4.0 RESULTS AND DISCUSSION

Presence of colour in the textile effluent is one of the most important and challenging problem in the aquatic environment. Nowadays the use of algae in textile industry for decolourisation has become an academic interest. In the present study, the potential of naturally occurring alga was explored for its efficiency in the removal of dye from aqueous solutions. The results of the present investigation entitled *"Bioremediation of methyl orange from aqueous solutions using Oedogonium subplagiostomum AP1"* are discussed under the following phases.

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PHASE VI

4.6 Reuse of dye desorbed algae and treated dye solution

4.6.1 Dye desorbed algae for compost production

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PHASE VII

4.7 Applicability of the Oedogonium subplagiostomum AP1 in treating textile dyeing effluent

PHASE VIII

4.8 In silico docking of azoreductase with methyl orange

PHASE I

4.1 IDENTIFICATION OF THE ALGA

4.1.1 Morphological identification

Choosing the algal species based on the uptake and growth efficiency in a given environmental conditions is the most essential step in the process of bioremediation. The algal species capable of decolourising methyl orange was identified based on morphological and molecular characteristics to detect their position in classification system.

Based on the morphological characteristics the isolated alga was identified as *Oedogonium* species which belongs to the class *Chlorophyceae* and family *Oedogoniaceae* (Plate 8). The voucher specimen number for the identified algae is BSI/SRC/5/23/2018/Tech./639 (Appendix 32).

Plate 8 - Morphology of *Oedogonium* species

Oedogonium is a genus of unbranched filamentous green algae made up of small cylindrical cells. This genus has a worldwide distribution and is a common component of natural ecosystems where it grows either attached to the substrate or as free floating mats. *Oedogonium* is a robust and competitively dominant genus that has been identified as a key target group for the bioremediation of freshwater waste streams and as a feedstock biomass for bioenergy applications (Cole *et al*., 2013, Lawton *et al*., 2013, Lawton *et al*., 2014, Roberts *et al*., 2013, Neveux *et al*., 2014 and Kidgell *et al*., 2014).

4.1.2 Molecular identification

The molecular phylogeny using 5.8S rRNA was used to further confirm the isolated alga. The 5.8S rDNA region of the isolated strain was amplified using 5.8S ITS primer and the PCR product was sequenced. The 5.8S rRNA of the isolated alga was submitted to NCBI GenBank under the accession number KY575148 (Figure 2). The algal strain was sequenced and the homologous sequence was searched using NCBI BLAST. The sequence of the alga is given below:

AAGGATCATTGAATCAGATCAACAACACTCGTGAACTTTAGCAAGTGCTTGTC
ACTTGCCGTTCAGGGCCAGGTTGCTTTGAAGTGGTAGGAAGGGAGCATGTCT
CTCATCCGAAGCTTGTGAGCGCTTGGTCTTGCAACTCGATCTTCTTCGAAGGT
CGTCTTGCGCTTTTTTCCAACCAAACACCCAACCTTGTTCATCTGAAGTGATGC
AATTGGCATTGCCGATTGTGTAACCCATCAAACAACTCTCAACAACGGATATC
TTGGCTCTCGCAACGATGAAGAACGCAGCGAAATGCGATACGTAGTGTGAAT
TGCAGAATTCCGTGAATCATCGAATCTTTGAACGCATATTGCGCTCGAGGCTT
CGGCCAAGAGCATGTCTGCCTCAGCGTCGGCATAACCCCTCACTCCCACTTC
ATGTGTGAGTGGAACTGGCAGTCTTAGTGGCATTAGTTACTAGGTCTGCTGAA
GAACAGAGGCTTGAGCATGGACCCAATAAGGGTAACAACTAGGTAGGCACGT
CTTCGGACTGCATAATCTTAGTTGGTGCCTGGGACTGTGCTGGAGGCCATGCA
GGACCAACCTTTGGTTGGAACACTCCATTTTCGACCTGAGCTCAGGCAGGCTA
CCCGCTGAACTTAAGCATAAGAACAGAGGCTTGAGCATGGACCCAATAAGGG
TAACAACTAGGTAGGCACGTCTTCGGACTGCATAATCTTAGTTGGTGCCTGGG
ACTGTGCTGGAGGCCATGCAGGACCAACCTTTGGTTGGAACACTCCATTTTCG
ACCTGAGCTCAGGCAGGCTACCCGCTGAACTTAAGCAT

Figure 2 - 5.8S rRNA sequence of *Oedogonium subplagiostomum* AP1 (651bp)

The homologous search showed that the isolated alga share 99% identity with *Oedogonium subplagiostomum* (DQ413054.1) and 92% identity with *Oedogonium* sp. M3 (DQ413059.1) and *Oedogonium pakistanense* (DQ413060) respectively.

The Weighted version Neighbour joining phylogenetic tree was constructed and the number at nodes depicts the bootstrap values obtained with 100 resampling analysis (Figure 3). The homologous search and Neighbour joining phylogenetic tree further confirm that the isolated strain was *Oedogonium* species.

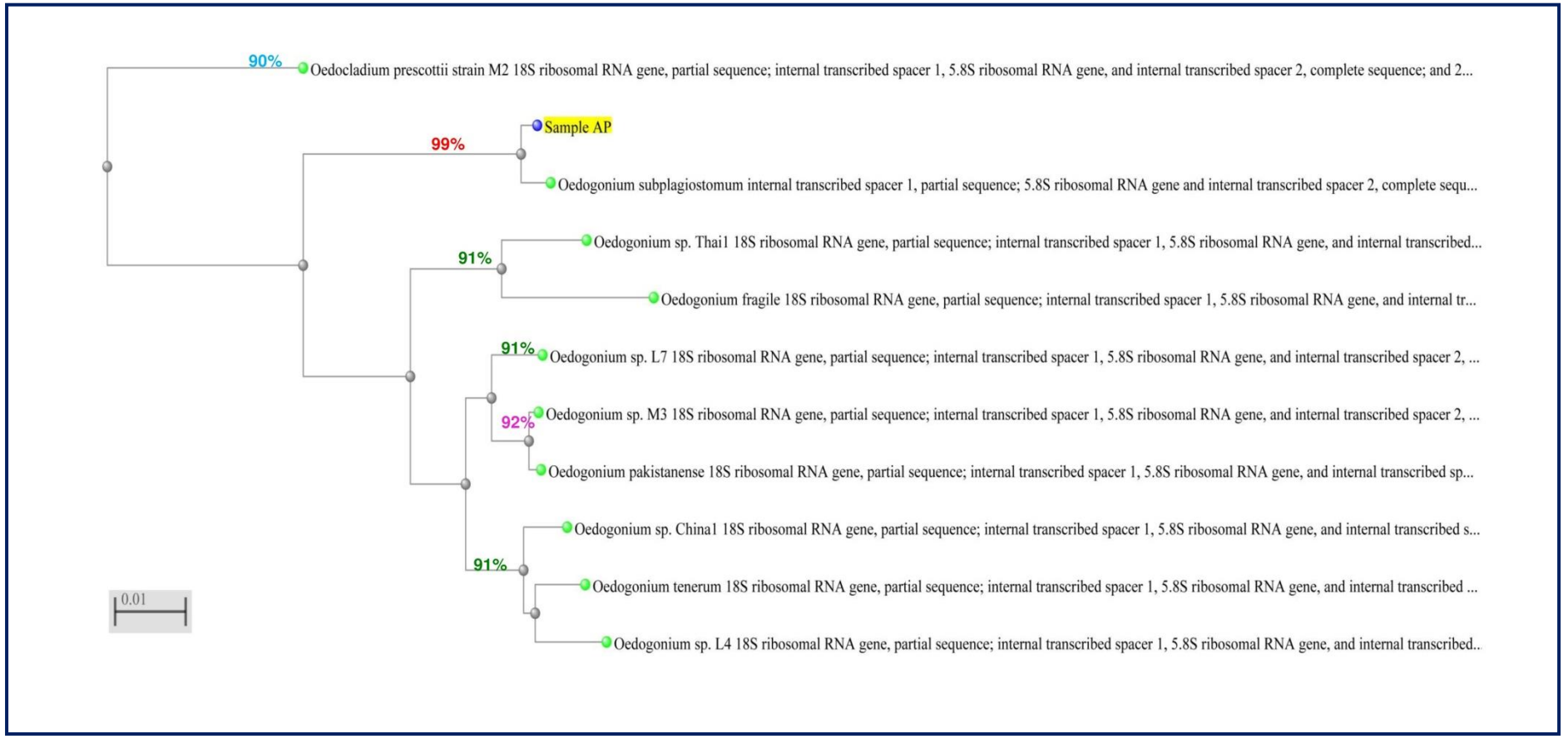


Figure 3 - Molecular phylogenetic analysis of the alga by Neighbour joining tree method

PHASE II

4.2. BATCH DECOLOURISATION AND DESORPTION EXPERIMENTS

4.2.1 Decolourisation of different dyes by *Oedogonium subplagiostomum* AP1

The effectiveness of dye decolourisation depends on the survival and adaptability of the algae. Dyes of different structures were used in the textile industry and the effluents released from these industries are markedly variable in composition. Hence, an attempt has been made to confirm the ability of the algae to decolourise different dyes selected the study. The ability of *Oedogonium subplagiostomum* AP1 to decolourise various dyes at an optimum concentration of 500mg/L was depicted in Figure 4. The alga was able to decolourise methyl orange to a maximum (97%) level followed by the other dyes in aqueous solutions within 5 days of incubation period.

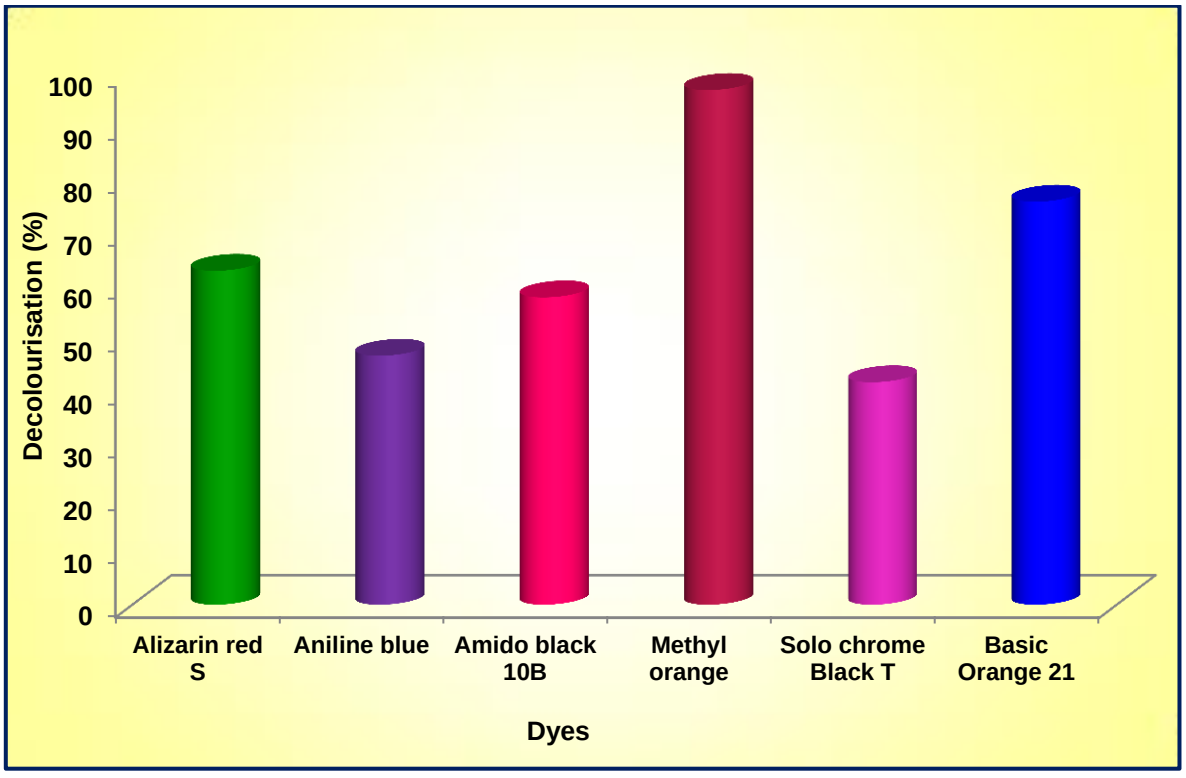


Figure 4 - Decolourisation efficiency of *O. subplagiostomum* AP1 in synthetic dye solutions

4.2.2 Optimization parameters for methyl orange decolourisation by *Oedogonium subplagiostomum* AP1

The biosorption of dyes is a complex process affected by diverse factors such as pH of the solution, biosorbent concentration, initial dye concentration, temperature and contact time. Hence, batch study was carried out to optimize the above factors for efficient sorption of the selected dye. Figure 5a – 5e represents the optimization parameters on decolourisation of methyl orange by *O. subplagiostomum* AP1.

Initial dye concentration

Initial concentration provides a significant driving force to overcome all the mass transfer resistance of the dye between the solid and aqueous phases and thereby increases the uptake. Hence dye concentration plays an important role in the removal of colour from the aqueous solution (Lv *et al*., 2013). The effect of varying dye concentration (100-900mg/L) on decolourisation and sorption capacity of the dye using *O. subplagiostomum* AP1 was investigated at pH 6 with 400mg/L biosorbent concentration for 5 days at 30^{o}C. Based on the data plotted (Figure 5a) it was observed that, when the initial dye concentration was increased from 100-400mg/L the uptake capacity and decolourisation percentage by *O. subplagiostomum* AP1 increased from 8 to 88mg/g and 78 to 92% respectively. The maximum removal and uptake of dye (97% and 120mg/g) was observed at 500mg/L which proves to be the optimum dye concentration. The phenomenon increase in the percentage removal of methyl orange with increase in dye concentration upto a certain level (500mg/L) and beyond 600-900mg/L may cause less removal of dye from the aqueous solution. Hence, the results showed that dye uptake and decolourisation increased linearly with increased dye concentration up to a certain extent and then it levels off.

The reduction in the dye removal at higher concentration might be due to the limitation of available binding sites for the dyes to adsorb the fixed mass of biosorbent in the aqueous solution, as well as the increase in the intraparticle diffusion (Bazrafshan *et al*., 2014). Similar reduction in uptake and removal of dye at higher concentration was observed in methylene blue by *Euchema spinosum* (Mokhtar *et al*., 2017) and basic red 46 by *Spirulina platensis* (Deniz and Kepekci, 2016) respectively. Thus the initial dye concentration can alter the dye removal efficiency of the biosorbent through an amalgamation of factors such as availability

of specific surface functional groups and the capability of those to bind dye ions (Mahmoud *et al*., 2017 and Gong *et al*., 2005).

Biosorbent concentration

In the process of biosorption a significant role was played by the biosorbent concentration. The biosorbent provides binding sites for the dyes and hence its concentration strongly affects the dye sorption from the aqueous solutions. Hence, to resolve the potential of biosorbent in the removal of dyes at specific concentration, its role in treatment studies is pivotal (Haddadian *et al*., 2013).

The percentage removal and adsorption capacity of methyl orange by *O. subplagiostomum* AP1 was analysed at varying biomass quantity (100-700mg/L), keeping the other parameters constant. The percentage removal and uptake of dye using *O. subplagiostomum* AP1 varied from 69-90% and 75-108mg/g respectively (Figure 5b). The dye removal increased when the amount of biosorbent was increased from 100 to 400mg/L and further increase in the biosorbent concentration from 500-700mg/L did not recover the removal and uptake of methyl orange. The maximum biosorption (97% and 120mg/g) occured with 400mg/L of alga dispersed in the aqueous solution amended with 500mg/L dye.

The results revealed that the biosorbent dose significantly influences the amount of dye adsorbed. In the present study, maximum dye removal and uptake was noted at 400mg/L biosorbent concentration which indicates that the surface area and binding sites for the attachment of dye molecules was high. Beyond 400mg/L, the dye removal was not appreciable which might be due to the aggregation of biosorbent at higher doses. This also leads to the blockage of binding sites on the biosorbent surface and thereby no further removal of dye was achieved at higher doses (Sibi, 2016, Khataee *et al*., 2009a, Gupta *et al*., 2012 and Shukla and Pai, 2005). The results of the present study corroborate with the findings of Aravindan *et al*. (2007) who reported that the decrease in the sorption capacity with increasing biosorbent concentration may be due to complex interactions of various factors such as solute availability, interference between the binding sites and electrostatic interactions.

Balasubramaniam *et al*. (2009) also reported that the percentage removal and sorption capacity are equally important in adsorption studies because these factors

decide the performance of a given biosorbent. According to Hussein *et al*. (2018), it was observed that the accumulation of methyl orange by *Nostoc carneum* was reduced at higher doses due to the reduction in surface area and availability of biosorption sites.

The optimum biosorbent concentration (400mg/L) of the present study coincides with the experiments conducted by various researchers on the removal of direct brown by *Spirogyra* (Sivarajasekar *et al*., 2009), malachite green by *Turbinaria conoides* (Kannan *et al*., 2010) and acid red 88 by *Lemna minor* (Balarak *et al*., 2015) respectively.

pH

pH is an important parameter considered during the process of dye sorption because it not only affects the biosorption capacity, but also the solubility of dyes and the colour of the dye solution (Das and Charumathi, 2012).

In the present study, the effect of pH on methyl orange removal and uptake by *O. subplagiostomum* AP1 has been investigated at various pH (2-11), keeping the other variables constant. The sorption capacity of methyl orange by the *O. subplagiostomum* AP1 was minimum at pH 2 (48% and 43mg/g) and increases monotonically upto pH 5 and further increase in pH from 6 leads to decrease in dye removal and uptake. The extent of raising the pH from 2-11 leads to a high increment in decolourisation and dye uptake which has reached a maximum value of 97% and 120mg/g at pH 6 (Figure 5c). According to the surface charge of the biosorbent, the degree of ionization of the adsorptive molecule and extent of dissociation of functional groups on the active sites of the biosorbent depends on the pH which is the prime factor influencing the sorption process (Nandi *et al*., 2009).

The charge on the biosorbent is dependent on pH because its surface has different functional groups such as carboxyl, hydroxyl, amino and phosphate. The reduction in the sorption of dye at low pH can be explained by the fact that, at acidic pH the surface of the biosorbent becomes protonated and acquires net positive charge and thereby causes an electrostatic attraction to increase the binding capacity of the anionic dye methyl orange to the surface of the biosorbent (Kannan *et al*., 2010). The results of the study concurs with the findings of Abd-El-Kareem and Taha (2012) who reported that at acidic pH the binding sites of the biosorbent

would be closely associated with H^+ ions which act as a bridging ligand between the biosorbent surface and the dye molecule (Srinivasan and Viraraghavan, 2010).

The reduction in the dye removal and uptake at high pH values (7-11) may be due to the increased net negative charge on the biosorbent surface causing electrostatic repulsion between the biosorbent and dye (Aksu and Donmez, 2003 and Pathomsiriwong and Reanprayoon, 2012).

The removal of methyl orange by *O. subplagiostomum* AP1 at optimum pH in the present study was in accordance with the reports observed in methylene blue by *Ulva lactuca*, *Caulerpa taxifolia*, *Chaetomorpha media* and *Enteromorpha intestinalis* (Deokar and Sabale, 2014), malachite green by *Pithophora* species (Kumar *et al*., 2006), crystal violet by *Chaetophora elegans* (Rammel *et al*., 2011) and *Spirulina platensis* (Nadeem *et al*., 2014) and methylene blue by *Enteromorpha* species (Ncibi *et al*., 2009) respectively.

Temperature

Temperature is a significant kinetic parameter which will change the sorption capacity of the biosorbent (Argun *et al*., 2008). The effect of temperature on the biosorption of methyl orange by *O. subplagiostomum* AP1 was studied at a temperature range of 20 - 60^{o}C. As the temperature was increased from 20 - 30^{o}C the dye removal and uptake capacity increased from 72 to 93% and 73 to 115mg/g respectively. Further increase in temperature from 35^{o}C to 60^{o}C leads to a gradual decline in dye removal and uptake, thereby indicating 30^{o}C as the optimum temperature for dye removal (Figure 5d).

The temperature profile indicates that the sorption capacity increases to a maximum value as the temperature increases and then decline suggesting the fact that the biosorbent loses its property at high temperature due to denaturation (Kannan *et al*., 2010). Alqaragully (2014) suggested that the biosorption between the algal biomass and dye involves a combination of chemical interaction and physical adsorption based on the structure and the functional groups available on the surface of the biosorbent.

The decrease in the dye uptake with raise in temperature (35^{o}C - 60^{o}C) might be due to the damage of active binding sites in the biosorbent or an increasing

tendency to desorb the dyes from the interface of the solution or altered surface activity of the biosorbent (Ozer and Ozer, 2003 and Saltali *et al*., 2007). It has also been documented by Al-Qodah (2000) that raises in temperature increases the rate of diffusion of the dye molecule across the external boundary layer and in the internal pores of the biosorbent as a result of decreased viscosity of the solution.

Nacera and Aicha (2006), Aksu and Tezer (2000), Chu and Chen (2002) also reported that the removal and uptake of dye decreases with increase in temperature indicates the adsorption reaction is exothermic in nature.

The dye removal and uptake by *O. subplagiostomum* AP1 at optimum temperature in the present study falls in line with the findings of various researchers who reported the removal of malachite green by *Turbinaria conoides* (Kannan *et al*., 2010), reactive red 198 by *Nostoc linckia* (Mona *et al*., 2011), remazol brilliant blue R by *Scenedesmus quadricauda* (Ergene *et al*., 2009), remazol black B by *Chlorella vulgaris* (Aksu and Tezer 2005), acid blue 9 by *Turbinaria conoides* (Rajeshkannan *et al*., 2010), acid red 274 by *Enteromorpha prolifera* (Ozer *et al*., 2005) and malachite green by *Pithophora* species (Kumar *et al*., 2005).

Contact time

Contact time plays a significant role between the dye molecules and the biosorbent for successful sorption in treatment process. In batch sorption studies, the optimum contact time and dye concentration are important key factors to determine the time required to bind the adsorbate to biosorbent sites (Haddadian *et al*., 2013).

The biosorbent experiment was carried out at different contact times (1-10 days) to assess the efficiency of *O. subplagiostomum* AP1 in dye removal. The percent removal and uptake of methyl orange by the alga increases during the initial stage of absorption and then continues to increase at a slow speed until a state of equilibrium is attained. The results depicted that the biosorption capacity of the biosorbent gradually increased with increase in contact time and 5 days was optimum to adsorb maximum amount of methyl orange (97% and 120mg/g) from the aqueous solution. Increase in the incubation period from 6-10days showed a sharp decline (93 to 52% and 115 to 48mg/g) in the removal of methyl orange from aqueous solution by *O. subplagiostomum* AP1 (Figure 5e).

In general, the decolourisation rate of biosorbent is rapid initially because of the availability of large number of vacant surface sites at initial stage and after a lapse of time the remaining sites are difficult to be occupied due to the repulsive force between the solute molecules and the solid-bulk phases (Bazrafshan *et al*., 2014 and Kannan *et al*., 2010). The decrease in the efficiency of the biosorbent with the time may also be due to the aggregation of dye molecules around the algae which may hinder the migration of dyes as the active sites are filled up and also resistance to diffusion of dye molecules develops as the biosorbent increases (Gupta *et al*., 2014 and Umpuch and Sakaew, 2013).

Experiments conducted by El-Sheekh *et al*. (2009) showed maximum decolourisation of methyl red by *Nostoc linckia*, orange I with *Lyngbya lagerlerimi*, basic cations with *Elkatotirix viridis*, G-red (FN-3G) with *Chlorella vulgaris* and basic fuschin with *Oscillatoria rubescens* on 5th day of incubation period which supports the findings of the present study.

Thus the uptake capacity of dyes by algae and the time required for decolourisation suggest the effectiveness of the biosorbent for wastewater treatment (Khataee *et al*., 2013).

Figure 5a - 5e - Optimization parameters for dye uptake and decolourisation using *O. subplagiostomum* AP1

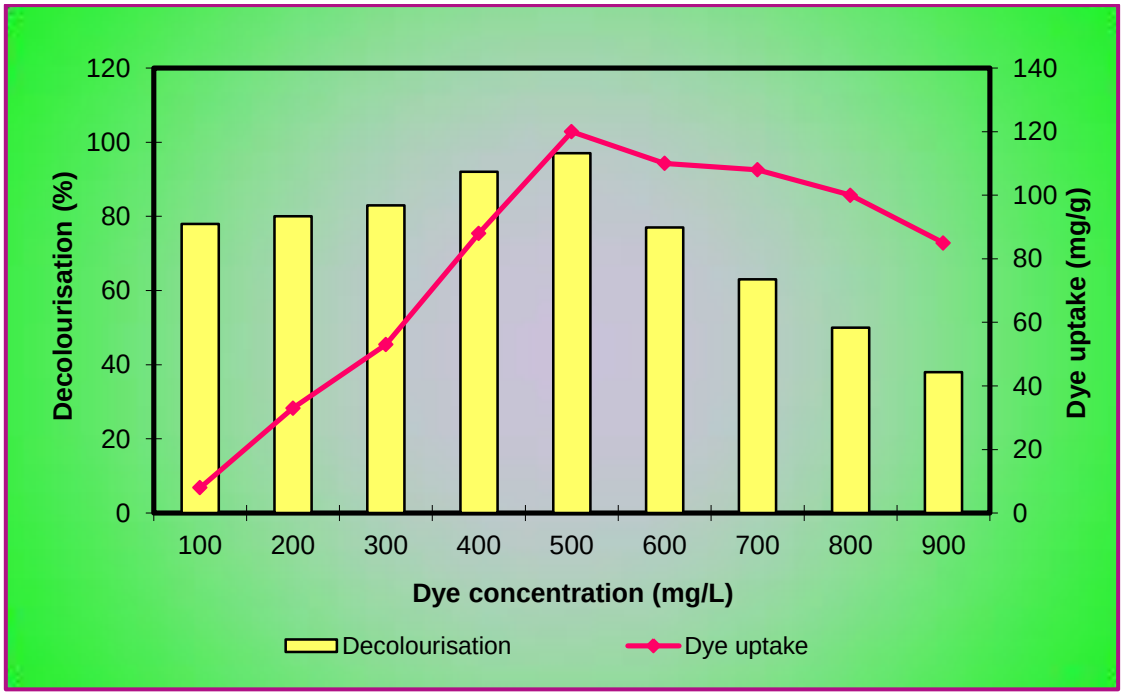


Figure 5a - Effect of different dye concentration on biosorption of methyl orange

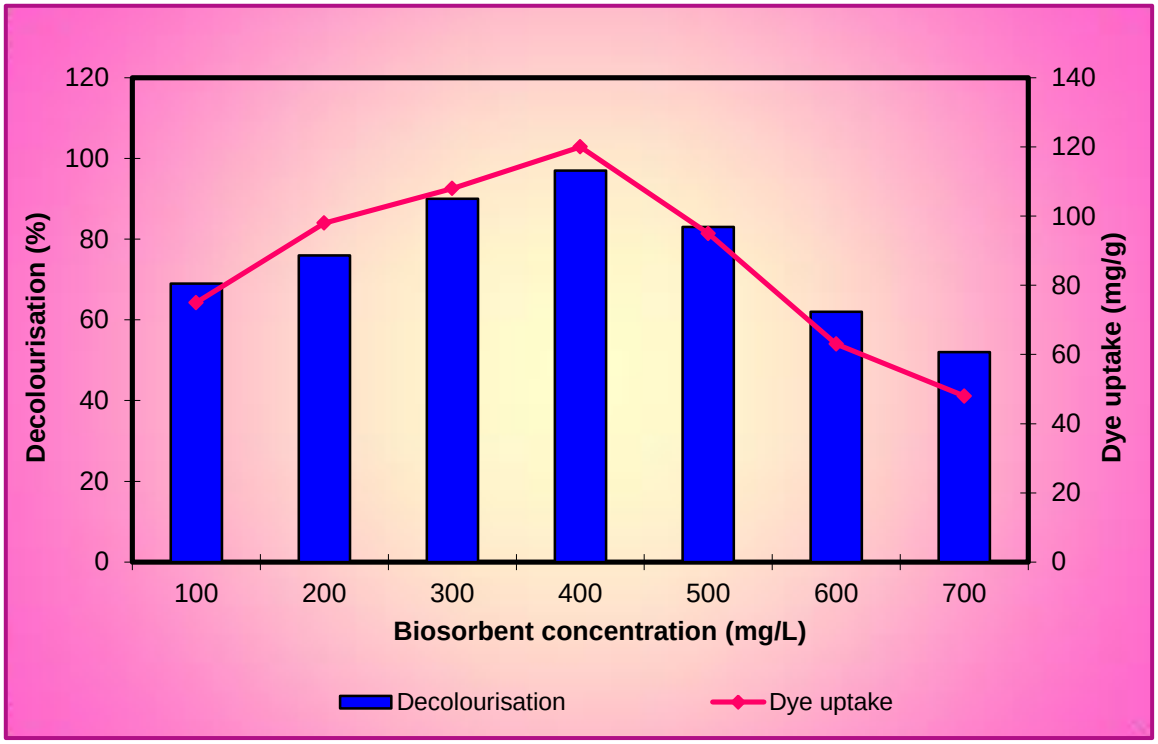


Figure 5b - Effect of varying biosorbent concentration on the biosorption of methyl orange

