International Journal of Environmental Bioremediation & Biodegradation, 2014, Vol. 2, No. 3, 117-124
Available online at http://pubs.sciepub.com/ijebb/2/3/4

DOI:10.12691/ijebb-2-3-4

Microbial Decolorization and Degradation of Remazol Black & Mordant Orange by Microbial Consortia Isolated from Common Effluent Treatment Plant

Maulin P Shah*, Kavita A Patel

Industrial Waste Water Research Laboratory, Division of Applied & Environmental Microbiology, Enviro Technology Limited, Ankleshwar, Gujarat, India
*Corresponding author: shahmp@uniphos.com

Received December 30, 2013; Revised May 08, 2014; Accepted May 08, 2014

Abstract Azo dyes are xenobiotic and recalcitrant against biodegradation, causing environmental problems. Under certain environmental conditions, microorganisms can transform dyes to non-coloured products or completely mineralize them. In the present study, the first attempt on dye decolourisation potentials of local microbial consortia isolated from dye contaminated soils in common effluent treatment plant were determined against two azo dyes. Decolourisation rate and kinetics were monitored by spectrophotometry under different conditions. Effect of process parameters: pH, dye concentration and inoculums size on dye decolourisation rate was optimised using the full factorial design. Microbial growth and decolourisation rate were higher in shaking than static conditions. The bacterial consortium gave highest decolourisation of 91.86% for Remazol Black and 93.75% for Mordant Orange within 48 hours in shaking cultures; 57.78% and 62.06% respectively after 48 hours under static condition, followed by the mixed and fungal consortium. Kinetics studies revealed the bacteria consortia had highest tendency towards decolourisation, with a greater half live value for Remazol Black (13.97 hours) than for Mordant Orange (10 hours). pH out of the range 7.2-8 and dye concentrations above 100mg/l reduced decolourisation rate by the bacteria consortia while increasing inoculums size increased it. Optimum decolourisation was achieved when pH and dye concentrations were kept low while the inoculums size was high.

Keywords: *Remazol Black, Mordant Orange, decolorization, common effluent treatment plant*

Cite This Article: Maulin P Shah, and Kavita A Patel, "Microbial Decolorization and Degradation of Remazol Black & Mordant Orange by Microbial Consortia Isolated from Common Effluent Treatment Plant." *International Journal of Environmental Bioremediation & Biodegradation*, vol. 2, no. 3 (2014): 117-124. doi: 10.12691/ijebb-2-3-4.

1. Introduction

Indiscriminate and uncontrolled discharge of industrial and urban wastes into the environmental sink has become an issue of major global concern in developing and densely populated countries like India. They are a main source of direct and often continuous input of pollutants into aquatic ecosystems with long-term implications on ecosystem functioning including changes in food availability and an extreme threat to the self-regulating capacity of the biosphere. In recent years, interest in environmental control of dyes has increased, due to their possible toxicity and carcinogenicity; this is because many dyes are comprised of known carcinogens, such as benzidine and other aromatic compounds [1]. There is a great need to develop an economic and effective way of dealing with the textile dyeing waste in the face of the ever-increasing production activities [2]. Environmental biotechnology is constantly expanding its efforts in the biological treatment of dye-contaminated wastewaters. Biological treatments of azo dyes are more attractive than physicochemical approaches because of their cost effectiveness, lower sludge production, and environmental friendliness [3]. The bacterial decolorization and degradation of these dyes has been of considerable interest since it can achieve a higher degree of biodegradation and mineralization, is applicable to a wide variety of azo dyes, is inexpensive and environmentally-friendly, and produces less sludge [4]. Nature is full of bacterial diversity. Azo dye decolorizing bacteria can be isolated from soil, water, human and animal excreta and even from contaminated food materials. However, other potential ecological niches for isolating such bacteria are colored effluents arising from dye manufacturing and textile industries. While much researched has been performed to develop effective treatment of dye containing wastewater, no single solution has been satisfactory for remediation of the broad spectrum of textile dye wastewater [5]. Several physicochemical decolourization techniques have been reported, few of them were accepted by the textile industries [5,6]. New processes for dye degradation and wastewater treatment and reutilization are being developed, [7]. In particular, systems based on biological processes using a large variety of bacterial strains, allow

for degradation and mineralization with a low environmental impact and without the use of potentially toxic chemical substances, under mild pH and temperature conditions [8,9]. Activated sludge is commonly used as an inoculum to initiate degradation, and it appears that many different microorganisms can decolorize azo dyes [10] but may require a mixed community to mineralize them [11,12]. Amongst these systems, several microbial strains including *Sphingomonas spp.*, *Pseudomonas luteola*, *Streptococcus faecalis*, *Klebsiella pneumoniae*, *Penicillium spp.*, *Aspergillus spp.* have been described as being capable of reducing azo dyes [13]. These microorganisms have the ability not only to decolourise dyes but also to detoxify it [14]. Furthermore, it has been demonstrated that local indigenous microflora biomass is significantly better at biodegradation/biodecolourisation than commercially obtained once [15], but to the best of our knowledge, no information exist about the biodecolourization potential of local microbial isolates from common effluent treatment plant of Ankleshwar, Gujarat, India. In view of these, the objective of this study was to evaluate the decolourization potential of textile dyes by local microbial consortia developed from common effluent treatment plant contaminated soils from Ankleshwar (Gujarat, India). Factors affecting the decolourization process: pH, temperature, inoculums size and dye concentration, were studied in view of optimizing the decolourization process and provide an affordable treatment technology.

