Bioremediation of methylene blue dye using *Bacillus subtilis* MTCC 441

Ganta Upendar, Susmita Dutta, Pinaki Bhattacharya and Abhishek Dutta

ABSTRACT

Methylene blue (MB) commonly found in the textile industry effluent has been chosen as a model dye to investigate bioremediation using *Bacillus subtilis* MTCC 441. Both free cells and calcium alginate immobilized cells have been used to remove MB from the effluent. The operating variables of initial concentration of dye (20–60 mg/L), inoculum size (4–8%) and temperature (25–35 °C) have been varied judiciously during the kinetic study in a batch contactor. A maximum removal of 91.68% is obtained when 20 mg/L MB solution was inoculated with 8% inoculum and cultured for 6 h at 30 °C. Continuous removal of MB has been studied in a fixed bed contactor using immobilized cells as packing materials. Influent concentration (10–30 mg/L) was varied and breakthrough parameters have been determined. With increase in influent concentration from 10 mg/L to 30 mg/L, percentage removal of dye decreases from 72.44% to 49.62%.

Key words | *Bacillus subtilis* MTCC 441, bacterial cells immobilization, continuous study, kinetic analysis, methylene blue

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NOMENCLATURE

A_0	Absorbance of dye solution before treatment
A_t	Absorbance of dye solution after treatment with cells at time 't'
C_0	Initial concentration (mg/L)
C_r	Concentration of the dye removed (mg/L)
C_t	Concentration at time t (mg/L)
F	Flow rate (1.0×10^{-3} dm^3/min)
H	The bed height (m)
K_M	Substrate saturation constant or Monod constant (g/L)
m_t	Total amount of dye entering the column (mg)
N	The number of experimental runs
q_{ce}	The equilibrium uptake capacity of the column (mg/g)
q_t	Total mass of the dye removed (mg)
S	Substrate concentration (g/L)
S_0	Initial substrate (glucose) concentration (g/L)
t_b	Breakthrough time (min)
V_{ef}	The volume of effluent (1.0×10^{-3} dm^3)
w	Mass of the bead as used in bed (g)
X_0	Initial biomass concentration (g/L)
$X_{\exp,i}$	Experimental biomass concentration (g/L)
$X_{sim,i}$	Simulated biomass concentration (g/L)
$Y_{X/S}$	Yield coefficient (g/g)
y	The percentage removal of dye
Z	The carboxyl or hydroxyl functional group
μ	Specific growth rate (h^{-1})
μ_{max}	Maximum specific growth rate (h^{-1})

INTRODUCTION

Dyes are extensively used in many industries such as leather, textile, pulp and paper production, food processing and cosmetics (Tan *et al.* 2007). With an ever-increasing world population, textile production remains one of the prominent industries that require large amounts of water and produce highly polluted wastewater (Berrios *et al.* 2012) which may cause severe environmental pollution if it is released to the environment without any proper treatment (Dutta *et al.* 2011). The main problem involved in treating textile wastewaters is the removal of colour, since there is no single process available for treating such effluents (Berrios *et al.*

doi: 10.2166/wst.2017.031

2012). Methylene blue (MB), a heterocyclic aromatic compound with the molecular formula $C_{16}H_{18}N_3SCl$, is widely used in different fields, especially in the textile industry (Guo *et al.* 2014). Although it is not regarded as a highly toxic dye, it can have various harmful effects on human beings and aquatic systems (Yang *et al.* 2011). According to the Bureau of Indian Standards the desirable and permissible limits of dye in wastewater are 5 and 25 Hazen units, respectively (Bureau of Indian Standard (BIS) 2012). The discharge of coloured waste into the environment not only affects the aesthetic nature but also affects the aquatic systems by obstructing the penetration of sunlight into streams and thereby reducing the photosynthetic action (Lata *et al.* 2007). To date various methods have been used for the removal of textile dye such as activated sludge process, flotation, coagulation/flocculation, filtration, ozonation, photocatalysis, electrolysis, Fenton-biological treatment process and adsorption on activated carbon (Vandevivere *et al.* 1998). Biological approaches are proven to be potentially effective. The main advantages of bioremediation of pollutant by various biological species are high selectivity, cost-effectiveness and good removal efficiency (Aksu *et al.* 2010). Bioremediation of pollutants occurs through two different routes, namely biosorption and bioaccumulation. While the removal using dead biomass of microorganisms, namely bacteria, fungus, algae, etc., occurs through a biosorption process, the removal using living microorganism takes place through both biosorption and bioaccumulation (Vijayaraghavan *et al.* 2008a; Dutta *et al.* 2015a, 2015b). The metabolic-independent binding of pollutants with the bonds present at the external surface of microbial cells is called biosorption, while metabolic-dependent transport of pollutant from the external environment to the interior of cells is called bioaccumulation. Several works on MB dye removal using different microorganisms such as fungi, bacteria (Santos *et al.* 2007), green algae and cyanobacteria (EI-Sheekh *et al.* 2009) have been published; however, detailed investigation on the application of *Bacillus subtilis* on MB removal is yet to be done. *Bacillus* sp. such as *Bacillus subtilis* and *Bacillus cereus* have several advantages such as high biomass growth rate, easy availability from standard microbial collection centres, and capability of treatment of industrial wastewater. For example, *B. subtilis* has been effectively used in the biodegradation of reactive red M5B dye (Gunasekar *et al.* 2013), and *Bacillus* sp. strain AK1 (Anjaneya *et al.* 2013) has been used to remove amaranth dye. Since utilization of immobilized cells has several advantages, such as easy separation of cells for further use, enhancement of chemical stability, etc., bioremediation using immobilized cells may be a preferred option. Although immobilization of *B. subtilis* using layered double hydroxide (LDH) for decolorization of MB was performed by Liu *et al.* (2014), the preparation of LDH is a rather complex method. In the present study, a relatively simple method of immobilization of *B. subtilis* MTCC 441 using calcium alginate by ionotropic gelation technique has been implemented. An effort has been made to remove MB from its aqueous solution using both free and calcium alginate immobilized cells of *B. subtilis* MTCC 441. Bioremediation of MB has been investigated with time in a batch contactor. Furthermore, to examine the efficiency of the process, a continuous column study has been performed using the immobilized bacterial cells as packing material.

MATERIALS AND METHODS

All the materials used were of AR grade and purchased from Merck, India.

