CHAPTER 3

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3.1 DECOLORIZATION OF TEXTILE DYES AND INDUSTRIAL EFFLUENT BY *Phanerochaete chrysosporium*

It is reported in literature (44), that *P. chrysosporium* has been used extensively as a model organism to study the physiological requirements and enzymes required for lignin biodegradation. Under culture conditions which favour ligninolysis, many xenobiotic compounds like polycyclic aromatic hydrocarbons, chlorinated phenols, pesticides and industrial dyes are oxidized and mineralized to varying extents by white rot fungus. So it was decided to use *P. chrysosporium*, a white rot fungus, for the decolorization and degradation of different industrial dyes including triphenylmethane dyes. Decolorization experiments were carried out with *P. chrysosporium* on agar plate and in liquid culture (static and shake) and the rates of decolorization were compared in a simple and complex medium.

Decolorization of **re red 120**, **re blue MR**, **direct black 22**, **crystal violet** and **acid green 20** in solid medium

A white rot fungus, *P. chrysosporium*, decolorized many kinds of textile dyes in a simple medium, showing a broad spectrum of decolorization. Re red 120, re blue MR, direct black 22 and crystal violet were decolorized on solid medium. Decolorization of different industrial dyes as **re red 120** (75 mg/I), **re blue MR** (200 mg/I), **direct black 22** (50 mg/I) and **crystal violet** (10 mg/I) was monitored on agar plates as shown in Fig 3. The rate of decolorization of crystal violet, a triphenylmethane dye (10 mg/I), was found to be very less in comparison to other dyes. On the solid medium, the fungus decolorized industrial dyes (re red 120, re blue MR and direct black 22) during 8-13 d incubation. Crystal violet was not decolorized even after 14 d incubation. It is shown that *P. chrysosporium*, decolorized a variety of dyes which are used extensively in textile
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dyeing and dye manufacturing industries, in a simple medium. The complete
decolorization of all dyes except crystal violet in a high concentration was observed in a
period of 10-13 d incubation at 30°C. Decolorization of acid green 20 (75 mg/l) by *P. chrysosporium* was also demonstrated on solid medium. Photographs of the
decolorized plates of acid green 20 on simple medium after 8-13 d incubation is shown in Fig 4. It clearly shows, the biotransformation of acid green 20 (Fig 4a) into an
unknown product (Fig 4b), then the intermediate is again transformed into another
colorless compound (Fig 4c). After long incubation, all the Acid Green 20 dye was
decolorized (Fig 4d). The biotransformation of acid green 20 in liquid culture was
carried out with 5 d old broth of *P. chrysosporium*. From time to time samples were
scanned in the visible range. The experimental data confirmed that during
biotransformation of acid green 20 by *P. chrysosporium*, initially it is converted to an
unknown product showing $\lambda_{\text{max}}$ at 522 nm and then the intermediate is again transformed into another colorless compound (Fig 5).

*Decolorization of red HE-8B, malachite green, navy blue HE-2R, magenta and crystal violet in shake and static culture*

The pattern of decolorization of various dyes by *P. chrysosporium* in static as well as in
shake culture is shown in Fig 6. More than 80% color of most of the dyes (red HE-8B, malachite green and navy blue HE-2R) were removed by the growing cells of *P. chrysosporium* in shake culture. Malachite green was more readily decolorized than the structurally similar dyes like crystal violet and magenta. Clearly, relatively small structural differences can markedly affect decolorization. Presumably, these differences were partly due to electron distribution and charge density and partly due to steric hindrance. There appears to have only one systematic study relating to dye structure

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Figure 5 Visible spectra of acid green 20 digested with *P. chrysosporium*; (a) spectrum at 0 h, (b) spectrum at 24 h, (c) spectrum at 60 h.
Figure 6 Decolorization of various dyes by *P. chrysosporium* (25%, v/v inoculum) in shake and static culture; (a) red HE-8B (10 mg/I), (b) malachite green (6 mg/I), (c) navy blue HE-2R (10 mg/I), (d) magenta (6 mg/I), (e) crystal violet (5 mg/I).
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on degradation by *P. chrysosporium* (108). They reported that the nature and position of substituents on one of the aromatic rings of azo dyes can markedly influence decolorization, although a simple and clear pattern was not established. They also demonstrated that the ligninolytic enzymes, i.e. ligninase and manganese peroxidase differed in terms of their ability to degrade different dyes. More studies of homologous series of dyes with different fungi are required to establish structure-degradability relationships. It may be mentioned that the medium used by us for the growth of *P. chrysosporium* was different than that reported by Tien and Kirk (109). We have assayed the activities of laccase, manganese peroxidase and ligninase in the medium, no activities were found (109-111). So decolorization may be due to some other unknown enzymes. When the rates of decolorization of various dyes were considered in shake as well as in static culture, it is evident that in static condition the rates of decolorization are lower compared to shake culture (Fig 6). This may be due to improper contact between cells and dye solutions. In the case of 25% (v/v) inoculum (Fig 6), the rate of decolorization of red HE-8B was maximum compared to other dyes used. In shake culture, 64% red HE-8B color was removed by *P. chrysosporium* in one day, while in the same period malachite green, navy blue HE-2R, magenta and crystal violet lost only 17, 48, 26 and 6% color, respectively. Almost 100% red HE-8B color was removed in 6 d with 25% inoculum. In the case of static culture only 13% decolorization was noticed with crystal violet in 4 d, while 28% decolorization took place with magenta. It is also evident from the Fig 6 that except red HE-8B all other dyes showed lower rate of decolorization in static culture than shake culture. In the case of 50, 75 and 100% inoculum (data not shown), sharp increase in the rates of decolorization was observed.
It is clear that the rates of decolorization are dependent on the inoculum concentration. Intimate mixing may be one of the reason for higher rates of decolorization in shaking condition. The idea of conducting static culture is to generate enough data for the ultimate use in higher scale (lagoon). With 50% inoculum the total decolorization of crystal violet and magenta was less in static culture. The rates of decolorization of red HE-8B and navy blue HE-2R dyes were not much less in static culture compared to shake culture. As the inoculum concentration was increased, the rates of decolorization both in static as well as in shake culture were increased. With 50% inoculum the rate of decolorization of magenta was less compared to other dyes both in static as well as in shake culture. This happened with higher percentage of inoculum also. With 75 and 100% inoculum the similar pattern of decolorization was observed. In shaking condition except magenta, all other dyes showed higher rates of decolorization while in static condition not much improvement of decolorization rates was observed. Here also it took 5 d for the half decolorization of crystal violet and magenta in static condition. In general it may be stated that with higher percentage of inoculum the benefit of quick decolorization was not much in the case of static culture compared to shake culture. Decolorization of the dye solution could be due to either adsorption on fungal biomass or by biodegradation. When adsorption occurred, examination of the light absorption spectrum revealed that all peaks had decreased approximately in proportion to each other. However, when degradation occurred, there was either complete removal of the major visible light absorbance peak or a significant spectral change (e.g. a change in the relative absorbance of two peaks or development of a new peak). Dye adsorption was also evident from the inspection of the mycelial mats; those adsorbing dyes were deeply colored, whereas those causing degradation remained white.
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Decolorization of textile and dye-stuff industrial effluent is shown in Fig 7. It is seen that the rate of decolorization increased with the increase in inoculum concentration. With 5, 10, 15 and 20% inoculum, the maximum decolorization was 24, 68, 95 and 98 %, respectively, in shake culture, while in the case of static culture 20, 56, 55 and 57% color were removed, respectively. In the case of static culture (which is feasible on higher scale) the ultimate color removal did not depend on the percentage of inoculum used except with 5% inoculum. This was not true with the shake culture.

Decolorization of synthetic effluent

*P. chrysosporium* was also used for decolorization of synthetic effluent containing a mixture of azo and benzidine dyes comprising of acid green 20, re red 120, re blue MR and direct black 22 (final concentration of 5 mg/ml). Figure 8 represents the repeated addition of synthetic effluent in the growing culture of *P. chrysosporium* (shown by black arrows). About 20 ppm synthetic effluent was decolorized during this repeated addition without a significant decline in activity up to the third addition of the effluent, after which the decolorization affected remarkably.