2. Materials and Methods

2.1. Dyestuffs and Chemicals

All chemicals were of highest purity and of an analytical grade. Microbiological media were obtained from Hi-media laboratory, India. The textile dyes Remazol Black B and Mordant Orange was obtained from local textile mill, Ankleshwar, India. The effluents were collected in airtight plastic container and filtered through ordinary filter paper to remove large suspended particles. The pH of the filtered effluent was adjusted to 7.0 and stored at 4 ± 1°C until use.

2.2. Isolation, Screening and Development of Dye Degrading Microbial Consortium

The microorganisms present in soil samples from effluent disposal site of a common effluent treatment plant of Ankleshwar, Gujarat, India were enriched in a growth medium according to the modified method of Li *et al*., [16]. The enrichment medium consisted of: glucose 0.1%, yeast extract 0.05%, peptone 0.5%, NaCl 0.5%, $(NH_4)_2SO_3$ 1%, K_2HPO_4 0.02%, KH_2PO_4 0.5% and $MgSO_4.7H_2O$ 0.5%, amended with 50 mg/L of the test dyes for adaptation of the microorganisms. The medium was autoclaved at 121°C for 30 min. After cooling, 10 g of soil samples were aseptically inoculated into three 250 ml conical flask containing 100 ml of the enrichment media. Nystatin (0.1 g/ml) was used in one flask to inhibit fungal growth for the development of the bacteria consortium, chloramphenicol (250 mg/ml) was used in the second flask to inhibit bacteria growth in the development of the fungal consortium while the third flask was used for the mixed/microbial consortium. The three flasks were incubated under shaking conditions at 180 rpm and at a temperature of 30°C. After 10 days incubation, the most effective decolourizing species were screened by 48 hours incubation on Plate Count Agar and Potato Dextrose Agar amended with 50 mg/L of the test dyes for the bacteria, mixed and fungal consortium respectively. Bacterial colonies that showed a clear decolourization zone around them were picked and reintroduced into 100 ml of freshly prepared enrichment media. Same was done for fungal species showing growth on Potato Dextrose Agar and the mixed consortium showing growth on Plate Count Agar. The three flasks containing the isolated and screened bacteria, fungal and mixed isolates were incubated at 30°C under agitation at 160 rpm for 3 days to develop the different consortiums.

2.3. Microbial Consortium, Media and Culture Condition

The bacteria, fungi and mixed consortium were grown and maintained on the enrichment media proposed by Li *et al*., [16] amended with 50 mg/l of the test dyes at a temperature of 30°C under agitation at 160 rpm. Decolourization experiments were carried out in 10 ml the this medium (glucose 0.1%, yeast extract 0.05%, peptone 0.5%, NaCl 0.5%, $(NH_4)_2SO_3$ 1%, K_2HPO_4 0.02%, KH_2PO_4 0.5% and $MgSO_4.7H_2O$ 0.5%) amended with 50 mg/L of the test dye. Different pH values, culture volume and dye concentration were used in the present study to investigate their effect on the decolourization rate.

2.4. Decolourization Experiments

2.4.1. Decolourization at Static and Shaking Conditions

Decolourization of individual dye by the different microbial consortium in the culture supernatants was determined using a photo spectrometer [17] under shaking and static culture at optimum pH (7.2) and temperature (30°C). 1ml inoculum from each consortium was individually introduced into separate 100 ml conical flask containing 10 ml growth medium amended with 50 mg/l of the individual dye. One set of flask was incubated under agitation at 160 rpm and temperature of 30°C while the second set was incubated under stationary condition at 30°C for a period of 48 hours. These served as the experimental. The control consisted flask without any microorganisms. All experiments were done in triplicates.

2.4.2. Analytical Methods for Dye Decolourization Studies

Aliquots (3 ml) of the culture media were withdrawn at time intervals of 6 hours over 48 hours and centrifuged at 8000 rpm for 10 min. Decolourization was quantitatively analyzed by measuring the absorbance of the supernatant using a UV–visible spectrophotometer (Shimadzu UV-1800, Japan) at maximum wavelength, λmax, of 595 nm for Remazol Black B and 385 nm for Mordant Orange. The decolourization rate was calculated using the equation [18].

$$\text{Dye Decolorization} = \text{Initial absorbance} - \text{Final absorbance} / \text{Initial absorbance} \times 100$$

2.4.3. Decolourization with Increased Dye Concentration, Inoculum Size and Different Ph Values

Various concentrations of dye (50, 100, 200, 300, 400 and 500mg/l), typical of those used in studies on treatment of azo-dye wastewater effluent [19] and inoculums sizes of 10% (1 ml), 20% (2 ml), 30% (3 ml) and 40% (4 ml)) were used to examine the effect of initial dye concentration and inoculums size on the decolourization rate. Incubation was done as described above under shaking condition. The effect of different pH values [3,5,7, 8,10] on decolourization rate was also investigated under shaking condition. pH was adjusted using either HCl (0.1M) or Na2CO3 (0.1M). The percentage decolourization was determined over 48hrs as described above.

