Isolation and screening of strains

The textile mills have usually an effluent treatment plant (ETP) where all the effluent from the different parts of the mill is collected and treated. In the present case liquid samples from the drain carrying effluent to the treatment plant and from the effluent treatment plant were collected. Different soil samples were also collected from the vicinity of the effluent treatment plant. Soil and liquid effluent samples so collected were screened using a suitable media containing different triphenylmethane dyes. The organisms were screened by incubating the soil and water samples on dye agar plates which contain varying quantities of yeast extract, ammonium sulphate, potassium dihydrogen phosphate, di-sodium hydrogen phosphate, agar, and triphenylmethane dyes and maintained at a pH ranging from 6.0 to 8.0. Plates containing samples were incubated at temperature in the range of 25-50^{0}C and the organisms growing on them were selected on the basis of the clear zone on plates. Out of several organisms isolated one very potent organism was picked up on the basis of percent decolorization for further studies. This organism was identified as *Bacillus sp.* and has been deposited in the Microbial Type Culture Collection and Gene Bank (MTCC), a National Facility located at the Institute of Microbial Technology, Chandigarh, under the accession No. MTCC B0006, where B denotes a bacterium.

Maintenance and growth of strain

The *Bacillus sp.* MTCC B0006 isolated as above was maintained on nutrient agar plates (containing nutrient agar and triphenylmethane dye). This organism was grown in liquid

medium in shake flasks. Cells at the exponential phase were used as inoculum for the large scale cultivation of cellmass in a fermenter using a suitable production medium. The cells from the early stationary phase were harvested and the cellmass itself was used as catalyst for the decolorization experiments.

Optimization of culture conditions and medium

The *Bacillus sp.* was grown in a medium having suitable ingredients for providing carbon and nitrogen sources, vitamins, amino acids, and metal ions in required quantity. Different carbon sources like glucose, fructose, maltose, sucrose, glycerol, lactose, and inositol were used for the production of cellmass. The carbon source was used along with different organic nitrogen sources such as peptone, soyapeptone, tryptose, tryptone, casamino acid gelatin, biopeptone etc. in combination with growth factors such as yeast extract, meat extract, malt extract, beef extract, and brain heart infusion. Since the dye decolorizing activity is cell associated, medium producing high cell density with good decolorizing activity was used for further studies. All these studies were carried out in shake flasks.

The best carbon sources were glucose and maltose which gave nearly equal cellmass with nearly equal decolorization activity. Minimum cell mass and activity were obtained when starch was used as carbon source. In order to find out the optimum concentration of glucose for growth and decolorization, different concentrations (0.25-2.0%, w/v) of glucose were used in the medium containing 1.0% (w/v) soyapeptone. Results showed that 1.0% (w/v) glucose was optimum for growth and decolorization activity.

Among all the nitrogen source used, soyapeptone was found to be most effective in terms of cellmass production and dye decolorization activity. Gelatin yielded the minimum cellmass with less decolorization activity. In order to find out the optimum concentration of soyapeptone for dye decolorization, different concentrations (0.25-2.0%, w/v) of soyapeptone was used in the medium containing 1% (w/v) glucose. 1.25-1.5% (w/v) soyapeptone concentration was found to be optimum in terms of cellmass yield and decolorization activity. Therefore, 1.25% (w/v) soyapeptone was used for all further studies.

Different inorganic nitrogen sources (ammonium sulphate, ammonium acetate, ammonium nitrate, ammonium chloride, and urea) were evaluated at a concentration of 0.5% (w/v) in a medium containing glucose (1.0%, w/v) and soyapeptone (1.25%, w/v). It was found that nitrogen supplied in terms of inorganic salts did not improve the cellmass production as well as dye decolorization in comparison to control having no nitrogen source. Urea showed the relatively good cellmass in all the inorganic nitrogen sources used but it has negative effect on dye decolorization activity. Various ammonium salts showed good decolorization activity with low yield of cellmass.

Optimization of pH, temperature, and inoculum size for growth of Bacillus sp.

The optimum pH for growth was found to be between 7.0-7.25. The optimum temperature for growth was 30^{0}C. Growth decreased drastically when temperature was increased up to 37^{0}C. In order to find out the effect of inoculum size on growth and dye decolorization activity 2.5-10% (v/v) inoculum was added in the cultivation medium. The age of inoculum used was 24 h. It was observed that with 10% (v/v)

inoculum size, good growth was obtained with maximum decolorization activity and lag time for growth was also found to be minimum.

Optimization of pH and temperature for decolorization activity

pH and temperature were optimized for dye decolorization activity. Optimum pH was 7.0 and optimum temperature was found to be 50^0C. The cell activity at high temperature suggested that biological activity was quite stable and good for textile effluent treatment because the temperature of effluent coming out from dye-house is sufficiently high (approximately 60^0C).

Decolorization of triphenylmethane dyes with growing and resting cells of Bacillus sp.

Growing and resting cells of *Bacillus sp.* were used for decolorization Crystal Violet, Malachite Green, Magenta. In the case of growing cells, the dyes were added at the time of inoculation. Interestingly it was found that the *Bacillus sp.* could grow and decolorize higher amount of dyes (upto 400 ppm) in 120 h. It was also observed that Magenta was least toxic to *Bacillus sp.* (in terms of growth inhibition) however, the rate of decolorization was less compared to other triphenylmethane dyes tested. Resting cells, harvested at the late exponential phase were found to be very active from the decolorization point of view. Only 0.5 g cellmass was needed to decolorize upto 100 ppm dyes by resting cells.