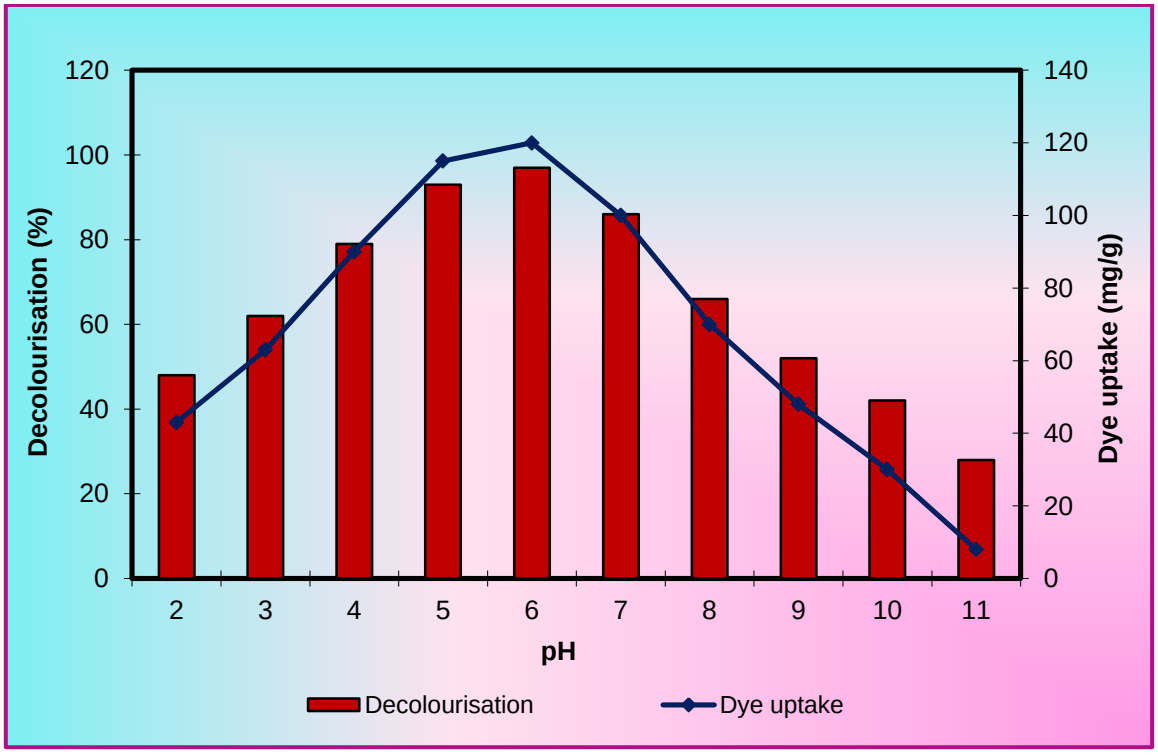


Figure 5c - Effect of varying pH on the biosorption of methyl orange

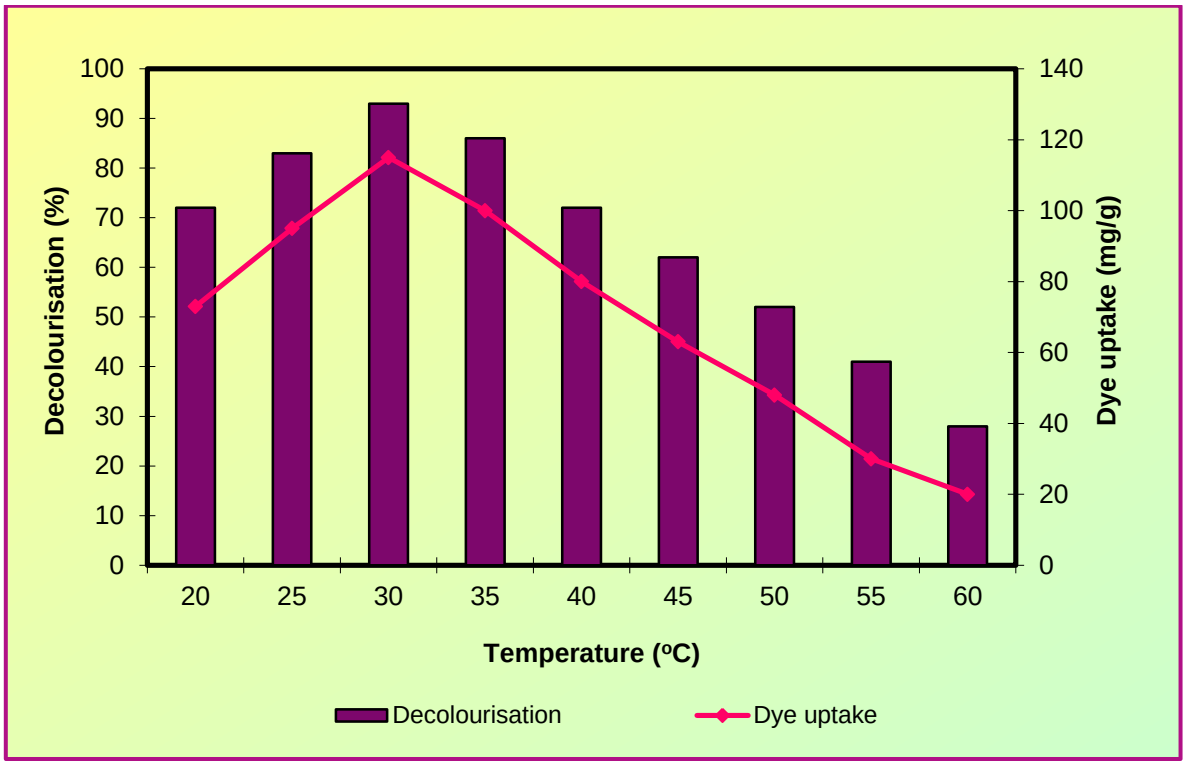


Figure 5d - Effect of varying temperature on the biosorption of methyl orange

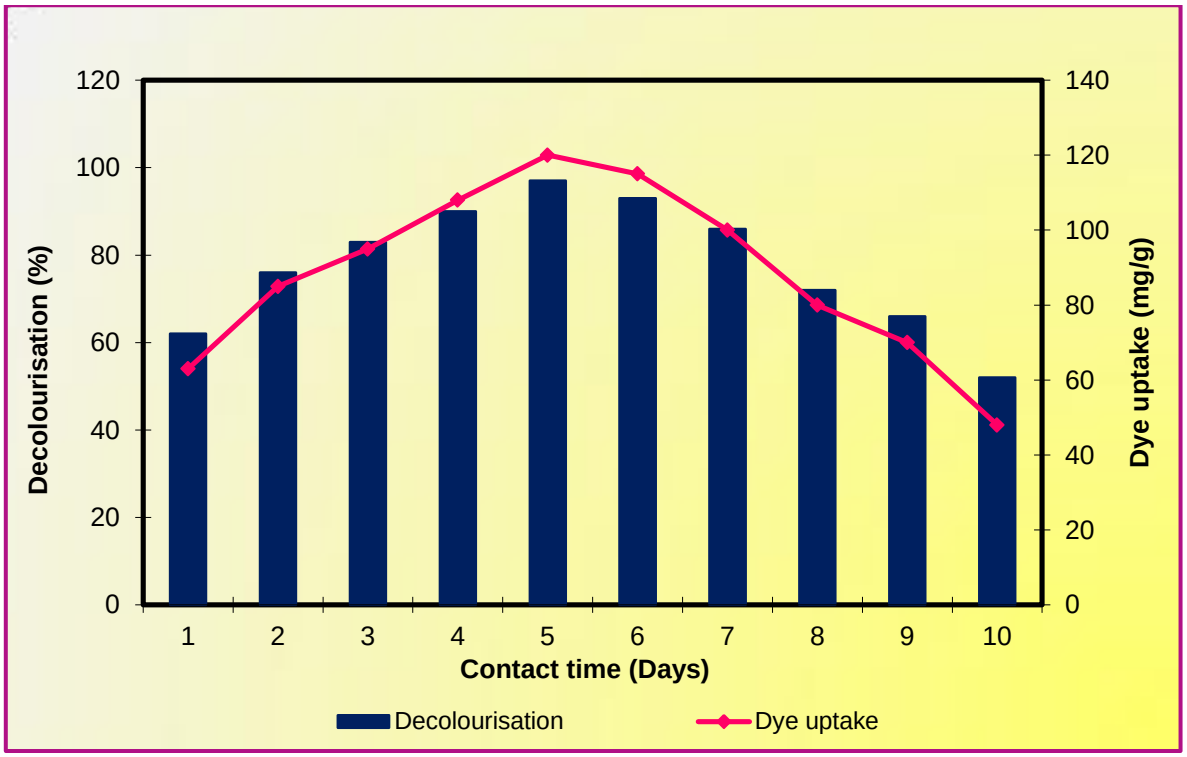


Figure 5e - Effect of varying contact time on the biosorption of methyl orange

4.2.3 Decolourisation of methyl orange under optimized conditions

The dye removal and uptake efficiency by *Oedogonium subplagiostomum* AP1 under optimised conditions was presented in Table 5. Plate 9 portrays the decolourisation of methyl orange using *Oedogonium subplagiostomum* AP1 under optimized conditions.

Table 5 - Optimum levels for methyl orange removal and uptake by *O. subplagiostomum* AP1

Parameters	Optimum level	Decolourisation	Dye uptake
Dye concentration	500mg/L	97%	120mg/g
Biosorbent concentration	400mg/L		
Contact time	5 days		
pH	6		
Temperature	30^{o}C		

Plate 9 - Decolourisation of methyl orange by *O. subplagiostomum* AP1 at optimized conditions

A) Before methyl orange decolourisation

B) After methyl orange decolourisation

Under the optimal environmental conditions, maximum decolourisation and uptake of dye was obtained which might be attributed to the accumulation of dyes onto the active sites of algae. The surface of algae possesses various functional groups which enhance the binding of the dye molecule and thereby helps in the removal of colour from the aqueous solutions (Mohan *et al.,* 2002 and Ozer *et al.,* 2006). Thus in the present study, the selected alga has a promising efficiency in decolourising the methyl orange from aqueous solutions. This paves a way to employ *Oedogonium subplagiostomum* AP1 in the decolourisation of textile effluent in large scale level.

4.2.4 Response Surface Methodology (RSM) approach for dye uptake and decolourisation

RSM was employed to determine the simple and interactive effects of process variables on dye uptake and decolourisation. It is more advantageous than single parameter optimization process in that, it saves time, raw materials and space (Sadhukhan *et al*., 2016).

In the present study RSM with Box-Behnken Design (BBD) was applied to optimize the decolourisation performance of the algae. A total of 29 experiments with different combination of concentration of dye (Factor A: 100-900mg/L), biosorbent concentration (Factor B: 100-700mg/L), pH (Factor C: 2-11) and incubation time (Factor D: 1-10 days) were performed.

The experimental design and the actual response values paired with process parameters (independent variables) on percent decolourisation and uptake of dye was summarized in Table 6.

Table 6 - Experimental design and Box-Behnken design matrix with variables

Run	Factors				Decolourisation (%)		Dye uptake (mg/g)	
	A	B	C	D	Expected	Predicted	Expected	Predicted
1	500	400	6.5	5.5	97	97	120	120
2	500	700	6.5	10	83	94	95	110
3	500	400	2	1	52	52	48	58
4	900	400	6.5	10	76	88	85	108
5	100	400	6.5	1	62	63	63	70
6	100	400	2	5.5	48	50	43	48
7	900	400	11	5.5	69	73	75	88
8	500	100	6.5	10	83	84	95	116
9	500	400	11	10	72	75	80	94
10	500	400	11	1	66	66	70	90
11	500	100	2	5.5	62	63	63	76
12	900	400	6.5	1	62	65	63	81
13	500	400	6.5	5.5	97	97	120	120
14	500	100	11	5.5	76	82	85	112
15	500	400	6.5	5.5	97	97	120	120
16	500	700	2	5.5	52	68	48	88
17	900	700	6.5	5.5	79	81	90	99
18	500	400	6.5	5.5	97	97	120	120
19	500	100	6.5	1	62	69	63	96
20	500	400	6.5	5.5	97	97	120	120
21	500	400	2	10	72	72	80	98
22	900	400	2	5.5	66	70	70	76
23	100	100	6.5	5.5	62	63	63	80
24	500	700	6.5	1	52	66	48	84
25	100	400	11	5.5	72	71	80	77
26	900	100	6.5	5.5	83	85	95	96
27	100	400	6.5	10	72	74	80	82
28	100	700	6.5	5.5	72	78	73	72
29	500	700	11	5.5	72	78	80	70

A: Concentration of dye (mg/L), B: Biosorbent concentration (mg/L), C: pH and D: Incubation time (Days)

By applying multiple regression analysis the following second order polynomial equation that could relate decolourisation and dye uptake to the process parameters studied was given as equation 13 and 14.

Decolourisation (%) $= +4.57 + 0.0755A + 0.0216B + 0.0894C + 0.1223D - 0.0654AB - 0.0772AC + 0.0355AD - 0.0316BC + 0.0392BD - 0.0494CD - 0.1607A^2 - 0.0718B^2 - 0.2325C^2 - 0.1504D^2$ ---------- (13)

Dyeuptake (mg/g) $= +120.00 + 9.92A - 4.42B + 7.25C + 10.75D + 2.75AB - 4.25AC + 3.75AD - 13.50BC + 1.50BD - 9.00CD - 24.08A^2 - 8.83B^2 - 24.33C^2 - 10.33D^2$ ---------- (14)

The statistical significance of the model was evaluated by analysis of variance (ANOVA) which confirms the goodness of the model. The model F value was calculated as the ratio of the mean square regression and mean square residual. The P values were used as a tool to check the significance of each coefficient which in turn was necessary to understand the pattern of mutual interactions between the process variables. Table 7 shows the data obtained from the ANOVA for decolourisation and dye uptake by Response Surface Model.

The ANOVA of the quadratic regression confirms the model is significant. In the present study, the model F value was 79.19 and 68.86 for decolourisation and dye uptake indicating the models were significant.

Smaller the magnitude of P, the more significant is the corresponding coefficient. P value less than 0.05 specifies that the model terms are significant. The model P value (P>F) was very low (0.0001) for all four responses which reiterates that the model was significant.

It was evident from the ANOVA table that effect of dye and biosorbent concentration, pH and contact time are more significant for the treatment of textile dyeing wastewater. Also, the interactive effects of the process variables are significant for and dye uptake and decolouriation.

Table 7 - Analysis of variance (ANOVA) for the fitted quadratic model of dye decolourization and dye uptake

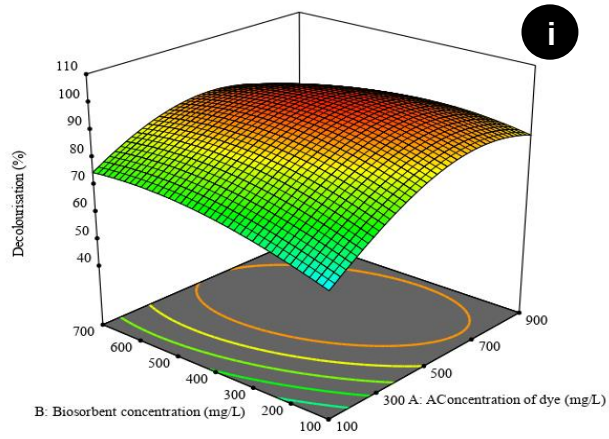
Source	Decolourisation (%)			Dye uptake (mg/g)		
	Coefficient factors	F-value	p-value	Coefficient factors	F-value	p-value
Model	4.57	79.19	< 0.0001	120.00	68.86	< 0.0001
A-Concentration of dye	0.0755	83.26	< 0.0001	9.92	102.51	< 0.0001
B-Biosorbent concentration	0.0216	6.84	0.0203	-4.42	20.33	0.0005
C-pH	0.0894	116.69	< 0.0001	7.25	54.79	< 0.0001
D-Incubation time	0.1223	218.42	< 0.0001	10.75	120.46	< 0.0001
AB	-0.0654	20.85	0.0004	2.75	2.63	0.1273
AC	-0.0772	28.99	< 0.0001	-4.25	6.28	0.0252
AD	0.0355	6.14	0.0266	3.75	4.89	0.0442
BC	-0.0316	4.86	0.0447	-13.50	63.33	< 0.0001
BD	0.0392	7.49	0.0160	1.50	0.7818	0.3915
CD	-0.0494	11.88	0.0039	-9.00	28.14	0.0001
A^2	-0.1607	203.84	< 0.0001	-24.08	326.81	< 0.0001
B^2	-0.0718	40.66	< 0.0001	-8.83	43.97	< 0.0001
C^2	-0.2325	426.86	< 0.0001	-24.33	333.63	< 0.0001
D^2	-0.1504	178.57	< 0.0001	-10.33	60.16	< 0.0001
Residual	0.0115	14	0.0008	161.17	14	11.51
Lack of Fit	0.0115	10	0.0012	161.17	10	16.12
Pure Error	0.0000	4	0.0000	0.0000	4	0.0000
Cor Total	0.9224	28		11258.97	28	
R^2	0.9875			0.9857		
Predicted R^2	0.9282			0.9175		
Adjusted R^2	0.9751			0.9714		
Std. Dev.	0.0287			3.39		
C.V. %	0.6635			3.69		
Adeq Precision	31.2393			28.6182		

A: Concentration of dye (mg/L), B:Biosorbent concentration (mg/L), C:pH and D:Incubation time (Days)

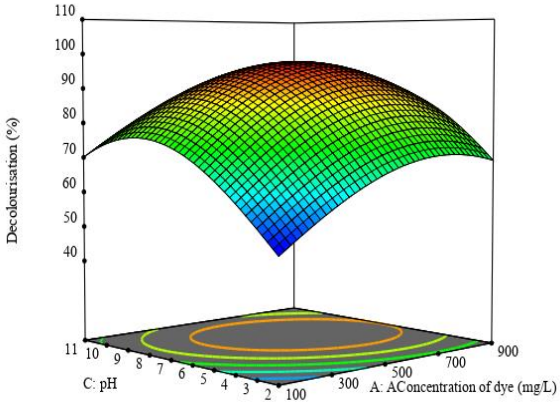
The coefficient of determination, R^2 value is always between 0 and 1, and a value > 0.90 indicates the suitability of model. For a good statistical model, R^2 value should be close to 1.0. In the present study, the fit of the model is also expressed by R^2, which was found to be greater than 0.90 for the four responses. The predicted R^2 of 0.9282 (decolourisation) and 0.9175 (dye uptake) was in reasonable agreement with the adjusted R^2 of 0.9751 (decolourisation) and 0.9714 (dye uptake). The fit of the model is also expressed by the coefficient of regression R^2, which was found to be 0.9875 for decolourisation and 0.9857 for dye uptake, indicating that more than 95% of the variability in the response could be elucidated by the model. This infers that the prediction of experimental data is relatively satisfactory. Adequate precision measures the signal to noise ratio. A ratio greater than 4 is desirable. In this study, the ratio was found to be 31.23 and 28.61 for decolourisation and dye uptake, indicating an adequate signal.The coefficient of variation (CV) represents the degree of precision with which the factors are compared. The higher value of CV indicates the reliability of the experiment is lower. In this study, the CV is less than 4 for the responses, which indicates the greater reliability of the experiments performed.

The magnitude of coefficient factors in Table 7 gives the positive effect of dye concentration, biosorbent concentration, pH and contact time for decolourisation. For dye uptake, dye concentration, pH and contact time showed positive effect, whereas biosorbent concentration had a negative effect.

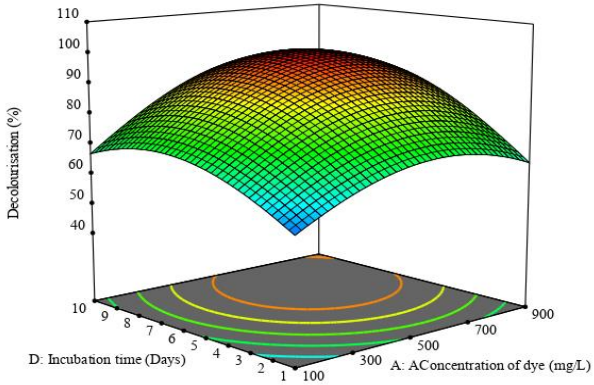
The response surface plots were drawn or generated for different interactions of any two independent variables while holding the values of other variables as constant. The 3D surface gives clear-cut geometrical illustration and affords valuable information about the performance of the system within the experimental design. The optimization of process variables were intended for finding the levels of independent variables which would provide percentage decolourisation and dye uptake. The 3D response surface plots for the decolourisation and dye uptake were shown in Figure 6a and 6b.



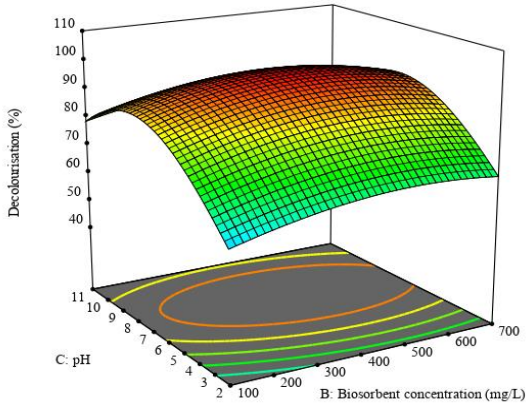
Biosorbent and dye concentration



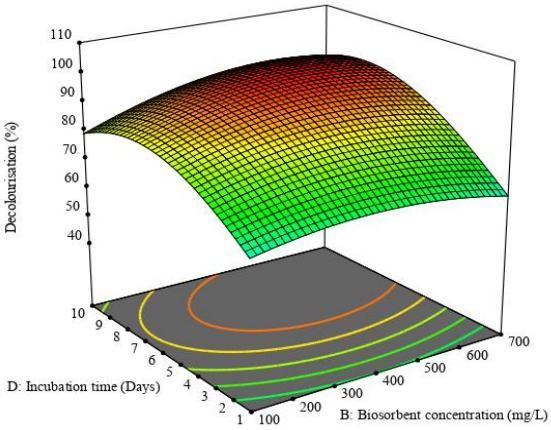
ii) pH and dye concentration



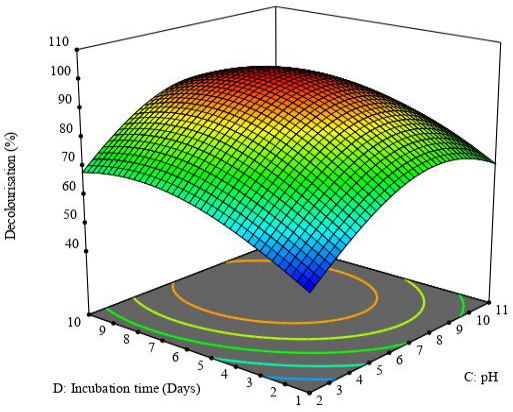
iii) Incubation time and dye concentration



iv) pH and biosorbent concentration



v) Incubation time and biosorbent concentration



vi) Incubation time and pH

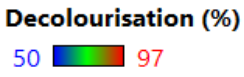
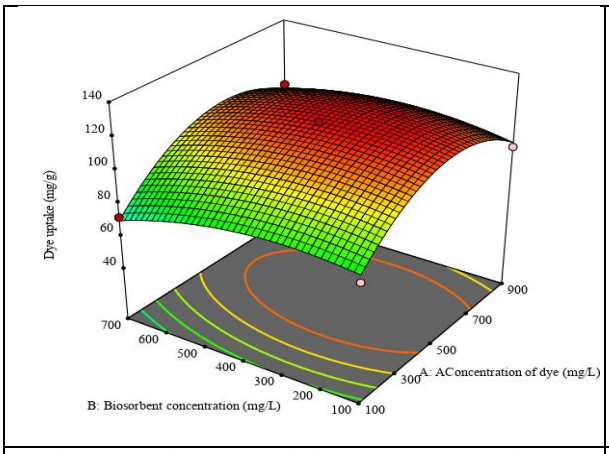
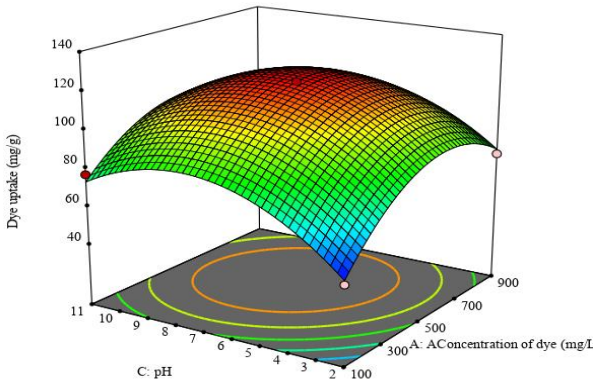


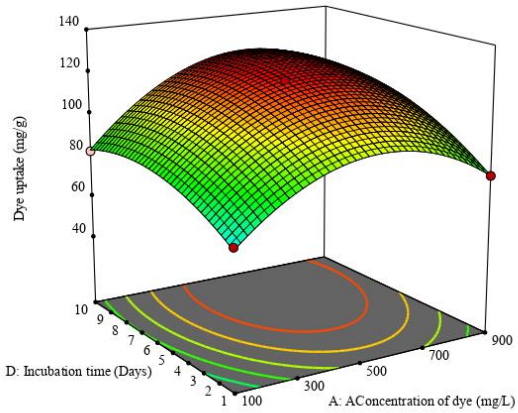
Figure 6a - 3D response surface plots of two variables on dye decolourisation



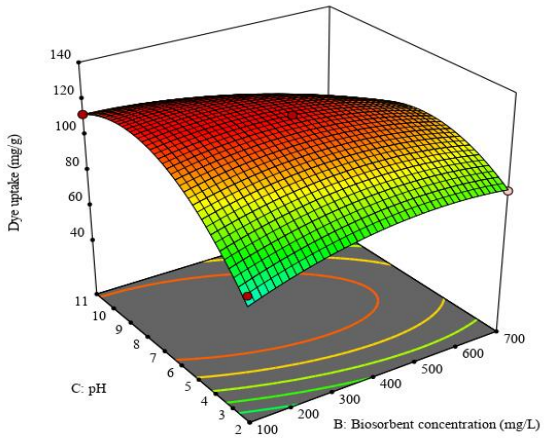
i) Biosorbent and dye concentration



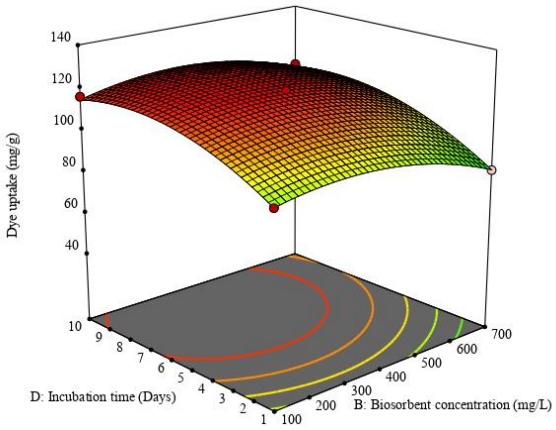
ii) pH and dye concentration



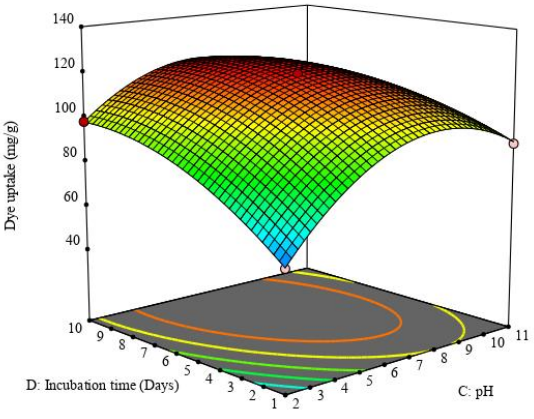
iii) Incubation time and dye concentration



iv) pH and biosorbent concentration



v) Incubation time and biosorbent concentration



vi) Incubation time and pH

Figure 6a - 3D response surface plots of two variables on dye uptake

The interaction between the variables depicts the nature of the response surface curve. The elliptical and circular shape of the curve indicates good and interaction between the two variables respectively. From the Figures 6a and 6b it was evident that the elliptical nature of the contour in all the graphs portrays the mutual interactions of all the variables. A relative significant interaction between every two variables was observed, and there was a maximum predicted decolourisation and dye uptake as pointed by the surface confined in the smallest ellipse in the contour plots. From the results of the optimization studies discussed earlier, it was recorded that the maximum decolourisation and dye uptake was obtained in the aqueous solution amended with 500mg/L dye, 400mg/L biosorbent concentration at pH 6 for 5 days of contact time. With these values, an interactive effect of biosorbent concentration with dye concentration, pH with dye concentration, contact time with dye concentration, pH with biosorbent concentration, contact time with biosorbent concentration and contact time with pH was carried out, while keeping the other variables constant and their responses were interpreted using RSM approach.