2.3.5. Biodecolourization Kinetics

Decolourization kinetics occurring in the natural environment is based on the empiric approach, reflecting the rudimentary level of the microbial population and their activity in an ecosystem. When the substrate is totally available, the biodegradation will solely depend on the microbial activity, following a logarithmic growth. On the other hand, the first order kinetics link to the population density is more realistic. This approach described by equation below was used to study the decolourisation kinetics:

$$-\frac{d(C)}{d(t)} = KC^n$$

Where C = concentration of the substrate (dye), t = time, K = rate constant of substrate disappearance, n = an appropriate parameter.

2.3.6. Full Factorial Design for the Optimization of the Decolourization Process

The full factorial design was used to evaluate the effects of the interaction between the pH, inoculums size and dye concentration on dye decolourisation rate. Decolourisation percentage, Y, (response) which depends on these factors was represented mathematically as:

$$Y = (X_1 X_2 X_3)$$

Where, X1, X2, X3 are pH, dye concentration and inoculums size respectively. The number of experimentation (N) for the full factorial design given by; $N=2^k + n_0$.

Where, k=number of factors, 3, and no repetition at the centre, 4. Thus, $N = 2^3 + 4 = 12$. In other to avoid the influence of the variable being dependent on the units used, the real variables were coded as represented on Table 1.

Table 1. Coded variables for full factorial design

Factors	Low level (-1)	Centre (0)	High level (+1)
pH	7.2	7.6	8
Dye concentration (mg/L)	50	75	100
Inoculum size	10	25	40

3. Results and Discussion

3.1. Screening of Decolourizing Bacteria, Fungi & Mixed Consortia

As also observed be Chen *et al.* (2003), screening on solid media after adaptation and enrichment gave effective dye decolourizing isolates; bacteria colonies with decolourization zone and fungi shows growth and decolourization on solid culture (data not sown) which were further picked out and enriched in the liquid media to develop the deferent decolorizing consortium.

3.2. Decolourization under Shaking and Static Conditions

With higher growth in shaking condition for all the consortiums, there was a corresponding higher decolourisation rate in shaking condition than static condition for both dyes (Figure 1). Dye decolourisation in shaking cultures varied from 55.89% to 93.75% while in static cultures, it varied from 48.41% to 62.06%. There is better oxygen transfer and nutrient distribution in shaking cultures than static cultures, therefore better microbial growth. In shaking conditions; fungi grow in spherical pellets which are uniformly suspended during agitation leading to greater contact with dye and nutrient while fungi growth in stationary cultures is in the form of mat of mycelia, which covers the whole interface of media and air, thus blocking exchanged of air [21].

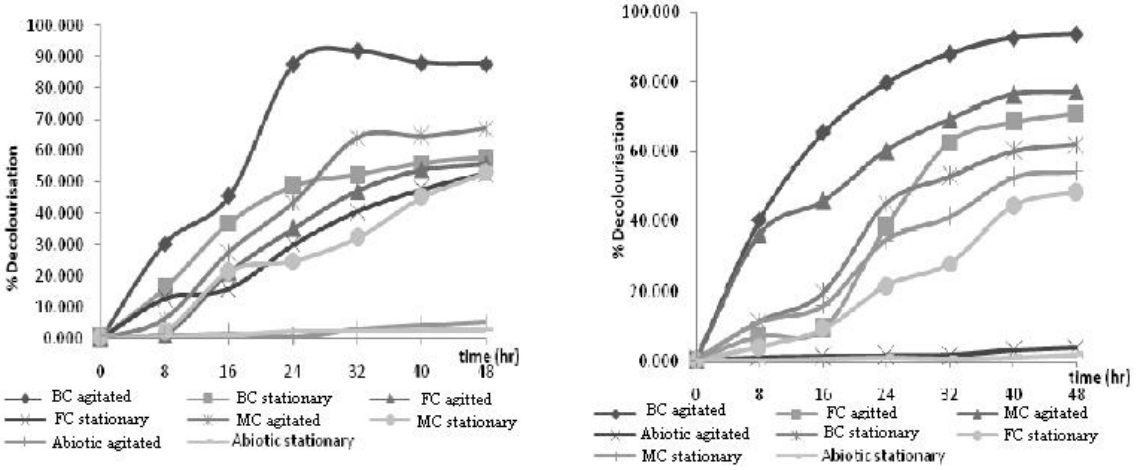

Figure 1. Decolorization under shaking and static condition for a) Remazol black and b) Mordant orange