Collection and culturing of microorganisms

Bacillus subtilis MTCC 441, the microbial strain used in the present study, was procured from Microbial Type Culture Collection and Gene Bank (MTCC), Chandigarh, India. The strain was cultured in nutrient broth medium (beef extract: 2.0 g/L, yeast extract: 1.0 g/L, peptone: 5.0 g/L, NaCl: 1.0 g/L) as prescribed by MTCC (Binupriya *et al.* 2010). Bacterial strains were maintained in a medium containing 2% nutrient agar.

Study of growth kinetics of *B. subtilis* MTCC 441 in synthetic substrate

The synthetic substrate (K_2HPO_4: 12.18 g/L, KH_2PO_4: 4.08 g/L, $(NH_4)_2SO_4$: 3.3 g/L, $MgSO_4$: 0.06 g/L, $MnSO_4$: 0.00151 g/L, $C_6H_{12}O_6$: 1.0 g/L) containing glucose as a sole carbon source is used for the study of growth kinetics of *B. subtilis* MTCC 441 (Kruyssen *et al.* 1980). Initially, 500 mL of medium was prepared and autoclaved at 15 psig for 20 min. The whole medium was equally distributed into several Erlenmeyer flasks aseptically. The medium was then inoculated with the strain from the agar plate using a platinum wire loop. The flasks were placed in a BOD incubator-shaker (Modern Instrument, Kolkata, India) and shaken at 110 rpm for 24 h at 30 °C. The flasks were collected after every 2 h and centrifuged at 6,000 rpm for 10 min using a laboratory centrifuge (ELTEK TC8100F, India). The weight of dry biomass was measured in each case following the standard method. To assess the effect of carbon source on the

growth of the said bacterial strain, the glucose concentration was varied in the range of 0.25 to 1.5 g/L in the medium. The growth kinetics of *B. subtilis* MTCC 441 was examined in terms of its dry biomass content.

Immobilization of *B. subtilis* MTCC 441 in calcium alginate bead

B. subtilis MTCC 441 was immobilized in calcium alginate beads for the removal of dye. Immobilization of alginate beads was done following a standard protocol (Daassi *et al.* 2014). Synthetic substrate was inoculated with the bacterial cells and the cells were allowed to grow in a BOD incubator-shaker at 30 °C with agitation speed of 110 rpm for 18 h. Since the log phase exists up to 18 h, the 18 h culture was harvested by centrifugation at 6,000 rpm for 10 min. The collected biomass was washed twice with sterile distilled water. The bacterial biomass (1.0845 g of biomass) was added to 150 mL of sterile 3% sodium alginate under aseptic conditions. The alginate–cells mixture was added dropwise into cold and sterile 0.2 M $CaCl_2$ solution (Benhouria *et al.* 2015). The resultant alginate beads were allowed to harden by resuspending in fresh $CaCl_2$ solution for 24 h at 4 °C. The excess calcium ion was then removed by washing the beads with distilled water. The washed beads were kept in water and stored at 4 °C.

Characterization of free and alginate immobilized *B. subtilis* MTCC 441

Fourier transform infrared (FTIR) spectroscopy (Nicolet iS10, Thermo Fischer Scientific, USA) study was done to determine the functional groups present in the bacterial cells. A simulated solution of MB (20 mg/L) was inoculated with 4% living free cells and shaken at 150 rpm for 14 h in a BOD incubator-shaker at 30 °C. The solution was centrifuged and the spent biomass was collected and free cells both before and after treatment with MB were used for FTIR analysis. At this stage, the free cells, before and after treatment of MB, were lysed through sonication. The lysed product was then centrifuged at 6,000 rpm for 10 min. The supernatant was collected and used for FTIR analysis. The purpose of using intracellular fluid obtained from free cells before and after MB treatment is to assess the mechanism of binding of MB with cells. In another experiment, the synthetic solution was contacted with 3.0 g alginate bead for 3 h in the same incubator at 30 °C. The beads were separated and used for FTIR analysis.

Bioremediation of MB using free cells of *B. subtilis* MTCC 441 in batch contactor

The culture medium was prepared and autoclaved at 120 °C for 20 min. MB solutions of different concentrations were prepared from the sterile stock solution (100 mg/L) by diluting it with sterile synthetic substrate under aseptic condition in a bio-safety cabinet (Lunar, India). The simulated solution of MB was then inoculated with the bacterial strain. The flasks were kept in a BOD incubator-shaker at 30 °C and shaken at 150 rpm for 6 h. The samples were collected after a particular time interval and centrifuged at 6,000 rpm for 10 min. The supernatant was analysed for residual MB concentration using a UV-Vis spectrophotometer (UV-VIS -2300, TECHCOM) at a wavelength of 660 nm. The equation for calculation of decolorization vis-à-vis percentage removal of MB is as follows:

$$\%\ \text{Removal of methylene blue} = \frac{(A_0 - A_t)}{A_0} \times 100 \quad (1)$$

Removal of MB in fixed bed contactor under continuous mode

For the continuous study, a fixed bed contactor (internal diameter: 2.36 cm and column length 12 cm) made up of borosilicate glass was used. The removal was carried out under ambient temperature of 30 °C. The *B. subtilis* MTCC 441 immobilized alginate beads were used as packing material in the contactor and the bed volume of 35×10^{-6} m^3 was maintained by keeping a constant bed height of 0.08 m. Removal of MB in the column contactor was studied with varying initial concentration of MB, keeping other variables like flow rate and bed height constant. The column was operated for 5 h. The effluent samples were collected from the top of the column at regular intervals of time. The samples were then analysed for residual MB.

Desorption study

For the desorption study, both MB-loaded free cells (0.1 g) and immobilized cells (1.0 g) were treated individually with 50 mL of 0.1 N HCl. The flasks were kept in a BOD incubator at 25 °C and shaken at 150 rpm for 2 h for free cells and 4 h for immobilized cells. The solutions obtained after separation of cells, either free or immobilized, were analysed for desorbed MB.

RESULTS AND DISCUSSION

Growth study of *B. subtilis* MTCC 441 in synthetic substrate

The growth of *B. subtilis* MTCC 441 in synthetic substrate in terms of its dry biomass content is shown in Figure 1(a). The concentration of glucose (i.e. the sole carbon source) was varied in the range of 0.25–1.5 g/L. From the figure, it is evident that the lag phase extended up to 2 h and the stationary phase started at 18 h. The biomass increased from 0.592 g/L to 0.948 g/L during the log phase when initial glucose concentration was kept at 1.0 g/L, the original concentration of glucose present in synthetic substrate.

