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Biological Decolorization of Crystal Violet by a Newly Isolated *Bacillus sp.* and Microbial Assessment of Toxicity of Untreated and Treated Dye

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Synthetic organic dyes are essential in satisfying the ever-growing demand, in terms of quality, variety and speed for coloration of a large number of substances. Due to the toxic nature, these materials present certain hazards and environmental problems. Toxicity of dyes varies with their structure. In this paper, a report is provided regarding a new *Bacillus sp.* which decolorizes one of the triphenylmethane dyes, Crystal Violet, very efficiently. Efforts were made to assess the toxicity of Crystal Violet and its decolorized product through 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 cell mass 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 rate (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 *E. coli* DH5α. Based on the results of the 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.

INTRODUCTION

A toxicant is an agent that can produce an adverse response (effect) in a biological system, seriously damaging its structure or function or causing death [1]. The assessment of toxicity of any chemical is necessary from the effluent management point of view. In contrast, the uptake of minute quantities of toxic chemicals may result in no apparent adverse effect. Therefore, one of the main concepts of toxicology is that, in general, no chemical is completely safe or completely harmful. The factor that determines whether a chemical agent is potentially harmful or safe is the relationship between the concentration (quantity) of the chemical to which an organism is exposed and the duration of the exposure.

Dyes are one of the most commonly used chemicals in various industries. The dye waste mainly comes from dyestuff manufacturing, dyeing and paper printing industries. Since most of the dyes used are relatively recalcitrant, they usually come out as such into the effluent. Crystal Violet, a triphenylmethane dye, is used extensively as human and veterinary medicine, a biological stain and a textile dye in the dyeing of acrylic fibres [2-4]. Generally, Crystal Violet presence in the wastewater causes inhibition of microbial growth and makes the biological decolorization process more difficult. Consequently, it is necessary to determine the toxicity level of the dye for the microorganisms to ease the biological decolorization process. To meet such a demand, various methods have been used, among

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which the most standardized one is using fish. Since toxicity testing with the help of fish takes a little longer, various alternative methods were considered using different types of test organisms. Algae and protozoa, invertebrates, mixed cultures of bacteria, yeast and pure bacterial cultures were used for toxicity test of chemical compounds. The ability of yeast to attack complex nitrogen compounds, fats, polysaccharides and related substances may be an important feature in the role it plays in the biodegradation and detoxification processes in the natural environment. Despite the slower growth, compared to most bacteria, other properties, such as production of organic acids, alcohol and antibiotics, make yeast a more useful organism for toxicity and biodegradation studies.

Yeasts are eukaryotic cells and most of the processes and pathways have been found to be highly conserved from yeast to man. These similarities also allow yeasts to be used for possible screening purposes against novel cytotoxic chemicals provided they are permeable to these compounds. *Saccharomyces cerevisiae* has been used as a model experimental eukaryote to study the interaction and response of eukaryotic cells to toxicants [5]. They have a greater genetic complexity than bacteria and, therefore, offer certain biochemical advantages over them. It has been shown that yeasts possess a wide tolerance to various environmental conditions. The fission yeast *Schizosaccharomyces pombe*, however, may be a better choice for toxicity studies. The major advantage in using *S. pombe* is the greater similarities seen between this yeast and higher eukaryotic cells, as compared to *S. cerevisiae*. Transcription in *S. pombe* is more similar to mammalian cells than *S. cerevisiae* [6]; mammalian and plant promoters function in *S. pombe* [7], but not in *S. cerevisiae*. Splicing and splicing signals are more conserved between *S. pombe* and higher eukaryotes [8]. *S. pombe* glycoproteins contain galactose, mannose and glucose residues while those of *S. cerevisiae* contain only glucose and mannose residues. Studies on the secretory and endocytotic pathways of fission yeast reveal greater similarities with higher eukaryotic cells. In addition, diphtheria toxin acts on *S. pombe* whole cells (although at a higher level than mammalian cells) but not on *S. cerevisiae*. Investigation on several other genes such as the heat shock factor gene indicates greater functional similarities of the *S. pombe* genes with their mammalian homologues [9]. These similarities of *S. pombe* with the mammalian system has led to use of this fission yeast in testing the toxicity of untreated and treated Crystal Violet.