3.3. Dye decolourization by the Different Consortium: Bacteria, Fungi and Mixed Consortia

The bacterial consortium exhibited highest decolourization rate, which was higher under shaking condition, 91.86% decolourization of remazol black after 32 hours (Figure 2a), and 93.75% decolourization of mordant orange after 48 hours (Figure 2c) than in static condition, 57.78% and 62.06% decolourization after 48 hours for remazol black and mordant orange respectively (Figure 2b and Figure 2d). The microbial/mixed consortium on its part exhibited a maximum decolourisation of 67.04% for remazol black and 77.20% for mordant orange after 48 hours under shaking conditions, with 52.94% and 54.31% decolourisation for remazol black and mordant orange respectively after 48hrs under static condition. The lowest decolourisation rate was exhibited by the fungi consortium (Figure 2) after 48 hours of incubation while the abiotic showed negligible decolourisation both in the static and the shaking conditions. Operational conditions (concentration of dye, pH and temperature) are serious drawback for biological treatment process (Fu and Viraraghavan, (2001). The ability of microorganisms to degrade azo dyes is generally correlated with the ability to synthesize enzymes, such as lignin-degrading exoenzymes, which are affected by environmental factors such as pH, temperature and substrate concentration [23]. While thermorphilic microorganisms have a growth temperature range of 10 to 45°C, bacteria and fungi have an optimum growth temperatures between 25–30°C. Fungi grow well under acidic pH values and their ligninolytic enzymes show maximal activity at low pH. The maximum degradation pH for many fungi species have been reported to be in the range of 3.5 – 4.5, while bacteria species perform well at neutral or slightly alkaline pH. Asgher *et al*., (2008) reported a fungi consortium of *Schizophyllum spp*. which decolourised 73% of solar golden yellow at a pH of 4.5 after 6 days and the efficiency decreased from 59% to 8% as pH was increased from 5 to 6 while a bacteria consortium of *Acinetobacter spp.*, *Citrobacter freundii* and *Klebsiella oxytoca* decolourised 92% of 4-nitroaniline (and structurally different azo dyes) at a pH of 7.2 within 42 hours under aerobic conditions with shaking, which decreased as pH varied below 7 or greater than 7.2 [25]. It can thus be concluded that, efficient dye decolourisation is observed at low pH for fungi while bacteria strive well under neutral or slightly alkaline pH at temperatures between 25-35°C. Contrary to report from some authors on the synergistic interaction between bacteria and fungi species that resulted in higher decolourisation of azo dyes [25], an antagonistic interaction in the mixed consortium, resulting to lower decolourisation rate compared to the bacteria consortium was observed. It is possible that the fungi inhibited the maximum growth of the bacteria consortium through competition for or excretion of byproducts that are toxic to the bacteria. A spectrometric scan of the supernatant after 48 hours decolourisation with the bacteria is represented on Figure 3.