To represent the growth of *B. subtilis* MTCC 441 in synthetic substrate, the classical Monod model was assumed (Kushwaha *et al.* 2014).

$$\mu = \frac{\mu_{\max}[S]}{K_M + [S]} \tag{2}$$

The values of $\mu_{\max}$ and K_M were found out by non-linear regression analysis and their values were 0.152 h^{-1} and 2.0 g/L, respectively. The values of dry biomass during the log phase were used for analysis of the growth kinetic model. The value of $Y_{X/S}$ was found to depend on the ratio of initial biomass concentration and initial substrate concentration. Mathematically it can be expressed as,

$$Y_{X/S} = 0.232 \exp\left[0.955 * \left(\frac{X_0}{S_0}\right)\right] \tag{3}$$

The growth kinetic equations to show the variation of biomass and substrate with time during the exponential phase for batch study can be expressed by the following differential equations:

$$\frac{dX}{dt} = \mu X \tag{4}$$

$$\frac{dS}{dt} = -\frac{dX/dt}{Y_{X/S}} \tag{5}$$

Initial condition: at $t = 0$, $X = X_0$ and $S = S_0$ (5a)

Equations (4) and (5) were solved simultaneously using the values at initial condition and the evaluated parameters such as $\mu_{\max}$, K_M and $Y_{X/S}$ with the help of the fourth order Runge Kutta method. The simulated biomass concentrations for different glucose concentrations obtained during the log phase have been shown in Figure 1(b). Experimental data were placed on the same figure. To check the validity of the equations, root mean square error (RMSE) values were evaluated using Equation (6).

$$\text{RMSE} = \frac{\sqrt{\sum_{i=1}^{N} (X_{\exp,i} - X_{\text{sim},i})^2}}{N} \tag{6}$$

The RMSE values were found to be in the range of 0.001477 to 0.1486, which proved the suitability of the model to analyse experimental data. The values of minimum, mean and maximum percentage error were found to be 0%, 7.24% and 29.88%.

Characterization study of *B. subtilis* MTCC 441

FTIR spectra of intracellular fluids obtained after sonication of bacterial cells before (Figure 2(a)) and after (Figure 2(b))

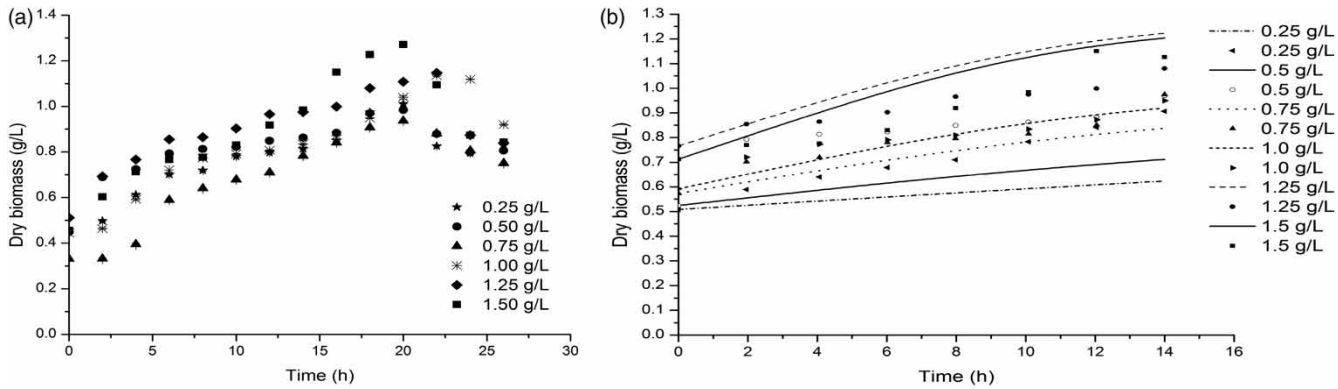

Figure 1 | (a) Study of growth kinetics of *B. subtilis* MTCC 441 in synthetic substrate at various glucose concentrations. (b) Experimental (points) and simulated (lines) values of dry biomass at various glucose concentrations during log phase only.