No inducers were used in the cultivation medium of *P. chrysosporium* for the production of laccase, polyphenol oxidases and peroxidases which are mainly responsible for the decolorization or degradation of dyes. The decolorization was neither due to simple adsorption nor due to any known enzymatic reaction. The decolorization may be due to one or more unknown enzymes. We have assayed the activities for enzymes known to be produced by *P. chrysosporium*, in all the media tested, but no activities were found. From the positive control runs it is evident that salts and other medium ingredients in simple media did not have any effect on the
transformation of acid green 20. It is well known from the literature that *P. chrysosporium* grows well in complex medium as reported by Tien and Kirk (109) while producing lignin and manganese peroxidases responsible for the decolorization or degradation of dyes. It may be by some other unknown enzyme/s which decolorized the dye solution.

**Comparison of decolorization of acid green 20 by *P. chrysosporium* in different media**

Simple and complex media (109) were used for the growth of *P. chrysosporium* in order to evaluate the rates of decolorization of acid green 20 in both the media. The medium used by us was a simple one containing (g/l): glucose, 20; malt extract, 20 and peptone, 10. It is seen from Fig 9 that the rate of decolorization with cells grown in the simple medium was more than that in complex medium used by Tien and Kirk (109). A higher percentage (83%) of decolorization was observed in the simple medium after 12 h incubation, while at the same time only 42% of the color was removed in the complex medium. It is evident from these findings that the cells of *P. chrysosporium* in a simple medium are not only capable of decolorizing acid green 20 but at a higher rate than that in the complex medium. These characteristics give fungus the potential to remove colored materials not only in a reactor type system but also in bioremediation in soil. The fungus is believed to attack the chromophores directly. The effectiveness of decolorization depends on the type of dye, dye structure and complexity.

3.2 DECOLORIZATION OF VARIOUS DYES BY *Geotrichum candidum*

*G. candidum* MTCC 1360, *G. candidum* MTCC 1735, *G. candidum* MTCC 1788 and *G. candidum* MTCC 2337 were grown on agar plates containing (g/l): malt extract, 3; yeast extract, 3; peptone, 5; glucose, 10; agar, 20 and 20 ppm crystal violet. The dye agar
Figure 7  Decolorization of industrial effluent by *P. chrysosporium* in shake and static culture with different percentage of inoculum (% v/v; numbers at curves)
Figure 8 Decolorization of synthetic effluent by *P. chrysosporium* in repeated addition mode (as shown by black arrows).

Figure 9 Comparison of rate of decolorization of acid green 20 by *P. chrysosporium* in different media; 1, Simple medium (glucose 20 g/l, malt extract 20 g/l, peptone 10 g/l; 2, Tien and Kirk medium (complex medium).
plates were inoculated by all the *G. candidum* strains and incubated at 30°C. The clear zone produced by *G. candidum* MTCC 1735 was bigger than other strains of *G. candidum*. This strain was also found to decolorize crystal violet at 30 mg/l, while other strains of *G. candidum* were inhibited at the same concentration of dye. So *G. candidum* MTCC 1735 was selected for the subsequent experiments. *G. candidum* MTCC 1735 was grown on petri plates in the above medium containing different industrial dyes (re red 120, 75 mg/l; re blue MR, 200 mg/l; direct black 22, 50 mg/l; red HE-8B, 30 mg/l and navy blue HE-2R, 30 mg/l). There was no decolorization even after a prolong period of incubation although enough growth was observed. Only triphenylmethane group of dyes were decolorized *G. candidum* MTCC 1735 (data not shown).

### 3.3 DECOLORIZATION OF TRIPHENYLMETHANE DYES BY NEW ISOLATES

There are few reports on the decolorization of triphenylmethane dyes by microorganisms (Table 1). Among the various organisms *Cyathus bulleri* (growth time 96 h), *Pseudomonas pseudomallei* 13NA (growth time 120 h) and *P. chrysosporium* (growth time 216 h) decolorized crystal violet (92-96%) in 96, 120 and 216 h, respectively. All the reports have shown that various organisms took longer time to decolorize the triphenylmethane dyes. So it was thought to isolate organisms which have less growth time and decolorize triphenylmethane dyes in a shorter period of time.

#### 3.3.1 Screening for triphenylmethane dye-decolorizing organisms by “dye agar plate” and “shake flasks soil slurry” method

All the reported literatures and above studies on decolorization of triphenylmethane dyes by *P. chrysosporium* and *G. candidum* clearly revealed that there is still a
necessity for screening of new and potent triphenylmethane dye-decolorizing organisms. Screening was done by (i) "dye agar plate" method and (ii) enrichment of soil samples in the presence of dye by "shake flask soil slurry" method. A total of 13 bacterial strains were examined for the growth and decolorization on agar plates containing crystal violet. Seven strains decolorized the plates to different extent. The best strain *Kurthia sp.* was selected on the basis of percentage of decolorization and used for further studies (Fig 10). By "shake flask soil slurry" method, three bacterial strains, UR1, UR2 and UR3 were isolated which were found to decolorize plates containing crystal violet.

### 3.3.2 Decolorization of crystal violet by *P. chrysosporium*, *G. candidum*, UR1, UR2, UR3 and *Kurthia* sp.,

Decolorization of crystal violet by the cells of *P. chrysosporium*, *G. candidum*, UR1, UR2, UR3 and *Kurthia* sp. is shown in Fig 11. The *Kurthia* sp., *G. candidum*, *P. chrysosporium*, UR1, UR2 and UR3 were grown for 14, 48, 144, 24, 24 and 36 h, respectively. The cells were harvested and washed thoroughly ( thrice) with 0.1 M phosphate buffer, pH 7.0. The washed cells were used for the decolorization of crystal violet. Five hundred ml flasks containing 1 g cellmass (ww) and 1 mg crystal violet in 100 ml 0.1 M phosphate buffer (pH 7.0) were incubated at 30°C (200 rpm) and decolorizing activity was measured from time to time. Figure 11 clearly indicates that *Kurthia* sp. took less time for the decolorization of crystal violet in comparison to all other strains. The rates of decolorization of crystal violet by *P. chrysosporium*, UR1 and UR2 were less compare to those of by UR3 and *G. candidum*. In the case of *P. chrysosporium* only 44% decolorization was achieved. *Kurthia* sp. decolorized 100% crystal violet in 60 min, while at the same time *G. candidum*, *P. chrysosporium*, UR1,
Figure 11 Comparison of rate of decolorization of crystal violet by *P. chrysosporium*, *G. candidum*, UR1, UR2, UR3 and *Kurthia sp.*

Dye concentration, 10 ppm; Cellmass, 10 g/l (ww)
UR2 and UR3 decolorized 64, 44, 16, 14 and 74% crystal violet, respectively. *Kurthia* sp. also decolorized higher concentration of crystal violet (30 to 60 mg/l), while *G. candidum, P. chrysosporium*, UR1, UR2 and UR3 were inhibited by 30 mg/l dye concentration (data not shown). The culture supernatant of all the organisms did not have any decolorizing activity. Only the cells exhibited decolorizing activity. The thin layer chromatographic resolution of crystal violet and the crude biotransformed products of crystal violet by *Kurthia* sp., *G. candidum*, UR1, UR2 and UR3 with authentic sample of leucocrystal violet is shown in Fig 12. It is evident from Fig 12 that all the strains transformed crystal violet in the same product. It may be mentioned that it was not possible to isolate the product from the digestion mixture of crystal violet with *P. chrysosporium*. *Kurthia* sp. was found to be superior over *G. candidum*, UR1, UR2 and UR3 because of following reasons:

(i) The growth time of *Kurthia* sp. was less compare to other organisms tested,

(ii) The rate of decolorization of triphenylmethane dyes by *Kurthia* sp. was higher in comparison to other organisms. More over, the biotransformed products of crystal violet by all the strains were the same. On the basis of above reasons *P. chrysosporium, G. candidum*, UR1, UR2 and UR3 were left behind and *Kurthia* sp. was used for further studies.

### 3.3.4 Decolorization of triphenylmethane dyes by the growing cells of *Kurthia* sp.

Decolorization of various triphenylmethane dyes (magenta, crystal violet, malachite green, brilliant green, pararosanilin and ethyl violet) by the growing cells of *Kurthia* sp. is shown in Fig 13. In the case of decolorization of magenta, the 9 h old broth of *Kurthia* sp. was used with an initial cell concentration of 0.54 g/l (dw). With the dye concentration of 1.0-4.0 mg/l, more than 90% of the color was removed in 30 min while
Figure 13 Decolorization of magenta, crystal violet, malachite green, brilliant green, pararosaniline and ethyl violet by the growing cells of Kurthia sp.
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at the same time only 40% of the color was removed when the dye concentration in the broth was increased to 10 mg/l. The rate of dye decolorization with lower concentration (1.0-4.0 mg/l) of dye was higher than with higher concentration of dye in the broth. The decolorization of magenta was totally stopped after 120 min when dye concentration in the broth was 10 mg/l.