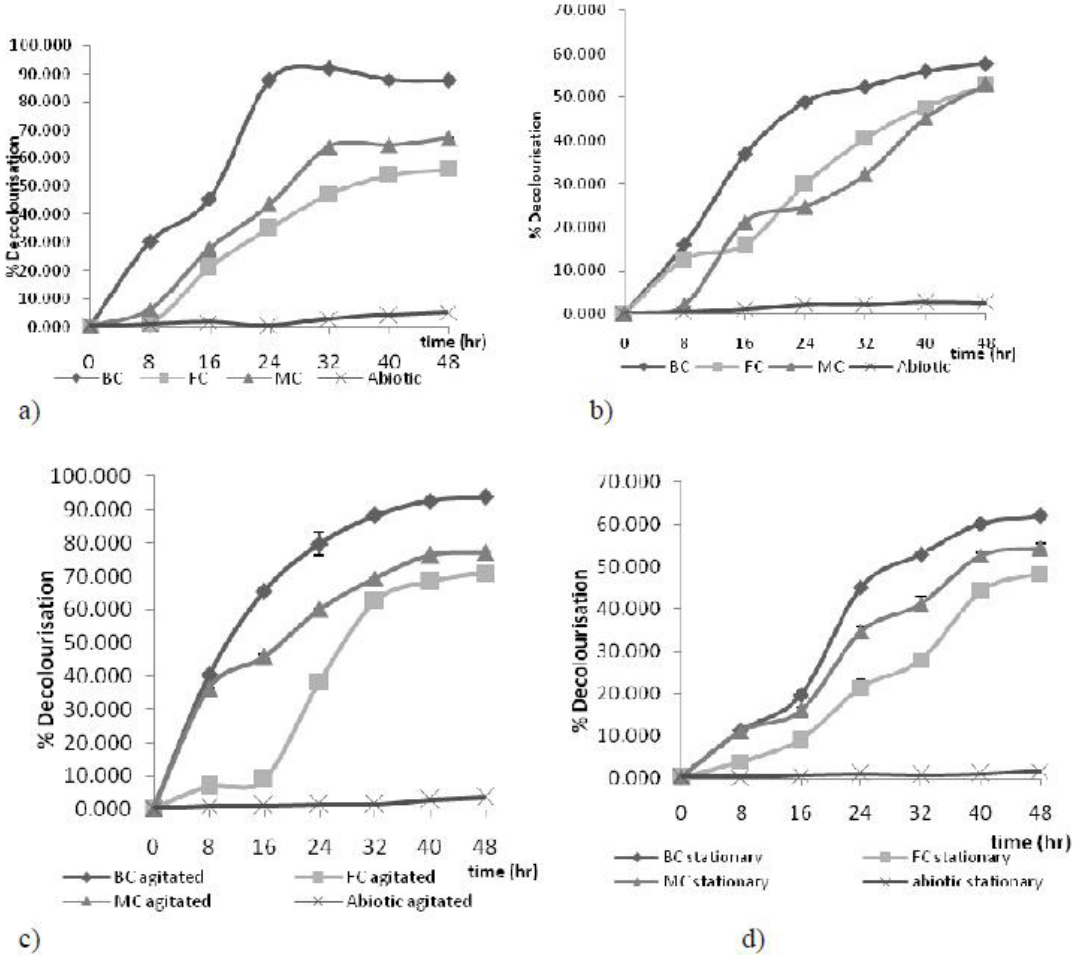

Figure 2. Percentage Decolorization of remazol black: a) Shaking condition b) Static condition and mordant orange: a) Shaking condition b) Static condition

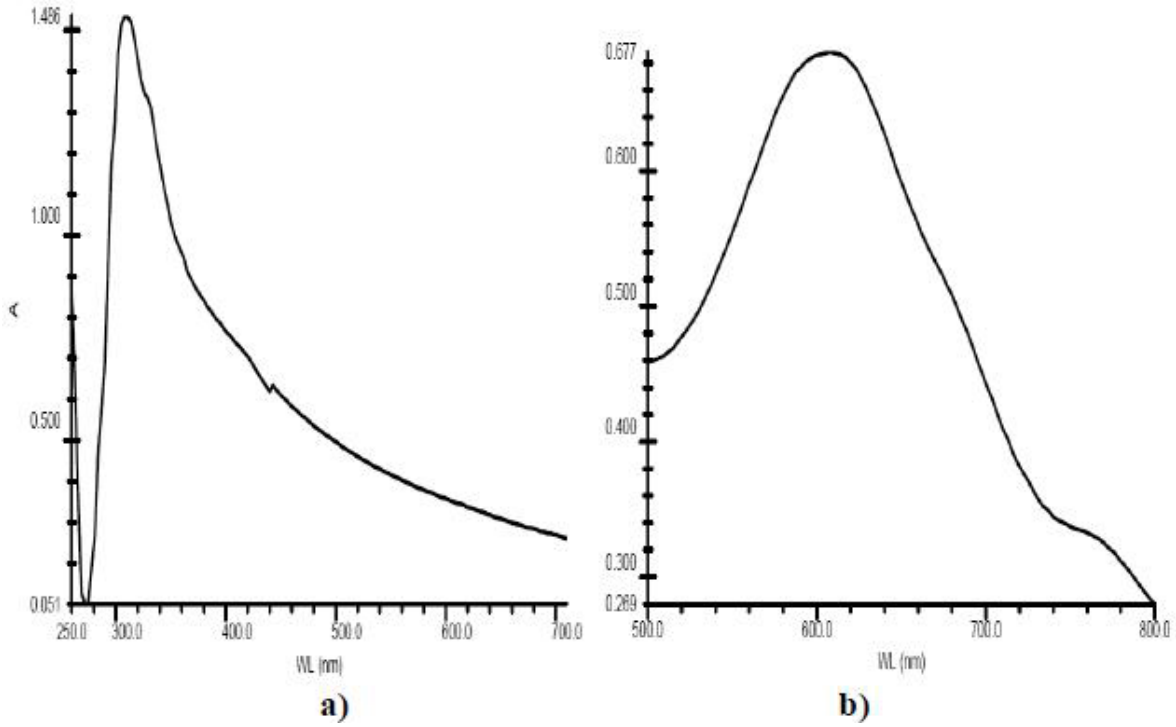

Figure 3. Spectroscopic scan of remazol black dye afte 48 hrs Decolorization: a) Bacterial consortium b) Abiotic

3.4. Kinetics Study of the Decolourization Process

The best kinetic model giving a good correlation coefficient for the dyes is the exponential model of the form;

$$\frac{C}{C_0} = Y_0 + Ae^{(-Bt)} \quad (2)$$

Where C and Co are dye concentration at any time, t, and at the initial time, t = 0hr, Yo, A and B are constant.

The kinetics models (Figure 4) and kinetic parameters (Table 1) show that both dyes are degraded by all three consortia but with different correlation factors. The decolourization of remazol black and mordant orange are closely tied to the bacteria consortium as shown by the high R2 values, with the shortest half life of 13.97 hours for remazol black and 10 hours for mordant orange.