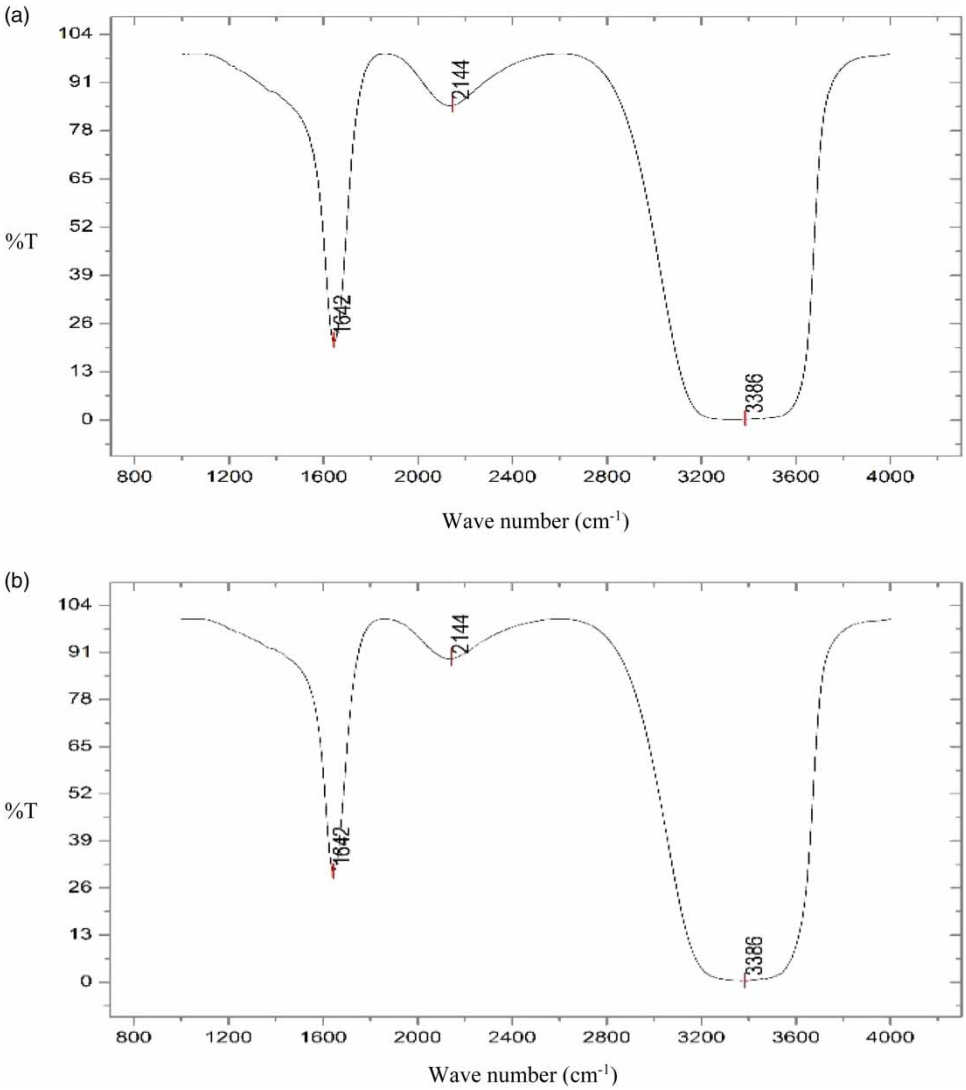

Figure 2 | FTIR graphs of intracellular fluid obtained after sonication of *B. subtilis* MTCC 441 (a) before treatment with MB (b) after treatment with MB.

treatment with MB did not show any variation; instead all the peaks were obtained at the same wave numbers. The peaks at the wave numbers of 1,642, 2,144 and 3,386 cm^{-1}, characterized by the C=C stretch, —C≡C-stretch, and —OH stretch (strong), were found in both the cases. From the results, it is apparent that MB did not penetrate inside the cells during treatment with MB solution.

FTIR studies of free cells before (Figure 3(a)) and after (Figure 3(b)) treatment with MB showed changes in position of peaks. The functional groups of carboxylic acid and C-O aromatic were seen to be present in the FTIR spectrum of free cells before treatment with MB at the wave numbers of 1,246 cm^{-1} and 1,547 cm^{-1}, respectively. However, disappearance of both of these groups in the FTIR spectra of MB-loaded cells implies the probable involvement of these groups in the binding of MB, leading to bioremediation of MB. The functional groups C—O stretch (strong), —CH bending (variable), C=C stretch, —CH stretch (strong), and —OH stretch (vibration of phenolic/carboxylic acid) were shifted from wave numbers of 1,052, 1,409, 1,653, 2,931, and 3,433 cm^{-1} as present in FTIR spectra of native bacterial cells to 1,058, 1,396, 1,641, 2,925, and 3,439 cm^{-1} respectively, in the case of MB-loaded bacterial cells. Several mechanisms may exist for decolorization of dye in bacterial systems. Olivella *et al.* (2012) reported that the interaction between negatively charged anion group (like COO^-) and cations (like MB^+) would be responsible for the shift of chemical bend as observed during biosorption of MB using vegetable wastes. The probable mechanism for bioremediation of MB using *B. subtilis* MTCC 441 has been shown in

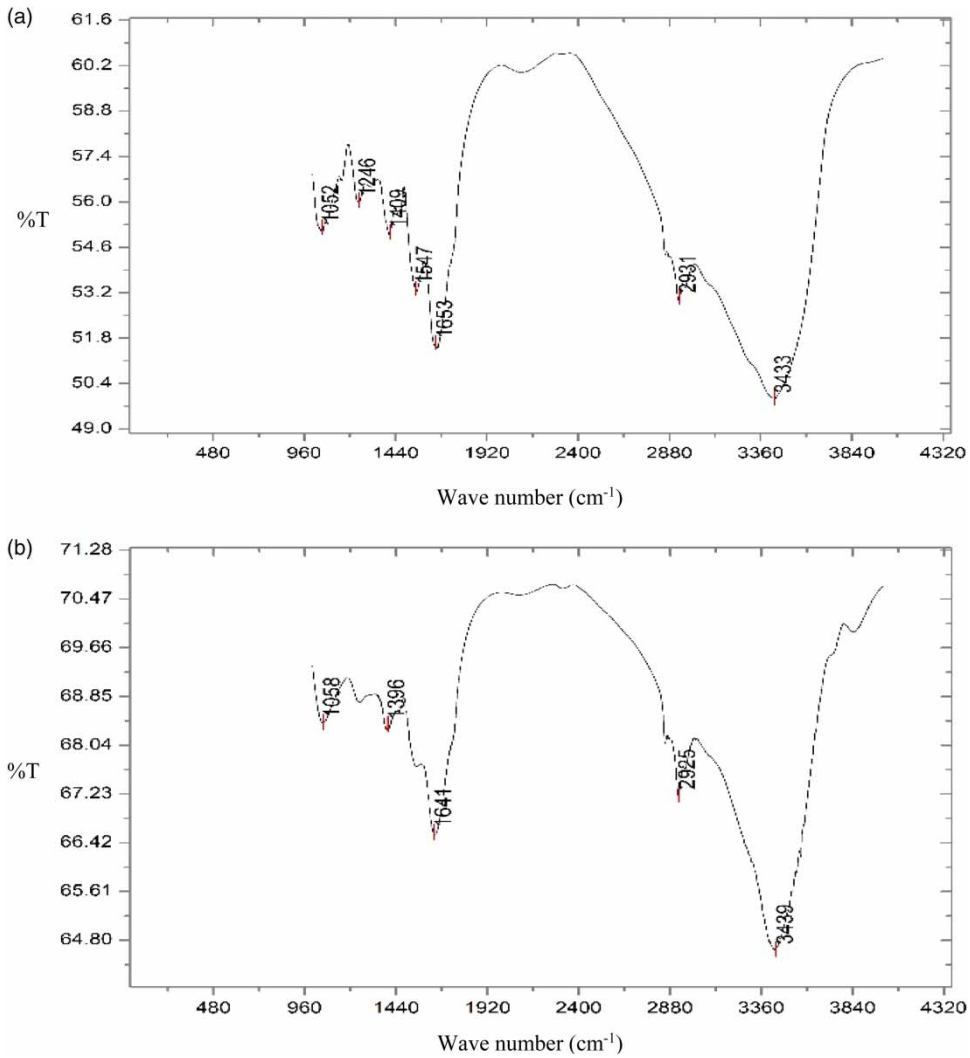

Figure 3 | FTIR graphs of *B. subtilis* MTCC 441 (a) before treatment with MB and (b) after treatment with MB.