It was evident from the figures; there was a positive interaction between the concentration of dye with incubation time, biosorbent concentration with incubation time and negative interaction between concentration of dye with biosorbent concentration, concentration of dye with pH, biosorbent concentration with pH, pH with incubation time for decolourisation experiments. While, a positive interaction was observed between the concentrations of dye with biosorbent concentration, concentration of dye with incubation time, biosorbent concentration with incubation time and a negative interaction between concentration of dye with pH, biosorbent concentration with pH, pH with incubation time was noticed. This was revealed in the contour lines and in the multiple regression equation 13 and 14.

Model validation

The results obtained from the statistical analysis of BBD were performed using RSM under optimal conditions for the process of verification. Further the statistical experimental strategies were confirmed for its validity with the experimental data. The results of the predicted response were well accorded with the experimental data which reflected the applicability and correctness of the RSM.

Moreover, it is also evident from Table 8 that the results of the experimental value (97% and 120mg/g) are similar with the predicted value (97% and 120mg/g). Hence, the outcome of the model developed was considered to be accurate and reliable.

Table 8 - Validation of experimental and predicted models for dye decolourisation and uptake using *O. subplagiostomum* AP1

Parameters	Validation results	
	Experimental	Predicted
Biosorbent concentration (mg/L)	400	400
Concentration of dye (mg/L)	500	500
pH	6	6.5
Contact time (Days)	5	5.5
Decolourisation (%)	97	97
Dye uptake (mg/g)	120	120

Thus, the results implicate that RSM based Box-Behnken design can save time and effort by the assessment of the optimization conditions for decolourisation and dye uptake.

4.2.3 Desorption and reuse efficiency of *O. subplagiostomum* AP1

Regeneration of biosorbent is necessary to make the process of biosorption economically feasible. The recycling of the dye loaded biosorbent was performed by desorption using highly efficient chemicals which does not damage the bisorbent structure (Bulgariu and Bulgariu, 2014 and Kwak *et al.,* 2015).

The recovery and regeneration of dye from the selected biosorbent is an important consideration for industrial applications. Hence for the ease of economic use the selected biosorbent must not only possess excellent biosorptive capacity, but it should also be easily regenerated and reused. Hence for the good desorption process the eluent, type of biosorbent and biosorption mechanism should be the basic requirement (Das *et al*., 2010a).

Desorption experiments were performed to elucidate the dye sorption mechanism and also to assess the regeneration capacity of the biosorbent under

optimized conditions. Three consecutive cycles of adsorption-desorption experiments were carried out in 500mg/L of methyl orange inoculated with 400mg/L of biosorbent separately using the desorbents or eluents namely 0.1N NaOH, 0.1N HNO_3 and 0.1N HCl under optimised conditions.

Maximum recovery of methyl orange was observed when 0.1N NaOH solution (83%) was used as an eluent on second day when compared with 0.1N HNO_3 (61%) and 0.1N HCl (43%) respectively.

Table 9 - Desorption of methyl orange from *O. subplagiostomum* AP1 using different eluents

Incubation period (Days)	Desorption efficiency by eluents (%)		
	0.1N NaOH	0.1 N HNO_3	0.1 N HCl
1	78	52	37
2	**83**	**61**	**43**
3	58	45	22
4	42	37	19
5	17	13	11

The values were mean of triplicates

Further increase in incubation period did not favour higher desorption efficiency (Table 9). Rachna and Sumathi (2008) reported that while using NaOH as an eluent for desorption studies, the pH of the medium increase and thereby the negative charges on the biosorbent gets increased. This in turn creates an electrostatic repulsive force between the negatively charged adsorbent and the anionic dye molecules and thereby causing desorption of dye molecules from the biosorbent. This might be due to the decomposition or damage caused by the alkaline solution on certain functional groups or adsorption sites present on the surface of the biosorbent.

Vijayaraghavan and Yun (2008) reported that complete desorption of methyl orange was not observed in aqueous solution indicating the entrapment of dye on the intrapores of the biosorbent and thereby difficult to release. The results of the

present study coincides with the findings of Hosseini *et al.* (2011) and Umpuch and Sakaew (2013) who reported that the recovery of methyl orange was maximum when alkaline NaOH solution was used as an eluent.

The desorption study aids to elucidate the adsorption mechanism whereas the recycling or regeneration of biosorbent makes the treatment process economical and more reasonable (Kavitha and Namasivayam, 2007).

To study the biosorption efficiency of dye desorbed *Oedogonium subplagiostomum* AP1, the adsorption – desorption experiments were performed for three repeated cycles at optimized conditions. The decolourisation efficiency of methyl orange by *Oedogonium subplagiostomum* AP1 was 59% in first cycle and it declined to 10% and 3% in the consecutive cycles.

Thus, the regeneration capacity of the biosorbent is quite important in decolourisation studies because it is eco-friendly, cost effective and reduces the secondary sludge formation. For effective regeneration, the selection of desorbing solvent or eluent is important which strongly depends on the type of biosorbent and the mechanism of biosorption (Bagchi and Ray, 2015).

4.2.6 Column studies for dye decolourisation

To evaluate the performance of biosorption process particularly for the treatment of wastewater on industrial and large scale level, flow rate is considered as an important parameter (Saha *et al.,* 2011). Therefore, the effect of flow rate in the biosorption of methyl orange by *O*. *subplagiostomum* AP1 was studied at various time intervals (10-120ml/h). Plate 10 shows the column setup for methyl orange decolourisation using *O. subplagiostomum* AP1 at lab scale level.

It was evident from Figure 7 that the decolourisation of dye was higher (97%) at lower flow rate (10 ml/h) and lower (2%) at high flow rate (120ml/h). Thus the biosorption efficiency of methyl orange was low at higher flow rate and when the flow rate was increased from 10-120ml/h the rate of dye decolourisation decreased.

Plate 10 - Column study for methyl orange decolourisation at lab scale

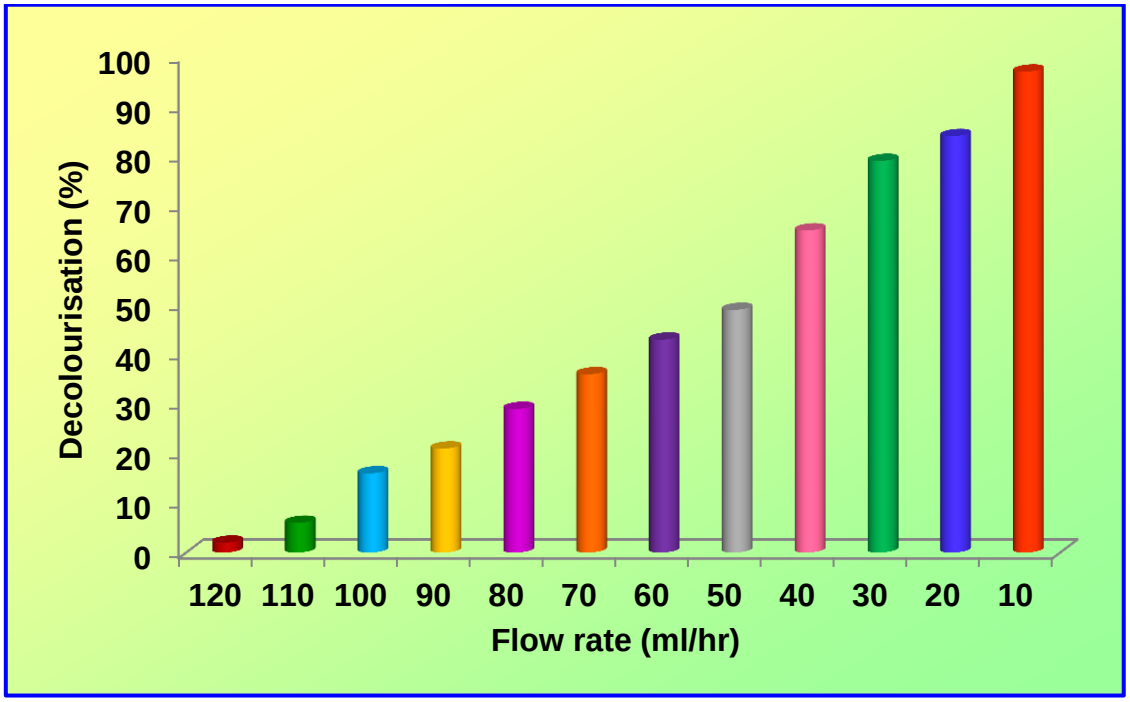


Figure 7 - Effect of flow rate on decolourisation of methyl orange

The decrease in the decolourisation efficiency of dye at higher flow rate might be due to the exposure of dye with the biosorbent surface for a short period. Therefore the dye molecules does not have sufficient time to bind with the active sites on the biosorbent surface or diffuse into the pores of the biosorbent leaving the column before attaining equilibrium (Sadaf *et al*., 2014).

When the flow rate was slow, the external mass transfer controlled the process which is ideal for intraparticle diffusion system. Thus, at lower flow rate, the diffusion process was more effective and higher was the resistance time of the biosorbent, which resulted in high sorption capacity (Vijayaraghavan *et al.,* 2004).

Thus the utilization of column for decolourisation of dye will be an economical tool for the treatment of textile dyeing effluent in the nearby future.

PHASE III

4.3 ADSORPTION MODELS FOR DYE REMOVAL

4.3.1 Isotherm models

Adsorption isotherm describes the equilibrium between the concentration of the adsorbate on the solid and the liquid phases. The experimental results of the sorption equilibrium data are important in order to accurately monitor the adsorption behaviour and to explore the isotherm data for design purposes (Boudechiche *et al.,* 2016 and Rammel *et al.,* 2011).

The Langmuir model describes the monolayer adsorption of the adsorbate on a homogeneous adsorbent surface to obtain a maximum adsorption capacity at a constant temperature. Once a site is filled, no further sorption takes place at that site which indicates that the surface reaches a saturation point where the maximum adsorption of the surface will be achieved (Langmuir, 1918).

The expression of Langmuir isotherm model is depicted in Eq. 15

$$q_e = \frac{q_{max} K_L C_e}{1 + K_L C_e} \quad \text{---------- (15)}$$

where, q_e is the amount of biosorbed dye at equlibirium (mg g^{-1}), q_{max} is the maximum biosorption capacity at equilibrium (mg/g), C_e is the dye concentration at equlibirium (mg L^{-1}) and K_L is the Langmuir constant (L mg^{-1}). The linearized form of Langmuir equation is expressed as follows,

$$\frac{C_e}{q_e} = \frac{1}{q_{max} K_L} + \frac{C_e}{q_{max}} \quad \text{---------- (16)}$$

The Langmuir constants K_L and q_{max} were calculated from the slope of plot between C_e/q_e verses C_e. The fitness of isotherm model describing the type of adsorption was determined by regression correlation coefficient value (R^2). The higher R^2 value (near to unity) represents the fitness of the isotherm model.

A dimensionless constant separation factor (R_L) of Langmuir isotherm was used to determine the favourability of the biosorption process. R_L is defined as below,

$$R_L = \frac{1}{1 + K_L C_o} \quad \text{---------- (17)}$$

where K_L is Langmuir constant (L mg^{-1}) and C_o is the initial dye concentration in liquid phase (mg L^{-1}). The R_L values between 0 and 1 indicates the shape of isotherm to be either favourable ($0<R_L<1$) or unfavourable ($R_L>1$) or linear ($R_L=1$) or irreversible ($R_L=0$) (Stephen *et al*., 2006).

The Freundlich model is based on the assumption that dyes are adsorbed on a heterogeneous surface through a multilayer adsorption mechanism and is not restricted for the formation of monomolecular layer (Freundlich, 1906). The Freundlich empirical equation is represented as follows

$$q_e = K_F C_e^{1/n} \quad \text{---------- (18)}$$

where K_F is the Freundlich constant related to adsorption capacity of adsorbent [(mg g^{-1}) (mg L^{-1})$^{-1/n}$] and n is the biosorption intensity (affinity of the adsorbate for adsorbent). The value of n falling in the range 0 - 1 indicates favourable sorption. The linearized form of Freundlich equation can be expressed as

$$\ln q_e = \ln K_F + 1/n (\ln C_e) \quad \text{---------- (19)}$$

The isotherm information extracted from the models were summarised in Table 10 and Figure 8 and 9 depicts the plotted models.

Table 10 - Isotherm parameters for adsorption of methyl orange onto *O. subpalgiostomum* AP1

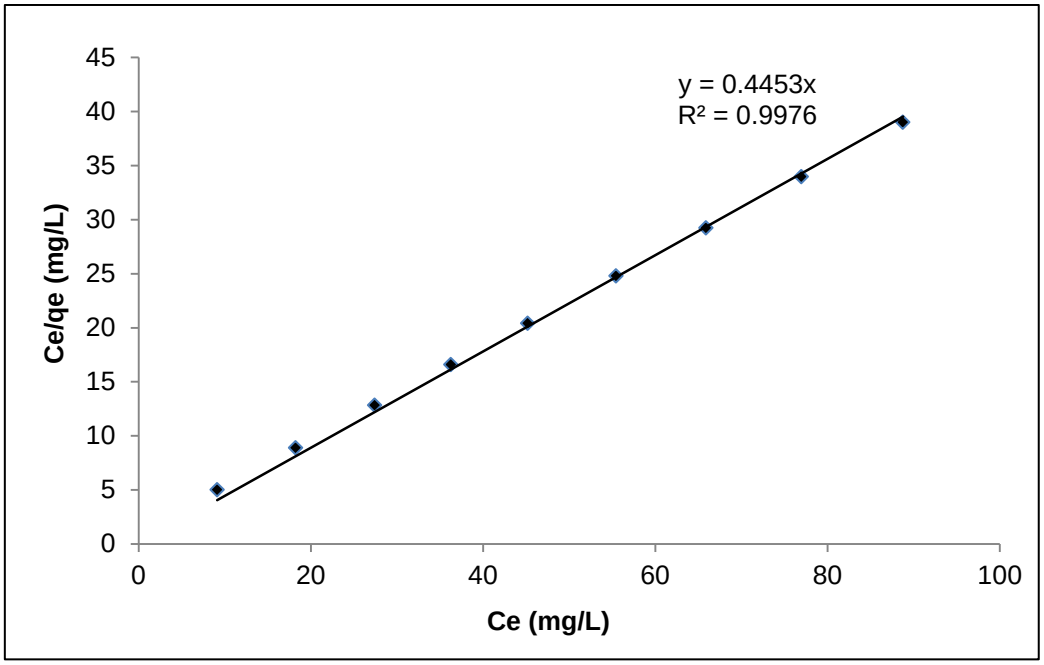
Langmuir Isotherm			Freundlich Isotherm		
K_L (L mg^{-1})	R_L	R^2	K_F (L g^{-1})	n	R^2
0.38	0.1	0.99	0.42	1.56	0.88

The results indicated that the Langmuir isotherm model best fitted the experimental data showing a straight line with a good correlation coefficient value (R^2= 0.99) indicating the applicability of model for dye-algal biosorption. The R_L value which is the basic and characteristic index of the Langmuir model was recorded as 0.1 which indicates the favourable adsorption of the dye onto the algal surface with the maximum adsorption capacity of 120mg/g.

In Freundlich isotherm model, the R^2 value was 0.88 which could not adequately describe the relationship between the amount of dye adsorbed by the alga and its equilibrium concentration in the aqueous solution. The n value of Freundlich isotherm was 1.56 which does not fall between 0 to 1 and thereby indicates that the model was not suitable for experimental data in the present study.

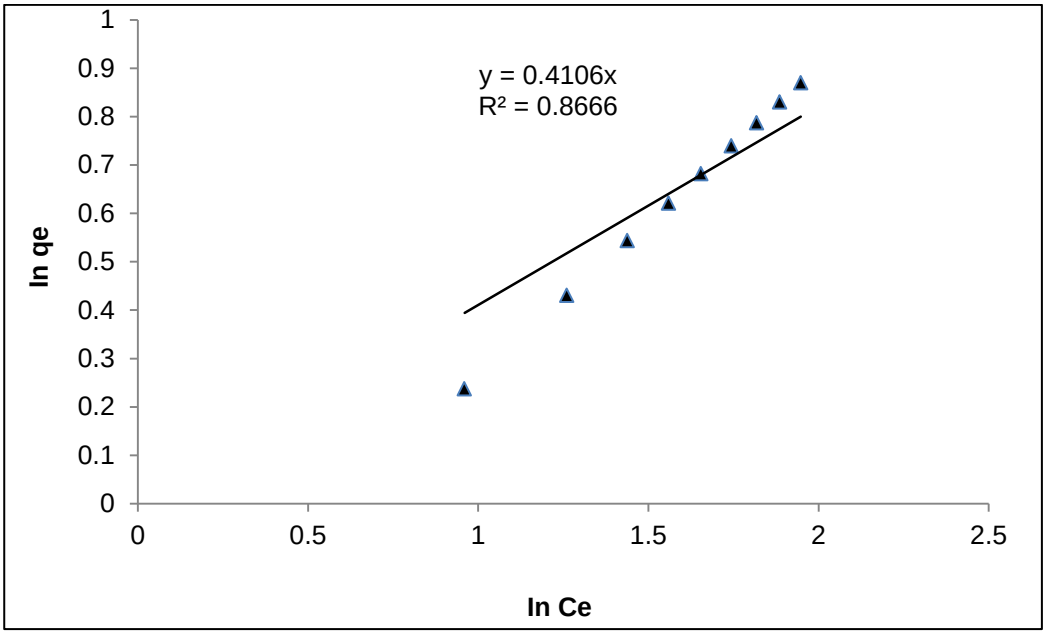
The results of the present study coincides with the findings of Hussein *et al*. (2018) who reported that Langmuir isotherm proves to be a better choice in interpreting the biosorption reaction between methyl orange and *Nostoc carneum*, basic orange dye onto the dried biomass of *Anabaena filiculoides* (Tan *et al*. 2010), acid red 88 onto *Lemna minor* (Balarak *et al*., 2015), reactive black 5 onto *Codium iyengarii* (Azmat, 2014) and acid red 66 onto *Acutodesmus obliquus* strain PSV2 (Sarwa *et al*., 2014) respectively.

Hence the results indicated the monolayer coverage of the dye on the active sites of the adsorbent with homogeneous distribution.



(Optimised conditions: Dye concentration - 500mg/L, Biosorbent concentration- 400mg/L, pH- 6, Temperature - 30°C and contact time – 5 days)

Figure 8 - Langmuir isotherm plot for the biosorption of methyl orange onto *O. subplagiostomum* AP1



(Optimised conditions: Dye concentration - 500mg/L, Biosorbent concentration- 400mg/L, pH- 6, Temperature- 30°C and contact time – 5 days)

Figure 9 - Freundlich isotherm plot for the biosorption of methyl orange onto *O. subplagiostomum* AP1

4.3.2 Kinetic models

Adsorption kinetics is important for understanding the treatment of aqueous solution since it provides information for designing the mechanism of biosorption process. The kinetic models are also used to investigate the potential rate controlling steps such as mass transfer sorption for large scale batch process (Jalil *et al*., 2012).

The experimental data was evaluated by pseudo-first and pseudo-second order kinetics to understand the mechanisms of biosorption process.

The pseudo-first order rate expression of Lagergren is generally described by the following equation

$$\ln (q_e - q_t) = \ln q_e - \frac{k_1 t}{2.303} \quad \text{---------- (20)}$$

where q_e and q_t (mg/g) are the amount of dye adsorbed at equilibrium and time (t in days) respectively and k_1 is the pseudo first order rate constant. The value of kinetic constant (k_1) obtained from the slope of the linear plot of ln (q_e - q_t) against t.

If the rate of sorption is a second order mechanism, the pseudo-second order kinetic model is expressed as

$$\frac{t}{q_t} = \frac{1}{k_2 q_e^2} + \frac{1}{q_e} t \quad \text{---------- (21)}$$

where k_2 (g mg^{-1} min^{-1}) is the pseudo second order kinetic constant which was derived from linear plot of t/q_t against t (Santhy and Selvapathy, 2006).

The plots of pseudo-first order and second-order kinetics equation were plotted in Figure 10 and 11 and the experimental values evaluated from these plots were tabulated in Table 11. The result depicts that the linear regression correlation coefficient (R^2) value was -1.3 and 0.94 for pseudo-first order and pseudo-second order kinetics respectively.

Table 11 - Kinetic parameters for methyl orange biosorption onto *O. subpalgiostomum* AP1

Pseudo first order Kinetics log (q_e-q_t)			Pseudo second order kinetics (t/q_t)	
q_e (mg/g)	k_1 (mg^{-1} min^{-1})	R^2	k_2 (mg^{-1} min^{-1})	R^2
118	0.14	-1.3	0.76	0.94

The higher regression value confirmed that the sorption data fits well with the pseudo second order kinetic model. Thus the uptake of dye by alga was assumed to be controlled by chemical process or chemisorption which involves balance forces through the exchange of sharing of electrons (Hameed, 2009 and Thinakaran *et al*., 2008).

Aksu and Tezer (2005) also computed kinetic models such as pseudo-first order and pseudo second-order for the adsorption of reactive dyes namely remazol black B, remazol red RR and remazol golden yellow RNL onto dried *Chlorella vulgaris* in a batch system. The results revealed that the dye sorption process onto *C. vulgaris* followed pseudo-second order kinetics which supports the findings of the present study.

The results of the present study coincides with the findings of Sivarajasekar *et al*. (2009) who observed that the biosorption of direct brown dye by *Spirogyra* species fits well with pseudo-second order kinetics and supports the assumption behind the present system indicating the sorption is by chemical process.

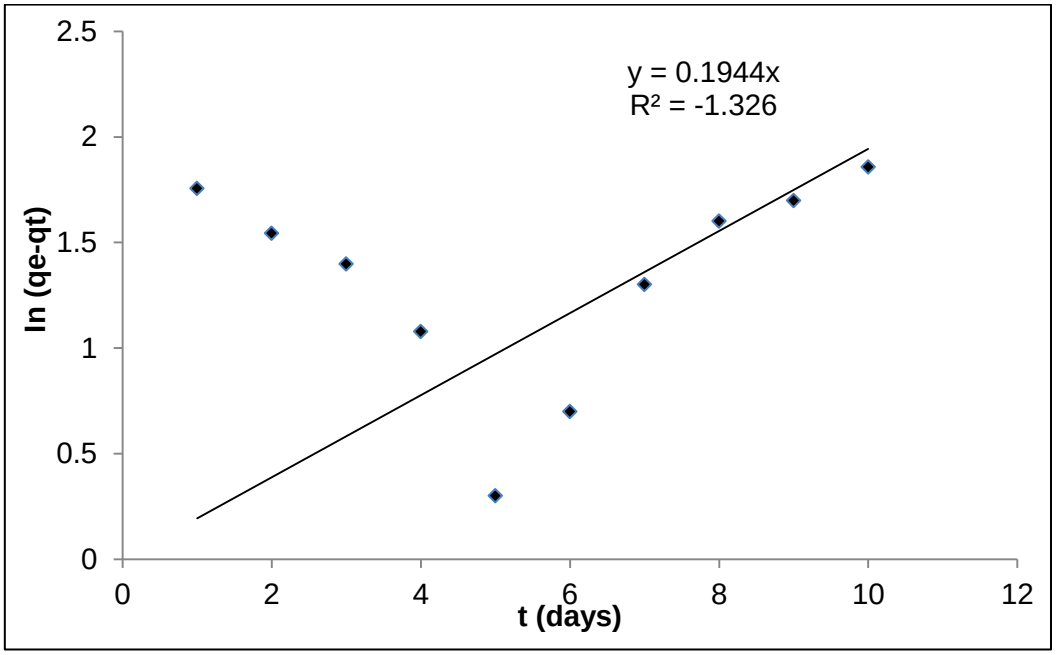


Figure 10 - Pseudo-first order kinetic plot for the biosorption of methyl orange onto *O. subpalgiostomum* AP1

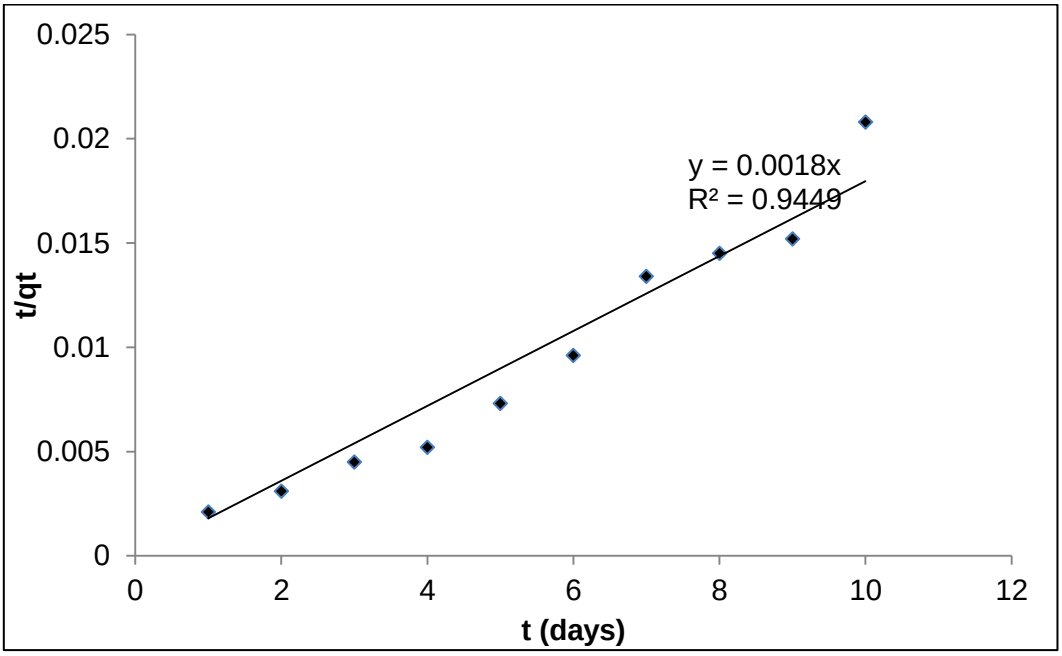


Figure 11 - Pseudo-second order kinetic plot for the biosorption of methyl orange onto *O. subpalgiostomum* AP1

The sorption data corresponded well with the pseudo-second order kinetic model of *Padina pavonica* for fast yellow dye (Fakhry, 2013) and *Microspora* species for methylene blue removal (Maurya *et al*., 2014) which corroborates with the findings of the present study.

Thus to conclude, the pseudo-second order kinetic model describes well the biosorption kinetics and the overall biosorption appears to be controlled by the chemisorption process.

4.3.3 Thermodynamic studies

Temperature is a significant physicochemical parameter which has a notable effect on the biosorption and thermodynamic parameters. In the present study the decolourisation of methyl orange by *O. subplagiostomum* AP1 was analysed at five different temperatures (293.15K, 303.15K, 313.15K, 323.15K and 333.15K) keeping all other experimental conditions constant [dye concentration (500mg/L), biosorbent concentration (400mg/L), pH (6.5) and incubation period (5 days)]. At the end of the incubation period (5^{th} day) the thermodynamic parameters for the adsorption process such as change in the standard free energy or Gibbs free energy (ΔG^o), enthalpy (ΔH^o) and entropy (ΔS^o) were determined.

The change in free energy (ΔG^o) is related to the equilibrium constant by the following relationship/equation

$$\Delta G^o = -RT \ln K_D \quad \text{---------- (22)}$$

where R is the universal gas constant (8.314 J/molK), T is the absolute temperature (K) and K_D is the adsorbate distribution coefficient (Lg^{-1}) (q_e/C_e).

The enthalpy (ΔH^o) and entropy (ΔS^o) parameters were estimated from the following equation,

$$\ln K_D = \frac{-\Delta G^o}{RT} = \frac{\Delta S^o}{R} - \frac{\Delta H^o}{RT} \quad \text{---------- (23)}$$

Accroding to Eq. (23), the ΔH^o and ΔS^o parameters can be calculated from the slope and intercept of the plot of ln K_D vs 1/T yield respectively. The results of the thermodynamic parameters namely ΔG^o, ΔH^o and ΔS^o were tabulated in Table 12.