This paper reports the decolorization of Crystal Violet by a newly isolated *Bacillus sp.* and the toxicity assessment of Crystal Violet and its decolorized product. In the present study, the logic to take *Escherichia coli* as the test organism is that the gram negative bacteria are the most dominant microbial population in the aquatic ecosystem, while the fermentative yeasts *S. cerevisiae* and *S. pombe* were selected on the basis of their capability of tolerance for toxicants.

MATERIALS AND METHODS

Chemicals

Crystal Violet, adenine, uracil, leucine, histidine and lysine were purchased from the Sigma Chemical Company, MO, USA. The media components were obtained from HiMedia, Mumbouo, India. All other chemicals were of analytical grade.

Microorganisms for Toxicity Test

Gram negative bacteria and fermentative yeasts were used as test organisms for the determination of toxicity of Crystal Violet and its decolorized product. Two *E. coli* strains (*E. coli* DH5α and *E. coli* JM109), two *S. cerevisiae* strains (*S. cerevisiae* BJ5418 and *S. cerevisiae* ABC261) and two *S. pombe* strains (*S. pombe* ABP4 and *S. pombe* ABP20) were provided by the Yeast Molecular Biology Laboratory of the Institute of Microbial Technology, Chandigarh, India.

Isolation of Dye Decolorizing Organism

Various soil and liquid effluent samples were collected from a textile dyeing industry and screened for dye decolorizing organisms. The screening of the strains for dye decolorization was carried out on agar plates containing (g/l): yeast extract 5; $(NH_4)_2SO_4$ 0.5; KH_2PO_4 2.66; Na_2HPO_4 4.32 and agar 20 [10]. The medium was supplemented with 2.5 μg/ml Crystal Violet. The pH of the medium was adjusted to 7. Organisms were selected on the basis of a clear zone on agar plate. The isolates growing on the plates and decolorizing the dye were selected and grown in the same liquid medium containing glucose (5 g/l). The cells were grown in 500 ml flasks containing 100 ml medium and incubating at 30°C for 48 h on a shaker (200 rpm). The organism selected for decolorization studies was identified as *Bacillus sp.* by Microbial Type Culture Collection and Gene Bank, Institute of Microbial Technology, Chandigarh, India. The organism was deposited under the accession no. MTCC B0006.

Decolorization Assay

Percent of dye decolorization was determined spectrophotometrically by the modified method of Yatome et al. [11]. One ml broth was centrifuged at 10000 rpm for 10 min at 4°C and after discarding the supernatant,

1.5 ml phosphate buffer (0.1 M, pH 7.0) was added to it. The cell suspension was placed in a water bath at 50°C. Pre-equilibrated (50°C) 0.5 ml Crystal Violet (0.12 mg/ml) was added to the cell suspension and incubated at 50°C. Two samples were taken from the reaction mixture, one at zero time and another after 5 min. The aliquots of the samples were immediately placed in 1 ml water saturated *n*-butanol to stop the decolorization reaction. Optical density of butanol fraction was determined at 584 nm and decolorization percentage was calculated as follows:

$$\% \text{ Decolorization} = \frac{\text{Initial OD - Final OD}}{\text{Final OD}} \times 100$$

Dye Decolorization for Toxicity Studies

The decolorization activity of *Bacillus sp.* is cell associated in nature.

Cells were grown as described above and centrifuged at 10000 rpm for 10 min and placed in phosphate buffer (0.1 M, pH 7). Twenty milligrams of suspended cells in 1 ml buffer were placed inside a test tube and in a water bath at 50°C. One ml pre-equilibrated (50°C) Crystal Violet (0.05 mg/ml) was added. Decolorization was monitored by decrease in optical density at 584 nm. Incubation was continued untill the color of Crystal Violet disappeared completely.

