 4.0 cm root length by *Bacillus* sp. There was 1.2 cm root length for untreated dye solutions (Figure 10).

The shoot length was observed in water irrigated plant was 9.6 cm. In *Bacillus* sp. treated dye solutions, it was 9.6 cm shoot length 3.2 cm shoot length by untreated dye solutions for reactive yellow 15 metabolites respectively (Figure 11).

Figure 9 Germination of millet seeds

Control (water) treated reactive yellow 15 untreated dye sample

Figure 10 Growth of millet root

Control (water) treated reactive yellow 15 untreated dye sample

Figure 11 Growth of millet shoot

Control (water) treated reactive yellow 15 untreated dye sample

Discussion

The present study focused on the good decolourization ability revealed by *Bacillus licheniformis* isolated from textile effluent and soil sample. Screening experiments measured the potential of bacterial strain isolated from textile effluent and soil for decolourizing reactive yellow 15 azo dye in dye fermentation agar medium (mineral salt medium revised with dye). A

number of bacteria having abilities to degrade reactive azo dyes have been isolated from the effluent contaminated site (Asad *et al*., 2007). The present study was carried out with whole bacterial cells rather than isolated enzyme. Present study similar to the findings of Sofia Nosheen (2010). In this present study the maximum removal of reactive dye was found at pH 9 after 7 days of incubation period. Temperature is one of the most influential environmental factors as it controls the activity of all microorganisms. A mesophilic organism favours a temperature range from 30-37°C (Fulekar, 2010).

In previous studies Dawker *et al*., (2008) reported that the addition of lignin peroxidase and laccase inducers such as $CaCO_3$, indole, veratrole, vanniline and toludine to the culture medium of microorganism may enhance lignin peroxidase and laccase production. Tom Sinoy *et al*., (2011) reported that TLC spots with different RF values were obtained as compared to spot of the standard dye sample. Asad *et al*., (2007) observed that the disappearance of the dye band in decolourized media. In this present study, *Bacillus licheniformis* used to degrade the reactive dye, involvement of lignin peroxidase enzyme in reactive yellow 15 by *Bacillus licheniformis* degradation leads to asymmetric cleavage by the bacterial isolates. Kalyani *et al*., (2008) reported that asymmetric cleavage of reactive red 2 by peroxidase enzyme.

Ester of phthalic acid like diethyl phthalate also formed in reactive yellow 15 biodegradation by the bacterial isolates. Low molecular weight aromatic compound phthalic acid formed during biodegradation of C. I reactive red 195 by *Enterococcus faecalis* strain YZ66 (Madhuri Sahasrabudhe Mate and Girish Pathade, 2012). In this present study low molecular weight aromatic compound, phenol was identified from the degradation of reactive yellow 15. Similar results were observed by Soundararajan *et al.*, (2012). Release of azo linkage and formation of aromatic amines, carboxylic acid, chain hydrocarbons, formation of phenyl radicals and amines as intermediates by lignin peroxidase enzyme produced by the bacterial isolate have potential to degrade the reactive azo dyes which indicates that *Bacillus licheniformis* have potential to degrade the dye reactive yellow 15.

Plant growth bioassays and seed germination are the most common techniques used to evaluate the phytotoxicity (Kapanen and Itavaara, 2001). In the present study pearl millet (*Pennisetum glaucum*) was used to assess the toxic nature of biodegradation. Seed germination percentage, root and shoot length was noted in the water (control), treated dyes (*Bacillus licheniformis*) and untreated textile dyes on pearl millet was analysed. Moawad and Wafaa, (2003) reported that the phytotoxicity of different textile dyes was assessed by measuring the reactive changes in seed germination of four plants: clover, wheat tomato and lettuce. In the present study 90 % of germination in the control (tap water), 81 % and 61 % of germination when reactive dyes treated with *Bacillus licheniformis* and 32 % of germination with untreated textile reactive azo dye reactive yellow 15 was observed. Brilliant blue G (4000 ppm) showed 70 % inhibition in the germination of *Sorghum biocolor* and *Triticum aestivum*, when compared to control (Sheetal *et al*., 2008).

In this study, the plant growth was observed in the experimental divisions (control, biodegraded products) but the growth of plant in the presence of biodegraded products was not

normal or equal as compared to the growth in tap water. In untreated dye samples, there was observable retardness in the growth of plant and the leaf and root length. Satish Kalme *et al.*, (2007) reported metabolites formed after complete decolourization have no germination inhibition in *Sorghum biocolor* and *Triticum aestivum*.

The phytotoxicity shows that bacterial degraded dye solutions decreased the dye toxicity at certain level and also shows the less toxic nature of the treated dye solutions. In previous studies Dawker *et al*., (2008) assessed the toxicity nature of biodegraded samples. This study supports the less toxic nature of the degraded products to the experimental plants. Sheetal *et al*., (2008) observed that detoxification of brilliant blue G with respect to germination of *Triticum aestivum* and *Sorghum biocolor*. Kalyani *et al*., (2008) also reported that phytotoxicity testing with seeds of *Sorghum vulgare* and *Phaseolus mungo* showed more sensitivity towards the untreated dyes and degraded products after dye decolourization does not have any inhibitory effects.

Conclusion

The present study concludes that dye-degrading bacterium *Bacillus licheniformis* from an effluent and soil contaminated site of textile dyeing industry have potential of decolourizing and detoxifying ability. This observation has established that the bacteria are adaptive in nature and can degrade the dye contaminants. The ability of the strain *Bacillus licheniformis* to endure, decolorize and degrade reactive yellow 15 azo dyes at high concentration gave it an advantage for treatment of textile industry wastewaters.

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