Table 12 - Thermodynamic parameters for the adsorption of methyl orange onto *O. subpalgiostomum* AP1

Temperature (k)	ΔG^o (kJmol^{-1})	ΔH^o (kJmol^{-1})	ΔS^o (kJmol^{-1})
293.15	-126.37	-14.37	0.04
303.15	-147.15		
313.15	-252.76		
323.15	-411.54		
333.15	-673.49		

The values of ΔG^o are negative for the experimental temperatures indicating the adsorption of methyl orange onto *O. subplagiostomum* AP1 might have occurred spontaneously without any energy gain from an external source. Also it was observed that the decrease in the negative value of ΔG^o with increase in temperature indicates a favourable adsorption of dye by algal biomass at higher temperatures (Zaki *et al*., 2000).

The ΔH^o value was negative which suggest that the biosorption was exothermic. This might be due to the fact that at higher temperatures there is an increased tendency of the dye molecules to escape from the surface of the biosorbent which pass onto the solution phase. Hence, increase in temperature results in decreased dye adsorption onto the algal biomass (Gupta *et al*., 2015).

The positive value in change in entropy (ΔS^o) suggest that the structural changes might have occurred on the biosorbent and the randomness at the solid/liquid interphase in the sorption system may increase during the process of adsorption (Gupta, 1998 and Khamparia and Jaspal, 2016).

Dotto *et al*. (2013) also observed negative values in ΔG^o, ΔH^o indicating the process of biosorption was spontaneous, favourable and exothermic. The positive values of ΔS^o reflected the increased randomness at the solid/liquid interphase during the adsorption of dye onto the algae.

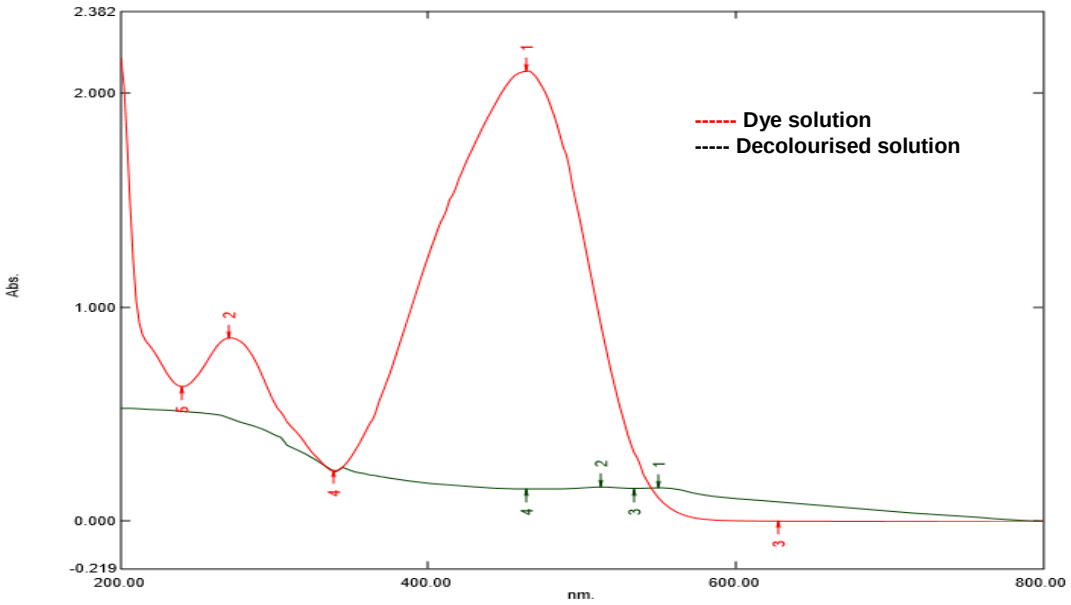
Thus according to thermodynamic properties (ΔG^o, ΔH^o and ΔS^o) adsorption of methyl orange onto *O. subplagiostomum* AP1 is spontaneous, exothermic and feasible in the temperature range of 293 to 333K.

PHASE IV

4.4 ANALYTICAL METHODS FOR DECOLOURISATION STUDIES

4.4.1 UV-Vis spectral analysis

UV-Vis spectral analysis was used to confirm the process of dye decolourisation which was due to degradation or biosorption (Aksu, 2003). The absorption spectra of methyl orange before and after decolourisation with *O. subplagiostomum* AP1 in spectral range of 200-800nm was depicted in Figure 12. As seen, the spectrum of the control (methyl orange) was characterised with two main peaks at 464nm (visible range) and 270nm (UV range). While in the absorption spectra of the dye treated with *O. subplagiostomum* AP1, the peaks were diminished or disappeared indicating the process of biodegradation (Figure 12).



Arrows indicates the absorption speaks at different wavelength (nm) that disappeared after decolourisation of dye

Figure 12 - UV-Vis spectra of methyl orange before and after decolourisation by *O. subplagiostomum* AP1

Bhatt *et al.* (2005) also reported that if dye removal is attributed to biodegradation either the major visible light absorbance peak would completely disappear or a new peak will appear. The results of the present study corroborates with the findings of Mubarak *et al.* (2011) who observed a decreased absorbance band when the textile dyeing effluent was treated with *Oscillatoria formosa* NTDM02.

Thus, the shift in λmax value of methyl orange to lower wavelength observed in the decolourised medium confirms the biodegradation of methyl orange.

4.4.2 Fourier-Transform Infrared Spectroscopy (FT-IR) analysis

The FT-IR analysis helps in elucidating the possible interaction between the dye and the functional groups present on the surface of the algal cell. The FT-IR spectra of dye unloaded and loaded algae were presented in Figure 13. The shifting and intensification of peaks indicates the involvement of *O*. *subplagiostomum* AP1 in the removal of dye.

The broad peak at 3379.29cm^{-1} was shifted to 3256.57cm^{-1} which indicates the involvement of hydroxyl group in the binding of dye to the algal surface. Also a shift from 3834.49cm^{-1} to 3849.92cm^{-1} was observed in the dye loaded biosorbent indicating the presence of hydroxyl group. Sari and Tuzen (2008) stated that the overlapping of amine and hydroxyl stretching vibrations indicates the presence of both N-H and hydroxyl groups in the biosorbent.

The shifting and intensification of peak from 2322.29cm^{-1} to 2254.58cm^{-1} indicates the carboxyl stretching vibrations. After decolourisation a shift in the IR peak was observed from 1813.09cm^{-1} to 1820.80cm^{-1} suggesting the degradation of dye. The IR peak in dye loaded biosorbent at 1602.35cm^{-1} was due to amine stretching or azo linkage, indicating the adsorption of methyl orange to the surface of the algae.

The absence of peak at 1388.75cm^{-1} for amine stretching vibration indicates the reductive cleavage of azo bond. Formation of new peaks (3741.90cm^{-1}, 2754.35cm^{-1}, 2677.20cm^{-1}, 2592.33cm^{-1}, 2461.17cm^{-1}, 1100.15cm^{-1} and 756.10cm^{-1}) and vanishing of peaks in the IR spectrum of dye loaded algae suggests the biotransformation of dyes into distinct metabolites.

The results of the present study coincides with the findings of Fakhry (2013) who reported that hydroxyl groups was responsible for the biosorption of acid fast yellow (azo dye) onto *Padina* biomass. Omar *et al*. (2018) reported the changes in the functional groups of algal biomass after treatment with malachite green and established the participation of these groups in dye removal. Similar such dye-adsorbent interaction was observed by Jayaraj *et al*. (2011) in malachite green treated with *Enteromorpha*.

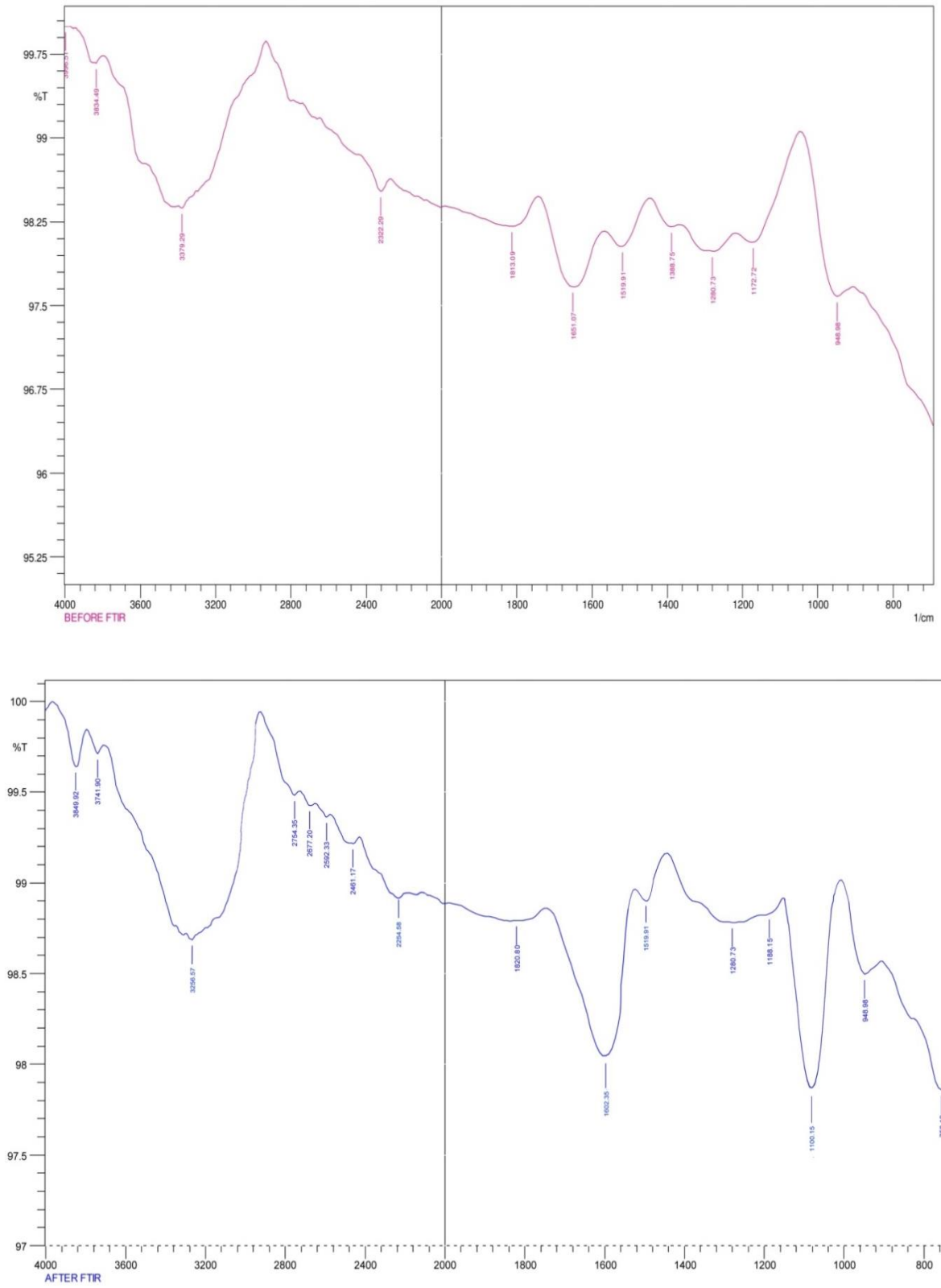


Figure 13 - FT-IR spectra of dye unloaded (a) and dye loaded (b) algae

The results of FT-IR analysis imply that dye adsorption might have occurred on the biosorbent by the presence of hydroxyl, amine and azo groups. Hence the presence of functional groups on the cell surface depends on the number of adsorption sites, their assemblage, chemical state and their affinity to bind with the dyes.

4.4.3 Scanning electron microscopy (SEM) with EDX analysis

The surface morphology of *Oedogonium subplagiostomum* AP1 was examined using scanning electron microscope (SEM) before and after biosorption of methyl orange and the corresponding SEM micrographs were potrayed in Figure 14.

The SEM micrograph of the biosorbent before and after biosorption showed morphological changes in the surface structure. The morphology of the biosorbent before adsorption was rough with numerous heterogeneous pores through which the dyes can be trapped and absorbed. While after dye sorption, the surface of the biosorbent reveals smooth texture indicating the entrapment of dyes (Figure 14). Similar changes in the surface porosity was observed in *Gelidium* due to methylene blue adsorption (Vilar *et al.,* 2006), tartrazine and allura red adsorption on *Spirulina platensis* (Dotto *et al.,* 2013).

The results of the present study were in accordance with Guarin (2018) who observed rough surface with high porosity in *Dicksonia antarctica* which accelerate dye adsorption. The EDX spectrum of *O*. *subplagiostomum* AP1 presented in Figure 15 shows the major elements such as C, O, Mg^{2+}, Si, Cl^{-}, K^{+} and Ca^{2+} respectively. When the algae interacted with methyl orange the elements such as Mg^{2+}, Cl^{-} and K^{+} were eliminated suggesting the possibility of the dye interaction with *O*. *subplagiostomum* AP1.

Aravindhan et al. (2004) reported the presence of calcium, magnesium, sodium, potassium, silicon, sulfur, carbon and oxygen in the EDX spectrum of Sargassum species. After treatment with chrome tanning solution, there was a disappearance or reduction of peaks of alkaline earth metals such as calcium, sodium, and magnesium and the appearance of a chromium peak indicating the mechanism of cations exchange for the accumulation of chromium onto the seaweed. EDX spectrum suggests the process of biosorption might have occurred through ion exchange mechanism.

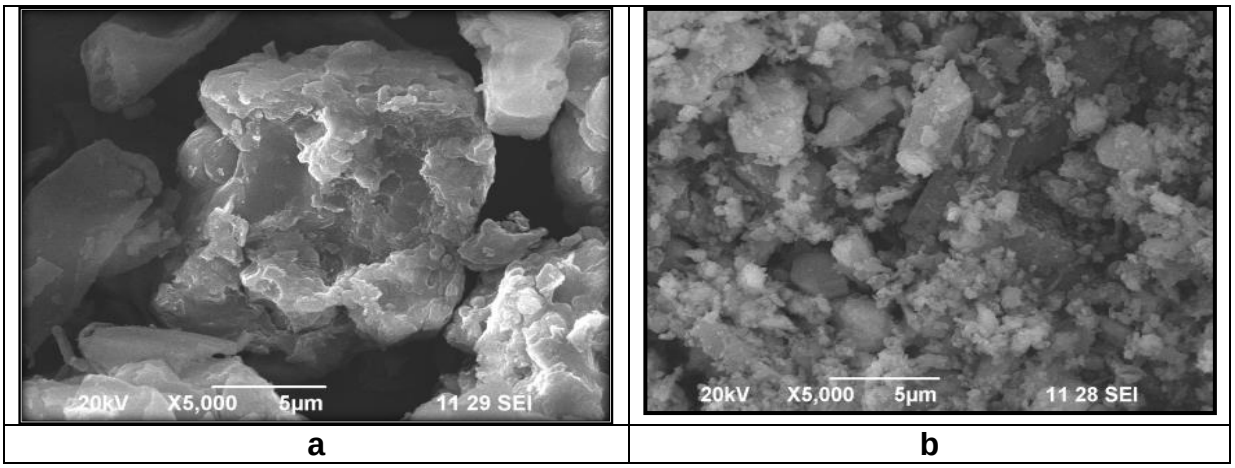


Figure 14 - Scanning electron micrographs of dye unloaded (a) and (b) loaded algae

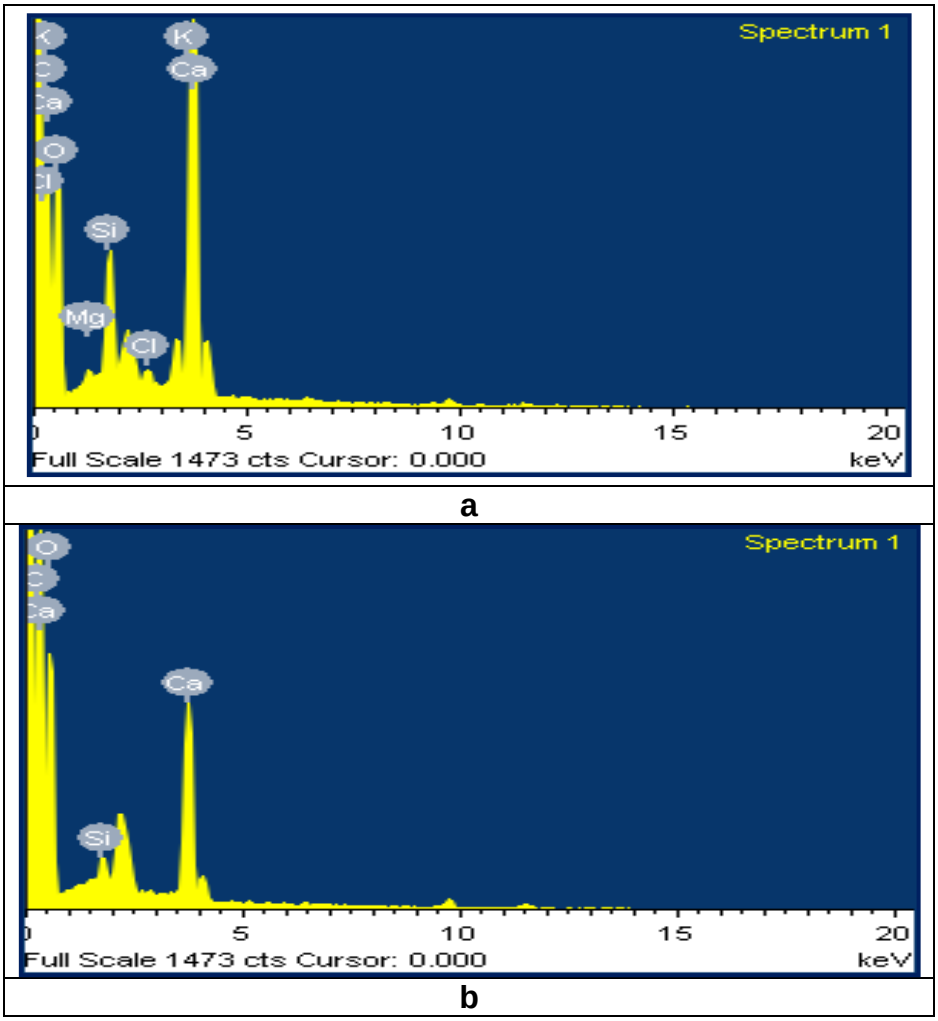
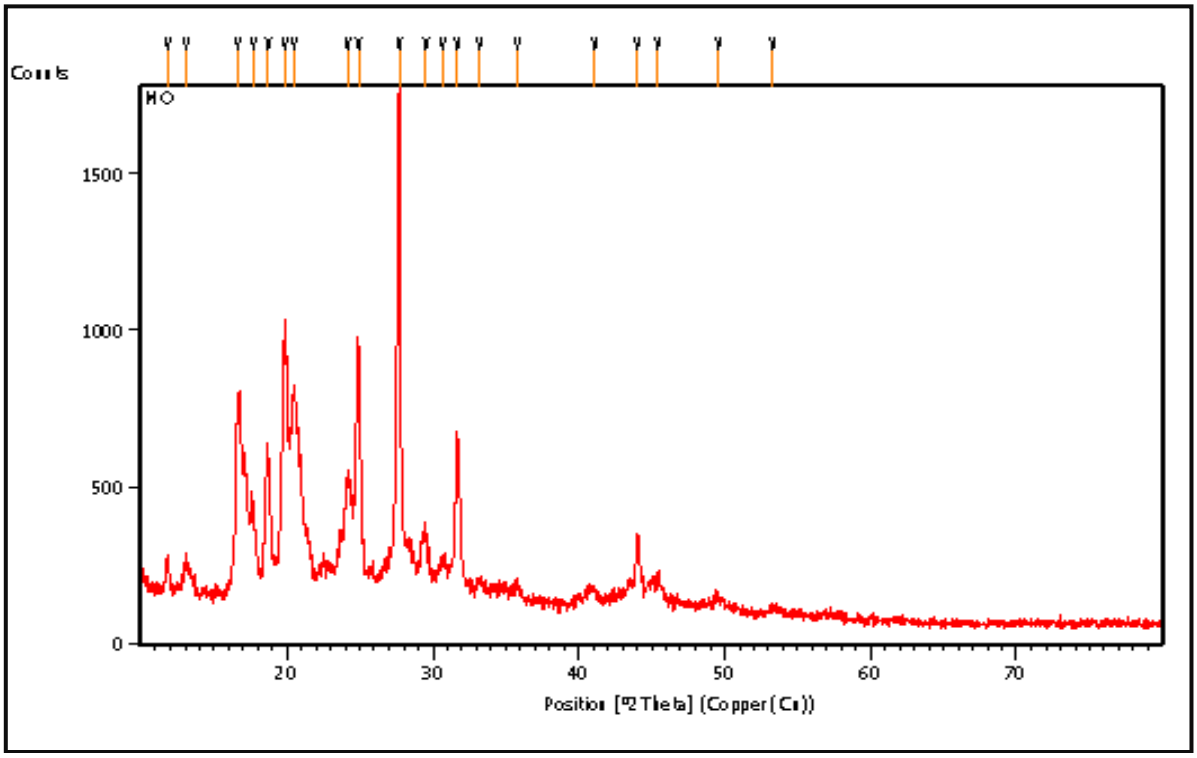


Figure 15 - EDX spectra of dye unloaded (a) and loaded (b) algae

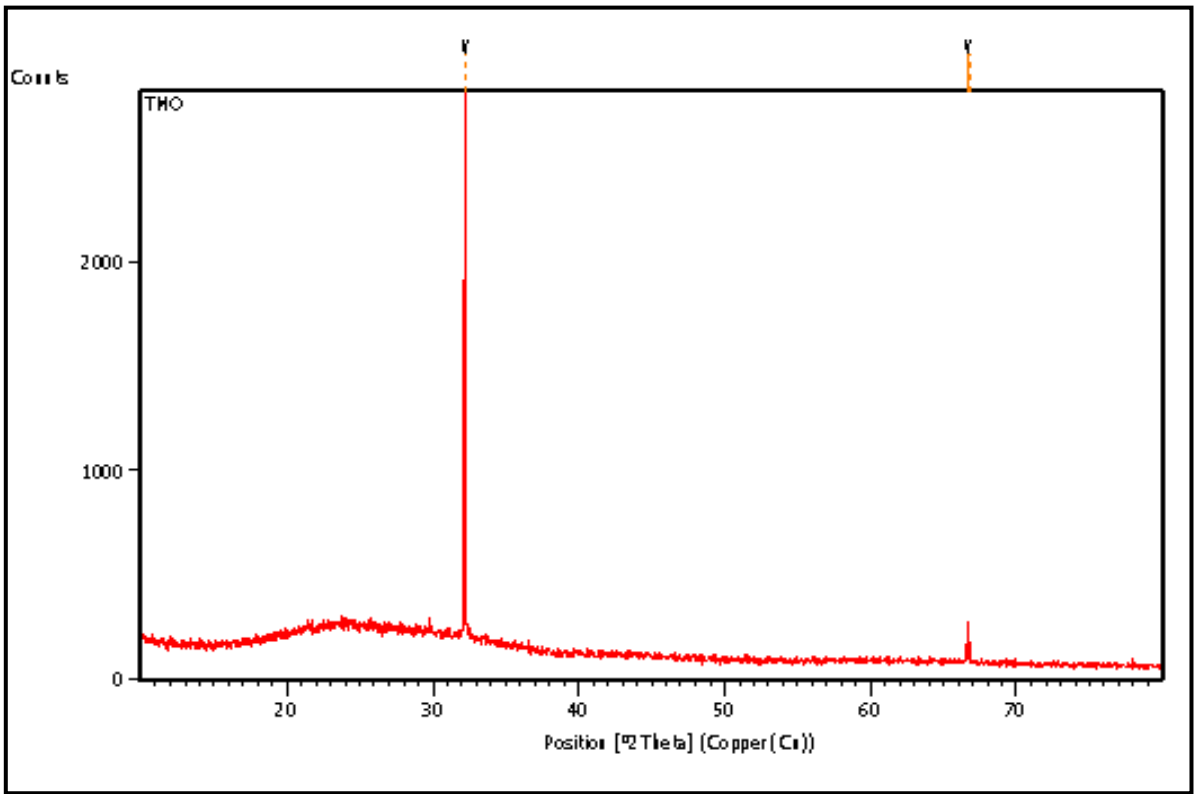
4.4.3.2 XRD analysis

X-ray diffraction technique is performed as a potent investigation tool to determine the nature of the biosorbent. Figure 16 illustrates the X-ray diffractogram of *Oedogonium subplagiostomum* AP1 loaded with and without methyl orange. The XRD spectrum of dye unloaded biosorbent displayed characteristic diffraction peaks at 2θ corresponding to 27.68, 19.84, 24.89, 20.51, 16.61, 31.68, 18.70, 24.17 and 17.17 with inter planar spacing (d spacing) of 3.22199, 4.47361, 3.6864, 4.32894, 5.33592, 2.82371, 4.74329, 3.57660 and 5.00475 respectively. With respect to the XRD spectrum of dye loaded biosorbent the diffraction peaks at 2θ were 32.17 and 66.62 with inter planar spacing (d spacing) of 2.78005 and 1.40250 respectively.

The presence of broad peaks in the spectrum of dye unloaded biosorbent indicates the predominance of crystalline nature which proves the suitability for biosorption. The presence of broad peaks in the biosorbent can be attributed to a heterogeneous complex matrix composed of various biomolecules such as protein, carbohydrate, lipids etc. (Saraf and Vaidya, 2016). The XRD pattern of dye loaded biosorbent exhibited exhibited amorphous nature allowing the dye molecules to adsorb on the surface through chmical process. The dyes might have been adsorbed onto the surface of the biosorbent and thereby alter the crystalline molecule structure by chemisorption process (Namasivayam and Kavitha, 2002). Similar such XRD pattern was observed in methyl orange loaded and unloaded *Nostoc carneum* biosorbent (Hussein *et al.,* 2018).



a



b

Figure 16 - XRD spectra of dye unloaded (a) and loaded (b) algae

PHASE V

4.5 TOXICITY STUDIES

Toxicity bioassays, provides an integrative information on the hazardous and toxic effects produced by the pollutants. In recent years, a wide range of indicators of different complexity have been employed for toxicity bioassays. Different organisms such as plants, fishes, microbes, worms, arthropods etc. have been employed as indicators in bioassay studies (Levy *et al*., 2007). Complex organisms are used for acute and chronic toxicity assessment due to their high susceptibility to pollutants. Hence, there is a need to check the toxicity of the degraded metabolites of the pollutants before disposing it into the environment.

In the present study, the toxicity of untreated methyl orange and their degraded metabolites formed after decolourisation using *Oedogonium subplagiostomum* AP1 has been studied on the growth of *Tagetes erecta* (Marigold), acute toxicity on *Labeo rohita*, inhibitory effects on microorganisms and also on the cytotoxicity and chromosomal aberrations in *Allium cepa*.

4.5.1 Phytotoxicity studies on Tagetes erecta

The untreated dyeing solution may cause deleterious environmental and health hazards when disposed into aquatic bodies. They also affect the agricultural lands and have direct impact on soil fertility (Kalyani *et al*., 2008). Biological treatment has gained much importance but the suitability of the treated waste water is still to be ascertained for the growth of plants. Thus, it is of great concern to assess the phytotoxicity of textile dye solutions before and after degradation (Kumar and Jaabir, 2013).

Hence in the present study, field level cultivation was conducted to assess the growth of *Tagetes erecta* using the degraded metabolites of methyl orange on 7^{th} and 60^{th} day respectively.

4.5.1.1 Effect of untreated and treated dye solution on seed germination and seedling growth of *Tagetes erecta*

The influence of tap water (T_1), untreated dye solution (T_2) and treated dye solution (T_3) on germination percentage, seedling length (shoot length and root length), vigour index and phytotoxicity index of *Tagetes erecta* was presented in Table 13. Plate 11 shows the growth of *T. erecta* seedling on 7^{th} day after sowing.

The mean germination percentage (98, 96 and 54), shoot length (3.95±0.65, 2.37±0.41 and 1.52±0.09), root length (1.91±0.86, 1.68±0.38 and 0.58±0.59) and vigour index (574, 389 and 113) were maximum in seeds exposed to tap water (T_1) followed by treated dye solution (T_3) and seeds exposed to untreated dye solution exhibited minimum growth (Table 13).

Table 13 - Biometric parameters of *Tagetes erecta seedlings* on 7th day

Treatments	Germination Percentage	Seedling length		Vigour index
		Shoot length (cm)	Root Length (cm)	
T_1	98	3.95±0.65	1.91±0.86	574
T_2	54	1.52±0.09	0.58±0.59	113
T_3	96	2.37±0.41	1.68±0.38	389
SED	-	0.19006	0.14627	-
CD (5%)	-	0.38281	0.29461	-

The values are mean ± SD

T_1 - Tap water (Control), T_2 - Untreated dye solution, T_3 - Treated dye solution

Seed germination and seedling growth are the essential stages for plant growth and its development. The process of reactivation of metabolic machinery of the seeds results in radicle and plumule emergence. Germination is a vital physiological process and also considered as a prime factor to assess the degree of pollution (Mishra and Pandey, 2002).