Decolorization of crystal violet by *Kurthia* sp. is also shown in Fig 13. Here 0.4 g/l (dw) cell concentration was used. At the same time (30 min) of incubation as before, crystal violet lost 94% of its maximum color at an initial dye concentration of 5 to 6.25 mg/l. When the dye concentration was increased to 20.8 mg/l, decolorizing activity was found to be inhibited. Yatome *et al.* (92) reported that the growth of *Nocardia corallina* cells was completely inhibited at higher concentration of crystal violet. Cell concentration in the case of decolorization of magenta was higher than that of crystal violet decolorization. With the same concentration of different dyes, the color intensity was different. Another triphenylmethane dye i.e. malachite green was used for decolorization by the cells of *Kurthia* sp. (Fig 13). With the same cell concentration (0.40 g/l) as in the case of crystal violet, experiments were carried out with malachite green. It is evident from figure 13 that 100% decolorization took place in 120 min incubation at a varied malachite green concentration of 5-35 mg/l. With higher concentration (55 mg/l) of malachite green, maximum decolorization was 59% after 120 min incubation. Compared to crystal violet, higher concentration of malachite green was decolorized with the same amount of cellmass. It may be due to the difference in structure of both the dyes (Fig 1). Malachite green has two dimethyl groups in two side chains whereas crystal violet is having three dimethyl groups in three side chains. It may be the reason that decolorization of more substituted triphenylmethane dyes took
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longer time. Decolorization of another triphenylmethane dye (brilliant green) is also discussed in Fig 13. Here also the same pattern of decolorization was observed as in the case of other triphenylmethane dyes. Cell concentration was little higher than other cases, however, brilliant green concentration used in the decolorization was also higher (2-10 times) than other cases of decolorization. It may be due to the simple structure of the dye concerned. Another two dyes were decolorized by the cells of Kurthia sp. (Fig 13). Same cell concentration was used in the decolorization of pararosanilnine and ethyl violet. In the case of pararosanilnine the rates of decolorization with lower concentration (upto 15 ppm) of the dye were more or less same. Inhibition of transformation started from 25 mg/l pararosanilnine. Upto 15 mg/l, there was no problem of decolorization of pararosanilnine, however, higher concentration inhibited the cells. Fig 13 also indicates the rate of decolorization of ethyl violet by Kurthia sp. Here the rates of decolorization are less than any other triphenylmethane dyes used. This may be due to the structure of the dye. Ethyl violet contains diethyl groups instead of dimethyl groups in the side chains (Fig 1). The concentration of ethyl violet used was also less compared to other triphenylmethane dyes.

Experiments were carried out with all the triphenylmethane dyes to find out the viable cell count after the decolorization was over. Viable cell concentration after the end of each experiment was counted by plate count method. The results are shown in Table 4. In the case of malachite green, pararosanilnine and crystal violet, viable cell concentration has reduced to 1.22 - 1.38x10⁶ cells/ml which is approximately 400 times less than the original number of cells. In the case of brilliant green, the number of viable cells after decolorization was very less (0.99x10⁵). With ethyl violet, the number of viable cells reduced drastically, infact, number of viable cells has reduced to only 11.
Table 4 Viable cell count during decolorization of triphenylmethane dyes by *Kurthia sp.*

<table>
<thead>
<tr>
<th>Dyes</th>
<th>Number of viable cells after decolorization</th>
</tr>
</thead>
<tbody>
<tr>
<td>Magenta</td>
<td>$1.26 \times 10^7$</td>
</tr>
<tr>
<td>Crystal violet</td>
<td>$1.327 \times 10^6$</td>
</tr>
<tr>
<td>Malachite green</td>
<td>$1.387 \times 10^6$</td>
</tr>
<tr>
<td>Pararosaniline</td>
<td>$1.221 \times 10^6$</td>
</tr>
<tr>
<td>Brilliant green</td>
<td>$0.986 \times 10^6$</td>
</tr>
<tr>
<td>Ethyl violet</td>
<td>11</td>
</tr>
</tbody>
</table>

Cells are grown in the medium containing (g/l): yeast extract 5; (NH₄)₂SO₄ 0.5; KH₂PO₄ 2.66; Na₂HPO₄ 4.32. Nine hour old broth of *Kurthia sp.* was used for decolorization with an initial dye concentration 5 mg/l. Decrease of viability was calculated by plate count method. Initial number of viable cells = $5.43 \times 10^9$ per ml.
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This indicates higher toxicity of ethyl violet compared to other triphenylmethane dyes used.

3.3.5 Decolorization of synthetic and industrial effluent by the resting cells of Kurthia sp.

Decolorization of synthetic and industrial effluent by the resting cells of Kurthia sp. is shown in Fig 14. In this experiment different triphenylmethane dyes (crystal violet, magenta, malachite green, brilliant green, pararosaniline and ethyl violet) were mixed at the same concentration and the synthetic effluent thus generated was decolorized by the cells of Kurthia sp. For this synthetic effluent $\lambda_{\text{max}}$ in 1-butanol was found to be 557 nm. Upto 3 mg/l mixed dyes, the rate of decolorization was very fast and almost 90% decolorization took place within 75 to 100 min incubation. With the initial synthetic effluent concentration 5 and 10 mg/l, about 39% color was removed after 20 min incubation. The rate of decolorization was retarded with the increase of synthetic effluent concentration. Decolorization of effluent from textile and dye-stuff industry is also shown in Fig 14. It is seen that the rate of decolorization of industrial effluent increased with the increase of cellmass concentration. With 0.31, 0.40, 0.60 and 0.88 (g/l) cellmass concentration, the maximum decolorization was 23, 48, 56 and 58%, respectively. In these experiments only cell concentration was varied keeping the constant quality of industrial effluent. It has been observed that maximum decolorization achieved was only 58% and the percentage decolorization could not be increased by increasing the cellmass concentration. With 0.60 g/l cellmass concentration, the maximum decolorization was 56%, while by increasing the cell concentration to 0.88 g/l, the proportionate increase of decolorization was not there.
Figure 14 Decolorization of synthetic and industrial effluent by the resting cells of *Kurthia* sp.
After complete decolorization of synthetic effluent, the decolorized product was extracted in 1-butanol and absorption spectrum was taken as shown in Fig 15. Absorption spectrum shows, very large reduction in the peak of the synthetic effluent after incubation with Kurthia sp. and it indicates the disappearance of the dyes. Dye decolorization may take place in two ways either by adsorption or degradation of the dyes by the cells. Dye adsorption may be evident from the inspection of the bacterial growth; those adsorbing dyes will be deeply colored whereas those carrying out degradation will remain colorless. With the Kurthia sp. no cells were found to be colored with any one of the dyes after transformation. Hence it was thought to be degradation/decolorization.

3.4 OPTIMIZATION OF PHYSICO-CHEMICAL CONDITIONS AND GROWTH MEDIA FOR MAXIMUM DECOLORIZING ACTIVITY

Kurthia sp. was grown in 2.5% w/v nutrient broth (beef extract, 1%; peptic digest of animal tissue, 1% and sodium chloride, 0.5%). A considerable amount of cellmass was obtained in nutrient broth medium. The decolorizing activity of Kurthia sp. is intracellular in nature and the activity is directly related with the amount of cellmass present. Hence more decolorizing activity is denoted by more amount of cellmass. So it was thought to optimize the media for maximum cellmass production before starting the detailed studies. The course of cultivation of Kurthia sp. was initially studied in shake flask.