Figure 4. MB, being a basic cationic dye, can bind with anionic functional groups like carboxyl or hydroxyl groups present on the cell wall of bacteria. Binupriya *et al.* (2010) reported the presence of such functional groups on the cell wall of *Bacillus subtilis*. In Figure 4, MB cation is associated with chloride ion. The negative functional groups such as carboxyl or hydroxyl group (Z^-) present on the cell wall can bind with cationic dye and lead to biosorption of MB. A similar mechanism was suggested by Vijayaraghavan *et al.* (2008a) for removal of MB using *Corynebacterium glutamium*. The FTIR study of calcium alginate immobilized bacterial cells before and after treatment with MB has been shown in Figure 5(a) and 5(b), respectively. The functional groups C—C, C—O stretch, amine group, O—C stretch, C=C stretch (medium), C=C, C=O, C—H stretch of alkyl acetyls, and —OH stretch corresponding to wave numbers of 969, 1,045, 1,126, 1,239, 1,546, 1,691, 2,086, 2,938, 3,295 cm^{-1} for native bacterial cells were shifted to wave numbers of 964, 1,045, 1,133, 1,220, 1,509, 1,671, 2,091, 2,925, and 3,270 cm^{-1}, respectively, for MB-loaded bacterial cells. The —OH stretch at 3,627 wave number disappeared after treatment with MB. From the result it is clear that alginate, being a polysaccharide, consists of several —OH groups. Therefore, —OH groups present at both the cell surface and immobilization matrix are responsible for binding of MB during treatment of MB solution with immobilized cells.

Bioremediation of MB using free *B. subtilis* MTCC 441 in a batch contactor

To observe the effect of initial concentration of dye on removal of MB using free cells of *B. subtilis* MTCC 441 in

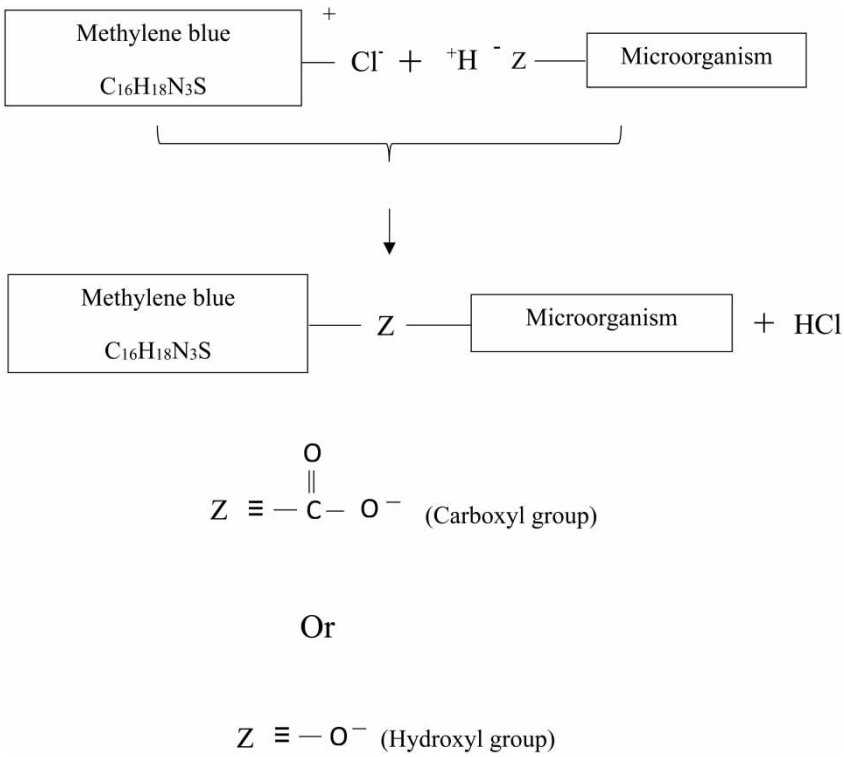

Figure 4 | Probable mechanism of binding of MB with functional groups of the bacterial cells of *B. subtilis* MTCC 441.

a batch contactor, the initial concentration of dye was varied from 20 to 60 mg/L, keeping the other variables constant: inoculum size 4%, temperature 30 °C, and shaking speed 150 rpm. The chemical oxygen demand, biochemical oxygen demand (BOD), pH and temperature of 20 mg/L dye solution was found to be 26.6 mg O_2/L, <2 mg/L, 7, and 25 °C, respectively. The percentage removal of MB decreased from 87.15 to 80.45% with increase in initial concentration from 20 to 60 mg/L (Figure 6(a)). The figure shows that most removal occurred within 2 h and the system attained equilibrium within 6 h. Since most of the removal happened in the lag phase, biosorption at the cell surface is the probable mechanism for MB removal. The cell wall of bacterial species contains several functional groups (such as —COOH and —OH) which are responsible for binding of the basic dyes such as MB. The less removal at higher concentration may be due to saturation of bacterial cells. Similar observation was made by Vijayaraghavan *et al.* (2008b), as they had reported that, with increase in initial concentration of dye, the percentage removal of dye decreased. Since maximum removal occurred at initial dye concentration of 20 mg/L and the equilibrium was attained within 6 h, the cellular growth at such concentration up to 6 h was observed. The growth was compared with that for pure synthetic substrate (Figure 6(b)). It has been found that dry biomass increased 1.67- and 1.50-fold when grown in pure synthetic substrate and in medium contaminated with MB dye, respectively, for the same time period. The growth in the second case suggests that the harmful effect of MB is less. Since there is no evidence of diffusion of MB in the cell interior, as seen in FTIR analysis of cellular fluid, it can be stated that MB may not have any direct effect on the cell growth. However, binding of MB with the functional groups present at the surface of the cell may interrupt the active transport of nutrient from exterior to interior and, thus, less growth is observed.