Seed vigour has to be assessed in order to enhance the germination and viability of a seed to gain insight in its performance in the field (Chandran *et al*., 2018). The decrease in vigour of seeds irrigated with untreated dye solution (T_2) might be due to the interaction of pollutants with the radical or it's entry into the membrane of embryonic axis rapidly (Bewley and Black, 1994).

Decrease in germination percentage in T_2 plants confirms the toxic effect of the pollutant which suppresses the plant growth. The reduction in the shoot and root length of the plant might be due to the inhibition of enzyme activation, water imbalance, nutritional deficiency or alteration in the hormonal status which alters the membrane permeability (Sharma and Dubey, 2005).

Plate 11 - Shoot length and root length of 7days old *Tagetes erecta* seedlings

T_1 – Tap water (Control)

T_2 – Untreated dye solution

T_3 – Treated dye solution

Yu *et al*. (2015) reported that the exposure of rice seedlings to methylene blue causes reduction in the plant growth rate which inturn also affects the transpiration rate. Sharma and Malaviya (2016) reported that the *Tagetes* seeds exposed to different concentrations of untreated and treated brewery-distillery effluent revealed maximum germination percentage, germination value, germination index, speed of germination and vigour index in 20% untreated and 60% treated effluent respectively.

The values of phytotoxicity were assessed in the ranges between 0 and 1. If the phytotoxicity index is greater or lower than 1 it indicates the toxic (negative) or stimulatory (positive) effect of the pollutant on the seed (Mekki *et al*., 2007). The results of phytotoxicity index in the present study revealed that the seeds of *Tagetes erecta* exposed to treated dye solution exhibited a value of 0.13 indicating the stimulatory effect of the dye degraded metabolites. In untreated dye solution the phytotoxicity index was recorded as 0.7 which was in close proximity to 1 indicating the toxic nature of the dye to the test plants (Rusan *et al*., 2015).

4.5.1.2 Yield attributes

The effect of various treatments (T_1, T_2 and T_3) on the yield parameters of *Tagetes erecta* is presented in Table 14 and Plate 12. T_1 plants recorded maximum and the number, size and weight of the flowers were also high followed by T_3 plants. T_2 plants exhbitied minimum yield attributes when compared to T_1 and T_3 plants indicating the toxic effect of the untreated dye solution (Table 14).

Reduction of flowering in T_2 plants might be due to the induction of high amount of abscisic acid in leaves which affects the movement of stomata and stimulates the senescence in leaves, thereby decreases the rate of photosynthesis which inturn inhibits the growth and yield of the plants (Kumar and Chopra, 2012b and Ahmad *et al*., 2011). Also the reduction in root and shoot length might be the major factors for the non-appearance of flower formation in *Tagetes erecta* irrigated with untreated dye solution.

Table 14 - Yield attributes of *Tagetes erecta* on 60th day

Treatment	Height of the plant (shoot and root length) (cm)	Number of Flowers	Size of the Flower (cm)	Weight of the flower (g)
T_1	61.13±0.27	18.88±0.97	6.85±0.25	6.71±0.11
T_2	11.21±0.17	1.89±0.32	2.38±0.63	2.76±0.39
T_3	59.81±1.64	17.56±1.19	6.52±0.76	6.23±0.33
SED	1.1985	0.8271	0.1427	0.6247
CD (5%)	2.9295	1.7749	0.6349	1.3184

The values are mean ± SD

T_1 - Tap water (Control), T_2 - Untreated dye solution, T_3 – Treated dye solution

Nivetha *et al*. (2014) observed the promotive effect on the morphological parameters of *Tagetes erecta* grown with textile effluent treated with *Oscillatoria subuliformis*. She also noted the presence of heavy metals in the untreated effluent which directly influenced the morphology of *T. erecta* and also results in reduced nutrient status which was contradictory in treated effluent and control.

The increase in the yield of flowers in T_3 plants might be due to the larger total leaf area which enhanced the duration of photosynthetase supply to the plants and flowers thereby contributing higher yield. Also the uptake of nutrients in T_3 plants from the soil may help to improve the rate of photosynthesis and provides energy to plants which in turn increases the yield of flowers (Vaithiyanathan *et al*., 2018).

Thus the treated waste water gives dampness required for crop growth, an alternative for traditional irrigation system and also provides plant sustenance supplements which form a substitute for costly chemical fertilizers. Treated waste water possesses a rich source of nutrient content and also provides nourishments to the plants. They contain higher amount of nitrogen which can be utilised in required amount by the plants and the rest are used for the vegetative development, prevents the delay in maturation of plants and for better yield.

Plate 12 - Yield of *Tagetes erecta* flowers on 60th day

T_1 – Tap water (Control)

T_2 – Untreated dye solution

T_3 – Treated dye solution

Thus the phytotoxicity studies indicated that the degraded metabolites of methyl orange had almost negligible effect on *Tagetes erecta* suggesting its non-toxic nature. Also the treated waste water provides an ideal supplement for the plant growth and careful agronomic management should be taken for the betterment of the ecosystem.

Cost economics for marigold yield per year

The cost economics for the flower yield in plants grown with treated dye solution was given below

The seeds are collected from the waste garldands in and around the surroundings.

Cost of 1kg marigold in market	= Rs. 50/-
Yield of marigold per plant	= 1kg
No. of plants grown per acre	= 1000 plants
Yield of marigold per acre	= 1000 X 1kg/ plant
	= 1000kg of flower
1 kg cost	= Rs. 50
∴ 1000 kg of flower cost	= Rs. 50,000 kg

Profit

Profit	= Total cost – Plant miscellaneous
	= Rs. 50,000 – Rs. 20,000
	= Rs. 30,000
The plant can be cultivated thrice in a year	= Rs.1,50,000 – Rs. 50,000
	= Rs. 1,00,000/-

4.5.1.3 Physicochemical characteristics of different treatment soil

At the end of 60th day of cultivation the soil samples from the T_1, T_2 and T_3 were collected and subjected to various physicochemical analysis and their results were tabulated in Table 15.

The mean pH and the electrical conductivity of the treated (T_3) and control (T_1) soil samples were recorded as 8.11±0.64 and 7.14±0.55 respectively whereas in T_2 (untreated) it was 12.37±1.00 which exceeded the tolerance limit (6.5-8.4) prescribed by Arnold (1984). The electrical conductivity of the soil treated with tap water (T_1) and treated dye solution (T_3) was 0.62±0.09 and 0.95±0.33mmhos/cm respectively whereas a maximum value of 3.81±1.24 was noticed in T_2 soil. The levels of electrical conductivity in the soil exposed to T_1 and T_3 are within the tolerance limit (<1 mmhos/cm) whereas in T_2 it exceeded the specified limit.

The macronutrients namely nitrogen, phosphorus, potassium, sodium and calcium were recorded to be 222.06±3.26, 32.04±8.16, 205.46±1.89, 279.97±4.90 and 17.34±0.26kg/ha respectively in T_1 soil while in T_3 soil it was recorded as 277.89±1.58, 21.69±4.78, 234.55±1.20, 239.62±1.48 and 8.09±0.37kg/ha respectively and the values were within the normal limit. In T_2 soil the values of macronutrients were found to be 131.28±2.13, 6.95±0.81, 85.77±3.09, 92.28±7.13 and 0.43±0.32kg/ha which were very low as prescribed by Gupta (2007).

The micronutrients namely iron, manganese, copper, zinc and nickel were recorded as 4.85±1.80, 0.66±0.03, 1.37±0.10, 1.91±0.41 and 21.09±4.48mg/kg in control soil (T_1) and 3.73±0.82, 0.51±0.10, 1.34±0.17, 1.18±0.32 and 27.58±3.92mg/kg in treated soil (T_3) whereas the level in T_2 were 1.59±0.37, 0.22±0.09, 4.84±0.47, 0.34±0.09 and 13.42±1.69mg/kg respectively which were below the limits prescribed by GUBRETAS (2010).

pH is an indicative measurement of the chemical properties of soil and electrical conductivity measures the total salt content in the soil. Continuous irrigation of the soil with untreated dye solution might increase the pH and electrical conductivity of the soil and may reduce the activity of beneficial microbes.

Table 15 - Physicochemical characteristics of experimental soil samples

Parameters	T_1	T_2	T_3	Tolerance limits
*pH	7.14±0.55	12.37±1.00	8.11±0.64	6.5- 8.4
*EC (mmhos/cm)	0.62±0.09	3.81±1.24	0.95±0.33	<1 (normal), 1-4 (critical),>4 (injurious to crop)
****Macronutrients (kg/ha)**				
Total Nitrogen	222.06±3.26	131.28±2.13	277.89±1.58	0-280 (low), 280-450 (medium), >450 (high)
Total Phosphorus	32.04±8.16	6.95±0.81	21.69±4.78	0-11 (low), 11-22 (medium), >22 (high)
Total Potassium	205.46±1.89	85.77±3.09	234.55±1.20	0-118 (low), 118-280 (medium), >280 (high)
Sodium	279.97±4.90	92.28±7.13	239.62±1.48	0-118 (low), 118-280 (medium), >280(high)
Calcium	17.34±0.26	0.43±0.32	8.09±0.37	0.5 (low), 5-10 (medium),10-20(normal), >20 (high-excess)
*****Micronutrients (mg/kg)**				
Iron	4.85±1.80	1.59±0.37	3.73±0.82	<2.5 (low), 2.5-4.5 (marginal) >4.5 (adequate)
Copper	0.66±0.03	0.22±0.09	0.51±0.10	0.72 (FAO, 1980)
Zinc	1.37±0.10	4.84±0.47	1.34±0.17	<0.2 (very low), 0.2-0.7 (low) ,0.8-2.4 (medium), 2.5-8.0 (high), >8.0 (too high)
Nickel	1.91±0.41	0.34±0.09	1.18±0.32	2-5 (WHO,1989)
Manganese	21.09±4.48	13.42±1.69	27.58±3.92	<4 (very low), 4-14 (low),15-50 (medium) , 51-170 (high), >170 (too high)

The values are mean ± SD,
* denotes the limits prescribed by Arnold (1984),
**denotes the limits prescribed by Gupta (2007),
*** denotes the limits prescribed by GUBRETAS (2010),
T_1 – Tap water (Control), T_2 - Untreated methyl orange dye soil, T_3 – Treated methyl orange dye soil

Plants require macronutrients for the growth and development which is provided by the soil. The presence of nitrogen helps the plants to prepare proteins through which they produce new tissues. High amount of nitrogen in the soil makes the plant to grow but they do not produce fruits or flowers. Similarly phosphorus stimulates the growth of roots, helps in flowering and increases the size of the seed. The soil microbe's activity and organic matter increases the availability of phosphorus. Potassium helps to improve the vigour, disease resistance and metabolic activities of plants. Calcium is required by plants in cell wall formation at growing points and to neutralize toxic materials. It improves the soil structure and helps to bind organic and inorganic particles. Iron regulates and promotes the growth of plant, manganese helps in photosynthesis, copper is an essential constituent for enzymes in plants, zinc production in plant hormone is responsible for stem elongation and leaf expansion (Zhang *et al*., 2010, Maser *et al*., 2002, Sato and Comerford, 2006, Setia *et al*., 2009, Giese *et al*., 2010, Yuan and Li, 2007, Foth, 1978, Haynes, 1985, Raij *et al*., 1986 and Nesse *et al*.,1998).

Jayathy *et al*. (2014) reported that the soil contaminated with dye showed reduced amount of macronutrients which may affect the growth of green gram. Reports on soil treated with dye solution were meagre and hence it is our utmost care to prevent the soil from such pollutants.

4.5.2 Zootoxicity studies on Labeo rohita

In recent years the treatment of waste water using biologicals has re-emerged. The cultivation of fishes in the recycled water has achieved to solve the environmental and sanitary problems in an ecofriendly and economical way. Literature reveals that the reuse of waste water for aquaculture operation using algae is meagre and hence in the present study an attempt was made to assess the impact of tap water (T_1), untreated dye (T_2) and treated dye (T_3) solutions on the behavioural response, mortality, haematological, biochemical, enzymological and histological examination of *Labeo rohita*.

4.5.2.1 Behavioural responses and mortality of *Labeo rohita*

The *Labeo rohita* exposed to different treatments selected for the present study showed a variety of behavioral changes. The irregularities observed prior to

mortality are a signal of depleted oxygen content due to high concentration of toxicant (Dahunsi and Oranusi, 2012).

Based on visual observation and the physical behaviour the fishes exposed to tap water (T_1) exhibited normal swimming, vision and proper body motion whereas those exposed to untreated dye solution (T_2) initially showed abnormal movements such as erratic swimming, hyper excitation and variation in opercular beat rates. After 24h, the fishes became weaker, showed sluggish movement and started to settle at the bottom of the trough. At the end of 7^{th} day, morphological changes such as reddening in the gills, faded body colour, gulping of air and shedding of scales from the skin were observed. This implies that the pollutant methyl orange directly affects the fishes and shows abnormalities in the growth and its behaviour.

Barot and Bahadur (2013) exposed *Labeo rohita* to direct green 6 which exhibited mucus secretion on the surface of the body, loss of appetite, dysfunctioning of gills which leads to respiratory distress causing suffocation, rapid opercular movement, gulping of air and accumulation of dye in gastrointestinal tract.

Katja *et al*. (2005) stated the gulping of air may help to avoid the contact to toxic medium while surfacing might be a demand of high oxygen level during the exposure period. Scott and Sloman (2004) and Kane *et al*. (2005) also noticed the absorption of environmental pollutants onto the skin of fish causing damage in the dermal layer and thereby lead to shedding of the scales.

Similar abnormal behavioral patterns such as erratic swimming, hyperventilation, rapid opening and closure of the operculum, hyperexcitation, excess mucous secretion and gasping was observed in *Oreochromis niloticus* exposed to acid dye 53 (Amwele *et al*., 2015) and *Labeo rohita* to acid orange 7 (Barot and Bahadur, 2015).

The fishes exposed to treated dye solution (T_3) showed normal swimming and stable movement indicating its tolerance level and depicting the reduction in the toxicity of dye.

Labeo rohita being a fresh water fish can tolerate disturbances and contaminants in the aquatic environment. The mortality of fishes mainly depends on the sensitivity and duration of exposure to the toxicant (Cavas and Gozukara, 2005).

However, introduction of fishes into the toxicant medium leads to severe damage of organ systems and causes mortality (Suvetha *et al*., 2015).

The percentage mortality of fishes was high in untreated dye solution (88%) when compared to fishes exposed to treated dye solution (33%) and tap water (1%) respectively (Figure 17).

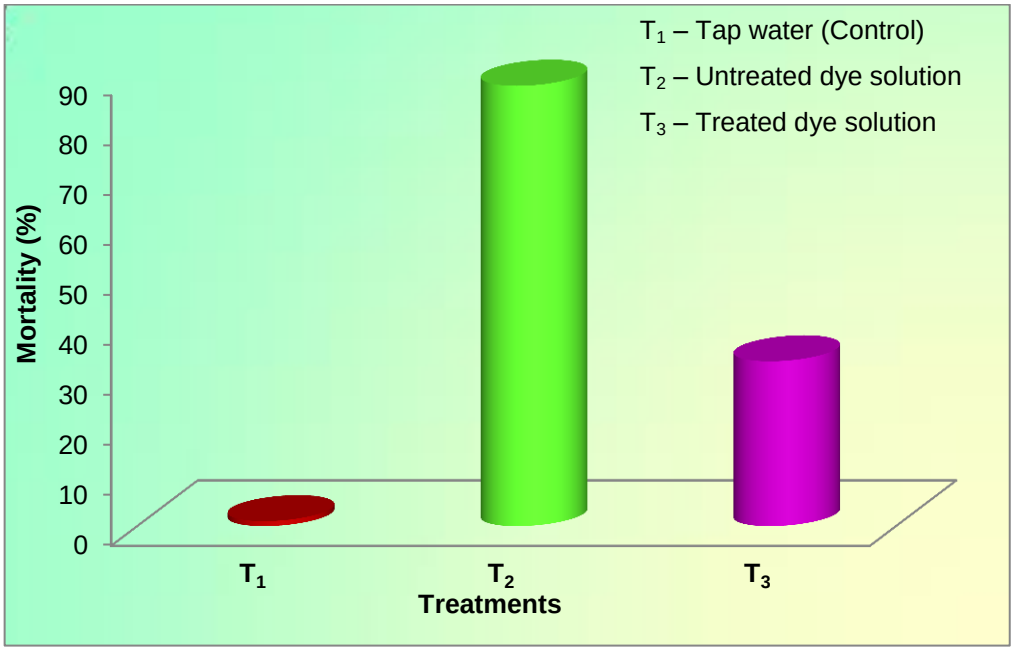


Figure 17 - Percentage mortality of *Labeo rohita* exposed to different treatments

Roopadevi and Somashekar (2012) observed the death of fishes stocked in higher effluent concentration for longer period and also the homeostatic behaviour of the test organism was eventually disturbed. This confirms with the results of the present study stating the toxicity of methyl orange to the experimental fish, *Labeo rohita*.

4.5.2.2 Haematological parameters

Blood is a pathophysiological reflector of the whole body and therefore its parameters are important in diagnosing the altered physiological status of fish exposed to toxicants. The haematological elements of fish are widely used as an indicator in toxicological research and environmental monitoring studies (Adhikari *et al*., 2004 and Carvalho and Fernandes, 2006).

After the exposure of fishes to different treatments (T_1, T_2 and T_3), the blood samples were collected at the end of experimental period (7^{th} day) and subjected to analyse the haematological (total RBC count, PCV, Hb, total WBC count, MCV, MCH and MCHC), biochemical (plasma glucose and plasma protein) and enzymological (AST and ALT) parameters. The heamatological profile, plasma glucose and plasma protein levels and enzyme activities were significant ($p<0.05$) in fishes exposed to treated dye solution when compared to untreated dye solution. The above parameters were significant ($p<0.05$) in control fishes when compared to treated fishes. The results of these parameters analysed in the experimental fishes were depicted in Table 16.

Table 16 - Haematological, biochemical and enzymological analyses in *Labeo rohita* exposed to different treatments

Parameters analysed	Treatments		
	T_1	T_2	T_3
Haematological parameters			
RBC (million/cu mm)	1.42±0.02a*	0.33±0.07b*	0.96±0.05c*
PCV (%)	16.31±0.57a*	7.02±0.21 b*	15.69±0.66c*
Hb (g/dl)	4.95±0.32a*	1.67±0.08b*	4.16±0.24c*
WBC (1000/cu mm)	1.37±0.13a*	2.23±0.24b*	1.50±0.02c*
Erythrocyte indices			
MCV (fl)	81.25±4.80[a]*	167.11±2.44b*	125.08±5.35c*
MCH (pg)	26.04±2.16[a]*	42.40±1.63b*	33.67±1.69c*
MCHC (g/dl)	23.86±0.47[a]*	17.65±1.63 b*	20.45±0.81c*
Biochemical parameters			
Plasma Glucose (mg/100ml)	56.37±2.49a*	25.15±1.63b*	55.76±2.05c*
Plasma Protein (µg/ml)	2.85±0.12a*	1.59±0.08b*	2.60±0.16 *
Enzymological parameters			
AST (IU/L)	78.21±2.86[a]*	143.69±5.79b*	119.25±11.2c*
ALT (IU/L)	15.22±0.81a*	58.91±0.70b*	36.73±2.59c*

The values are mean ± SD
T_1 - Tap water (Control), T_2 - Untreated dye solution, T_3 - Treated dye solution
a - T_1 vs T_2; b – T_2 vs T_3; c – T_1 vs T_3 * - Significant at 5% level ($p<0.05$)

The results revealed that *Labeo rohita* exposed to untreated dye solution (T_2) showed a notable decrease in the levels of RBC, PCV, Hb and MCHC when compared to T_1 and T_3 fishes. The WBC, MCV and MCH levels increased in the T_2 fishes when compared with T_1 and T_3 fishes (Table 16).

Decrease in the haematological parameters in T_2 fishes depicts its anemic condition which might have resulted from the hemolysis or damage caused in the gills leading to impaired respiratory capacity by the pollutant, methyl orange. The significant reduction of RBC content in T_2 fishes might be due to the failure of erythrocyte production, impaired osmoregulation, internal hemorrhage and inhibition of oxygen production during stress conditions (Kaoud *et al*., 2011, Kavitha *et al*., 2010, Joshi *et al*., 2002). Tariq *et al*. (1996) observed an increase in the treatment and carbon dioxide content in the blood of fishes exposed to pollutants. He also noticed the swelling of RBCs, cytotoxic effect on erythropoietic tissues leading to disturbances in bone marrow and alteration in the cell cycle and erythropoiesis.

PCV is a measure of percentage of RBCs present in a volume of whole blood. Decrease in the PCV of fishes exposed to untreated dye solution may be due to anemia or destruction of red blood cells or nutritional deficiency, disturbances occurred in metabolic and hemopoietic activities leading to impairment in hemopoietic organs (Sharma and Langer, 2014).

Thus PCV appears to be positively correlated with erythrocyte count where decrease in the number of RBCs followed by PCV confirms anemia in *Labeo rohita* (Palanisamy *et al*., 2011). The results are in agreement with the findings of Srivastav and Roy (2015) who observed a significant decrease in PCV of fishes exposed to malachite green when compared to the control and treated fishes.

Hb seems to be reliable and best blood indicator of environmental stress. Increase in its concentration could be a persistent sign of an adaptational improvement in oxygen transporting capacity of blood (Khalesi *et al*., 2014 and Cazenave *et al*., 2005).

Lower level of haemoglobin in T_2 fishes might decrease the ability to enhance the activities required to meet demands such as seeking of food and escaping from predators (Barot and Bahadur, 2013).The depletion or reduction in Hb content in T_2 fishes could also be attributed to the production of reactive oxygen species under the influence of toxicant resulting in the destruction of red blood cell membrane and its function, inhibition of the enzymes involved in Hb synthesis (Pamila *et al*., 1991), impaired intestinal absorption of iron or transferrin dysfunction (Joshi *et al*., 2002) and impairment in the immunological reactions to produce antibodies to cope up with stress induced by the toxicant (Ramdas, 2013).

Similar decrease in the amount of RBC, Hb and PCV could be corroborated with the findings of earlier investigations in *Tilapia mossambica* exposed to textile dyeing effluent (Karthik *et al*., 2016 and Deepika and Noorjahan, 2018), *Catla catla* exposed to acid red 97 (Avni and Jagruti, 2017), *Carassius auratus gibelio* exposed to azo red 120 (Al-Sabti, 2000) respectively.

Leucocytes or white blood corpuscles are cells of immune system which play a vital role in both specific and non-specific immune responses in protecting the body against toxicants (Ramesh *et al*., 2014 and Bujjamma and Padmavathi, 2018). One of the most elementary ways to assess the immune system is to explore the changes in WBC count (Nussey *et al*., 2007 and Pimpao *et al*., 2007).

The WBC count was observed to be high in T_2 fishes when compared to T_1 and T_3 fishes where the count was decreased or lowered. Increase in the WBC count in T_2 fishes can be attributed to the stimulation of the immune system in response to tissue damage caused by the dye. Witeska (2004) and Tiago *et al*. (2008) also reported that, the increase in antibody production helps in the survival and recovers the fishes exposed to toxicants. Significant increase in the total WBC count was observed in *Heteropneustes fossilis* exposed to malachite green and pyceze (Srivastav and Roy, 2015) which supports the findings of the present investigation.

For examining the health status, blood indices analyses has proven to be a valuable method which provides reliable information on metabolic ailments,

deficiency and chronic stress status (Zhou *et al*., 2009). The RBC indices namely MCV, MCH and MCHC are the part of whole blood count which express the size and Hb content of erythrocyte (Praveena *et al*., 2013). MCV is one of the important blood parameter which gives an indication of the status of the size of RBC and reflects the normal or abnormal cell division during erythropoiesis (Wepener *et al*., 1992).

In the present study, fishes exposed to untreated dye solution showed higher level of MCV and MCH when compared to the control and treated fishes. This increase might be due to the enlargement or swelling of RBCs as a result of osmotic disturbances or hypotonic condition or uptake of electrolytes and water into the cells accompanied by acidification of the cytoplasm of RBCs leading to macrocytic anemia in fishes (Suvetha *et al*., 2015). Ferrando and Moliner (1991) also reported that higher concentration of smaller immature erythrocytes in the circulation due to hyperplasia in erythrocyte forming sites leads to increased MCV. The decreased MCHC value in the present study may be due to the binding of the pollutant to Hb which prevents the oxygen carrying capacity and leads to metabolic stress and death of fishes (Lemly, 1993).

These results coincides with the findings of Amte and Mhaskar (2013), Barot and Bahadur (2014) and Afaq and Rana (2009) who observed an increase in the values of MCV and MCH in *Oreochromis mossambicus*, *Labeo rohita* and *Cirrhinus mrigala* exposed to textile dyeing effluent, direct green 6, bismarck brown and acid leather brown respectively.

Dhanve *et al*. (2014) observed anxious movement, high mortality, abnormalities in the shapes of erythrocytes and formation of micronuclei in *Acantopsis choirorhyncus* exposed to untreated textile effluent. In contrast, the effluent treated with *Exiguobacterium* sp. RD3 (biodegradation products) did not induce any observable hazardous health effects on the fish.

Thus haematological parameters also determine the changes in the levels of biomarkers namely the enzymes, normal functioning and histomorphology of the organs (Oyedemi *et al*., 2011).

4.5.2.3 Biochemical parameters

To monitor the presence of toxicants in aquatic media, biochemical analysis offers as an important bioindicator (Qiu *et al*., 2009). Carbohydrates and proteins serve as an energy precursor for fish under stress condition. Plasma glucose has been extensively used as a sensitive biochemical indicator to study the stress of fish under unfavorable environment (Sancho *et al*., 2000). Glucose is one of the most important compounds of carbohydrates which serve as an immediate and major metabolic fuel for all the biological activities (Pal and Reddy, 2018).

The decreased level of plasma glucose in untreated fishes (T_2) might be due to the hypoxic condition caused by the toxicant which reflects an excess utilization of stored carbohydrate during treatment period (Agrahari *et al*., 2007). Also the accumulation of the untreated dye in the kidney may cause renal injury which inturn reflects the concentration of glucose during stress conditions.

Protein is an important biochemical parameter which has been used to understand the general state of health and biological mechanism and metabolism under the stress of a toxicant (Saravanan and Ramesh, 2013).

Decrease in the plasma protein in T_2 fishes might be due to necrosis or liver cirrhosis or alteration in the enzyme involved in the biosynthesis of proteins (Nandhi *et al*., 2005, Yousef *et al*., 2008, Palaniappan and Vijayasundaram, 2009). The decrease in the plasma glucose and plasma protein in the present investigation coincides with the studies carried out in *Clarias gariepinus* and *Oreochromis niloticus* exposed to textile dye industry wastewater (Agbon *et al*., 2014), *Oncorhynchus mykiss* treated with malachite green (Atamanalp, 2007) and *Cyprinus carpio* with textile industrial effluent (Dhanalakshmi *et al*., 2018) respectively.

4.5.2.4 Enzymological parameters

Various physiological activities are regulated by the vital organ, the liver, which performs metabolism, storage, secretion and detoxification in fishes (Reethamma and Joseph, 2016). Enzyme activities in the blood serum are considered as an important biochemical indicator in hepatic dysfunction and damage (Jung *et al*., 2003). Hence, enzyme assays are widely used to assess the health of an organism in aquatic toxicology (Gul *et al*., 2004). Among the battery of enzymes,

aspartate transaminase and alanine transaminase are widely used as pathological and potential markers to detect the function of liver or toxicant induced hepatotoxicity (Huang *et al*., 2006).

In the present study, the activity of serum AST and ALT were high in T_2 fishes when compared to T_3 and control fishes. When the fishes were exposed to untreated dye solution the hepatic parenchyma cells were damaged, liberated AST and ALT into the blood stream and their activities were elevated. Moss *et al*. (1986) reported that the activities of these enzymes in serum were considered as a sensitive indicator of minor cell damage because the level of these enzymes exceeds those of the extracellular fluid by more than threefold increase.

Javed *et al*. (2016) reported that increased activity of the marker enzymes, AST and ALT indicated the increased rate of transamination. As a result, proteins are broken into free amino acids which are further utilized in glycogenic pathway. Alteration in the activity of AST and ALT is also reflected in the nitrogen metabolism and on the energy yielding TCA cycle (Beyer *et al*., 1996).