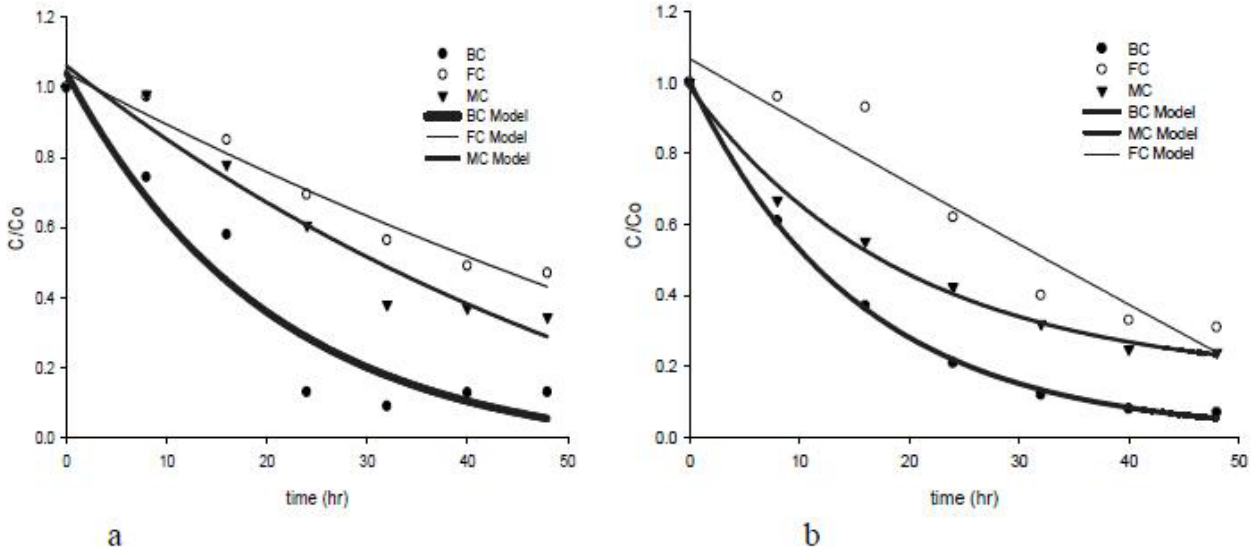

Figure 4. Kinetics of remazol black (a) and mordant orange (b) Decolorization by three microbial consortia

3.5. Decolorization with Increased Dye Concentration, Inoculum Size and Different Ph Values

The bacteria consortium was unable to efficiently decolorize the increasing dye concentration from 50 mg/l to 500 mg/L with the decolorizing efficiency initially increasing from 87.45% at 50 mg/l to 93.91% at 100 mg/l before dropping to 30.08% at 500 mg/l (Figue 5a). The percentage of decolourization increased as inoculums size was increased (Figure 5b) while the highest decolourization rates of 84.60% and 88.70% were obtained between pH of 7.2 and 8 (Figure 5c) and decreased as the pH varied away from this range.