Removal of MB using free cells of *B. subtilis* MTCC 441 in a batch contactor was done by varying the inoculum sizes from 4 to 8%, keeping the other variables constant: initial concentration 20 mg/L, temperature 30 °C, and shaking speed 150 rpm. The percentage removal of MB increased from 87.15 to 91.68% with increase in inoculum size from 4 to 8% (Figure 7(a)). With increase in inoculum size, the number of microorganisms increases and thereby the surface area for biosorption increases, and higher removal occurs. Liu *et al.* (2014) observed that with increase in inoculum size of *B. subtilis* the percentage removal of MB increased. Since highest removal was obtained with 8%

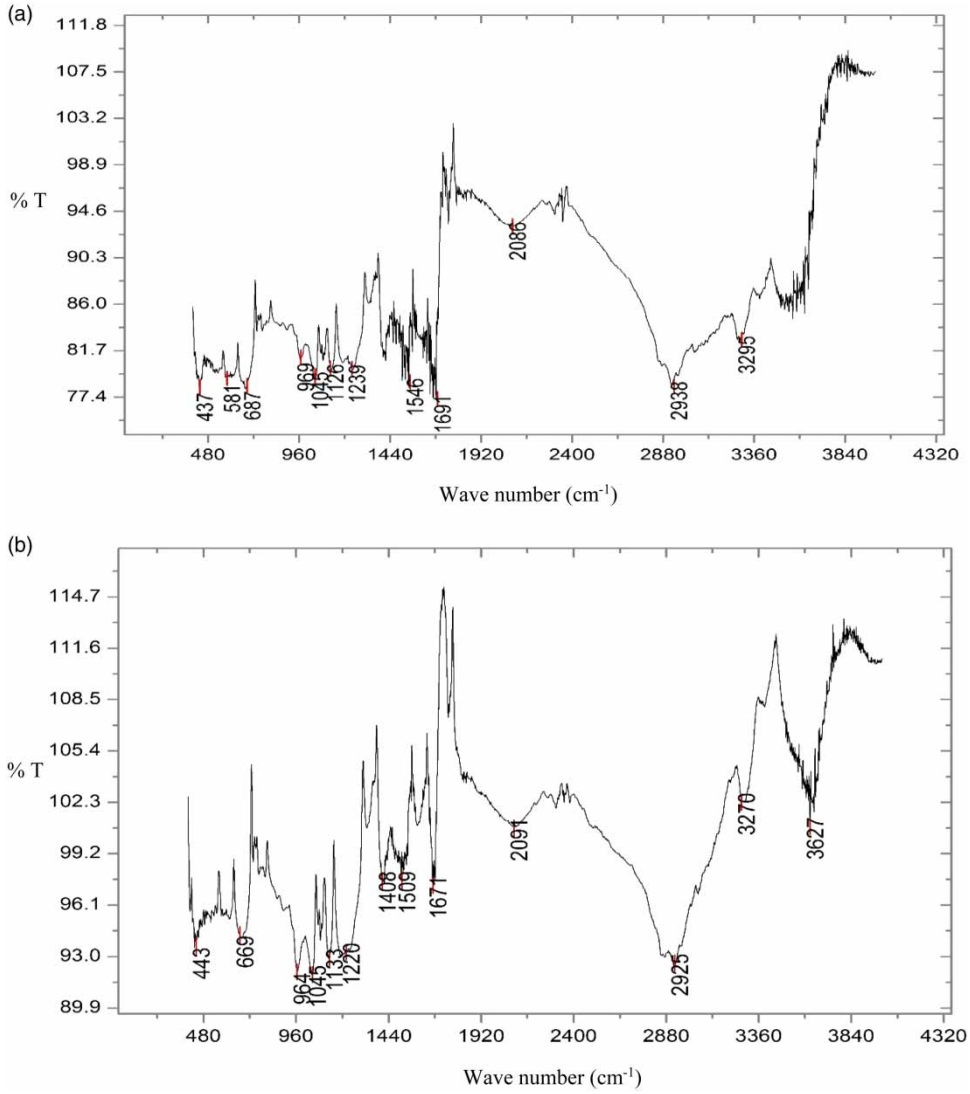

Figure 5 | (a) FTIR graph of immobilized *B. subtilis* MTCC 441 before treatment with MB. (b) FTIR graph of immobilized *B. subtilis* MTCC 441 after treatment with MB.

inoculum, the growth of bacterial cells was observed in 20 mg/L dye solution inoculated with 8% inoculum at 30 °C for 6 h. Growth of bacterial cells was also observed in pure synthetic substrate inoculated with 8% inoculum under identical condition and compared with the previous one (Figure 7(b)). It is seen that dry biomass increased 1.323- and 1.23-fold when grown in pure synthetic substrate and in medium contaminated with MB dye, respectively, for the same time period. This may be because of the interruption of active transport of nutrient from exterior to interior due to blockage of functional groups present at the cell wall after binding with MB.

To assess the effect of temperature on the removal of MB using free cells of *B. subtilis* MTCC 441, the temperature was varied from 25 to 35 °C, and other variables were kept constant as initial concentration 20 mg/L, inoculum size 4%, and shaking speed 150 rpm. The percentage removal of MB increased from 84.21 to 88.91% with increase in temperature from 25 to 35 °C (Figure 8(a)). The greater removal at higher temperature may be due to increase in diffusion rate of MB from bulk solution to the surface of the cell wall. Liu *et al.* (2014) reported the same – higher removal of MB at higher temperature. Since maximum removal was obtained at 35 °C, the growth of bacterial cells in the MB dye solution at such temperature was observed for 6 h. The growth of bacterial cells in dye solution was compared with the growth of bacteria in synthetic substrate at the same temperature under identical condition