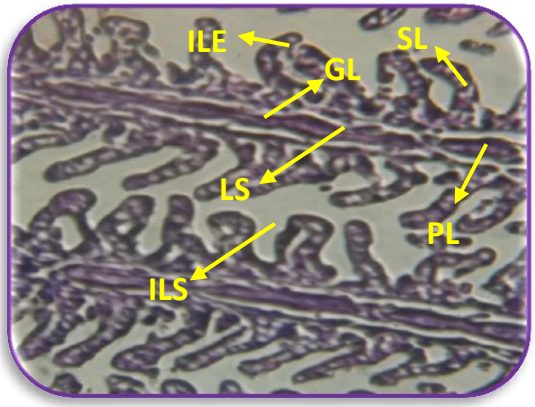
4.5.2.5 Histopathological examination

Histopathological investigations reveal the effect of pollutants on fish as it affords the direct transformation of toxic xenobiotic which influence the vital anatomical functions (Wester and Canton, 1991). Histopathological examination is a susceptible and sensitive parameter that provides a clear image on the cellular alteration that has occurred in target organs such as the gills, muscle, liver and kidney under stress conditions (Gernhofer *et al*., 2001). Thus histopathological investigation proves to be a cost effective tool in determining the health position of fish which thereby reflects the health of the entire aquatic ecosystem (Thophon *et al*., 2003). The histopathological changes in the target organs also leads to the alterations in physiological and biochemical parameters of the experimental fishes. Hence, histopathological studies are indispensable for the depiction and assessment of potential lesions in aquatic organisms exposed to various pollutants in aquaculture (Meyers and Hendricks, 1985 and Camargo and Martinez, 2007).

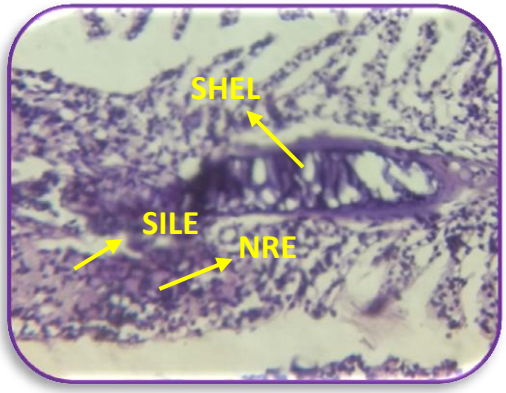
Histological changes in the gill of *Labeo rohita*

Gills are a good indicator of water quality and possess respiratory, osmoregulatory and excretory functions. Absorption of pollutants from an aquatic medium through gills makes fish a vulnerable target to its toxicity (Fanta *et al*., 2003 and McDouald, 1983).

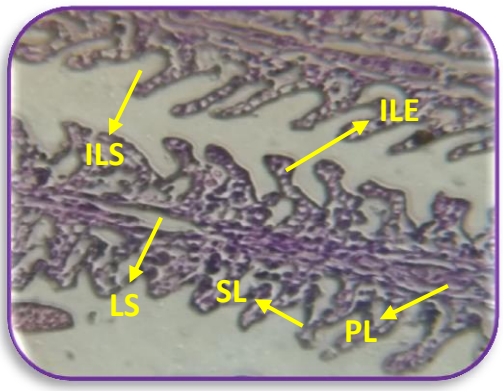
Plate 13 - Histology of gills in *Labeo rohita*



13a – Gill section of *L. rohita* - Control



13b-Section of gills in *L. rohita* exposed to untreated dye solution



13c– Section of gills in *L. rohita* exposed to treated dye solution

PL – Primary Lamellae
SL – Secondary Lamellae
ILE – Inter Lamellar Epithelium
ILS – Inter Lamellar Space
LS – Lamellar Space
GL – Gill Filament
SILE – Swelling of Inter Lamellar Epithelium
NRE – Necrosis of Respiratory Epithelial cells
SHEL – Severe Haemorrhage and Erosion in the Lamella of gills
RRE – Regeneration of Respiratory Epithelium

Histological observation on the gills of *Labeo rohita* exposed to tap water (T_1) showed normal architecture of gill filaments such as primary and secondary gill lamella (Plate 13a). The gills of *Labeo rohita* fingerlings exposed to untreated dye solution (T_2) showed necrosis of secondary lamella, swelling in the inter lamella and severe haemorrhage and erosion in the lamella of gills (Plate 13b). These histopathological changes may be a reaction to pollutant intake or an adaptive response to prevent their entry through the gill surface. The necrosis of respiratory epithelial cells can adversely affect the gaseous exchange and ionic regulation in fishes. Similar such necrosis and haemorrhage in the gills of fishes exposed to untreated dye solution was also evidenced in *Catla catla* to reactive red 120 (Avni and Jagruti, 2016), *Labeo rohita* to textile mill effluent (Nikalje *et al*., 2012) and *Tilapia mossambica* to textile dyeing effluent (Ravisankar and John, 2012) respectively.

However, the fingerlings exposed to treated dye solution did not show any deformities in the gills and the regeneration of respiratory epithelium of the gills were observed (Plate 13c). Similar such findings were reported in *Catla catla* exposed to treated sago effluent (Ramesh and Nagarajan, 2014) and *Etheostoma olmstedi* exposed to *Bacillus pumilus* treated textile wastewater (Watharkar *et al.,* 2014) respectively.

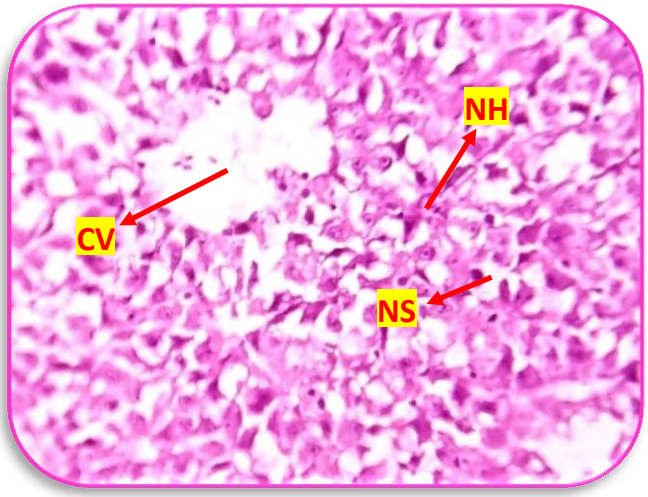
Histological changes in the liver of *Labeo rohita*

Liver plays an important role in the detoxification of contaminants because it acts as a main storage organ for many substances. Hence the accumulation of toxicants may affect its functions and thereby decreases the blood supply to all parts of the body (Nagai, 2002).

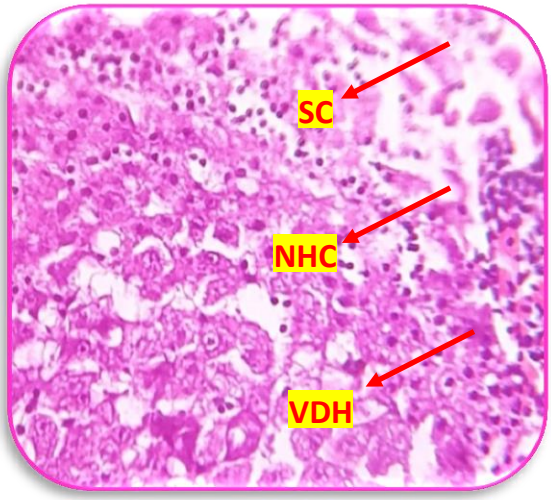
The histology of liver in control fishes showed normal hepatocytes arrangement. The hepatocytes were located among the sinusoids which form a cord like structure and possess large nuclei cords (Plate 14a). No remarkable changes were observed in the hepatocytes of fishes exposed to treated dye solution and the histo architecture was similar to that of control fishes (Plate 14c). Fishes exposed to untreated dye solution showed vacuolar degeneration, sinusoidal congestion,

necrosis and haemorrhage of hepatocytes (Plate 14b). Intoxication of the pollutant in the fish changes the general architecture of liver indicating the degree of structural heterogeneity which is enhances with the inhalation of the toxicant.

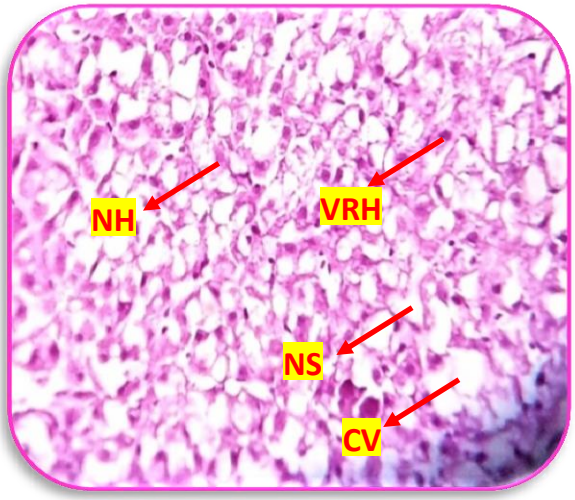
Plate 14 – Histology of liver in *Labeo rohita*



14a – Liver section of L. rohita - Control



14b – Section of liver in *L. rohita* exposed to untreated dye solution



14c – Section of liver in *L. rohita* exposed to treated dye solution

CV – Central Vein
NH – Normal Hepatocytes
NS – Normal Sinusoids
SC – Sinusoidal Congestion
NHC – Necrosis of Hepatic Cells
VDH – Vacuolar Degeneration of the Hepatocytes
VRH – Vacuolar Regeneration of Hepatocytes

The histo architecture of the fishes exposed to untreated dye is in confirmation with the findings of *Poecilia reticulata* exposed to textile dyeing industry effluent (Selvaraj *et al*., 2015), and *Channa punctatus* exposed to vat blue 4 and vat green 1 (Olaganathan and Patterson, 2012) respectively.

Histological changes in the kidney of *Labeo rohita*

Kidney is a primary organ important for the elimination of waste and osmoregulation in fish. Histology of kidney in control fishes showed well characterised glomeruli and renal tubules (Plate 15a). Fishes exposed to untreated dye solution showed highly degenerative and necrotic changes in the renal tubules, congested and shrunken glomeruli (Plate 15b).

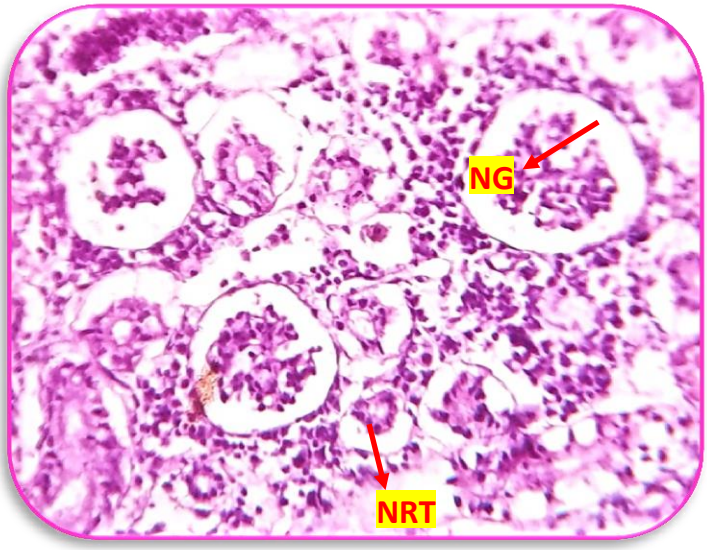
Degeneration of renal tubules and necrotic changes in the kidney has also been observed in *Labeo rohita* exposed to direct green 6 (Barot and Bahadur, 2013), *Clarias lazera* exposed to dyestuff wastewater (Abdel-Moneim *et al*., 2008), *Cyprinus carpio* exposed to textile industrial effluent (Dhanalakshmi *et al*., 2018) and *Labeo rohita* exposed to textile dyeing effluent (Rana and Raizada, 2000) respectively.

Kl-Neweshy and AbouSrag (2011) observed necrotic changes in the tubular epithelium of kidney exposed to acid orange 7. The intoxication of the dye may result in necrotic cell death which might be caused by the swelling and eventual failure of cell membrane, leading to rupture of the renal cells and release of its content into the extracellular space.

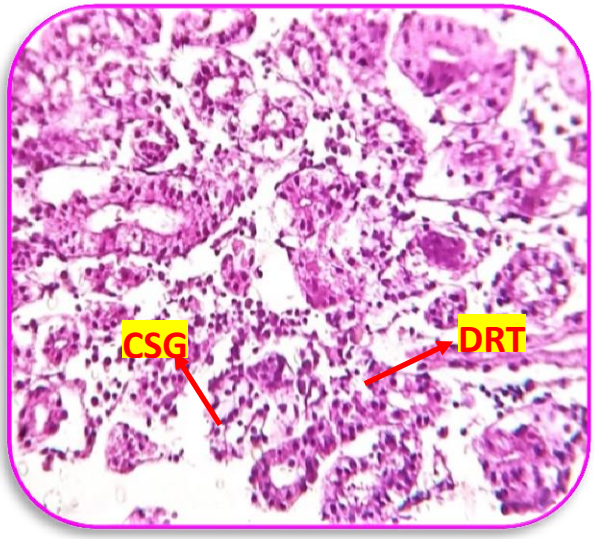
The renal tubules and glomeruli were regenerated in the fishes exposed to treated dye solution (Plate 15c). The structural features were found to be alike of those observed in the control fishes.

Literature on haematological, biochemical, enzymological and histological parameters analysed in fishes exposed to treated dye solution are meagre and the present study forms a platform to carry out research in this area.

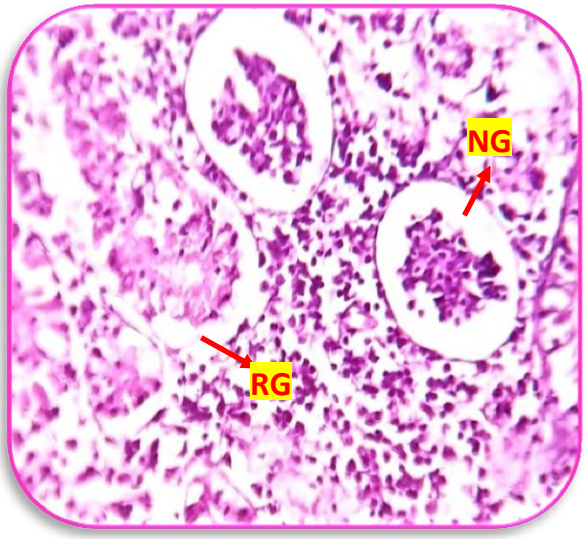
Plate 15 - Histology of kidney in *Labeo rohita*



15a – Kidney section of *L. rohita* - Control



15b – Section of kidney in *L. rohita* exposed to untreated dye solution



15c – Section of kidney in *L. rohita* exposed to treated dye solution

NG – Normal Glomeruli

NRT – Normal Renal Tubules

DRT – Degeneration of Renal Tubules

CSG – Congested and Shrunken glomeruli

RG – Regeneration of Glomeruli

4.5.3 Microbial toxicity studies

Microbial bioassay represents a quick, fast and reliable response in the assessment of a toxicant or pollutant when compared to higher or complex organisms, since they require long incubation period. Moreover, it is also easy to handle, since the metabolism of microbes are fast, they are versatile and proves to be best choice in the assessment of toxicity determination (Hassan *et al*., 2016). The results of microbial toxicity of methyl orange and its degraded metabolites against the tested bacterial and fungal isolates were depicted in Table 17. Plate 16a and 16b reveals the zone of inhibition of microbes against the untreated dye and its degraded metabolites.

The size of the inhibition zone of methyl orange ranged from 8-12mm and 10-14mm for the selected bacterial and fungal isolates respectively indicating the toxic nature of the dye. There was no significant growth inhibitory zone in tap water and dye degraded metabolites indicating their non-toxic nature against the tested bacterial and fungal isolates (Table 17).

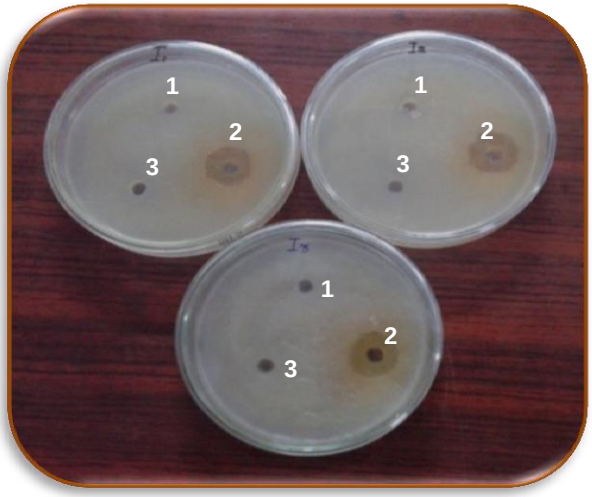
Similar such inhibition zone was not observed against the degraded metabolites of acid black 210 treated with *Providencia* species (Agarwal *et al*., 2014), reactive red M8B treated with *Bacillus subtilis* (Arulazhagan, 2016), green HE4BD, golden yellow HE4R and orange 3R treated with *Proteus vulgaris* and *Micrococcus glutamicus* (Saratale *et al*., 2010), synozol red HF-6BN and congo red treated with *Aspergillus niger* (Iiyas and Rehman, 2013 and Asses *et al*., 2018), reactive red BS treated with *Pseudomonas aeruginosa* NGKCTS (Sheth and Dave 2009) and methylene blue treated with *Stenotrophomonas maltophilia* (Kilany, 2017) and crystal violet and malachite green treated with *Staphylococcus aureus* (Sandhiya *et al*., 2016), *Bacillus* species (Ayed *et al*., 2009) and *Kocuria rosea* MTCC 1532 (Parshetti *et al*., 2006) respectively.

Hence the degraded metabolites of methyl orange by *Oedogonium subplagiostomum* AP1 confirm the non-toxic nature to the microbes.

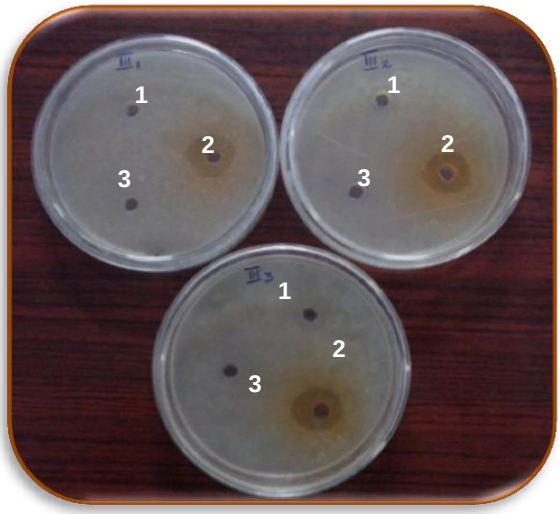
Table 17 - Microbial toxicity of methyl orange and its degraded metabolites

S. No.	Microorganisms	Zone of inhibition (mm)		
		Tap water	Methyl orange	Dye degraded metabolites
Bacterial isolates				
1.	*Klebsiella pneumoniae*	NI	11.7±0.30	NI
2.	*Vibrio cholerae*	NI	10.2±0.11	NI
3.	*Pseudomonas aeruginosa*	NI	11.5±0.16	NI
4.	*Shigella* species	NI	11.2±0.50	NI
5.	*Bacillus cereus*	NI	13.7±0.18	NI
6.	*Proteus vulgaris*	NI	11.3±0.75	NI
7.	*Staphylococcus aureus*	NI	11.2±0.20	NI
8.	*Escherichia coli*	NI	9.5±0.55	NI
9.	*Salmonella enteritis*	NI	8.5±0.30	NI
10.	*Enterococcus faecalis*	NI	12.2±0.20	NI
11.	*Yersinia enterocolitica*	NI	11.3±0.15	NI
12.	*Acinetobacter* species	NI	10.2±0.11	NI
Fungal isolates				
13.	*Aspergillus flavus*	NI	12.1±0.25	NI
14.	*Aspergillus niger*	NI	10.5±0.31	NI
15.	*Acremonium* species	NI	12.5±0.72	NI
16.	*Rhizopus* species	NI	13.7±1.10	NI
17.	*Trichoderma viride*	NI	14.6±1.54	NI

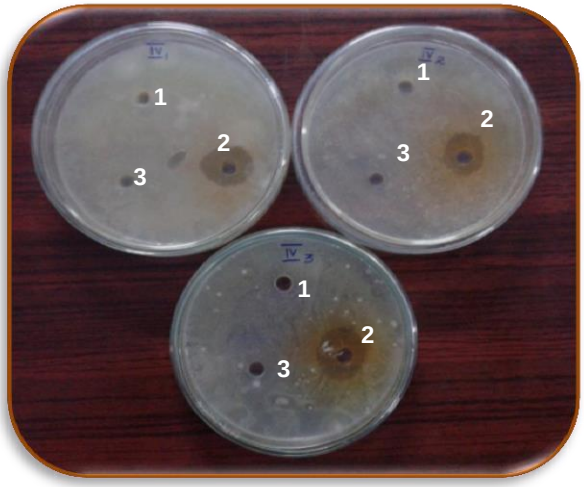
The values are mean±SD, NI-No inhibition zone



Klebsiella pneumoniae

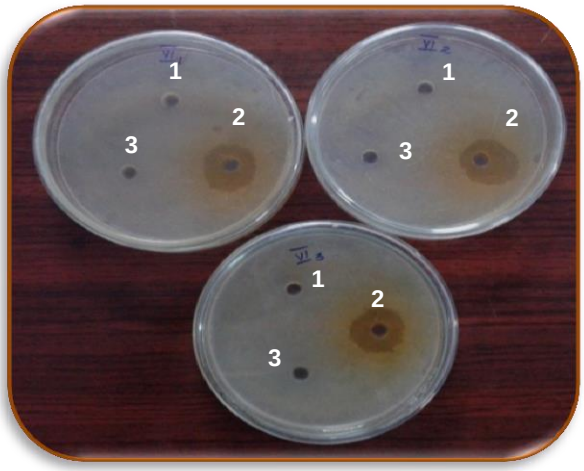


Vibrio cholera

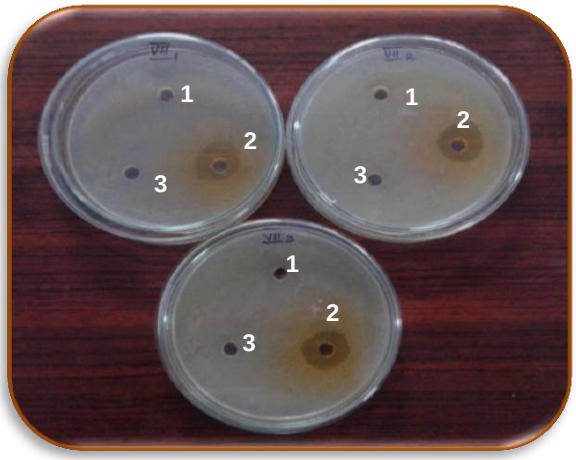


Pseudomonas aeruginosa

***Shigella* species**



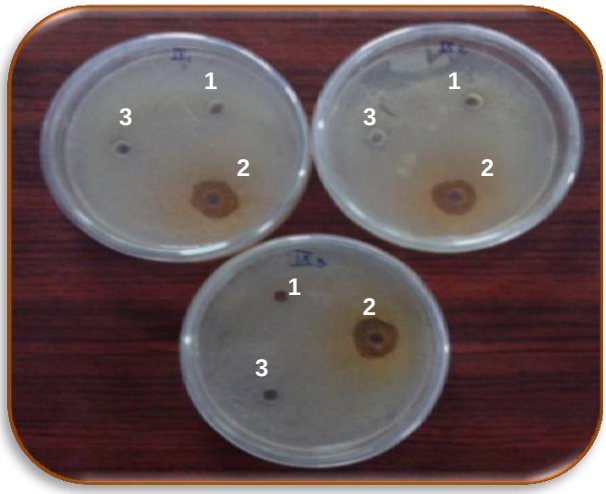
Bacillus cereus



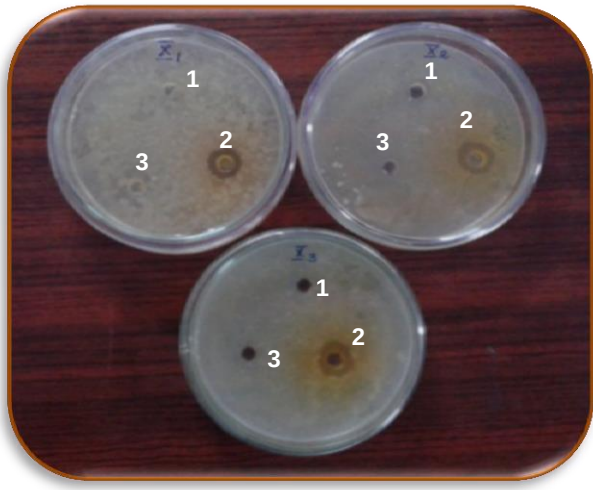
Proteus vulgaris

1- Tap water, 2 - Methyl orange, 3 - Degraded metabolites

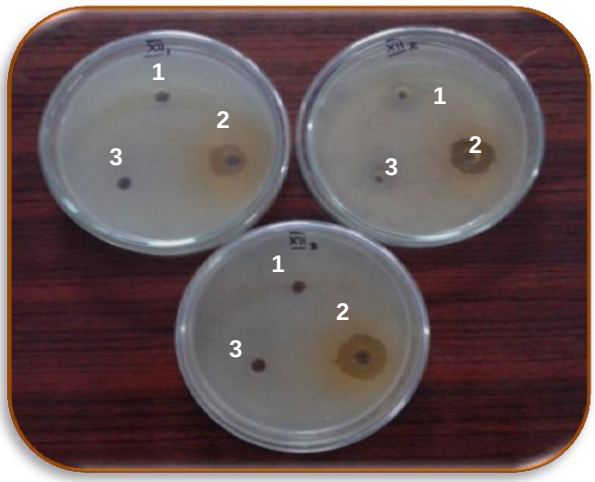
Plate 16a - Growth inhibitory zone against the selected bacterial isolates



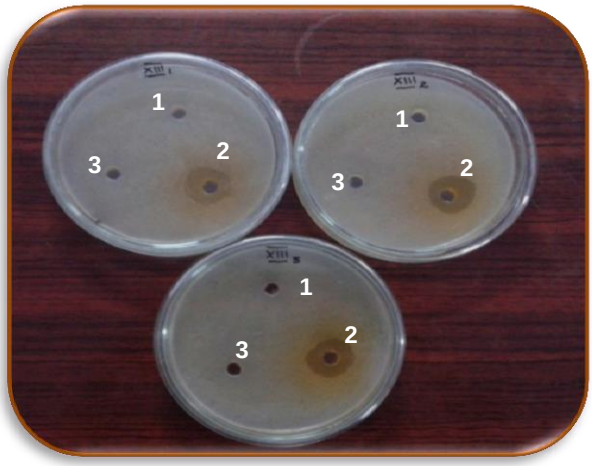
Streptococcus aureus



Escherichia coli

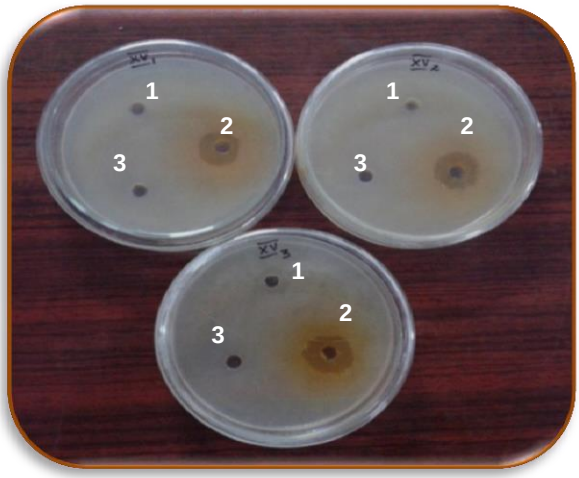


Salmonella enteritis



Enterococcus faecalis

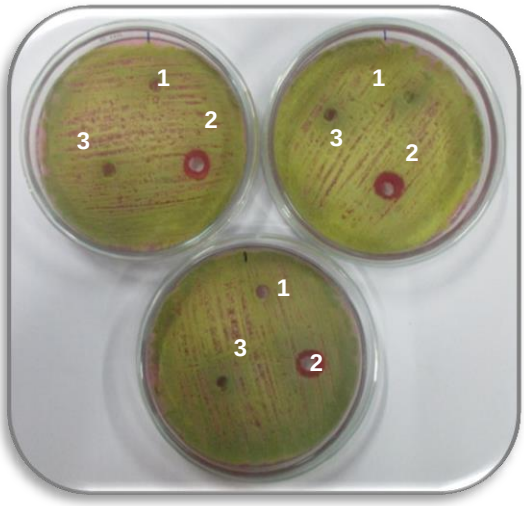
Yersinia enterocolitica



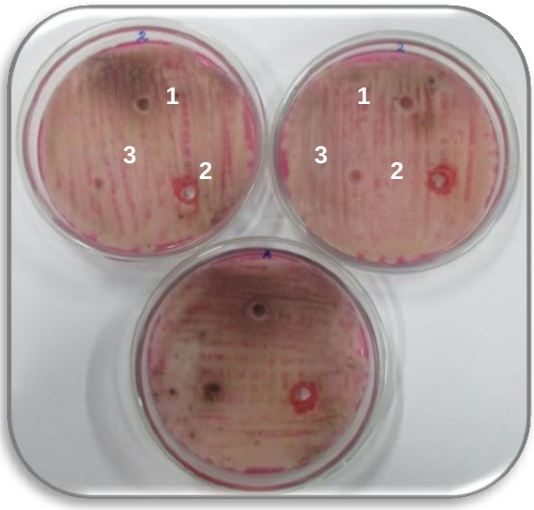
***Acinetobacter* species**

1- Tap water, 2 - Methyl orange, 3 - Degraded metabolites

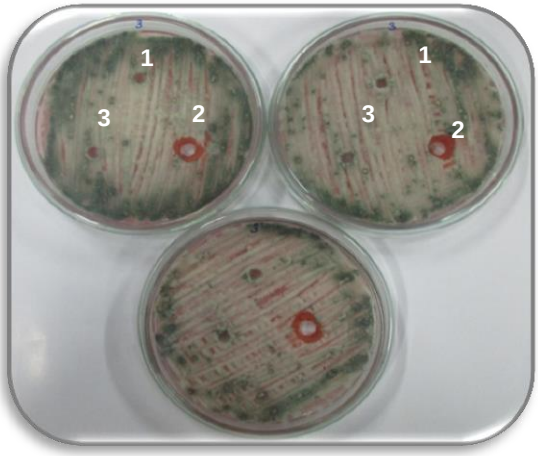
Plate 16a - Growth inhibitory zone against the selected bacterial isolates