3.4.1 Course of cultivation of Kurthia sp. with respect to decolorizing rate

The course of cultivation of Kurthia sp. with respect to decolorizing rate is shown in Fig 16. It is evident from the figure that the decolorizing rate of crystal violet is dependant on cellmass up to 12 h thereafter it decreased very sharply. The cellmass concentration
Figure 15  Visible spectra of synthetic effluent digested with Kurthia sp.; (a) spectrum at 0 min, (b) spectrum at 140 min
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increased up to 14 h and attained a maximum value of 19.7 g/l (ww). The data clearly shows that the decolorization of crystal violet was dependent on the initial cell concentration and growth phase of precultured cells. This supported the dependence of decolorization of crystal violet on the growth phase of the organism. Decolorization of crystal violet by Kurthia sp. in repeated addition mode is shown in Fig 17. In this experiment cells were grown in 500 ml shake flasks having 100 ml nutrient broth medium for 12 h at 30°C (200 rpm). The initial cell concentration in the flask was 7.9 g/l. A definite quantity of crystal violet solution was added in the flask to have a final concentration of 75 ppm. The flasks were again put in the incubator shaker at 30°C (200 rpm) till it is completely colorless. In the second addition, dye was again added to have a final concentration of 75 ppm. About 225 ppm dye was decolorized by the growing cells of Kurthia sp. in 4 h without any significant decline of decolorizing rate. After 4 h, time taken for complete decolorization started increasing. After three additions (after complete decolorization of 225 ppm crystal violet), the fourth and fifth additions took 7 and 37 h, respectively for complete decolorization. After the fifth addition, further addition was not possible as crystal violet was not decolorized. The number of viable cells also decreased with number of additions and this is shown in Fig 18. The number of viable cells decreased very sharply after 48 h (Fig 18). In control, the number of viable cells decreased with a diminished rate. The decrease of number of viable cells of Kurthia sp. with crystal violet is because of toxic effect of crystal violet. Kurthia sp. is Gram positive bacteria and have lower isoelectric point in comparison to Gram negative bacteria. So it contain more acidic components than Gram negative bacteria. Hence this bacteria combine with crystal violet (cationic dye) very rapidly.
Figure 16 Course of cultivation of Kurthia sp. with respect to decolorizing rate.

Figure 17 Decolorization of crystal violet by Kurthia sp. in repeated addition mode.

Figure 18 Viable cell count during decolorization of crystal violet by Kurthia sp.
3.4.2 Effect of inoculum size, initial pH and temperature on decolorizing rate by *Kurthia* sp.

To find out the effect of inoculum size on the growth and decolorizing activity by *Kurthia* sp., different levels (1 to 10%, v/v) of inoculum were used. The higher level of decolorizing activity and reduced lag phase were observed with 5% (v/v) inoculum (data not shown). Hence for all the subsequent experiments a 5% v/v inoculum level was used. Effect of initial pH on the decolorizing rate is shown in Fig 19. The maximum decolorizing rate (98 ppm/h/mg cell) was observed when the initial pH of the medium was adjusted to 8.5. So the initial pH 8.5 was used in all subsequent experiments. No significant difference in decolorizing rate was observed when the initial pH of the medium was adjusted to 8.5 and 9.0. With the increase of initial pH, the decolorizing rate started decreasing. At pH 10, the decolorizing rate had decreased to a minimum value. To see the effect of initial temperature on the decolorizing rate, the organism was grown at different temperatures (30, 37 and 45°C). There was no growth of *Kurthia* sp. at 45°C (Fig 20). The decolorizing rate at 37°C increased upto 13 h and thereafter it decreased very sharply. The maximum decolorizing rate of crystal violet was observed at 30°C.

3.4.3 Effect of growth factor on the decolorizing rate by *Kurthia* sp.

Effect of growth factors on the decolorizing rate by *Kurthia* sp. is shown in Fig 21. Addition of growth factors (brain heart infusion, meat extract, malt extract, potato extract and yeast extract) were found to greatly influence the decolorizing rate of crystal violet by *Kurthia* sp. Maximum decolorizing rate with malt extract was approximately 25% higher compared to potato extract which performed in a more or less similar fashion. From Fig 21, it is evident that the decolorizing rate by *Kurthia* sp. with malt
Figure 19 Effect of initial pH on the decolorizing rate by *Kurthia* sp.

Figure 20 Effect of temperature on the decolorizing rate by *Kurthia* sp.
Figure 21: Effect of growth factors on the decolorizing rate by *Kurthia sp.*

Figure 22: Effect of carbon sources on the decolorizing rate by *Kurthia sp.*
Results and discussion

extract was very high in comparison to other growth factors and it did not attain peak even after 24 h, while with other growth factors, it started decreasing after 18 h. The decolorizing rate with brain heart infusion, meat extract, yeast extract is not satisfactory. Meat extract and yeast extract both behaved in the same way in the production of cellmass and decolorizing activity. Brain heart infusion as a growth factor did not have any positive influence on the decolorizing rate. As we know that the growth factors contain nitrogen, the variation of production of cellmass with different growth factors may be due to the varied nitrogen content of the growth factors. So for all the subsequent experiments, malt extract was always used in the cultivation medium of Kurthia sp. for the maximum decolorizing rate.

3.4.4 Effect of carbon source on the decolorizing rate by Kurthia sp.

Effect of carbon sources on the decolorizing rate by Kurthia sp. is shown in Fig 22. Various carbon sources (described in section 2.3.7) were added at a concentration of 1% (w/v), except molasses, 5% (v/v) in nutrient broth (2.5%, w/v). Molasses was found to be the most effective carbon source for yielding maximum decolorizing rate, giving a high value of 178 ppm/h/mg cell and did not attain peak even after 32 h. Other carbon sources like glucose, fructose, maltose and starch exhibited negative effect on the decolorizing rate. It has been found that with molasses as a carbon source, the decolorizing rate is on the higher side. It may be due to the presence of different metal ions (Ca²⁺, Mg²⁺, K⁺ etc.) in the molasses.

3.4.5 Effect of nitrogen source on the decolorizing rate by Kurthia sp.

Effect of inorganic nitrogen source

Inorganic nitrogen sources on the decolorizing rate is shown in Fig 23. Different inorganic nitrogen sources (described in section 2.3.7) were evaluated at a
Figure 23 Effect of inorganic nitrogen source on the decolorizing rate by *Kurthia sp.*

- Control
- Ammonium sulphate
- Ammonium oxalate
- Urea
- Ammonium chloride
- Ammonium dihydrogen phosphate
- Ammonium peroxodisulphate

Figure 24 Effect of organic nitrogen sources on the decolorizing rate by *Kurthia sp.*

- Control
- Peptone
- Gelitone
- Gelatine
- Soyapeptone
- Biopeptone
Results and discussion

concentration of 0.2% (w/v) with 1% (w/v) malt extract, 2.5% (w/v) nutrient broth and 5% (v/v) molasses. Ammonium peroxodisulphate was found to be most effective for maximum decolorizing rate (228 ppm/h/mg cell). After 36 h, the decolorizing rate did not attain a peak value with ammonium peroxodisulphate. The sampling was stopped after 36 h, because of insufficient amount of culture broth in flasks. With the ammonium dihydrogen phosphate, ammonium sulphate and urea, the decolorizing rate started decreasing after 24 h, but in the presence of ammonium oxalate, it increased up to 161 ppm/h/mg cell. Ammonium sulphate, ammonium dihydrogen phosphate and urea showed negative effect on decolorizing rate as compared to control.

Effect of organic nitrogen source

Effect of organic nitrogen sources on the decolorizing rate by Kurthia sp. is shown in Fig 24. Various organic nitrogen sources (described in section 2.3.7) were used at a concentration of 0.5% (w/v) in a medium (pH 8.5) containing 2.5% (w/v) nutrient broth, 1% (w/v) malt extract, 5% (v/v) molasses and 0.2% (w/v) ammonium peroxodisulphate. Among all the organic nitrogen sources used, biopeptone was found to be the most effective for the decolorizing rate (240 ppm/h/mg cell). The decolorizing rate with other organic nitrogen sources was not satisfactory and they behaved in a more or less similar way with control. Peptone, gelitone, gelatine, soyapeptone and tryptose were found to exert negative influence on the decolorizing rate. There was no considerable differences in the decolorizing rates when the organism was grown with peptone, gelitone, gelatin, soyapeptone and tryptose. All organic nitrogen sources except biopeptone showed same effect on the decolorizing rate.
3.4.6 Effect of metal ions and chemicals on the decolorizing rate by *Kurthia* sp.

Table 5 shows the effect of metal ions and chemicals on the decolorizing rate by *Kurthia* sp. Various metal ions and chemicals (described in section 2.3.7) were used with final concentration 0.5 and 2.0 mM in growth medium (pH 8.5) containing 2.5% (w/v) nutrient broth, 1% (w/v) malt extract, 5% (v/v) molasses, 0.2% (w/v) ammonium peroxodisulphate and 0.5% (w/v) biopeptone. Mg$^{2+}$, K$_2$HPO$_4$, KI and K$_3$[Fe(CN)$_6$] had no stimulatory effect on the decolorizing rate by *Kurthia* sp. Ag$^+$ and Co$^{3+}$ completely inhibited the growth of *Kurthia* sp. and hence decolorizing rate at 2.0 mM level whereas Cu$^{2+}$ inhibited the decolorizing rate completely at the concentration of 2.0 mM after 60 h of growth. Ca$^{2+}$ at the 0.5 mM level had a stimulatory effect on the decolorizing rate. Maximum decolorizing activity (110%) was obtained at 36 h of growth in the presence of 0.5 mM Ca$^{2+}$. Each ions and chemicals at the higher concentration level (10 mM) exhibited poor growth and hence low decolorizing rates (data not shown).