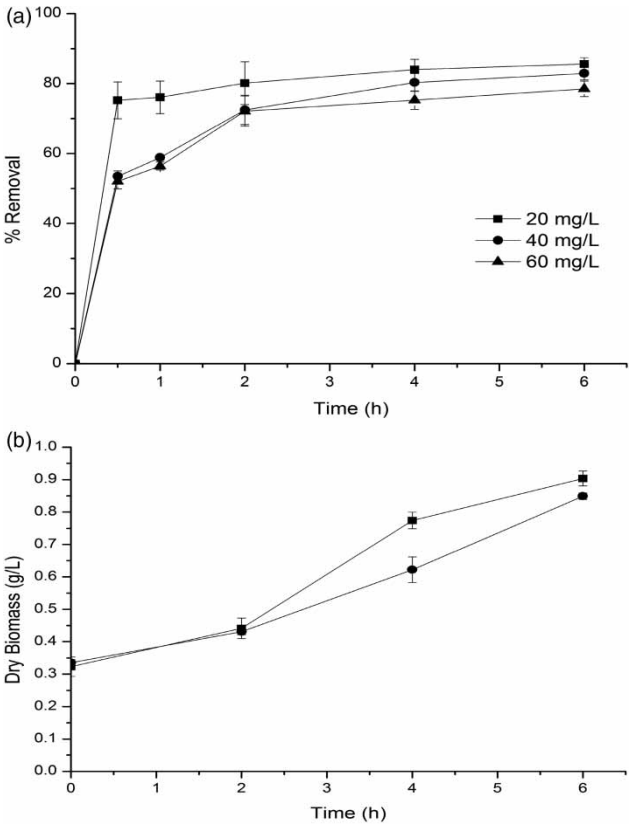

Figure 6 | (a) Percentage removal of MB using *B. subtilis* MTCC 441 at various initial concentrations of MB. (b) Comparison of growth of bacteria in synthetic substrate and synthetic MB solution. Concentration = 20 mg/L, inoculum size = 4%, temperature = 30 °C.

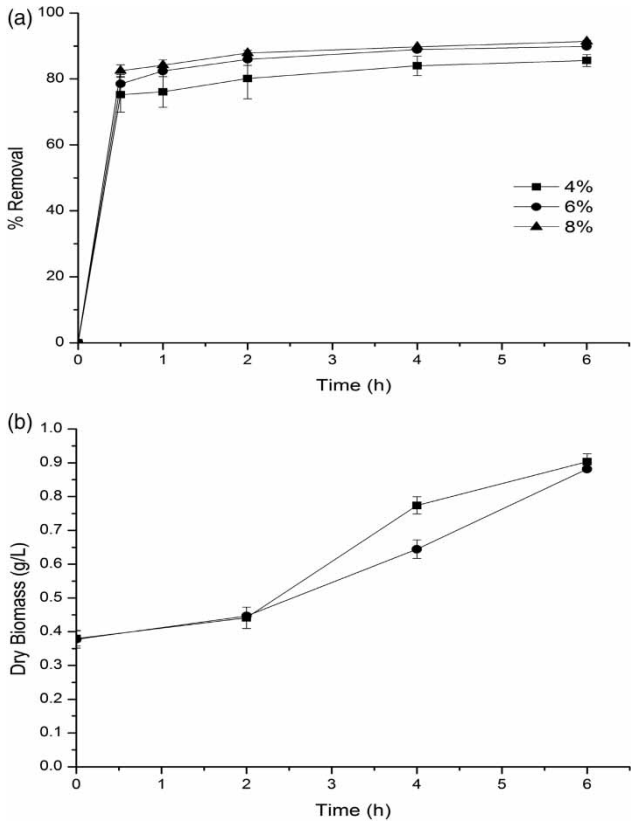

Figure 7 | (a) Percentage removal of MB using *B. subtilis* MTCC 441 at various inoculum sizes. (b) Comparison of growth of bacteria in synthetic substrate and synthetic MB solution. Concentration = 20 mg/L, inoculum size = 8%, temperature = 30 °C.

(Figure 8(b)). Although growth was observed in unsupplemented dye media, the difference in amount was not significant. Dry biomass increased 1.53 times and 1.4 times when grown in pure synthetic substrate and in medium contaminated with MB dye, respectively, for the same time period. It can therefore be inferred that temperature does not have significant effect on growth under the range studied.

Removal of MB using immobilized *B. subtilis* MTCC 441 in continuous contactor

To assess the applicability of treatment with immobilized cells, removal of MB in a fixed bed contactor was studied in continuous mode with a bed volume of 35×10^{-6} m^3 using immobilized cells as packing material. A schematic sketch of the experimental set-up is seen in Figure 9.

The breakthrough time is defined as the time when the effluent concentration reaches 50% of the influent concentration (Liang *et al.* 2014). The time required for the effluent concentration to reach 90% of the influent concentration is known as exhaustion time. Removal of MB using immobilized cells of *B. subtilis* MTCC 441 in A column contactor was also studied with varying initial concentrations of MB from 10 to 30 mg/L, keeping other variables constant: flow rate 1.0 mL/min (1.0×10^{-3} dm^3/min) and bed height 0.08 m. The effluent samples were analysed to determine the residual MB concentration. The breakthrough curves have been shown in Figure 10, and the corresponding parameters are given in Table 1.

The percentage removal of MB decreased from 72.44 to 49.62% and breakthrough time increased from 41 to 90 min with an increase in initial concentration of dye from 10 to 30 mg/L as shown in Figure 10. This may be due to the saturation of the bed at higher concentration of dye. Similar findings were reported by Vijayaraghavan *et al.* (2008b). According to their study, with increase in initial concentration of dye, the uptake of dye increased; however, the percentage removal of dye decreased. At higher concentrations of MB dye, the sorption sites decrease compared to the moles of MB dye present.

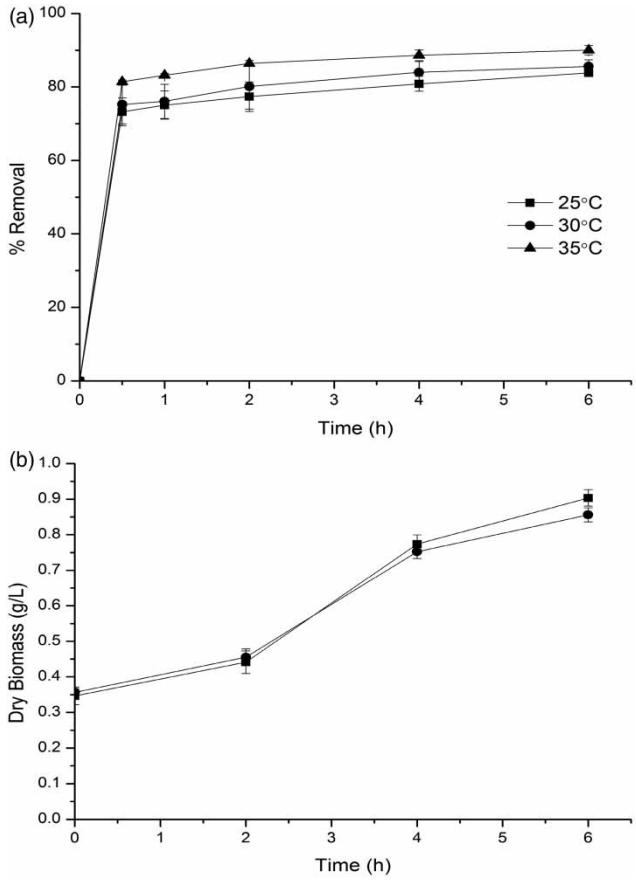

Figure 8 | (a) Percentage removal of MB using *B. subtilis* MTCC 441 at various temperatures. (b) Comparison of growth of bacteria in synthetic substrate and synthetic MB solution. Concentration = 20 mg/L, inoculum size = 4%, temperature = 35 °C.