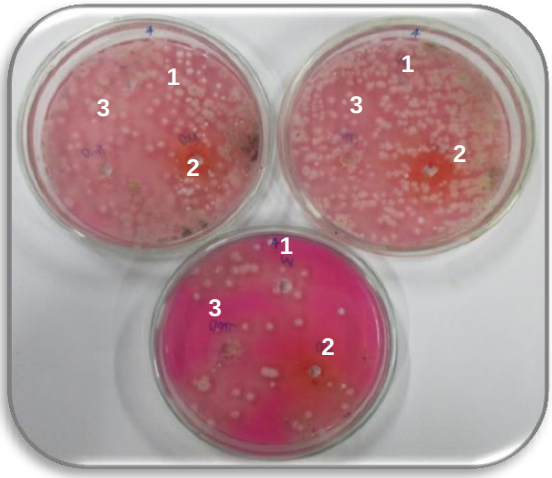
Aspergillus flavus



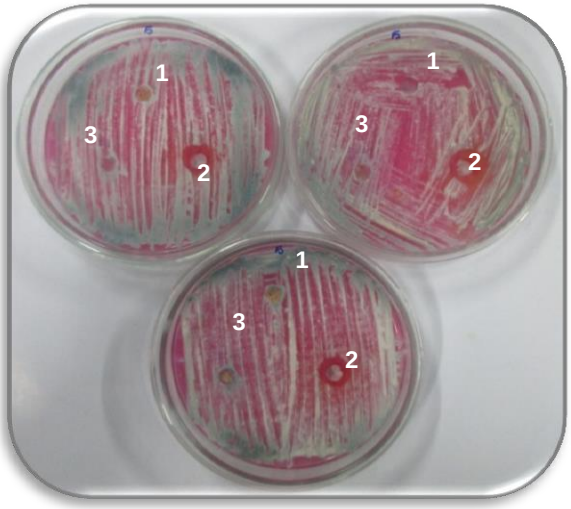
Aspergillus niger



***Acremonium* species**



***Rhizopus* species**



Trichoderma viride

1- Tap water, 2 - Methyl orange, 3 - Degraded metabolites

Plate 16b - Growth inhibitory zone against the selected fungal isolates

4.5.4 Cytogenotoxicity studies on *Allium cepa*

Plants are considered as essential and sensitive model to check the cytogenotoxicity of various environmental pollutants when compared to animal models (Fiskesjo, 1981 and Turkoglu, 2009). Moreover plant bioassay studies prove to be cost effective and exhibit a good correlation with different *in vitro* or *in vivo* mammalian cell line (Gerstner and Huff, 1997, Vig, 1978 and Lerda *et al*., 2010).

The cytotoxicity level of a pollutant can be studied based on the increase or decrease in the mitotic index, which serves as a basic indicator in environmental monitoring (Alimba *et al*., 2013 and Kincl *et al*., 1996). Scoring the incidence of chromosomal aberrations in the root tip cells also provides an easy method to study the impact of pollutants (Tripathy and Patel, 2014).

Numerous bioassay studies have been carried out using *Allium cepa* to assess the chromosomal aberration of pollutants. Root tip is the first and foremost part of a plant that comes into contact with the pollutants present in the water or soil. Toxicological assay using the root tip system of *Allium cepa* was universally recognized for assessing the effect of pollutants on root growth, mitotic index, mitotic inhibition and DNA damage due to its short cycle duration, simplicity, sensitivity and cost effectiveness (Nielsen and Rank, 1994 and Evseeva *et al*., 2003).

Thus the present study was embodied to evaluate the cytogenotoxic effect of tap water (T_1), untreated dye solution (T_2) and treated dye solution (T_3) employing *Allium cepa* root chromosomal assay.

Root growth

Root growth occurs as apical meristematic cells near the tip of the root which pass through interphase and mitotic phase to complete the cell cycle (Nilsen and Orcutt, 1996). Therefore, mitotic index can be used as a reflection of cell proliferation and also to determine the rate of root growth. Table 18 represents the cytogenotoxic analysis of *Allium cepa* root tips exposed to different treatments.

The statistical analysis of cytogenotoxicity test showed that the root growth, root length, mitotic index and mitotix depression of *Allium cepa* root tip cells exposed to treated dye solution was significant ($p<0.05$) when compared to untreated dye solution.

Table 18 – Cytogenotoxic analysis of *Allium cepa* root tips exposed to different treatments

Treatment	Root growth (%)	Root Length (cm)	No. of dividing cells	Mitotic index	Mitotic depression/ inhibition
T_1	100	11.07±0.31a*	935.2±3.9 a*	85.89±0.87a*	4.98±0.24 a*
T_2	30	3.31±0.10b*	161.7±0.2 b*	12.20±0.32b*	58.92±1.39 b*
T_3	93	10.33±0.46c*	789.4±.1 c*	71.23±0.93 c*	6.86±0.33 c*

The values are mean ± SD
T_1 - Tap water (Control), T_2 - Untreated dye solution, T_3 - Treated dye solution
a - T_1 vs T_2; b – T_2 vs T_3; c – T_1 vs T_3 * - Significant at 5% level ($p<0.05$)

Allium cepa grown with control (T_1) exhibited 100% of root growth followed by treated dye solution (93%) and minimum root length was noted in T_2 (30%). The *Allium cepa* grown with untreated dye solution (T_2) resulted in stunted and wilting of roots, suppression of mitotic activity and occurrence of chromosomal aberrations indicating the toxicity of the dye. Plate 17 depicts the root growth of *Allium cepa* exposed to different treatments.

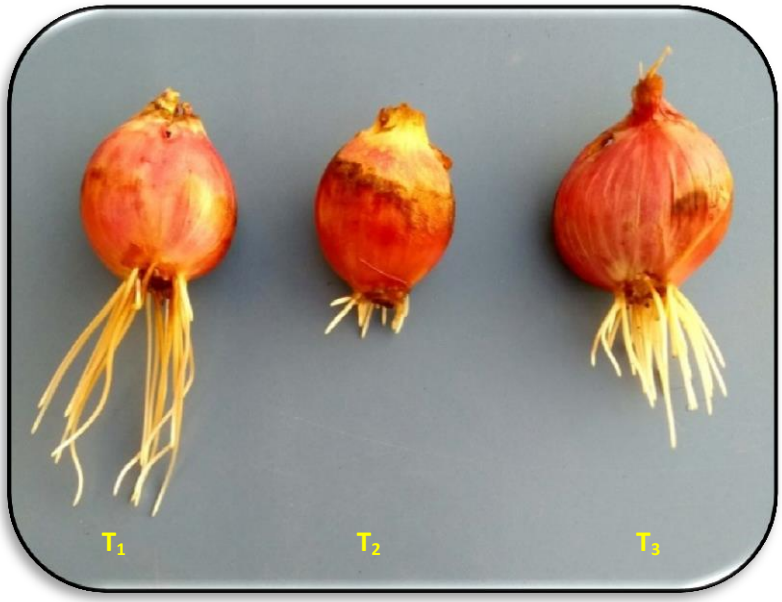


Plate 17 - Root growth of *Allium cepa* exposed to different treatments

Root length

Root length was considered as the macroscopic parameter for testing cytotoxicity of methyl orange. The mean root length was observed to be maximum in *Allium cepa* exposed to tap water (11.07±0.31cm) followed by treated dye solution (10.33±0.46cm) and minimum in untreated solution (3.31±0.10cm).

Decrease in the root length of *Allium cepa* exposed to untreated dye solution (T_2) may be due to the action of independent events that lead to cell elongation rather than cell differentiation in the proximal region of root tip or mitotically active meristematic region (Webster and McLeod, 1996 and Fusconi *et al*., 2006) which may result from the inhibition of protein synthesis (Seth *et al*., 2007).

Mitotic index

Mitotic index explains the potential of a cell to divide and also a method to biomonitor the effect of pollutant on cell division. It also measures the properties of cells in mitotic phase of a cell cycle and its inhibition could be interpreted as cellular death (Marcano *et al*., 2004).

In the present study, mitotic index was low in root tips exposed to untreated dye solution (12.20±0.32) when compared to those grown with treated dye solution and control (71.23±0.93 and 85.89±0.87). The increased rate of mitotic index is associated with the growth of root length. The cytotoxicity level can be determined by the decreased or increased rate of the mitotic index. Mitotic index below 22% and 50% of control indicates the sublethal (Antosiewicz, 1990) and lethal (Sharma and Vig, 2012) effects on the test organism respectively. In the present study, the decrease in mitotic index induced by untreated dye solution on *Allium cepa* when compared to control was below 22% suggesting this severe toxicity might be inflicted by the dye on the test plant.

The reduction in the mitotic index may also be due to the interference of pollutants with mitosis and preventing the cells from entering prophase or blockage of mitotic cycle interphase or inhibition of DNA/protein synthesis and chromosomal behaviors (Metin and Burun, 2010, Yuzbasioglu *et al*., 2003 and Glinska *et al*., 2007)

Mitotic depression/inhibition

The mitotic inhibition is a measure indicating the rate of mitosis prevented by a toxicant (Sajitha and Thoppil, 2018).The mitotic inhibition of *Allium cepa* exposed

to untreated dye solution was 58.92±1.39 and those exposed to treated dye solution was 6.86±0.33 which was close to that of control 4.98±0.24.

The decrease in mitotic inhibition in treated dye solution (T_3) when compared with the untreated dye solution (T_2) exhibits the potential of *Oedogonium subplagiostomum* AP1 to remove methyl orange and also to maintain the normal mitotic inhibition in *Allium cepa*. An increased mitotic inhibition in cells exposed to methyl orange may be related with uncontrolled or increased cell division, tumour formation or detrimental effects of cells (Hoshina, 2002 and Jadhav *et al*., 2010a).

The decreased root growth, root length, mitotic index and increased mitotic inhibition in untreated dye solution coincides with the findings of various researchers in *Allium cepa* exposed to direct brown (Alex *et al*., 2013), reactive turquoise blue (Tripathy and Patel, 2014), orange red (Tripathy and Rao, 2015) and textile dyeing industrial effluent (Alimba *et al*., 2013) respectively.

Allium cepa exposed to treated dye solution exhibited maximum root growth, root length, mitotic index and minimum mitotic inhibition which was in accordance with the results of reactive red 35 degraded with *Pseudomonas aeruginosa* ARSKS20 (Soni *et al*., 2015), textile effluent degraded with *Providencia rettgeri* strain HSL1 and *Aspergillus erythrocephalus* NCTBT124, *Aspergillus fumigatus* NCTBT126, *Cladosporium herbarumand* NCTBT142 and *Fusarium oxysporium* NCTBT156 (Lade *et al*., 2016 and Abubacker *et al*., 2018) respectively.

Thus the results collectively suggest that *Allium cepa* has great sensitivity towards methyl orange and hence can be used as an environmental monitoring agent.

Chromosomal aberrations

Chromosomal aberrations are characterized by the change in the total number of chromosomes or in their structure by the formation of a break or exchange of chromosomal materials or genetic damage produced by mutagenic agents (Qari, 2010). It provides an important measure to assess the genotoxic potential of toxicants. The chromosomal abnormalities were evaluated in different stages of cell cycle namely prophase, metaphase, anaphase and telophase.The results of scoring and percentage of chromosomal aberrations were depicted in Table 19 and their microphotographs were portrayed in Plate 18.

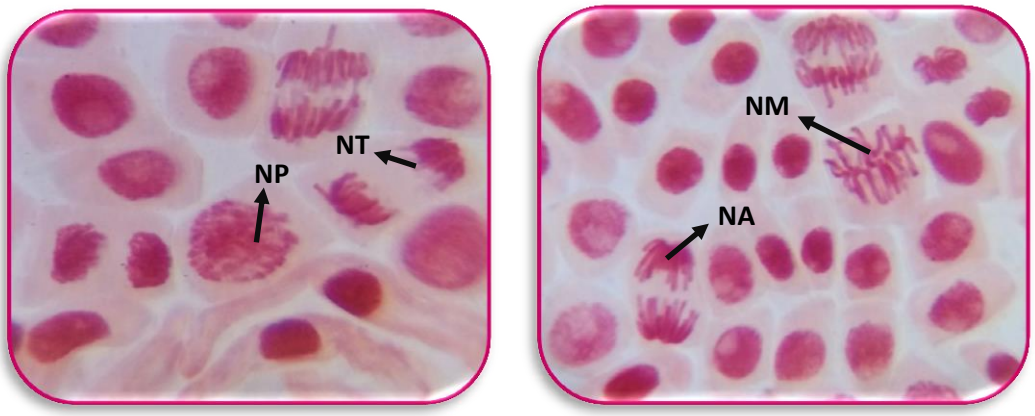
Chromosomal aberrations (per 1000 cells)	Treatments			Total Chromosomal aberrations	Aberrations (%)
	T_1	T_2	T_3		
Sticky chain and disturbed metaphase	-	45.40±1.63	2.11±0.4	47	5.02
Pulverized and disturbed anaphase	-	112.76±2.0	-	112	11.97
Chromosomal displacement in anaphase	-	19.15±1.63	-	19	2.03
Abnormal telophase	-	65.65±1.63	-	65	6.95
Chromosomal loss and bridge in telophase	-	145.31±1.2	-	145	15.50
Disoriented chromosomes	-	58.70±0.81	1.36±0.4	59	6.31
Abnormal grouping of chromosomes	-	66.06±1.24	1.86±0.4	67	7.16
Vagrant and laggard chromosomes	-	103.90±1.6	-	103	11.01
Spindle disturbances	-	12.20±0.81	2.11±0.4	14	1.49
Binucleate cells	-	160.20±0.81	-	160	17.11

The values are mean ± SD

T_1 - Control (tap water), T_2 - Untreated dye solution, T_3 - Treated dye solution

Table 19 - Genotoxic analysis of *Allium cepa* root tips exposed to different treatments

Plate 18 - Mitotic stages and chromosomal aberrations of *A. cepa* exposed to different treatments



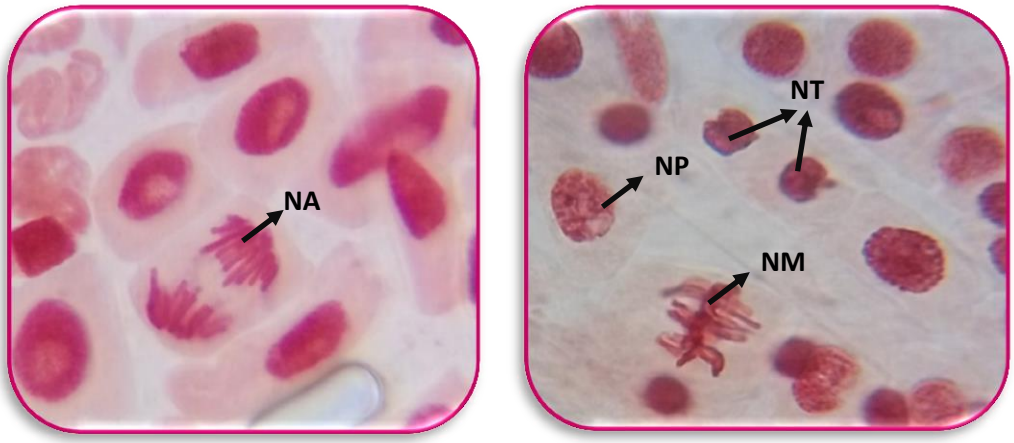
18a - Mitotic stages on *A. cepa* exposed to tap water (T_1)

NP – Normal Prophase

NM – Normal Metaphase

NA – Normal Anaphase

NT – Normal Telophase



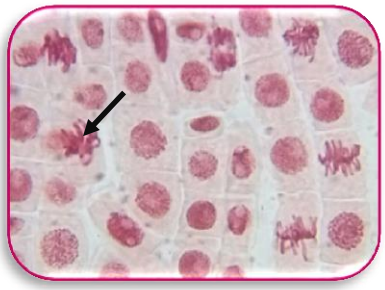
18c - Mitotic stages on *A. cepa* exposed to treated dye solution (T_3)

NP – Normal Prophase

NM – Normal Metaphase

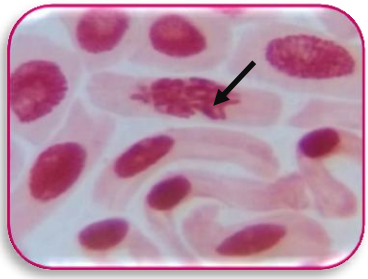
NA – Normal Anaphase

NT – Normal Telophase



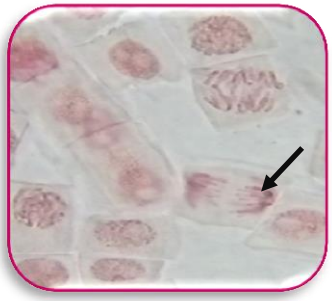
Disturbed and Sticky chain metaphase

Disturbed anaphase

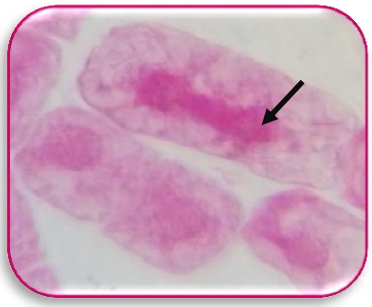


Pulverized anaphase

Chromosomal displacement in anaphase



abnormal telophase

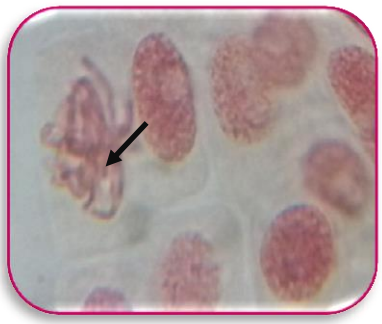


Chromosome bridge at telophase

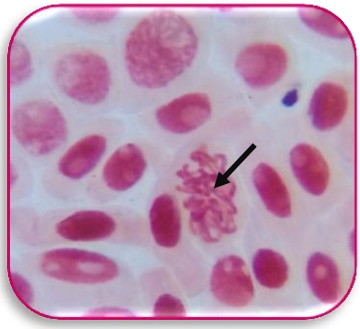
Binucleate cells

Spindle disturbances

Chromosome loss



Disoriented and abnormal grouping of chromosomes



Vagrant chromosomes

Laggard chromosome

18b - Chromosomal aberrations of *A. cepa* exposed untreated dye solution (T_2)

The root tips of *Allium cepa* exposed to treated dye solution (T_2) induced different types of mitotic abnormalities such as disoriented and abnormal grouping of chromosomes, vagrant and laggard chromosomes, sticky chain and disturbed metaphase, pulverized and disturbed anaphase, chromosomal displacement in anaphase, abnormal telophase, chromosomal bridge and loss in telophase, spindle disturbances and binucleate cells (Plate 18b).

The *Allium cepa* roots exposed to untreated dye solution (T_2) showed a significant increase in chromosomal aberration indicating the strong cytotoxic and genotoxic effect of methyl orange on the mitotic cells. On contrary, the number and frequency of chromosomal aberrations significantly reduced in the treated dye solution (T_3) (Plate 18c) and no chromosomal aberrations were observed in cells treated with tap water (T_1) (Plate 18a). Analysis of chromosomal aberrations not only allows the estimation of genotoxic effects, but also enables the evaluation of their clastogenic and aneugenic actions (Leme and Morales, 2009).

Root tip of *Allium cepa* exposed to untreated dye solution display stickiness in the chromosomal architechture. The sticky and condensed nature of the chromosome results in the delay of not reaching the poles and thereafter remained dispersed in the cytoplasm (Kong and Mao 1999). Stickiness of chromosomes may cause incomplete separation of the daughter chromosomes as a result of cross linkage of chromoproteins (Kong and Ma, 1999) or might have resulted from increased chromosomal contraction and condensation, depolymerisation of DNA, partial dissolution of nucleoprotein (Mertz, 1969), immediate reactions with DNA during its inhibition period causing DNA-DNA or DNA-protein cross linkage (Amin, 2002), alteration in the physicochemical properties of nucleic acids or nucleoproteins through lysosomal reactions (El-Ghamery and Mousa, 2017), incomplete replication of chromosomes by defective or less active replication enzymes (Sinha, 1979) or by late replicating DNA sequences of telomeric heterochromatin (Lima-de-Faria and Jaworkska, 1972 and Bennet, 1977).

Stickiness also indicates a highly toxic, irreversible chromosomal abnormality leading to cell death which might be caused by physical adhesion of proteins to the chromosome (Konuk *et al*., 2007).The interchromosomal chromatin fibers are

affected peripheral parts such as DNA topoisomerase II gets entangled and also leads to stickiness (Datta *et al*., 2018). Moreover sticky chromosome can also cause loss of genetic material, irregular cell division, non-adherence of chromosome to the assembled chromosomal complex which is being lost during the cell cycle (El-Ghamery and Mousa, 2017).

The disoriented metaphase observed in the T_2 roots of *Allium cepa* might be due to the inhibition of respiratory pathways resulting in low energy production necessary for chromosomal movement (Ajay and Sarbhoy, 1987), irregular distribution of chromosomes over the distributed spindle apparatus and non-alignment of chromosomes in the spindle of the root tip cells of *Allium cepa* exposed to untreated methyl orange.

The occurrence of chromosomal bridges in the methyl orange root tip cells grown with untreated dye could be attributed either to chromosomal stickiness or fusion between the chromatids which can be initiated by the synchronized breakage of two chromatids or by the loss of telomere capping (Gilley *et al*., 2005). It is also ascribed to the unequal exchanges that lead to the formation of dicentric chromosomes which are equally pulled at both poles in anaphase (Gomurgen, 2005) or by the breakage and fusion of chromosomes and chromatids respectively or by the incomplete replication of chromosomes by defective or less active replication enzymes (Sinha,1979).

Chromosomal bridge formation also occurs when the heterochromatin blocks are unable to complete the DNA replication even when the nucleus is ready to divide (Somashekar and Gowda, 1984). It also leads to a number of different outcomes namely polyploidy, aneuploidy and cell cycle arrest (Pampalona *et al*., 2016). Thus the induction of the chromosome bridges is an evident for the clastogenic effect of methyl orange.

Chauhan and Gupta (2005) and Kong and Ma (1999) conversed the relationship between stickiness and chromosomal bridges. They reported that the entanglement of interchromosomal chromatin fibre may result in stickiness which leads to subchromatid connections between chromosomes as a result of

chromoprotein cross linkage. These two may subsequently result in the failure of free anaphase separation.

Occurrence of disoriented chromosome might have been brought by the action of methyl orange on the microtubules. The dye might have caused the failure of chromosome to align at equatorial plate because of the dysfunction of spindle and energy deficiency causing delay in the division of centromeric region thereby resulting in distorted chromosomes (Jain and Sarbhoy, 1988). Fenech and Crott (2002) also reported the failure of cell plate formation during the process of cell division which leads to disorientation of chromosomes.

The *Allium cepa* root tips exposed to untreated dye solution results in the formation of vagarant chromosomes. Normally, the anomalies in spindle lead to the formation of vagrant chromosomes. These chromosomes move ahead from its chromosomal group towards poles and leads to unequal distribution or irregular separation of chromosomes in the daughter cells resulting in non-disjunction (Evseeva *et al*., 2005). Presence of vagrant chromosomes seem to have a weak c-mitotic (chromosome formation mitotic) effect indicating the risk of aneuploidy (Sondhi *et al*., 2008).

Rank (2003) stated that laggards or vagrant chromosomes are indicators of spindle poisoning. El-Ghamery *et al*. (2003) also observed the induction of vagrant chromosomes and laggards in *Allium cepa* root tip cells exposed to zinc which leads to the separation of unequal number of chromosomes or irregularly shaped nuclei.

Laggards are resulted from the direct breakage and fragmentation of chromosome which lead to the loss of centromeres and restricts their movement (Gari *et al*., 1998). Laggard chromosomes obtained in the *Allium cepa* root tip cells exposed to untreated dye solution might have resulted due to the failure or disturbances of the chromosomes or acentric fragments to get attached to spindle fiber and to move to either of two poles (Tripathy and Patel, 2014, Pulate and Tarar, 2014 and El-Ghamery and Mousa, 2017).

Laggard formation could be either attributed to the failure of the spindle apparatus to organize and function in normal mode. The prometaphase movement accompanied by the adhesion of the centromere(s) of one or more chromosomes to

the inner surface of the plasma membrane and the movement of the other chromosomes towards the equatorial plate may also results in the appearance of such lagging chromosomes (Patil and Bhat, 1992 and Turkoglu, 2007).

The induction of spindle disturbances in the cells of *Allium cepa* exposed to untreated dye solution may lead to aneuploidy or formation of micronucleus after cell division. This arises from irregular separation of chromosomes at anaphase and thereby helps the chromosomes to reach the poles (Albertini *et al*., 2000).

The formation of binucleate cells was observed in *Allium cepa* root tips exposed to untreated dye solution. Formaition of binucleated cells seems to affect phragmoplast, a complex assembly of microfilaments and endoplasmic reticulum and also a plant cell specific structure formed during late cytokinesis which serves as a scaffold for cell plate assembly and new cell wall formation by separating two daughter cells (El-Ghamery *et al*., 2003).

Various researchers have reported that the maximum chromosomal aberrations such as elongated nucleus, DNA vacuolation, diagonal anaphase and metaphase, chromosomal bridges, binucleate cells, disorientation of chromosomes, bridges in anaphase, sticky chromosomes (metaphase), micronuclei, chromosome breaks, laggard and vagrant chromosomes in *Allium cepa* exposed to untreated dyes namely reactive lanasol black B (Carita and Marin-Morales, 2008), eriochrome red B (Hassan and Yassein, 2014), lemon yellow and orange red (Vazhangat and Thoppil, 2016), disperse blue 373, disperse violet 93 and disperse orange 37 (Camargo *et al*., 2011) which support the findings of the present investigation.

Decrease or minimum chromosomal aberrations in *Allium cepa* exposed to treated dye solution was observed by Waghmode *et al*. (2012) in rubine GFL, Phugare *et al*. (2011) in red HE3B which harmonizes with the findings of the present study.

Thus, the *Allium cepa* treated with degraded metabolites of methyl orange or treated dye solution showed significantly less alteration in chromosomal DNA than those exposed to untreated dye solution indicating the effectiveness of *Oedogonium subplagiostomum* AP1 not only for degradation but also for its detoxification.

PHASE VI

4.6. REUSE OF DYE DESORBED ALGAE AND TREATED DYE SOLUTION

4.6.1 Dye desorbed algae for compost production

In the modern era of development and industrialization, there is a growing global interest in the use of algae as a renewable fuel for waste management. Algae also act as a biological solar panel to fix carbon dioxide for growth and production of intracellular storage compounds.

Composting is one of the alternative methods for the utilization of waste biomass and the compost obtained from algae finds its application as an substitute to conventional fertilizer (Michalak and Chojnacka, 2013). Algal compost provides essential nutrients, trace elements and also induces disease resistance to plants (Haroun and Hussein, 2003).