Decolorization studies in packed column reactor

Active cells were successfully immobilized in calcium alginate beads for repetitive use in dye decolorization experiments. Bead diameter was 3.0 mm. Two columns of different diameters were packed with the cells immobilized in calcium alginate matrix and used for the decolorization studies of synthetic dyes. It was found that dye can be efficiently decolorized to 100% for more than 10 cycles without any significant loss in activity. Beads after decolorization were broken in n-butanol and OD of butanol fraction was measured at respective wavelengths showing zero OD indicating that dyes were not adsorbed on beads and hence completely decolorized.

Decolorization studies in reactor

Optimization studies for decolorization of triphenylmethane dyes were carried out with Crystal Violet, Malachite Green, and Magenta at 100 ppm level in a LKB fermenter (working volume 3.0 liter). Different parameters like aeration (0.25 to 1.0 vvm), agitation (50 to 300 rpm), decolorization temperature (35 to 55^{0}C), and pH (6 to 8) were optimized for the maximum decolorization of dyes. It was found that the maximum decolorization in a batch reactor took place at the following conditions- pH 7.0; temperature 50^{0}C; aeration rate 0.5 vvm; agitation 200 rpm.

Microbial assessment of toxicity of untreated and treated dye

Due to the toxic nature, triphenylmethane dyes present certain hazards and environmental problems. Toxicity of dyes varies with the structure. Efforts were made to assess the toxicity of Crystal Violet, Malachite Green, and its decolorized product by microbiological means. The test organisms used were various strains of *Escherichia*

coli, *Saccharomyces cerevisiae*, and *Schizosaccharomyces pombe*. Toxicity was measured in terms of inhibition of growth of test organisms. Various parameters like lag period, maximum cellmass concentration, specific growth rate (μ) and inhibitive ability (n) were determined for the assessment of toxicity. In the presence of Crystal Violet (5 μg/mL), the specific growth rates (μ) for *E. coli* DH5α, *S. cerevisiae* BJ5418 and *S. pombe* ABP20 decreased from 1.25, 0.229, and 0.262 h^{-1} to 0.125, 0.125, and 0.044 h^{-1}, respectively. The specific growth rates did not decrease in the presence of treated dye. The inhibitive ability of Crystal Violet was maximum (2.188) with *S. cerevisiae* BJ5418 while it was minimum (0.688) for the *E. coli* DH5α. Based on the results of above parameters, it was found that Crystal Violet is toxic to all the organisms tested but when decolorized by *Bacillus sp.*, it was found to be non toxic.

For Malachite Green (5 μg/mL), the specific growth rates decreased from 0.2495 and 0.262 h^{-1} to 0.15 and 0.185 h^{-1} in the case of *S. pombe* ABP4 and *S. pombe* ABP20, respectively, while with treated dye no change in specific growth rates was observed. With Malachite Green, the maximum inhibitive ability was 2.25 for *S. cerevisiae* ABC261 and minimum value (0.714) was for *S. cerevisiae* BJ5418. The maximum ϕ value was for *E. coli* JM109 and minimum value was with *S. pombe* ABP20. In the peresence of Crystal Violet with both the *E. coli* strains, the inhibitive abilities (n) were found to be minimum, however, with Malachite Green, minimum inhibitive ability was with *S. cerevisiae* BJ5418 (0.714). This data relate to the earlier reports that Crystal Violet and Malachite Green are toxic to the higher eukaryotic system, however, the treated Crystal Violet and Malachite Green were found to be non-toxic for all the strains of test organisms.

Estimation of COD values of untreated and treated dyes

The COD values of different dyes and its decolorized products were estimated as per American Public Health Association (APHA) standard procedure. The COD values of the Crystal Violet, Malachite Green, and Magenta (100 ppm each) were found to be 248, 225, and 245 ppm, respectively. The decolorized dyes exhibited significant decrease in the COD values. Crystal Violet showed a 83% decrease in COD value, while for Magenta and Malachite Green percentage reduction of COD was 70 and 74%, respectively.

Decolorization of triphenylmethane by laccase

Decolorization of Crystal Violet, Malachite Green, and Magenta were carried out with extracellular fluid (containing laccase) and with the growing cells of *Pleurotus ostreatus* MTCC1802. *Pleurotus ostreatus* MTCC1802 was grown in potato, dextrose, yeast extract medium. After 8 d of growth, dyes were added to each flasks. Malachite Green was decolorized more rapidly than other two dyes under static condition while Magenta was decolorized at a very slow rate. This decolorization was supposed to be carried out by laccase, as no lignin peroxidase and manganese peroxidase was detected in culture filtrate. Cell free culture filtrate of 18 d old *P. ostreatus* was filter sterilized and used for the decolorization of various triphenylmethane dyes. The decolorization was observed without an external addition of H_2O_2. Furthermore, dye decolorization was found to be carried out by ultrafiltered and dialyzed extracellular culture filtrate. In contrast, boiled culture filtrate could not decolorize the dyes. The continuing dye decolorization could be because of the presence of active laccase in ultrafiltered and dialyzed extracellular fluid, whereas,

laccase was denatured in boiled fluid. *P. ostreatus* successfully decolorized the dyes in a medium without any nitrogen limitation. The ability of *P. ostreatus* to decolorize Crystal Violet, Malachite Green, and Magenta to a higher extent in a relatively simple medium without any external supply of H_2O_2, and without any nutrient limitation could be exploited for dye waste water treatment, however, the longer growth period and less dye decolorization are the major disadvantages of *P. ostreatus*.