3.4.7 Effect of surface active agent on the decolorizing rate by *Kurthia* sp.

Table 6 shows the effect of surface active agents on the decolorizing rate by *Kurthia* sp. In order to find out the effect of surface active agents on the decolorizing rate by *Kurthia* sp., polypropylene glycol and Tween 80 were used in the cultivation medium (pH 8.5) containing 2.5% (w/v) nutrient broth, 1% (w/v) malt extract, 5% (v/v) molasses, 0.2% (w/v) ammonium peroxodisulphate, 0.5% (w/v) biopeptone and 0.5 mM CaCl$_2$. It was found that with 0.1% v/v polypropylene glycol, the decolorizing rate increased, while there was no significant increase in the decolorizing rate at higher concentrations of 0.3 and 0.5% (v/v). So for all the subsequent experiments polypropylene glycol at a concentration of 0.1% (v/v) was added in the cultivation medium. Tween 80 at all concentrations inhibited the decolorizing rate. The inhibition of decolorizing rate
Table 5 Effect of metal ions and chemicals on the decolorizing rate by *Kurthia* sp.

<table>
<thead>
<tr>
<th>Chemicals</th>
<th>Concentration 0.5 mM</th>
<th>Concentration 2.0 mM</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>12h</td>
<td>36h</td>
</tr>
<tr>
<td>ZnSO₄·7H₂O</td>
<td>77</td>
<td>102</td>
</tr>
<tr>
<td>MnCl₂·4H₂O</td>
<td>106</td>
<td>101</td>
</tr>
<tr>
<td>MgSO₄·7H₂O</td>
<td>108</td>
<td>106</td>
</tr>
<tr>
<td>CoCl₂·6H₂O</td>
<td>75</td>
<td>0</td>
</tr>
<tr>
<td>CuSO₄·5H₂O</td>
<td>57</td>
<td>43</td>
</tr>
<tr>
<td>CaCl₂·2H₂O</td>
<td>108</td>
<td>110</td>
</tr>
<tr>
<td>FeSO₄·7H₂O</td>
<td>84</td>
<td>79</td>
</tr>
<tr>
<td>NiCl₂·6H₂O</td>
<td>81</td>
<td>83</td>
</tr>
<tr>
<td>AgNO₃</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>EDTA</td>
<td>81</td>
<td>79</td>
</tr>
<tr>
<td>K₂HPO₄</td>
<td>103</td>
<td>103</td>
</tr>
<tr>
<td>KI</td>
<td>105</td>
<td>103</td>
</tr>
<tr>
<td>K₃[Fe(CN)₆]₃</td>
<td>101</td>
<td>101</td>
</tr>
</tbody>
</table>

The decolorizing rate is expressed as a percentage of the decolorizing rate in the absence of chemicals.
Table 6 Effect of surface active agents on the decolorizing rate by *Kurthia* sp.

<table>
<thead>
<tr>
<th>Surface active agents</th>
<th>Concentration (% v/v)</th>
<th>24 h</th>
<th>48 h</th>
<th>72 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>Polypropylene glycol (PPG)</td>
<td>0.1</td>
<td>109.4</td>
<td>110.3</td>
<td>109.9</td>
</tr>
<tr>
<td></td>
<td>0.3</td>
<td>102.7</td>
<td>101</td>
<td>95.5</td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td>97.4</td>
<td>98.7</td>
<td>102.8</td>
</tr>
<tr>
<td>Tween 80</td>
<td>0.1</td>
<td>62.7</td>
<td>98.9</td>
<td>86.5</td>
</tr>
<tr>
<td></td>
<td>0.3</td>
<td>41.7</td>
<td>51.7</td>
<td>62.2</td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td>38.8</td>
<td>53.2</td>
<td>58.7</td>
</tr>
</tbody>
</table>

The decolorizing rate is expressed as a percentage of the decolorizing rate in the absence of surface active agents.
increased with the increase in concentration of Tween 80 in the medium. Minimum
decolorizing rate (38.8%) was obtained with 0.5% (v/v) Tween 80 in the medium.

3.4.9 Effect of temperature and pH on the decolorizing activity by Kurthia sp.
The optimum temperature for maximum decolorizing activity was 40°C (Fig 25). The
effect of temperature on the decolorizing activity by Kurthia sp. was studied in the
range of 10 to 70°C. As the temperature increased, the decolorizing activity increased
upto 40°C with a sharp increase between the temperatures 35 and 40°C, after that the
decolorizing activity started decreasing very sharply up to 45°C and then became
constant. It is evident from the figure that sharp increase in decolorizing activity had
started only after an incubation temperature 35°C below which the increase in
decolorizing activity was not abrupt. At 35 and 45°C, the relative decolorizing activities
were 88 and 81%, respectively. Fig 26 shows the optimum pH for the maximum
decolorizing activity to be 8.5. In the acidic side upto pH 7.0, there was no significant
increase in decolorizing activity and after that it increased very sharply and attained a
peak value at pH 8.5. The pH of the reaction mixture did not affect the decolorizing
activity as shown in this figure. In the control experiment, heat killed cells were used in
the pH range between 3.0 to 10 to find out the effect of pH on crystal violet
decolorization. Variation of pH between 3.0 to 10 did not have any effect in the
decolorization of crystal violet with dead cells whereas with the live cells (having
biological activity) the pH had tremendous effect. The optimum pH for the maximum
decolorizing activity was found in the alkaline region. The broad pH optima (7.5 to 10)
and slight higher temperature optima (40°C) may be suitable for the application of this
organism (Kurthia sp.) in the treatment of textile and dye-stuff industrial waste
containing triphenylmethane dyes.
Figure 25 Effect of pH on decolorizing activity by Kurthia sp. (Maximum activity obtained was taken as 100% and relative activity was calculated accordingly)

Figure 26 Effect of temperature on decolorizing activity by Kurthia sp. (Maximum activity obtained was taken as 100% and relative activity was calculated accordingly)
3.5 FERMENTER STUDIES FOR THE GROWTH AND DECOLORIZING ACTIVITY
BY Kurthia sp. IN OPTIMIZED MEDIUM

Production of Kurthia sp. was carried out in a 7 l fermenter (Chemap AG, Switzerland), with a working volume 5 l (Fig 27). The fermenter was equipped with different controllers such as pH, temperature, agitation, antifoam, dissolved oxygen. In order to obtain the maximum cellmass of Kurthia sp. in a stirred tank reactor, different parameters (agitation and aeration) were optimized.

Effect of agitation

The 7 l reactor was run with a working volume of 5 l at 30°C. Agitation rates were varied from 100 to 400 rpm keeping the aeration rate constant (1 vvm). Fig 28-31 shows the cultivation of Kurthia sp. in a batch reactor with a varied agitation of 100-400 rpm and constant aeration of 1 vvm. Without pH control, the cultivation of Kurthia sp. for the production of cellmass showed many interesting characteristics in optimized medium (pH 8.5) containing 2.5% (w/v) nutrient broth, 1% (w/v) malt extract, 5% (v/v) molasses, 0.2% (w/v) ammonium peroxodisulphate, 0.5% (w/v) biopeptone, 0.5 mM CaCl₂ and 0.1% (v/v) polypropylene glycol. In the initial phase of fermentation, the active cell growth was continued up to 10 h of cultivation and then decreased with reduced specific growth rate. This is true for fermenter runs with an agitation of 100 to 300 rpm. At an agitational speed of 400 rpm, the maximum amount of cellmass (38 g/l, ww) was obtained at 20 h of fermentation. The maximum specific growth rates (μ) for Kurthia sp. at different agitation rates were calculated from the basic growth equation. It has been observed that with the increase of agitational speed from 100 to 400 rpm with an interval of 100 rpm, the maximum specific growth rates were increased up to 300 rpm agitation and then with the increase of agitation (400 rpm), the maximum specific growth rates were decreased.
Figure 28 Course of cultivation of *Kurthia* sp. in a batch reactor (rpm, 100; aeration, 1 vvm)

Figure 29 Course of cultivation of *Kurthia* sp. in a batch reactor (rpm, 200; aeration, 1 vvm)
growth rate was reduced. The maximum specific growth rate values were 1.9, 1.25, 4.04 and 2.83 h\(^{-1}\) when the reactors were run at 100, 200, 300 and 400 rpm, respectively. Maximum specific growth rate (4.04 h\(^{-1}\)) was obtained when the reactor was run at 300 rpm speed. At higher agitation (400 rpm), the specific growth rate was reduced. This may be due to the cell lysis at higher agitational speed. The pH of the culture broth did not change as the medium was highly buffered. The sugar consumption rate was maximum when the reactors were run with 300 and 400 rpm agitational speed. At lower mixing rates (100 and 200 rpm), the rates of sugar consumption were less. This may be due to the insufficient mixing in slowly agitated reactors. In less agitation an insufficient mixing takes place due to which mass transfer limitation in terms of substrate and dissolved oxygen predominates.