Growth Conditions of Test Organisms

S. cerevisiae strains and *S. pombe* strains were grown in the medium containing (g/l): glucose (30), yeast extract (5), adenine, leucine, uracil, histidine and lysine (75 mg each). The pH of the medium was 5.5. Fifty milliliter medium was placed in a 250 ml flask and after inoculation, the flasks were incubated at 30°C on a shaker (200 rpm) for 48 h. Yeast strains were maintained on the same medium containing agar (2%, w/v). For the growth of *E. coli*, Luria Bertani broth (LB 2%, w/v, pH 7) was used. *E. coli* was grown in a 250 ml flask having 50 ml medium at 37°C (200 rpm) for 12 h.

Toxicity Assessment of Untreated and Treated Crystal Violet

Growth was monitored to study the toxicity of Crystal Violet and its biotransformed (colorless) form. Experiments were carried out in 250 ml flasks having 50 ml medium. To the autoclaved medium, Crystal Violet (filter sterilized) was added, with a varying final concentration of 5 to 25 μg/ml. The control flask did not contain any dye. For the toxicity assessment of decolorized dye, the same concentration of dye

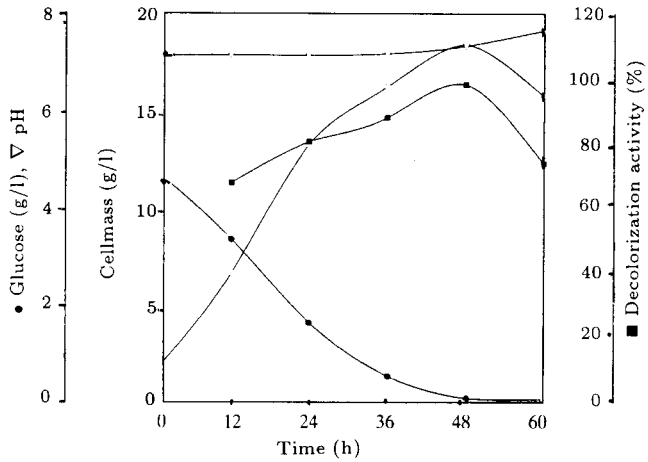

Figure 1. Course of cultivation of *Bacillus sp.* with decolorizing activity.

was decolorized (as described before) and, after filter sterilization, was added to the growth medium. All the flasks were inoculated with the test organisms and incubated under desired conditions. Periodical samplings were done under sterile condition to monitor the growth of organisms. The growth was measured by taking the cell weight (wet weight).

RESULTS

Course of Cultivation of *Bacillus sp.*

Figure 1 shows the course of cultivation of *Bacillus sp.* having decolorization activity. The organism grew very well up to 48 h of fermentation and the maximum cell mass obtained was 18.6 g/l (ww). Glucose consumption started from the very beginning and within 36 h of fermentation, almost all of the glucose was consumed. The medium contained salts having buffering effect and, hence, the pH of the medium increased very little towards the alkaline side (from 7.3 to 7.7) at the end of fermentation. The decolorization activity increased after 12 h of growth and maximum decolorization activity (99%) was found to be at 48 h. The decolorization activity of *Bacillus sp.* was found to be cell associated. The decrease in the decolorizing activity at 60 h of growth seems to be due to the death of cells.

Growth of the Test Organisms in the Presence of Dye and Transformed Dye

It is evident from Figure 2 that *E. coli* JM109 grew well in the medium without dye and the maximum cell mass obtained was 4.8 g/l (ww) in 6 h. When Crystal Violet was added to the medium and inoculated with *E. coli* JM109, the rate of growth of *E. coli* decreased with the increase in the Crystal Violet concentration in the medium. Practically, with higher concentrations

Table 1. Inhibitive ability, lag period and maximum cell mass obtained at different concentrations of Crystal Violet using various test organisms.