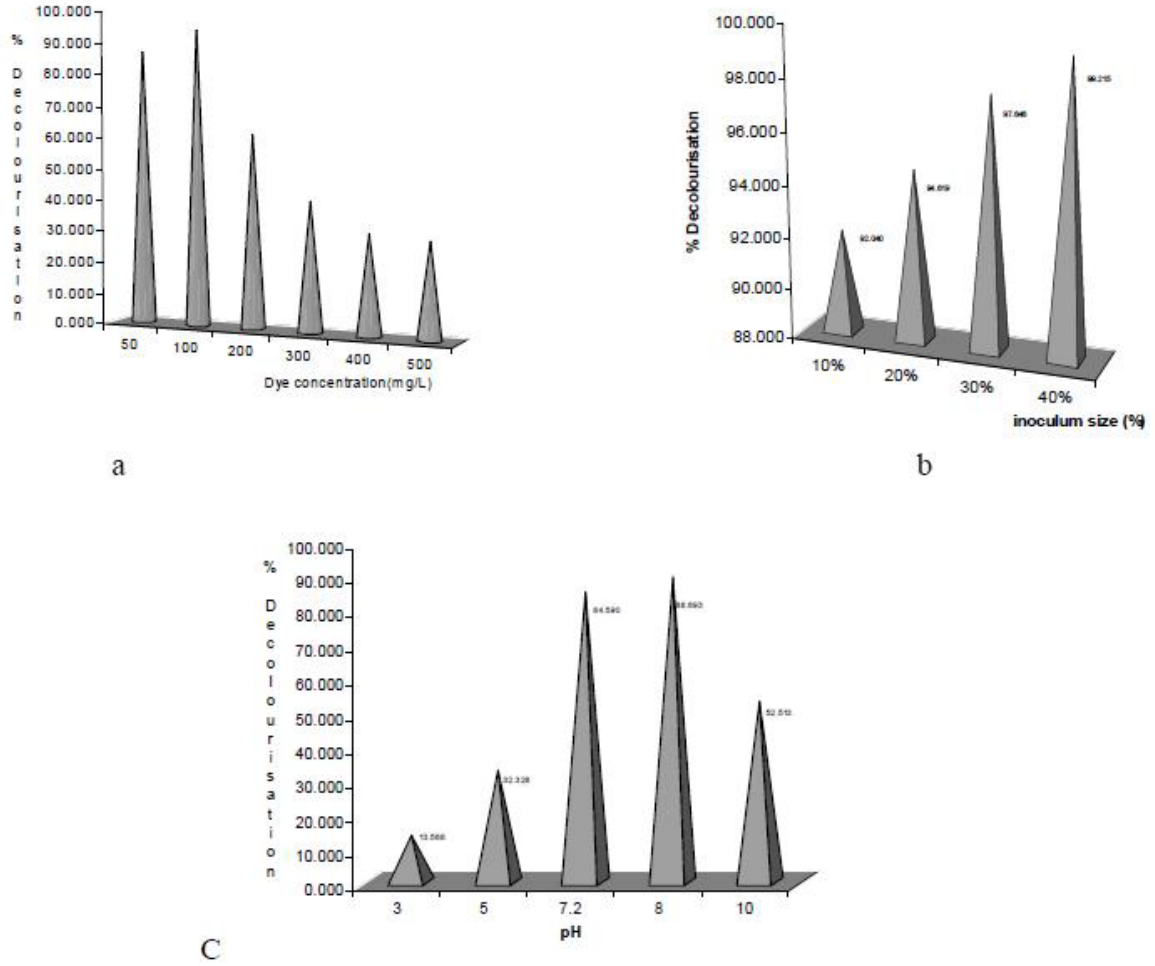

Figure 5. Effect of initial dye concentration (a), inoculums size (b) and pH (c) on decolorization rate

3.6. Full Factorial Experimental Plan to Optimize Remazol Black Dye Decolourisation by the Bacteria Consortium

3.6.1. Determination of the Model Equation

The decolourisation rate from the experimental design varied from runs to runs (Table 2). The first order mathematical model with interaction representing the decolourisation rate was given by the equation:

$$y = 86.554 + 3.223x1 - 10.775x2 + 13.842x3 + 1.060x1x2 - 2.744x1x3 + 0.608x2x3.$$

$$(R2 = 97.36\%)$$

The significant effects of the variable are given by the Pareto plot (Figure 6), at a confidence level of 95%.

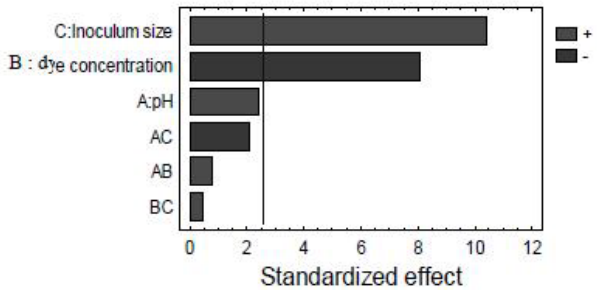

Figure 6. Pareto plot of effects of dye concentration, inoculums size and pH

Table 2. Kinetic parameters of remazol black and mordant orange

Parameter	Bacterial consortium (BC)		Fungal consortium (FC)		Mixed consortium (MC)	
	Remazol Black	Mordant Black	Remazol Black	Mordant Black	Remazol Black	Mordant Black
R-Square	0.9864	0.9991	0.9657	0.9201	0.9433	0.9922
Y_O	-0.0471	0.0095	-0.8880	-12.5695	-0.4530	0.1621
A	1.0894	0.9947	1.9291	13.6367	1.5150	0.8245
B	0.0493	0.0648	0.0079	0.0013	0.0149	0.0511
$T_{1/2}$ (hr)	13.97	10	31.14	32.59	41.58	17.46

3.7. Interaction of the Dye Concentration, Inoculums Size and pH

Three dimensional response of the interaction of the dye concentration, inoculums size and pH are shown on Figure 7.