Dissolved oxygen (DO) concentration in the fermenter also decreased with rapid growth and increased along with the depletion of sugar level and decrease in specific growth rate. From all the figures (28-31), it is evident that dissolved oxygen concentration remained constant up to 10 h of fermentation and then with the increase of growth, DO concentration started decreasing. After the active growth of Kurthia sp., DO concentration started increasing throughout the fermentation. Minimum DO% was obtained in the reactor where the agitational speed was maintained at 300 rpm. It was of particular interest to note that the time at which the DO of the culture broth began to increase coincided with that of sugar depletion in the medium except very slowly agitated (100 rpm) reactor. The turning point in the DO profile may therefore be an indication of sugar depletion and maximum growth during fermentation. The decolorizing activity started increasing with growth of cellmass and continued to increase till the specific growth rate started decreasing. It was also observed that
Figure 30 Course of cultivation of *Kurthia sp.* in a batch reactor (rpm, 300; aeration, 1 vvm)

Figure 31 Course of cultivation of *Kurthia sp.* in a batch reactor (rpm, 400; aeration, 1 vvm)
production of decolorizing activity was associated with cellmass growth. The fermentation time at which the activity reached its maximum, in general did not coincide with that of cellmass, however, decolorizing activity is associated with cellmass. It was also noted from the shake flask experiments (agitation effect) that a high oxygen transfer rate (OTR) in the culture medium was important to maintain a high level of cellmass production. Therefore, dissolved oxygen concentration of the culture broth was maintained almost at saturation level during the fermentation period at the specified agitation and aeration rate.

**Effect of aeration**

From the optimization of mixing rate (agitation), it has been found that an agitation of 300 rpm yielded maximum decolorizing activity, when the aeration rate was kept constant (1 vvm). Hence it was decided to optimize the aeration rate for the maximum decolorizing activity. Effect of aeration rate on the decolorizing activity by *Kurthia sp.* is shown in Figs 32 and 33. Reactors were run at two different aeration rates (0.25 and 0.75 vvm). The decrease of DO concentration was maximum (40%) at an aeration rate of 0.75 vvm, while at the same time maximum decrease in DO concentration was 70% at an aeration rate of 0.25 vvm. The maximum specific growth rate values at three different aeration rates (0.25, 0.75 and 1 vvm) were 3.94, 3.78 and 4.04 h⁻¹, respectively. The maximum amount of sugar was consumed when the reactor was run with an aeration rate of 1 vvm. Sugar consumption was minimum at an aeration rate of 0.25 vvm. Although, sugar consumption was maximum with 1 vvm of aeration, maximum decolorizing activity was obtained at an aeration rate of 0.75 vvm.
Figure 32 Course of cultivation of *Kurthia* sp. in a batch reactor (rpm, 300; aeration, 0.75 vvm)

Figure 33 Course of cultivation of *Kurthia* sp. in a batch reactor (rpm, 300; aeration, 0.25 vvm)
3.6 BIOTRANSFORMATION OF TRIPHENYLMETHANE DYES IN STIRRED TANK REACTOR

For optimization of biotransformation of triphenylmethane dyes (crystal violet, malachite green and magenta), the Kurthia sp. was grown in a medium (pH 8.5) containing 2.5% (w/v) nutrient broth, 1% (w/v) malt extract, 5% (v/v) molasses, 0.2% (w/v) ammonium peroxodisulphate, 0.5% (w/v) biopeptone, 0.5 mM CaCl$_2$ and 0.1% (v/v) polypropylene glycol in 15 l fermenter (Chemap AG, Switzerland) with agitation (300 rpm) and aeration (1.0vvm) at 30°C. Twenty two hour old culture was harvested and centrifuged. Cells were washed with 0.1 M phosphate buffer (pH 7.0) and cell slurry was made in 0.025 M glycine-NaOH buffer (pH 8.5). This cell slurry was used for biotransformation of triphenylmethane dyes in 6 l reactor (LKB, Bromma, Sweden) having working volume 3.0 l with different aeration (0.5-1.5 vvm) and agitation (100-500 rpm) rates at 40°C (Fig 34). The biotransformation of crystal violet (40 ppm) by Kurthia sp. in stirred tank reactor is shown in Fig 35. For crystal violet decolorization 6.0 g/l (ww) cellmass was used. Figure 35 (a) clearly shows that maximum decolorization was achieved with 300 rpm agitational speed in the reactor. Seventy six percentage decolorization was obtained after 10 min of agitation (300 rpm), while at the same time 18, 24, 58 and 54% decolorization were observed at 100, 200, 400 and 500 rpm agitation, respectively. The rate of decolorization at 100 rpm was very less and this may be due to the improper mixing at low agitation. At 100 rpm, the maximum decolorization was only 49%. At higher agitation (500 rpm), the reactor was full with foam and this had retarded the rate of decolorization. As the agitation was increased in the reactor, the rate of decolorization of crystal violet also increased upto 300 rpm, after that with the increase of agitation the percentage decolorization started decreasing. At lower agitation with a
defined reactor working volume (3.0 l), the proper mixing may have taken place, while at higher agitation overmixing of the reactor contents may be suspected. At higher mixing rate, a lot of foam was formed in the reactor and a larger portion of cells resided in the foam than in the reaction mixture and thus reducing the rate of decolorization.

The effect of aeration on the biotransformation of crystal violet by Kurthia sp. is shown in Fig 35 (b). The higher rate of decolorization was observed with 1.0 vvm aeration rate. In case of 1.5 vvm aeration, the reaction mixture was full of foam and hence retardation in the rate of decolorization was observed. In order to re-use the cellmass in the same reactor, experiments were conducted in repeated addition mode as shown in Fig 35 (c). Initially, the reactor was run with 40 ppm crystal violet in the presence of 6 g/l cellmass in optimized conditions (300 rpm, 1 vvm). After the decolorization was over, again dye concentration in the reactor was made to 40 ppm and decolorization was continued. In this way the repeated addition experiments were carried out. By repeated addition of dye (with each addition of 40 ppm), 120 ppm dye was decolorized in 110 min without any significant decline in decolorizing activity. After third addition, the decolorizing activity started reducing and it took 130 min to decolorize and the decolorization rate drastically reduced thereafter. The reduction of decolorization rate with the number of additions may be due to the toxic effect of crystal violet on the cells on long exposure.

The biotransformation of malachite green (40 ppm) by Kurthia sp. in stirred tank reactor is shown in Fig 36. For the decolorization of malachite green same amount (6 g/l) of cellmass was used. It has been observed that the decolorization rate of malachite green was low in comparison to crystal violet. It is evident from the Fig 36 (a) that the rate of decolorization of malachite green with 300 rpm was maximum. After 12
Figure 35  Biotransformation of crystal violet in stirred tank reactor
Figure 36  Biotransformation of malachite green in stirred tank reactor

Aeration, 1.0 vvm; Dye concentration, 40 ppm, Cell concentration, 6 g/l (ww)

- 100 rpm
- 200 rpm
- 300 rpm
- 400 rpm
- 500 rpm

Agitation, 300 rpm; Dye concentration, 40 ppm, Cell concentration, 6 g/l (ww)

- 0.5 vvm
- 1.0 vvm
- 1.5 vvm

Agitation, 300 rpm; Aeration, 0.5 vvm; Cell concentration, 6 g/l (ww)

Each addition 40 ppm malachite green

Time (min)
Results and discussion

min, 52% decolorization was achieved at 300 rpm agitation while at the same time 8, 9, 18 and 25% decolorization occurred at 100, 200, 400 and 500 rpm, respectively. The rate of decolorization at 100 rpm was very low and the dye was not decolorized even after 70 min, this may be explained due to the improper mixing at lower agitation. At higher agitation (500 rpm), the reaction mixture was full of foam and this had retarded the rate of decolorization. The effect of aeration on the biotransformation of malachite green by *Kurthia sp.* is shown in Fig 36 (b). The higher rate of decolorization was observed with 0.5 vvm aeration. In case of higher rate of aeration (1.5 vvm), percentage decolorization had reduced. To find out the effect of dye concentration on cellmass (constant amount), malachite green was added in the reaction mixture in repeated addition mode as shown in Fig 36 (c). By repeated addition of dye (with each addition of 40 ppm), 120 ppm dye was decolorized in 180 min without any significant loss of activity. After third addition of dye, the decolorizing rate was reduced and 40 ppm dye was decolorized in 135 min. From the fifth addition, the process became very slow.