Organisms	Inhibitive Ability (n)	Concentration of Inhibitive Substance at H=0.5 (Φ, mol/l)	Lag Period (h) at Different Dye Concentrations (μg/ml)						Maximum Cell Mass (g/l) Obtained at Different Dye Concentrations (μg/ml)					
			Control	5	10	15	20	25	Control	5	10	15	20	25
E. coli DH5α	0.688	0.872×10^{-5}	0.17	1.0	1.0	1.2	2.0	3.0	5.2	1.7	1.3	1.2	0.9	0.8
E. coli JM109	0.75	2.88×10^{-5}	0.15	0.5	1.0	2.0	3.0	4.5	4.9	2.4	1.7	1.4	0.9	0.8
S. cerevisiae BJ5418	2.188	2.29×10^{-5}	2.5	8.0	10.0	12.0	14.0	-	5	2.8	1.5	1.0	0.9	-
S. cerevisiae ABC261	1.875	2.19×10^{-5}	1.6	3.0	6.0	8.0	8.0	-	8.2	6.0	1.7	1.2	1.1	-
S. pombe ABP20	1.75	6.31×10^{-6}	2.5	10.0	20.0	-	-	-	10.6	1.7	1.0	-	-	-
S. pombe ABP4	1.25	3.16×10^{-6}	2.0	12.0	-	-	-	-	11.9	1.8	-	-	-	-

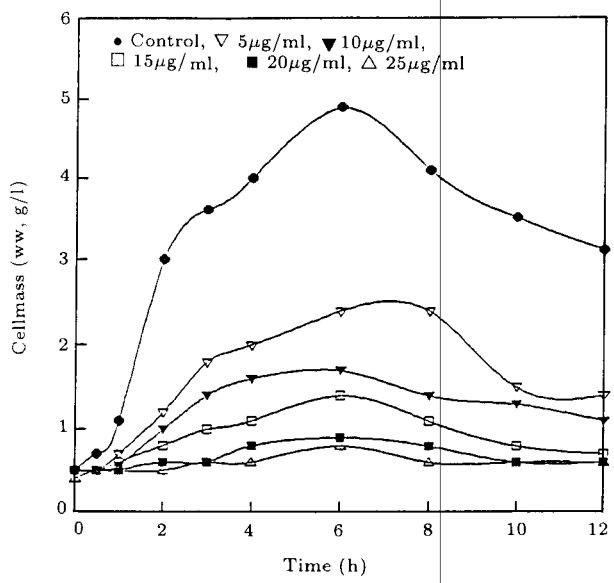

Figure 2. Growth of *E. coli* JM109 at different concentrations of Crystal Violet.

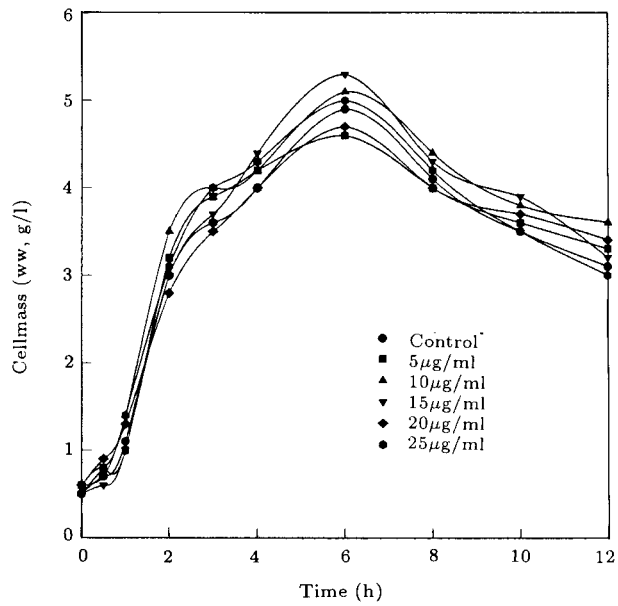

Figure 3. Growth of *E. coli* JM109 at different concentrations of treated Crystal Violet.