Many research works have been focused on composting organic waste materials using earthworm and no information is available on the degradation of dye desorbed algae by earthworms and also the analysis of physical and chemical constituents in compost. Hence an attempt has been taken in the present study to develop a simple, ecofriendly technology for the conversion of dye desorbed alga into compost through vermitechnology, to obtain a natural fertilizer and a biostimulant for plant growth. After 60th day of nurture, pH, EC (mmhos/cm), moisture content (%), total nitrogen (%), total phosphorus (%), total potassium (%) and organic carbon (%) were analysed in the algalcompost (Table 20).

Table 20 - Physicochemical constituents of the algal compost

Parameters analysed	Algal compost
pH	8
EC (mmhos cm^{-1})	1.88
Moisture content (%)	43
Organic Carbon (%)	32.78
Total Nitrogen (%)	1.15
Total Phosphorus (%)	0.78
Total Potassium (%)	0.91

The values are mean of triplicates

pH seems to be an important parameter for effective composting process since its variation may affect the microbial activity on the organic waste. An optimum range of pH should be maintained for a good control in the process of composting. In the present study, the pH of the algal compost was recorded as 8.

Nedgwa and Thamson (2000) pointed out that the alkaline pH might be related to the mineralization of the nitrogen and phosphorous into nitrates or nitrites and orthophosphates and bioconversion of the organic material into intermediate products of the organic acids. Earthworm alter the chemical properties of the substrates and indirectly promote microbial propagation and colonization regulating in more prominent mineralization rate the in algal composting beds than those by general composting.

Majlessi *et al*. (2012) stated that in the latter stages of vermicomposting, pH seems to be increased which might be due to the water used to maintain the moisture content of the compost pit. Thus partially alkaline pH values are usually the indicators of stable vermicomposts.

Electrical conductivity (EC) reflects the amount of salts present in the compost. It is also used to detect the maturity of compost. The EC was 1.88 (mmhos cm^{-1}) in the algal compost which indicates the release of mineral salts by the degradation of desorbed dye in the algae.

The EC level in the algal compost may be attributed by the release of different mineral ions such as phosphate, ammonium and potassium through decomposition of organic substances or shredding of organic materials by earthworm (Sharma, 2003 and Garg *et al*., 2006).

Moisture content is an environmental variable which provides the transport of the dissolved nutrients required for the metabolic and physiological activities of microorganisms in compost. Moisture content is a critical parameter for the compost material since the changes in microbial activities are strongly affected and oxygen supply is directly related to it. Rodriguez *et al*. (1995) suggested that when the moisture content falls below 25% or rises higher than 70%, it affects the microbial activity and restricts aeration. The moisture content in the present study was 43% which falls within the desirable limit and favours microbial activities.

Organic carbon is utilized for the metabolism of microbes and they utilize it as the source of energy. The organic carbon content was observed as 32.78% in the algal compost.

In the present study, the macronutrients such as nitrogen, phosphorus and potassium were observed as 1.15%, 0.78% and 0.91% respectively.

Earthworm enhances the nitrogen level of vermibed by adding excreta and other secretions. Also, mucus a polysaccharide is secreted by earthworm to moisten the body surface which also enriches the vermibeds with nitrogen fixers. Earthworm also alters the microclimatic conditions of vermibeds which consequently promotes the microbial populations responsible for nitrogen enrichment (Singh and Suthar, 2012).

The phosphorus level within algal cells may fluctuate widely depending on whether the algae are growing under phosphorus limited conditions or not. Even at very low levels in the environment the algae can accumulate and store phosphorus (Mackereth, 1953).

During composting, a small amount of phosphates are converted to more available forms by the gut enzymes (phosphatase) of earth worm and also increases microbial activity in the cast. The presence of large number of microflora in the gut of earthworm might play an important role in increasing P and K content in the process of vermicomposting (Kaviraj and Sharma, 2003). The phosphorus mineralization in algal compost suggests the suitability of waste substrate as a feed for earthworm and microbial propagation (Singh and Suthar, 2012).

Suthar (2007) has reported that an earthworm processed waste material contains higher concentration of exchangeable potassium due to enhanced microbial activity during the process of vermicomposting, which consequently enhances the rate of mineralization. The production of acid during decomposition of organic matter by the microorganisms is the key mechanism for solubilization of insoluble total potassium (Adi and Noor 2009).

Cole *et al*. (2015) reported that the value of NPK was 52.33±1.0, 30.50±5.67 and 1.59±0.11g Kg^{-1} in *Ulva ohnoi* mixed vermicompost which supports the findings of the present study.

Earth worm can utilize algae as a feed or supllimentary food along with cowdung and also enhance the reproductive and growth rates since they have high nutrient content. The utilization of desorbed algae in the process of vermicomposting allows the transfer of waste into valuable end product. The compost obtained can be used as a natural fertilizer and an also an organic soil conditioner to enhance the plant growth.

Cost economics for the algal compost

Cost of 1kg compost in market = Rs. 15 Algal compost production per year = 5000 kg ∴ 5000 kg of algal compost cost = 15 X 5000 = Rs. 75,000 **Profit** = Total cost – miscellaneous = Rs. 75,000 – Rs. 25,000 **= Rs. 50,000**	

4.6.2 Reuse of treated dye solution for dyeing fabrics

The reuse of treated dye solution in dyeing fabrics helps to minimise pollution and protects the environment. The physical parameters of the fabrics dyed using tap water and treated dye solution are presented in Table 21. Plate 19 potrays the fabrics dyed with tap water and treated dye solution.

Table 21 - Physical parameters of the dyed fabrics

Parameters	Undyed fabric (Control)	Fabric dyed using tap water	Fabric dyed using treated dye solution
Fabric weight (gsm)	1.64	1.92	1.93
Fabric thickness (mm)	0.33	0.36	0.37
Fabric strength (kg/cm^3)	57	55	56
Fabric elongation (inch)	1.60	2.0	1.94
Fabric stiffness			
Warp	2.24	2.59	2.72
Weft	2.14	2.31	2.42
Colour fastness (Grey scale rating)			
Sunlight	-	4	4
Pressing			
Wet	-	4	4
Dry	-	5	5
Crocking			
Wet	-	4	4
Dry	-	4	4

The values are mean of three replicates

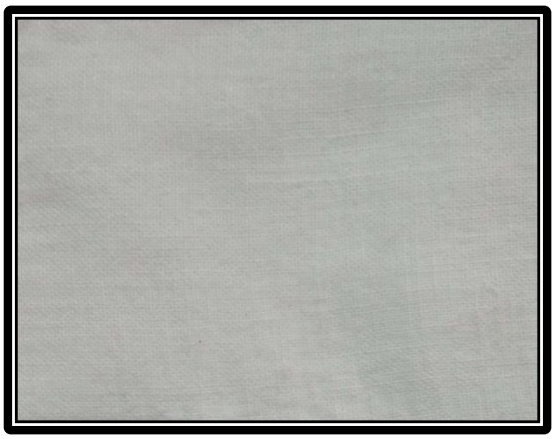
From the above table, it is clear that the fabric weight of the dyed samples increased when compared to the undyed control sample. The result showed that there was no difference in fabric weight between the dyed samples. The weight of the fabric dyed using treated dye solution was in close proximity with tap water dyed

sample. The results proved that the treated dye solution could be effectively utilized for dyeing.

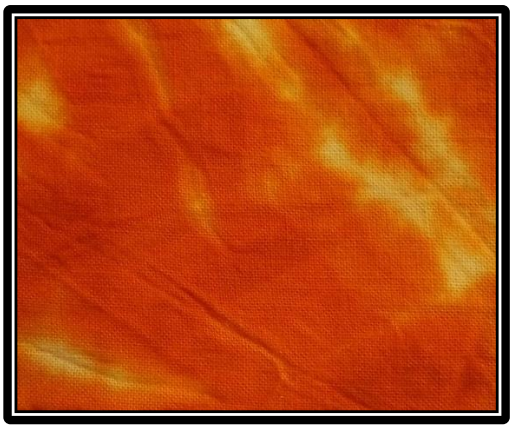
Fabric thickness was found to be increased in both the dyed samples when compared with the undyed fabric. The increase in thickness might be due to the deposition of dye particles on the surface of the fabric. Fabric stiffness increased in all the dyed samples in both warp and weft directions, over the undyed fabric. Maximum stiffness is observed in fabric dyed using treated dye solution (2.72 in warp and 2.42 in weft direction) and this increase in stiffness might be attributed by dyeing. The fabric strength of both the dyed samples decreased when compared with the undyed fabric. The minimum decrease is seen in sample dyed using treated solution (56 kg/cm^2). The decrease in the strength of the fabric may be due to the chemical present in dyes which could have reacted with the fiber components, resulting in strength decrease. The elongation of the dyed fabrics increased when compared with the undyed fabric. Maximum elongation was noticed in fabric dyed with tap water (2 inch).

Colour fastness results of dyed fabrics to sunlight, pressing and crocking were presented in Table 21. From the table, it is evident that samples dyed using tap water and treated water exhibited good colour fastness to sunlight. With regard to pressing, the dyed fabrics showed excellent fastness in dry condition whereas good fastness in wet state. The dyed samples showed good fastness to both wet and dry crocking. Colour fastness results showed that the fabrics dyed using treated dye solution exhibited similar fastness properties as that of the fabrics dyed using tap water.

From the results, it could be concluded that the physical properties of the fabrics dyed using algae treated water is comparable with that of tap water dyed fabric proving the utilization of treated water in textile industries (Plate 19).



Cotton Fabric (Control)



Fabric dyed using tap water

Fabric dyed using treated dye solution

Plate 19 - Fabrics dyed with tap water and treated dye solution.

PHASE VII

4.7 *APPLICABILITY OF Oedogonium subplagiostomum AP1 IN TREATING TEXTILE DYEING EFFLUENT*

Photosynthetic organisms produce oxygen that augments the biological degradation of the organic matter in the effluent (Hodaifa *et al*., 2010). Thus, the effluent should be treated before its discharge into aquatic or terrestrial ecosystem. The treated effluent can be utilized for the agricultural and aquaculture purpose which proves to be an eco-friendly approach to mankind. The physicochemical characteristics of untreated and treated textile dyeing effluent were presented in Table 22. Plate 20 reveals the textile dyeing effluent treated with *Oedogonium subplagiostomum* AP1.

A **B**

A Before decolourisation
B After decolourisation

Plate 20 – decolourisation of textile dyeing effluent using O. subplagiostomum AP1

Table 22 - Physicochemical characteristics of textile dyeing effluent

Characteristics	Untreated textile dyeing effluent	Treated textile dyeing effluent	Tolerance limits
Physical characteristics			
Colour	Dark orange	Light green	-
Odour	Bad odour	Obnoxious	-
pH	10.76	7.74	5.5-9.0 (BIS, 1981)
Electrical conductivity (µmhos/cm)	6.12	2.1	<2.25 (BIS, 1981)
Turbidity	Turbid	Clear	-
Chemical characteristics (mg/L)			
Alkalinity	470	64.97	270 (BIS,1993)
Total Dissolved Solids	7650	1840	2100 (BIS, 1981)
Total Suspended Solids	312	95	100 (BIS, 1981)
Total Solids	7962	1935	-
Total hardness	895	140	250 mg/l (BIS,1974)
Biological Oxygen Demand	95	24	30 (BIS, 1981)
Chemical Oxygen Demand	878	223	250(BIS, 1981)
Chloride	3569	950	1000 (BIS, 1981)
Sulphate	1896	507	1000 (BIS, 1981)
Nitrate	159	25.58	50 (BIS, 1981)

The values are mean of triplicates
BIS - Bureau of Indian standards

Physical parameters

The colour of the untreated textile dyeing effluent appeared to be dark orange with a bad odour and that of the treated effluent was light green with obnoxious odour.

The intensity of the effluent colour mainly depends on the pollutants that enter into it and this inturn increases the BOD and COD and makes oxygen unavailable to aquatic organisms (Chaudhy *et al*., 1998). Presence of contaminants, chemicals and addition of disinfectants such as chlorine produce bad odour to the effluent (Goel, 1997). Singh and Singh (2017) reported that algae can utilise three different mechanisms for azo dye decolourisation. Daneshvar *et al*. (2007) reported that the use of chromophore for algal biomass production through assimilation, production of CO_2 and water during the conversion of coloured molecules to non-coloured molecules and adsorption of chromophore by the algal biomass.

The untreated effluent was turbid which may be due to the presence of salts used in dyeing process whereas in treated effluent the algae might have absorbed the salts leading to a clear solution.

The pH of effluent affects physical and chemical properties of water which in turn adversely affects the biota of ecosystem and also changes the soil permeability thereby polluting underground resources of water (Rump and Krist, 1992). The pH and EC of the untreated textile effluent was 10.76 and 6.12 (µmhos/cm) whereas it was 7.74 and 2.1 (µmhos/cm) in treated textile effluent which falls within the limits prescribed by BIS. Higher pH and EC value was observed by Sivakumar *et al.* (2011) and Ahmed and Nizamuddin (2012) in textile dyeing effluent and their findings are in accordance with the present study.

The reduction in pH and EC was also reported in textile effluent treated with *Nostoc* species, *Eichhornia crassipes* and *Pistia stratiotes* (Roy *et al*., 2010) and *Anabaena fertilissima, N. muscorum, Phormidium fragile and Wollea* sp. (Ghazal
et al., 2016) respectively.

The total suspended solids recorded in the untreated textile effluent was 30mg/L whereas it was 95mg/L in treated effluent which satisfies the specified limits

of BIS (100mg/L).The amount of TDS present in the untreated textile effluent (7650mg/L) of the present study which was four times greater than that of treated effluent (1840mg/L) which was within the tolerance limit (2100mg/L) prescribed by BIS. Similar trend was observed in total solids in untreated and treated textile dyeing effluent (Table 22). Higher amount of TSS may interfere with sunlight penetration and prevents photosynthesis, elevates the density and turbidity of water which in turn affects osmoregulation and cause distress among livestock and human being (Kalita *et* al., 2003).

Increase in TDS is mainly due to the presence of suspended matter or the colloidal particle which does not settle or discharge solid waste from the catchment areas (Rajurkar *et al.,* 2003). The findings of the study falls in line with the report of Rao *et al.* (1993) who observed high amount of TDS and TS in textile dyeing effluent.

The reduction of TSS, TDS and TS in the textile effluent treated with *Oedogonium subplagiostomum* AP1 concurs with the studies conducted in textile dyeing industrial effluent treated with *Chlorococcum vitiosum, Spirulina platensis* (Chitra *et al.,* 2013), *Chlorella vulgaris* (Sivakalai *et al.*, 2013) and *Scenedesmus obliquus* (Elumalai *et al.,* 2013) respectively.

The BOD and COD in the untreated textile effluent (95 and 878 mg/L) was high when compared to treated textile effluent (24 and 223 mg/L) which falls within the tolerance limit (30 and 250mg/L) set by BIS for the discharge of effluent into inland surface waters. Release of textile dyeing effluent with increased BOD and COD into ecosystem may percolate and affect the quality of ground water (Islam *et al*., 2015 and Savin and Butnaru, 2008).

The values of EC, BOD and COD in untreated textile waste water was high and after treatment with *Anabaena flosaquae*, *A. variabilis, Nostoc ellipsosporum*, *N.linkia* and *Chlorella vulgaris* a successful decrease of the above tested parameters was observed. This reduction indicates that algae were able to carry out photosynthesis which increased the oxygen concentration of effluent. This oxygen in turn helps in microbial decomposition and biodegradation of organic materials which further decreased the levels of BOD and COD (Chen *et al*., 2003).

Similar such reduction in BOD and COD was observed in textile waste water effluent treated with *Spirogyra gracilis* (Alaguprathana and Poonkothai, 2015) and dye industry effluent with *Oscillatoria brevis* and *Westiellopsis prolifica* (Vijayakumar and Manoharan, 2012). Colak and Kaya (1988) reported the reduction of BOD and COD in dye industrial waste water treated with algae. Reduction in BOD and COD was also reported in textile effluent treated with *Chlorella vulgaris* (El-Kassas and Mohamed, 2014), *Nostoc* species (Roy *et al*., 2010) and *Spirulina platensis* (Sivakalai *et al*., 2013) which gives supportive evidence for the present study.

The total hardness in untreated textile effluent was recorded as 895mg/L whereas it decreased to 140mg/L in effluent treated with *O. subplagiostomum* AP1 which was below the specified limits (250mg/L) prescribed by BIS.

Higher level of hardness in the untreated effluent may be due to the presence of metal ions or minerals dissolved in water, which increases its boiling point and inhibits lather formation with soap (Janaki, 2001). Similar increase in total hardness was observed in textile effluent (Ohioma *et al.*, 2009) and reduction in hardness was also noticed in paper mill industrial effluent when treated with *Spirulina platensis* (Boominathan, 2000 and Olguin, 2003) which supports the findings of the present investigation.

The levels of anions namely total alkalinity, chloride, sulphate and nitrate estimated in the untreated textile effluent was 470 mg/L, 3569 mg/L, 1896 mg/L and 159 mg/L respectively while it was low in textile effluent treated with *Oedogonium subplagiostomum* AP1 (64.76mg/L, 750mg/L, 507mg/L and 25.58 mg/L) and these values falls within the limit prescribed by BIS (Table 22).

High levels of alkalinity, chloride, sulphate and nitrate in untreated textile effluent may lead to corrosiveness and affects water quality, damages the crop plants, harms the seedling stage and maturity of plants and causes disorders to human beings (Hussein, 2013). Suriyaprabha and Fulekar (2018), Manikandan *et al*. (2015), Mahajanashetti and Mise (2017) and Elango *et al*. (2017) observed higher amount of anions in the textile dyeing effluent which supports the results of the present study. The microalgae have a capacity to degrade dyes for nitrogen source by removing nitrogen, phosphorus and carbon from water. This inturn helps to

reduce eutrophication in aquatic ecosystem and reduces BOD and COD (Chitra *et al*., 2013). Removal of maximum amount of anions from textile effluent was reported by Saha*et al.* (2018) using *Nostoc carneum* and paper mill effluent using *Cyanobacterium* (Nagasathya and Thajuddin, 2008) which was in accordance with the findings of the present study.

The decolourisation percentage (86%) was observed in textile dyeing effluent treated with *O. subplagiostomum* AP1 under optimised conditions.

Cost economy of *O. subplagiostomum* AP1

The *O. subplagiostomum* AP1 can be sold at a rate of Rs. 10 per kg. The alga has the capacity to ramify by the process of photosynthesis. Hence minimum quantity of algae is sufficient for large scale wastewater treatment process.

Thus, the alga *O. subplagiostomum* AP1 can be successfully employed in the treatment of methyl orange bearing textile dyeing industrial effluent or wastewater.

PHASE VIII

4.8 *IN SILICO* ANALYSIS AND MOLECULAR DOCKING STUDIES ON DYE SORPTION

The mode of interaction of azo dyes with proteins such as peroxidase, azoreductase, laccase etc. is meagre, henceforth the investigation of the ligand azo dye binding against potential receptors involved in bioremediation remains significant. The discharge of synthetic dyes and their metabolites into the ecosystem is predominant in recent days and studies about remediation of azo dyes using *in silico* method is very limited (Kandelbauer and Guebitz, 2005).

In silico biology has witnessed tremendous advancement in the effective utilization of computational algorithms for virtual screening of molecular interactions. Hence, molecular docking analysis is a computational tool used to determine the interaction of a ligand to the dynamic site of a receptor (Schneider, 2010 and Shakil *et al*., 2013).

The *in silico* method seems to be advantageous since it is simple, cost effective, higher reproducibility with low compound synthesis requirements, reduced

utilization of animal models, less time consumption and thereby assist in providing metabolic information on protein ligand interaction (Meng *et al*., 2012).

Methyl orange was subjected to *in silico* studies for their efficiency against the target protein (azoreductase) involved in bioremediation using the commercially available tool Glide (SchrÖdinger software suite). The 2D and 3D structure of the ligand methyl orange was depicted in Figure 18 respectively.

In the present study, the methyl orange was docked into the receptor site of the prepared protein using Glide standard precision mode. The results were interpreted based on the glide score, glide energy, hydrogen bonds and Van der Waals interaction which indicate the binding affinity of ligand towards target molecule. The docking results showed that methyl orange effectively interacted with the target protein involved in bioremediation process as depicted in Table 23.

Table 23 - Glide parameters of methyl orange with azoreductase

Target	Glide Score	Glide H bond	Glide VDW	Glide E Model	Glide energy	Glide Conformer index
Azoreductase	-5.734	-0.914	-1797.97	-60.018	-44.359	1

The hydrogen bond interaction of methyl orange with azoreductase was shown in Table 24 and Plate 21.

Table 24 - Hydrogen bond interaction of amino acid residues of azoreductase with methyl orange

S. No	Donor... Acceptor	Distance
1.	Ser10…..…O-H-O……ligand	2.76
2.	Arg12……...N-H-O…….ligand	2.94
3.	Ser18……N-H-O……ligand	2.75
4.	Ser18……O-H-O……...ligand	3.38
5.	Ser16……N-H-O……ligand	2.82

It is evident from the docking study that there was good binding affinity between the ligand and the receptor site of the target protein. The binding affinity shows high negative values which revealed the high feasibility of the interaction between the ligand and the target as evidenced from Table 23.

The docking study showed that the binding mode of methyl orange was stabilized by 6 hydrogen bonds which might be involved in the effective interaction of the ligand (methyl orange) with the target protein (azoreductase) leading to undergo degradation.

The hydrogen bond interaction from the docking studies indicates that the amino acid such as SER10, ARG12, SER18, SER18 and SER16 plays an important role as catalytic residues in azoreductase. The removal of methyl orange by *O*. *subplagiostomum* AP1 was to a higher extent in the present study which could probably provide better information on understanding the molecular mechanisms involved in bioremediation process.

Thakuria *et al.* (2015) reported that the higher affinity of ligand was presumably attributed to the formation of hydrogen bonds which plays a potential role in dye degradation which supports the present investigation.

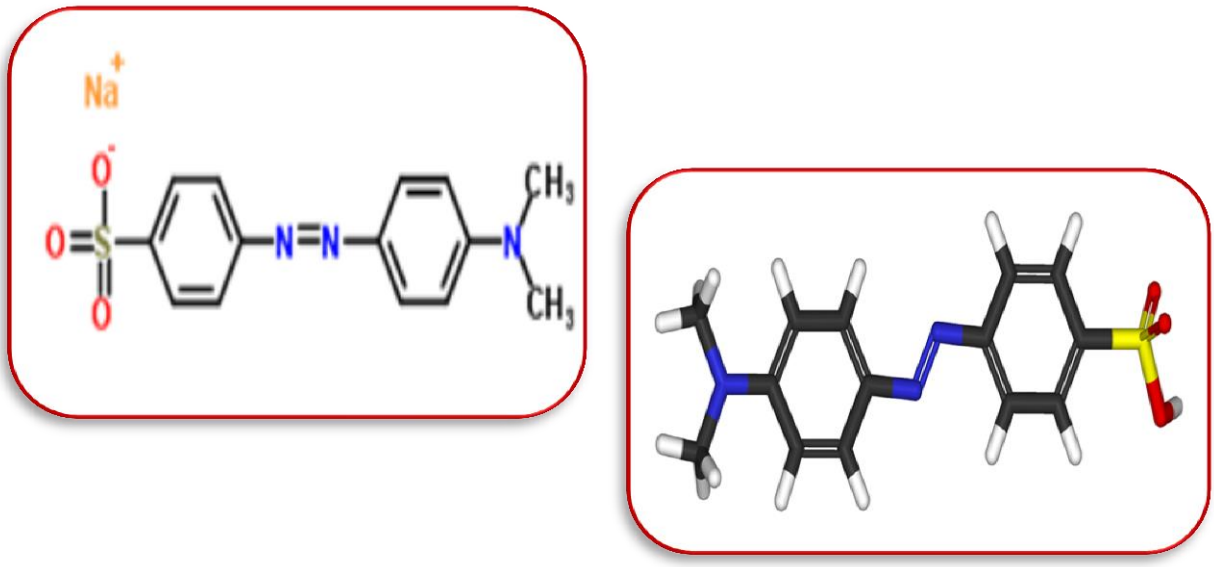
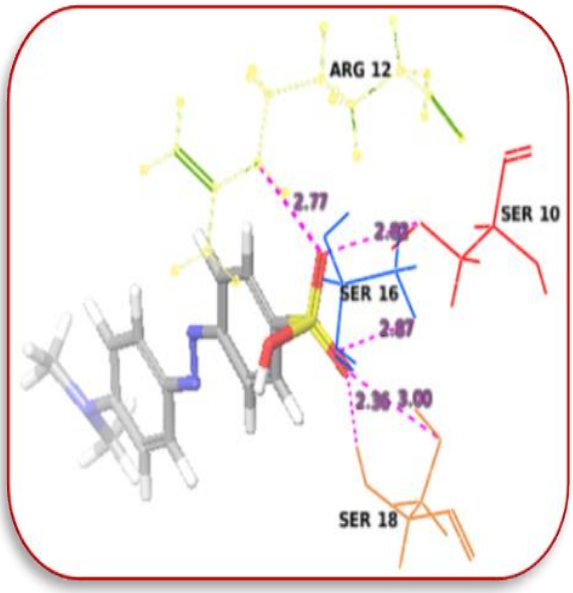
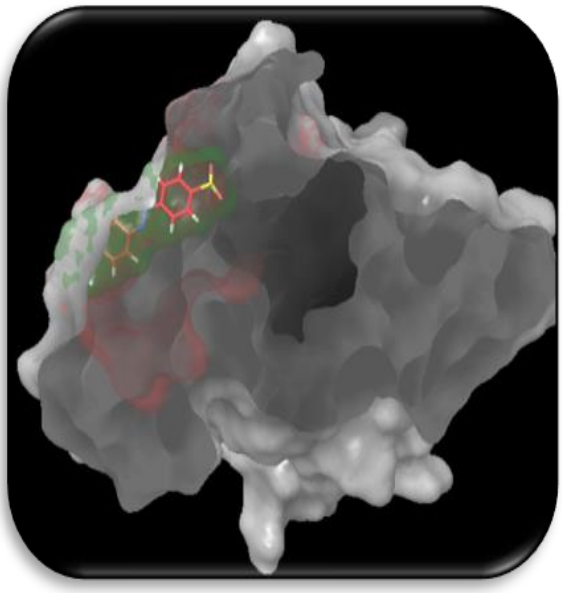
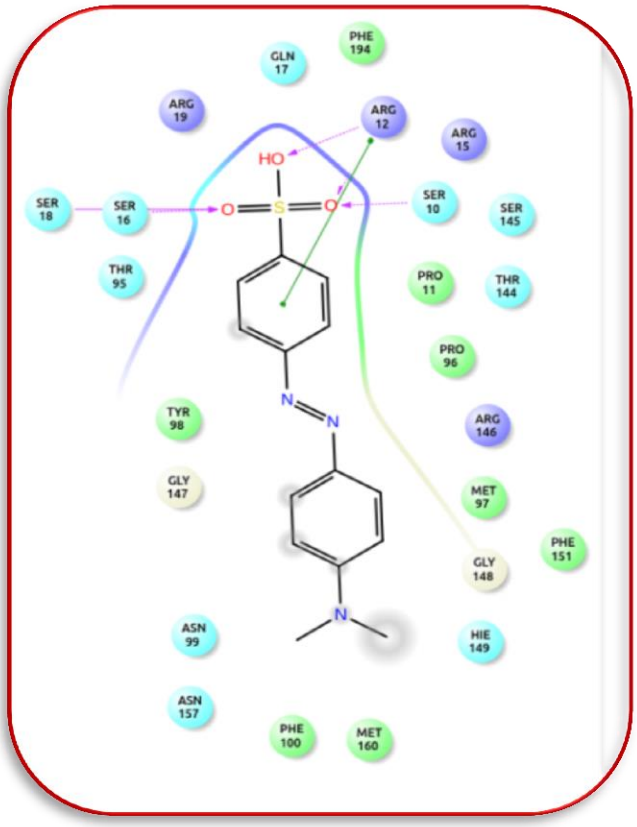


Figure 18 - 2D (a) and 3D (b) structure of methyl orange





21a **21b**



21c

Plate 21 - The interaction of methyl orange with azoreductase

21a: H-Bond interactions between ligand and target

21b: Docking interaction of methyl orange dye and *O. subpalgiostomum*AP1

21c: Amino acid interactions with methyl orange

Hence the results provide mechanistic information on the molecular interaction of azoreductase with methyl orange suggesting that algae secreting azoreductase can be explored in the bioremediation of wastewater.

Thus to conclude, the statistical analysis and the results of the present study clearly supported the rejection of null hypotheses and the acceptance of alternate hypotheses stating the efficiency of *Oedogonium subplagiostomum* AP1 in the removal of methyl orange from aqueous solutions.