The biotransformation of magenta by *Kurthia sp.* in a stirred tank reactor is shown in Fig 37. Effect of agitation and aeration on the decolorization of magenta showed more or less the same picture as that of crystal violet and malachite green. Here also at higher rpm, the rate of decolorization was less compare to lower agitation rate. The effect of aeration on the biotransformation of magenta by *Kurthia sp.* is shown in Fig 37 (b). Here also the higher rate of decolorization was observed with 1.0 vvm aeration. To find out the effect of dye concentration on cellmass, magenta was added in the reaction mixture in repeated addition mode as shown in Fig 37 (c). It is evident from the figure that repeated addition of magenta in the reactor did not yield same result as that
Figure 37  Biotransformation of magenta in stirred tank reactor
of crystal violet and malachite green. It took 80 and 180 min for the complete
decolorization of 40 ppm dye in first and second addition, respectively. From the third
addition onwards the process became very slow.

### 3.7 ISOLATION, PURIFICATION AND IDENTIFICATION OF BIOTRANSFORMED
PRODUCT OF CRYSTAL VIOLET

The biotransformed product of crystal violet was isolated and purified as described in
Materials and methods (section 2.6) and identified as leucocrystal violet by thin layer
chromatography, $^1$H and $^{13}$C NMR, DEPT-90, DEPT-135, mass spectra and IR spectra.
The UV-Visible absorption spectra of the transformed product of crystal violet by
*Kurthia* sp. was observed to similar that of an authentic sample of leucocrystal violet
(data not shown). The crystal violet, product extracted from crystal violet dye-digested
cells, purified biotransformed product and authentic compound were subjected to DC-
Alufolien kiesel gel 60 F$_{254}$ plate (9 cm X 17 cm) and developed in two different solvent
systems as shown in Fig 38 A and B. Spots were detected immediately after exposing
plates by iodine vapors. Lane 1 shows in Fig 38 A and B, the spots of crystal violet in
two different solvent systems with Rf values of zero and 0.47, respectively. Lane 2
shows in both Fig 38 A and B, the movement of impure biotransformed product. Three
bands were observed with Rf values of 0.39, 0.22 and 0.18, respectively as shown in
Fig 38 A. Lane 3 and 4, in both Fig 38 A and B show the spots of purified
biotransformed product and authentic compound with same Rf values of 0.39 and 0.15,
respectively.

The purified product has a melting point 177°C, which was found to be the same
as reported for leucocrystal violet. Its structure was further supported by appearance of
molecular ion (M+) peak at 374 in mass spectral data (Fig 39 A) and its spectra was identical with those of authentic sample of leucocystal violet (Fig 39 B). Its structure was confirmed by NMR spectral data. Its $^1\text{H}$ NMR (Fig 40 B) showed methyl protons as singlet at $\delta$ 2.89, methine proton as singlet at $\delta$ 5.29 and aromatic protons as doublets (J 8.6 Hz) $\delta$ 6.64 and (J 8.6 Hz) at $\delta$ 6.98. Its $^{13}\text{C}$ NMR showed (Fig 41 A) methyl carbons at $\delta$ 40.88, methine carbon at $\delta$ 54.11, tertiary aromatic carbons at $\delta$ 112.65 and $\delta$ 129.95 and quarter aromatic carbons at $\delta$ 133.81 and $\delta$ 148.90. Its NMR spectral data was identical with those of authentic sample of leucocystal violet (Fig 40 C and 41 B). The biotransformed purified product’s DEPT-90 spectra (shows only --CH peaks) showed methine carbon at $\delta$ 54.11 and tertiary aromatic carbon at $\delta$ 112.65 and $\delta$ 129.95 (Fig 42 A). Its DEPT-135 spectra (shows --CH and --CH$_3$ peaks in one phase and --CH$_2$ in opposite phase) showed methyl carbon at $\delta$ 40.88, methine carbon at $\delta$ 54.11, tertiary aromatic carbons at $\delta$ 112.65 and $\delta$ 129.95 (Fig 42 B). No peak was observed in opposite direction, hence it again confirmed the structure of the compound. Finally, the structure was confirmed by taking IR spectra which was identical with the authentic sample of leucocystal violet (Fig 43 A and B).

3.8 ISOLATION, PURIFICATION AND IDENTIFICATION OF BIOTRANSFORMED PRODUCT OF MALACHITE GREEN

The biotransformed product of malachite green was isolated and purified as described in Materials and methods (section 2.7) and identified as leucomalachite green by thin layer chromatography, $^1\text{H}$ and $^{13}\text{C}$ NMR, mass spectra and IR spectra. The UV-Visible absorption spectra of the transformed product of malachite green by Kurthia sp. was observed to be similar that of an authentic sample of leucomalachite green (data not
The malachite green, product extracted from malachite green dye-digested cells, purified biotransformed product and authentic compound were subjected to DC-Alufolien kiesel gel 60 F\textsubscript{254} plate (9 cm X 17 cm) and developed in two different solvent systems (methanol : acetic acid : isopropyl alcohol :: 2 : 2 : 1 [1]; hexane : ethyl acetate :: 2 : 1 [2]). Spots were detected immediately after exposing the plates by iodine vapors. The Rf values of purified biotransformed product in solvent systems [1] and [2] were 0.55 and 0.45, respectively. The same Rf values were also obtained in solvent systems [1] and [2] for the authentic sample of leucomalachite green.

Its structure was further supported by appearance of molecular ion (M\textsuperscript{+}) peak at 330 in mass spectral data (Fig 44 A) and it was identical with those of authentic sample of leucomalachite green (Fig 44 B). Its structure was confirmed by NMR spectral data. Its \textsuperscript{1}H NMR (Fig 45 A) showed methyl protons as singlet at $\delta$ 2.9, methine proton as singlet at $\delta$ 5.38 and aromatic protons as multiplets at $\delta$ 7.2, doublets at $\delta$ 7.0 (J 11.57 Hz) and $\delta$ 6.68 and (J 11.66 Hz). Its \textsuperscript{13}C NMR showed (Fig 46 A) methyl carbons at $\delta$ 40.80, methine carbon at $\delta$ 54.99, secondary aromatic carbons at $\delta$ 128.11, $\delta$ 128.4 and $\delta$ 132.87 tertiary aromatic carbons at $\delta$ 112.54 and $\delta$ 113 and quarter aromatic carbons at $\delta$ 112.54 and $\delta$ 148.96. Its NMR spectral data was identical with those of authentic sample of leucomalachite green (Figs 45 B and 46 B). Finally, the structure was confirmed by taking IR spectra which was identical with the authentic sample of leucomalachite green (Fig 47 A and B).
Figure 3 Photographs showing decolorization of textile dyes by *P. chrysosporium* in a simple medium; (a) re red 120 (75 mg/l) after 8 d incubation, (b) re blue MR (200 mg/l) after 10 d incubation, (c) direct black 22 (50 mg/l) after 12 d incubation and (d) crystal violet (10 mg/l) after 12 d incubation.
Figure 4 Photographs showing decolorization of acid green 20 (75 mg/l) by P. chrysosporium in a simple medium; (a) control, (b) formation of unknown product after 8 d incubation, (c) formation of colorless product after 9 d incubation, (d) complete decolorization after 13 d incubation.
Figure 10 Photograph showing the Bird's feather like growth of *Kurthia sp.* and decolorization of crystal violet.
Figure 12 Thin layer chromatographic resolution of crystal violet (a); crude biotransformed products of *Kurthia* sp. (b); *G. candidum* (c); UR1 (d); UR2 (e); UR3 (f) and leucocrystal violet (g). (The developing solvent system, n-hexane : ethyl acetate; 2 : 1, v/v).
Figure 27 Photograph showing the cultivation of *Kurthia sp.* in a stirred tank reactor (Chemap AG, Switzerland)
Figure 34 Photograph showing the biotransformation of triphenylmethane dye in a stirred tank reactor (LKB, Bromma, Sweden)
Figure 38 Thin layer chromatographic resolution of 1, crystal violet; 2, crude biotransformed product; 3, purified biotransformed product; and 4, authentic compound.