of Crystal Violet (20-25 μg/ml), there was no growth. This indicates that Crystal Violet is toxic to *E. coli* JM109. With very low concentration of Crystal Violet (5 μg/ml), the organism grew with a reduced growth rate and maximum cell mass obtained was less (2.4 g/l) than that of the medium having no Crystal Violet. The growth characteristic of *E. coli* JM109 is shown in Figure 3, in the medium containing different concentrations of treated dye. It is clear that the decolorized products of Crystal Violet did not have any inhibitory effect on the growth of *E. coli* JM109. The product concentrations were expressed in terms of the amount of dye taken initially for decolorization. The cell mass obtained with the highest concentration of product (25 μg/ml) was 4.4 g/l (ww), whereas with the same concentration (25 μg/ml) of Crystal Violet in the medium, the organism did not grow. Experiments were also carried out with other organisms (data not shown). In Table 1, the lag periods of growth and maximum cell mass obtained with different concentrations of Crystal Violet are provided. It is evident from Table 1 that all the tested organisms have a high lag period in the presence of dye compared to the control containing no dye. It is also evident that increasing the concentration of dye in the growth medium results in longer lag periods. For *E. coli* DH5α and *E. coli* JM109, the maximum lag period differed by 1.5 h at 25 μg/ml Crystal Violet. *E. coli* DH5α was less sensitive compared to *E. coli* JM109, although in both cases the organism did not grow well with higher concentration of Crystal Violet. A similar case was observed for *S. cerevisiae* strains. It seems that *S. cerevisiae* ABC261 is more resistant to dye compared to *S. cerevisiae* BJ5418. The lag period for S. cerevisiae ABC261 at 5 μg/ml Crystal Violet concentration was 3 h, while it was 8 h in the case of *S. cerevisiae* BJ5418. In addition to demonstrating longer

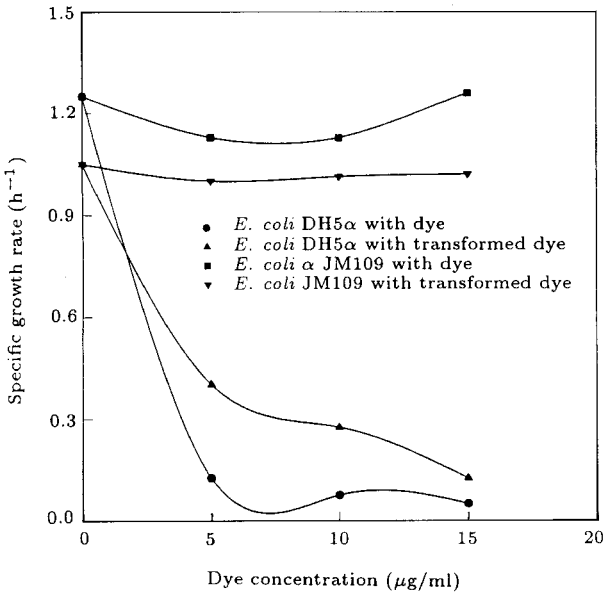

Figure 4. Effect of Crystal Violet concentration (untreated and treated) on the specific growth rate (h^{-1}) of *E. coli* DH5α and *E. coli* JM109.

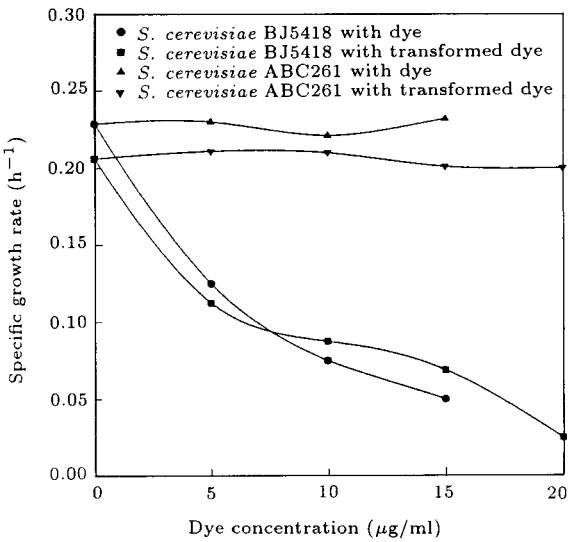

Figure 5. Effect of Crystal Violet concentration (untreated and treated) on the specific growth rate (h^{-1}) of *S. cerevisiae* BJ5418 and *S. cerevisiae* ABC261.