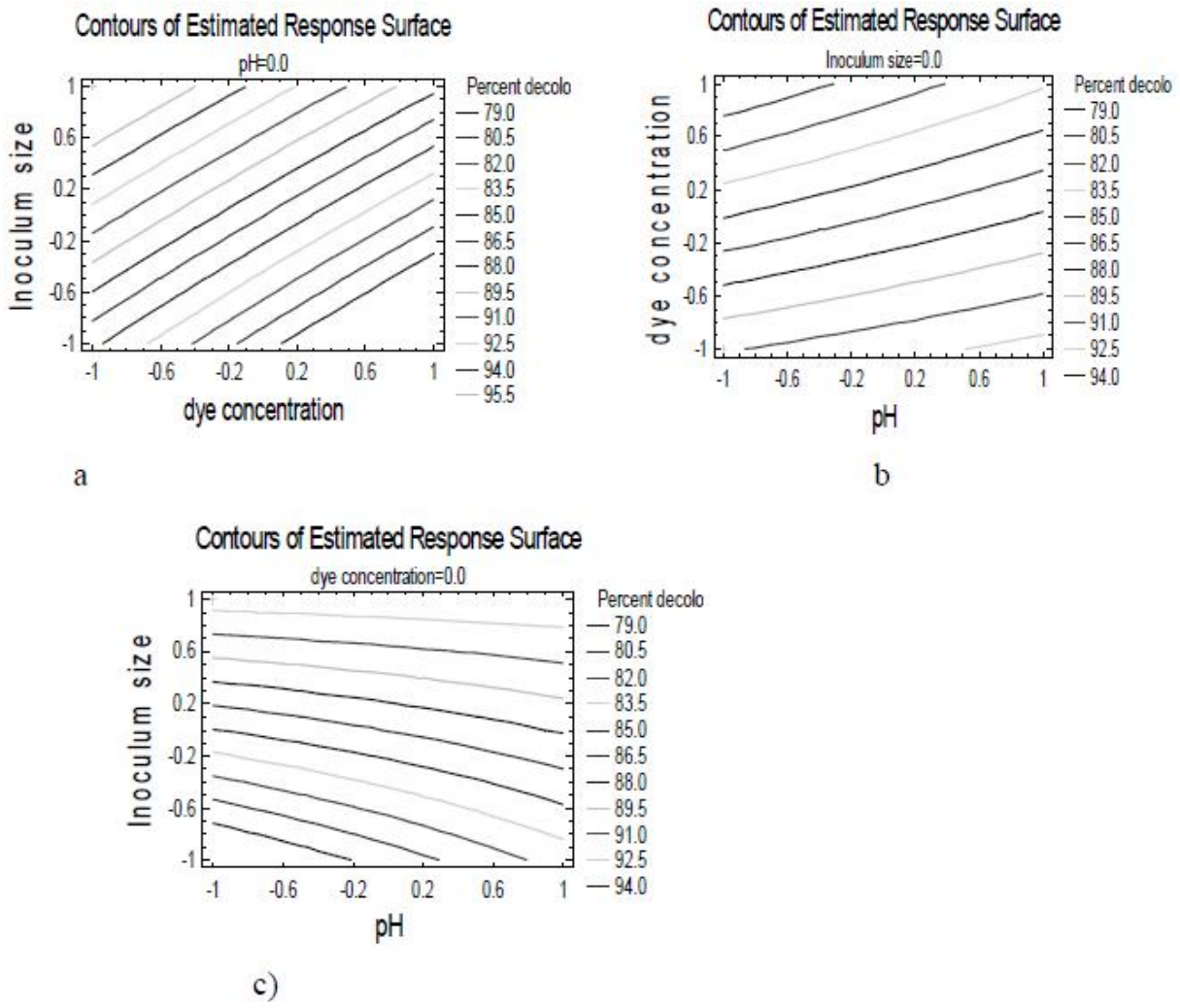

Figure 7. Response surface of interaction of the dye concentration, inoculums size and pH: a) inoculums size vs dye concentration b) Dye concentration vs pH and c) Inoculum size vs pH

Table 3. Experimental plan and the observed response

Experiment	Coded variable			Real variable			Response
	X_1	X_2	X_3	X_1	X_2	X_3	Y
1	-1	-1	-1	7.2	50	10	83.997
2	1	-1	-1	8	50	10	88.322
3	-1	1	-1	7.2	100	10	70.972
4	1	1	-1	8	100	10	78.581
5	-1	-1	1	7.2	50	40	99.394
6	1	-1	1	8	50	40	99.394
7	-1	1	1	7.2	100	40	88.747
8	1	1	1	8	100	40	89.706
9	0	0	0	7.6	75	25	85.121
10	0	0	0	7.6	75	25	84.689
11	0	0	0	7.6	75	25	85.208
12	0	0	0	7.6	75	25	84.516

3.8. Study and Validation of the Mathematical Model

A regression plot of the theoretical and the experimental results Y theoretical = F(Y experimental) (Figure 8) gave a regression equation of the type;

$$y - b * x + u$$

Where we have y = 0.974x + 2.286.

The high value of R2 = 97.358, indicates a strong relationship between the two variables, thus the mathematical model can be validated.

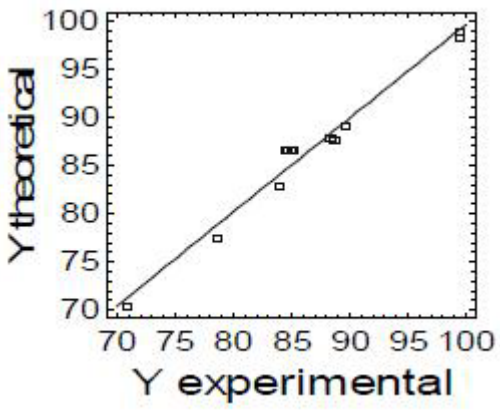

Figure 8. Regression plot of the fitted model: $Y_{theoretical} = f(Y_{experimental})$

4. Conclusion

The principal objective of this work was to evaluate the biodecolourisation potentials and kinetics of microbial consortium developed from common effluent treatment plant contaminated soils. Factors affecting the decolourisation process where investigated and used to optimise the decolourisation process. The two microbial consortia and the mixed consortium all showed potentials of decolourising the tested dyes, with the decolourisation rate varying from one consortium to another. A higher decolourisation rate was observed under shaking than static condition. The mixed consortium showed the highest decolourisation rate in both shaking (93.75 %) and static conditions (62.06%) within 48hr, followed by the pseudomonas consortium and finally the bacillus consortium. The exponential decay kinetics showed a higher tendency of decolourisation by the mixed consortium with the shortest half life. Furthermore, the process increased with increase in inoculums size, decreased with increase in dye concentration while it was high within a pH range of 7.2 to 8. The full factorial design gave an optimal decolourisation of 98.85% when the pH and the dye concentrations were kept low while the inoculums size was high. It can be concluded that adapted microbial isolates from common effluent treatment plant contaminated sites can effectively be used in the aerobic treatment of these effluents before discharging into the environment.

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