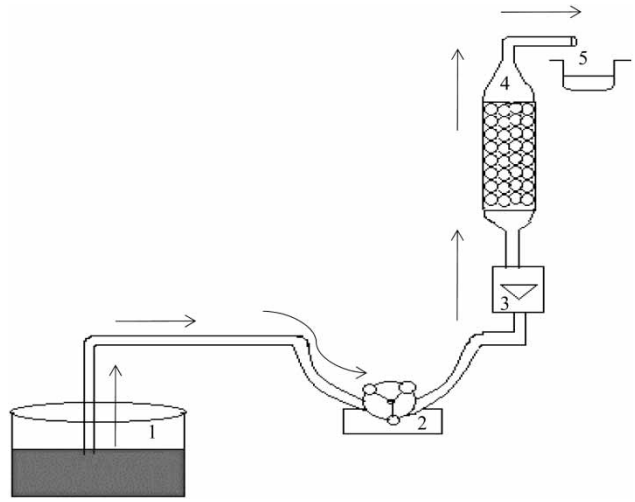

Figure 9 | The schematic representation of experimental set-up for column study. 1. MB stock solution, 2. peristaltic pump, 3. rotameter, 4. fixed bed contactor, 5. effluent.

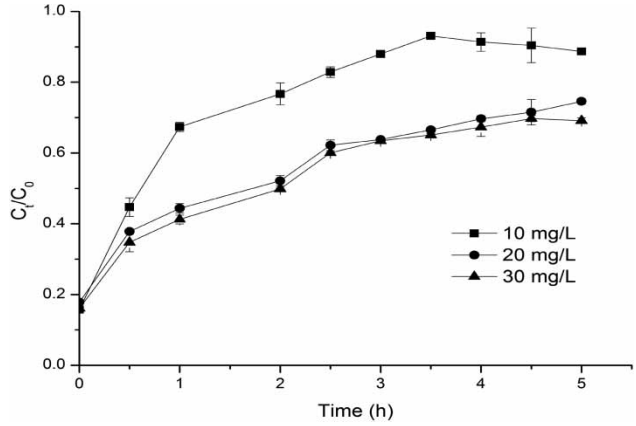

Figure 10 | Effect of influent concentration of MB on breakthrough curve at constant bed height (0.08 m) and influent flow rate (1.0×10^{-3} dm^3/min).

The empty bed contact time (EBCT) can be written as:

$$\text{EBCT} = \frac{\text{Bed volume}}{F} \tag{7}$$

The volume of effluent can be calculated from the following equation:

$$V_{ef} = F * t_b \tag{8}$$

Concentration of dye removed at time t can be written as:

$$C_r = C_0 - C_t \tag{9}$$

The value of the total mass of dye removed can be calculated from the area under the breakthrough curve as:

$$q_t = \frac{F}{1000} \int_0^{t_b} C_r dt \tag{10}$$

The equilibrium uptake capacity of the column can be calculated as follows:

$$q_{ce} = \frac{q_t}{w} \tag{11}$$

Total amount of dye entering the column is calculated as:

$$m_t = \frac{C_o V_{ef}}{1000} \tag{12}$$

Table 1 | Values of column parameters (see Nomenclature for definition of parameters)

C_0	F	H	EBCT	w	t_b	V_{ef}	q_t	m_t	q_{ce}	y
10	1.0	0.08	35	27.42	41	41	0.238	0.41	0.0086	72.44
20	1.0	0.08	35	27.42	83	83	0.3160	0.60	0.0115	52.66
30	1.0	0.08	35	27.42	90	90	0.4465	0.90	0.0163	49.62

Table 2 | Comparison between free and immobilized cells of *B. subtilis* MTCC 441 for MB removal

Removal agent	Initial conc. (mg/L)	% Inoculum or amount of bead (g)	Temperature (°C)	Max. time of operation (h)	Maximum removal of MB (%)
Free cells	20–60	4–8	25–35	6	91.39
Immobilized cells	10–30	27.42	30	5	72.44

The percentage removal of dye can be calculated from the following equation:

$$y = \frac{q_t}{m_t} \times 100 \tag{12a}$$

Finally, a comparison between free cells and alginate immobilized cells for removal of MB has been shown in Table 2.

Desorption study

After treatment with 0.1 N HCl, the percentage desorption of MB from free cells and immobilized cells were found to be 10% and 25%, respectively. The higher desorption with immobilized cells may be due to leaching of bound MB from cells as well as from the calcium alginate matrix. The low desorption value for both free cells and immobilized cells may be attributed to the strong binding of MB dye with cells. The extent of desorption of MB may be increased by increasing the strength of HCl and contact time. However, MB-loaded free cells can be used later for methane production by the anaerobic digestion method.

Supplementary material

Photographs of free cells and immobilized cells before and after loading of MB can be found in the accompanying online Appendix.

CONCLUSION

Both free and immobilized *B. subtilis* MTCC-441 strain have been used for the removal of MB dye from a simulated solution. Using free cells, it is seen that most biodegradation is achieved within 2 h and it remains almost constant beyond 6 h. The pattern of the curve indicates the biosorptive removal of dye. FTIR studies of intracellular fluid show that there is no penetration of dye into the cell. Disappearance of carboxyl group after treatment with dye, using free cells, proves the surface binding of carboxyl groups present with the cell wall. The maximum removal of 91.68% is achieved when 20 mg/L of dye solution is inoculated with 8% inoculum at 30 °C. A fixed bed contactor is used to remove MB dye from its simulated solution in continuous mode using alginate immobilized cells as packing material. A higher removal of MB is obtained at lower initial concentration of dye. It can be concluded that *B. subtilis* MTCC 441 is efficient in the removal of MB dye. However, application of this method needs a detailed study with actual industrial wastewater.

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First received 16 September 2016; accepted in revised form 16 December 2016. Available online 23 January 2017