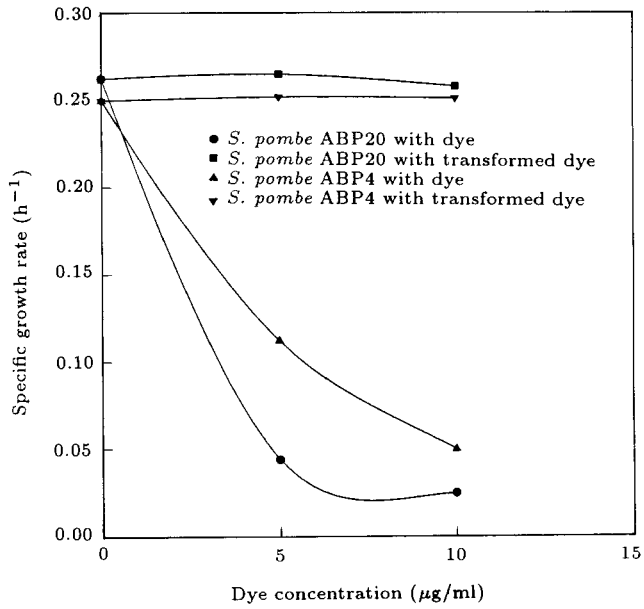

Figure 6. Effect of Crystal Violet concentration (untreated and treated) on the specific growth rate (h^{-1}) of *S. pombe* ABP20 and *S. pombe* ABP4.

lag periods, maximum cell mass concentrations were reduced by a considerable amount when the organisms were grown in the presence of dye. The specific growth rate values were calculated from the growth data obtained for each individual organism. In the case of *E. coli* DH5α, the specific growth rate decreased sharply with 5 μg/ml Crystal Violet and remained more or less the same with the higher concentration of dye. The specific growth rate also decreased (1.13 h^{-1}) with 5 μg/ml decolorized Crystal Violet and its value remained more or less constant with the higher concentration of decolorized dye. A similar situation was obtained with *E. coli* JM109. Experiments were also conducted with different strains of *S. cerevisiae* and *S. pombe*. Reduction in specific growth rates in the presence of Crystal Violet was observed in both cases. It is evident from Figures 4 to 6 that with higher concentration of Crystal Violet, the specific growth rates become noticeably lower. As the concentration of Crystal Violet in the medium is increased, the specific growth rate values start decreasing; however, with the same concentration of treated dye, the specific growth rates do not decrease. This clearly illustrates that the decolorized form of Crystal Violet does not have any inhibitory effect on the growth of test organisms used.

Toxicity in Terms of Inhibition of Growth

The degree of growth inhibition, H, is defined [12] as:

$$H = 1 - (k_d/k_o), \tag{1}$$

where k_d and k_o are the mean specific growth rate constants with and without dye, respectively. It has been shown experimentally for many kinds of inhibitive substances that the degree of inhibition is:

$$H = G^n/(\Phi^n + G^n), \tag{2}$$

where n is the exponent indicating inhibitive ability, G is the concentration of inhibitive substance and Φ is the concentration of the inhibitive substance at $H = 0.5$ [13]. Equation 2 can be rewritten as:

$$\log[H/(1-H)] = n\log G - n\log\Phi. \tag{3}$$

H-values were calculated from the growth curves and Equation 1; moreover, a graph was plotted between $\log[H/(1-H)]$ and $\log G$ (Figure 7). The values of n and Φ were determined, respectively, from the slope of the straight line and G value at $\log[H/(1-H)] = 0$ and are listed in Table 1 for the different test organisms used. It is known that Crystal Violet has a strong sterilizing property [14,15] and strongly inhibits the cell growth. The mean growth rate at the logarithmic phase and the cell concentration at the stationary phase decreased with an increase in the concentration of dye. Out of six strains tested, both *S. pombe* strains were very sensitive to the dye. For both *S. pombe* strains, the Φ values were very less compared to other strains tested. This also corroborates the findings that at 5 μg/ml Crystal Violet, the lag period was maximum (10-12 h). As a result, minimum cell concentration was obtained with both *S. pombe* strains. *S. cerevisiae* strains were moderately sensitive to the dye but they did not grow well at the dye concentration of 10 μg/ml. The inhibitive ability (n) with both strains of *S. cerevisiae* was found to be higher than that of the other organisms tested and Φ values were also higher than *S. pombe*, similarly, cell growth was on the higher side with *S. cerevisiae*. Both strains of Gram negative bacteria (*E. coli* DH5α and *E. coli* JM109) demonstrated a gradual decrease in mean growth rate of the cell population at logarithmic phase and also in cell concentration at stationary phase with an increase in dye concentration from 5 to 25 μg/ml. In case of *E. coli* strains, the inhibitive ability (n) was found to be minimum. Out of six strains tested, the lowest Φ values were obtained for the *S. pombe* strains.

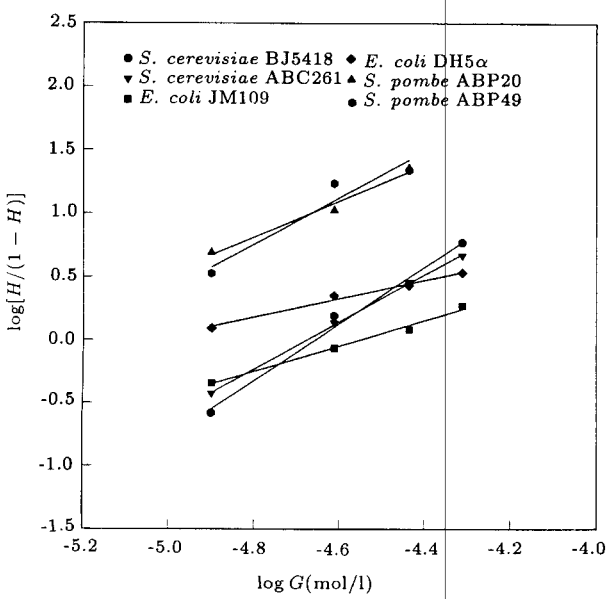

Figure 7. Relationship between $\log G$ and $\log[H/(1-H)]$ for different test organisms with Crystal Violet.

DISCUSSION

It is well-known that Crystal Violet, a typical triphenylmethane dye, is recalcitrant in nature [16]. Crystal Violet has a strong sterilizing property [14] and due to its toxic nature it is very difficult for many bacteria to degrade it. In 1991, Yatome et al. [11] reported the degradation of Crystal Violet by *Bacillus subtilis* IFO13719. They observed that at a very low level of dye concentration (below 7×10^{-6} mole/l), the decolorization of Crystal Violet occurs, however, they did not mention the toxicity status of the treated Crystal Violet. There are no report available in the literature regarding the toxicity of the treated dyes by various microorganisms. In this paper, the toxicity of Crystal Violet (on the basis of inhibition of growth) for various test organisms has been shown and also the toxicity of treated Crystal Violet (decolorized by *Bacillus sp.* MTCC B0006) has been tested under the same condition by the same test organisms.

Toxicity is a relative property of a chemical which refers to its potential to have a harmful effect on living organisms. It can be expressed as a function of chemical concentration and the duration of exposure. It has been found, in general, that bacteria are one of the most insensitive organisms to the toxicants [17]. In fact, there are very few reports of chemical toxicity to microorganisms at a concentration below which the higher animals are adversely affected. The relative insensitivity of bacteria to toxicants is primarily due to the bacterial metabolic processes. Bacteria are small organisms whose metabolic processes have been reduced to a minimum so that they efficiently utilize carbon and energy sources and reproduce rapidly. The simplicity of their metabolism and macromolecular organization is not generally the characteristic of higher organisms in which the metabolic and structural complexity and specialization are common. It is often due to this specialization that certain higher organisms are more sensitive to toxicants. The widespread existence of bacteriostatic and bactericidal agents clearly indicates that the bacteria are susceptible to the action of various chemicals which depending on their nature, concentration, etc., either inhibit bacterial growth or kill bacteria.