A. The developing solvent system - n-hexane : ethyl acetate (2 : 1, v/v).
B. The developing solvent system - methanol : acetic acid : isopropyl alcohol (2 : 2 : 1, v/v).
Figure 39  A. Mass spectra of biotransformed product of crystal violet  
B. Mass spectra of authentic compound (leucocrystal violet)
Figure 40: A. Proton NMR spectra of crystal violet
B. Proton NMR spectra of biotransformed product
C. Proton NMR spectra of authentic compound (leucocystal violet)
Figure 41  A. C\textsuperscript{13} NMR spectra of biotransformed product of crystal violet
B. C\textsuperscript{13} NMR spectra of authentic compound (leucocrystal violet)
Figure 42 A. DEPT-90 spectra of biotransformed product of crystal violet
B. DEPT-135 spectra of biotransformed product of crystal violet
Figure 43  A. IR spectra of biotransformed product of crystal violet
B. IR spectra of authentic compound (Leucocrystal violet)
Figure 44 A. Mass spectra of biotransformed product of malachite green
B. Mass spectra of authentic compound (leucomalachite green)
Figure 45  A. $^1$H NMR spectra of biotransformed product of malachite green
B. $^1$H NMR spectra of authentic compound (leucomalachite green)
Figure 46  A. $^{13}$C NMR spectra of biotransformed product of malachite green  
B. $^{13}$C NMR spectra of authentic compound (leucomalachite green)
Figure 47  A. IR spectra of biotransformed product of malachite green
B. IR spectra of authentic compound (leucomalachite green)
3.9 ASSESSMENT OF ACUTE TOXICITY OF TRIPHYLMETHANE DYES AND BIOTRANSMFORMED PRODUCTS

The acute toxicity of the crystal violet, leucocrystal violet and biotransformed product were found out based on the toxic effect of the test substances on cell killing and growth inhibition. It is evident from Fig 48 that cell concentration increased with time in both the cases (with and without crystal violet), however, the rate of increase of cell growth in the presence of crystal violet, is less compared to control. Experiments were conducted with higher concentration of crystal violet and the rates of growth were found to be much less compared to control. The experimental data from every test series (triplicate) were evaluated graphically by plotting the log-concentration of the compound under test versus the percent inhibition of cell growth which yields an exponential growth curve as shown in Fig 49 (with means and standard deviations). Linear transformation of the data yielded a straight line as seen as the insert in Fig 49. The IC\textsubscript{50} can simply be read of from this plot. The value of IC\textsubscript{50} for crystal violet was calculated 1.146 ppm from the insert of Fig 49. Upto 600 ppm, leucocrystal violet had shown no inhibition effect on yeast cell growth. There was also no significant growth inhibition with the biotransformed product (Fig 50). Cell free supernatant (5-25%, v/v) was found to be free from acute toxicity (data not shown). It was reported (87, 92) that the growth of Bacillus subtilis and Nocardia corallina were completely inhibited by the crystal violet at 2.84 ppm level. The biotransformed product of crystal violet by N. corallina and B. subtilis was 4,4'-bis(dimethylamino)benzophenone (irritant, mutagen and cancer suspect agent) which was extracted from supernatant. In our report, the biotransformed product, leucocrystal violet, having high chronic toxicity (9), was found to be cell bound. So the strain, Kurthia sp. can be used as decolorizing agent and for
Figure 48  Linear growth of yeast cells with and without crystal violet (The growth rate of yeast without any substance, is taken as 100%, the reduced growth rate by crystal violet, is expressed as percent of control)
Figure 49 Dose activity curve of crystal violet: means and standard deviation from three identical experiments; Insert; Linear transformation of dose activity curve by adjusting the regression line to the experimental points using the least square method.

Figure 50 The growth of yeast in the presence of biotransformed product, leucocrystal violet.
Figure 51 Dose activity curves of malachite green, ethyl violet, pararosaniline, brilliant green and magenta; means and standard deviation from two identical experiments; Insert: Linear transformation of dose activity curves by adjusting the regression line to the experimental points using the least square method.
Results and discussion

removal of toxicity from the effluent containing triphenylmethane dyes. The IC_{50} value, as determined with the yeast test, is a good measure for the acute toxicity of a chemical compound. It compares well to the LD_{50} found in vertebrates. Since the traditional LD_{50} test in laboratory animals has many shortcomings which are not found with the yeast test, the technique can be classified as advantageous among the so-called alternative methods. The acute toxicities of malachite green, magenta, brilliant green, ethyl violet and pararosaniline were determined by the same procedure as described above for crystal violet. The dose activity curves of all dyes are shown in Fig 51. The IC_{50} values of malachite green, magenta, brilliant green, ethyl violet and pararosaniline were 1.502, 30.002, 10.021, 0.7847 and 8.738 ppm, respectively.

3.10 ASSESSMENT OF CHEMICAL OXYGEN DEMAND IN CELL FREE SUPERNATANT AND TEXTILE AND DYE-STUFF EFFLUENT

Table 7 shows the reduction of COD values in cell free supernatant of triphenylmethane dyes and industrial effluent digested with the cells of Kurthia sp. For each triphenylmethane dye and synthetic and textile dye effluent, experiments were carried out in 500 ml flasks having different amounts of cellmass in a total volume of 100 ml. Dyes and effluents were added with constant stirring at 30°C till complete decolorization occurred. The cells were separated and COD values of the supernatant were estimated. It is evident from table 7, that Kurthia sp. had a great potential to transform different triphenylmethane dyes including synthetic and textile dye-stuff effluent to colorless compound. It is reported in literature (79) that Pseudomonas pseudomallei 13NA (growth time 20 h) decolorized 2.04 mg crystal violet in 120 h with 1 g cellmass (dw), while Kurthia sp. (growth time 14 h) decolorized 216 mg crystal violet
Table 7 The reduction of COD values in cell free supernatant

<table>
<thead>
<tr>
<th>Dyes</th>
<th>Cellmass (g/100 ml, ww)</th>
<th>Total dye decolorized (mg)</th>
<th>Dye decolorized (mg/g cell mass, ww)</th>
<th>Dye decolorized (mg/g cell mass, dw)&lt;sup&gt;6&lt;/sup&gt;</th>
<th>COD reduction (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Magenta</td>
<td>50</td>
<td>230</td>
<td>4.6</td>
<td>33.8</td>
<td>88.3</td>
</tr>
<tr>
<td>Crystal violet</td>
<td>48</td>
<td>216</td>
<td>4.5</td>
<td>33.1</td>
<td>94.7</td>
</tr>
<tr>
<td>Malachite green</td>
<td>49</td>
<td>210</td>
<td>4.3</td>
<td>31.6</td>
<td>93.2</td>
</tr>
<tr>
<td>Pararosaniiline</td>
<td>47</td>
<td>230</td>
<td>4.9</td>
<td>36.1</td>
<td>87.9</td>
</tr>
<tr>
<td>Brilliant green</td>
<td>38</td>
<td>200</td>
<td>5.26</td>
<td>38.7</td>
<td>88.2</td>
</tr>
<tr>
<td>Ethyl violet</td>
<td>40</td>
<td>105</td>
<td>2.63</td>
<td>19.3</td>
<td>70.1</td>
</tr>
<tr>
<td>Synthetic effluent</td>
<td>24</td>
<td>160</td>
<td>6.67</td>
<td>49.0</td>
<td>90</td>
</tr>
<tr>
<td>Textile and dye-stuff effluent</td>
<td>6</td>
<td>#</td>
<td>----</td>
<td>----</td>
<td>56</td>
</tr>
</tbody>
</table>

@, Kurthia sp. contain 86.4% water; #, concentration of dyes in textile and dye-stuff effluent was not known.
in 6 h with 6.6 g cellmass (dw). It is also evident that 1 g cellmass (dw) of Kurthia sp. can decolorize 32.73 mg crystal violet in 6 h while 2.04 mg crystal violet was decolorized by 1 g cellmass of Pseudomonas pseudomallei 13NA in 120 h. In the case of ethyl violet, amount of dye decolorized per gram of cellmass was less compare to other dyes. The reduction of COD of the cell free extract was around 88 to 94% in all the cases. In the case of textile and dye-stuff effluent, the reduction of COD was found to be 66%, while with synthetic effluent, the COD reduction was 90%. This may be due to the fact that textile and dye-stuff effluent may contain dissolved solids other than dyes.