Gram negative bacteria are generally more tolerant to toxic chemicals than Gram positive bacteria (like *Bacillus sp.* MTCC B0006). This tolerance has been attributed to a penetration barrier outside the cytoplasmic membrane [18]. Gram negative organisms such as *E. coli* have high isoelectric point and contain less acidic components than Gram positive bacteria; therefore, they combine with Crystal Violet less readily and resist more to dye [14]. Wild type strain of *E. coli* K-12 adsorbs Crystal Violet on the cell surface,

but the dye is not transported into the cytoplasm. However, when some mutants, which have an altered outer membrane, are exposed to Crystal Violet, the dye is also found in the ribosomal fraction [19]. The rate of transport of the dye into the cytoplasm is much lower for mutant cells at stationary phase than the exponentially growing cells. Crystal Violet is a cation and binds to negatively charged particles like ribosomes [20] and its uptake by *Neisseria gonorrhoea* was the result of both cell envelope adsorption and cell membrane permeability. About half of the Crystal Violet taken up by the cells was found in the cytoplasm. In the experiment conducted here, *E. coli* was found to be the most resistant to the Crystal Violet in comparison to *S. cerevisiae* and *S. pombe*. The inhibitive abilities (n) for both *E. coli* strains were found to be minimum. The lowest Φ values were observed for both *S. pombe* strains.

Crystal Violet slows the growth of microorganisms and at higher concentration inhibits it altogether. With increasing dye concentration in the test organisms growing medium, the lag period increases and cell concentration decreases (Table 1). It was found that decolorization rate was directly proportional to the cell mass. When the cell mass concentration is increased, cells start growing at their maximum specific rate (μ_{max}). This phenomenon was observed in the case of decolorization of Crystal Violet and other triphenylmethane dyes by the growing cells of *Bacillus sp.* MTCC B0006 (data not shown). The theory concerning bacterial response to the growth inhibitors like Crystal Violet is directly based on the kinetics of the enzyme inhibition and leads to a rather general assumption that *Bacillus sp.* MTCC B0006 behaves as an enzyme. This theory was supported by the fact that when the inhibitor (Crystal Violet) was decolorized, biocatalyst (*Bacillus sp.* MTCC B0006) started behaving normally. This model is not competitive in nature as Crystal Violet did not compete with the growth limiting substrate (glucose) for uptake.

In the present study, the specific growth rates of *S. cerevisiae* and *S. pombe* strains were calculated in the presence of untreated and treated Crystal Violet from the growth data. In the case of *S. cerevisiae* BJ5418, the specific growth rate decreased from 0.229 to 0.125 h^{-1} in the presence of 5 μg/ml Crystal Violet but it remained the same in the presence of treated Crystal Violet (up to 15 μg/ml). *S. pombe* ABP20 showed a similar pattern, but decrease in the specific growth rate was higher than *S. cerevisiae* (decrease was observed from 0.262 to 0.044 h^{-1} in the presence of Crystal Violet (5 μg/ml) in the growth medium). From these specific growth rate data, it is very clear that Crystal Violet is significantly toxic to *S. pombe*. This data relates to the earlier reports stating that Crystal Violet is toxic to higher eukaryotic systems; however, treated Crystal Violet was found to be non-toxic for the *S. pombe* strains.

In addition to the effect of Crystal Violet on specific growth rate, it may also depress cell mass yield coefficient by uncoupling growth from carbon energy substrate oxidation to varying degrees. Such mechanisms are clearly advantageous in wastewater treatment where minimization of sludge production is an established process objective. Consequently, *Bacillus sp.* MTCC B0006 can be used to treat wastewater containing Crystal Violet at slower growth rate with less cell mass (hence, less sludge) production and the resulting treated dye will be non-toxic in nature as tested microbiologically.

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