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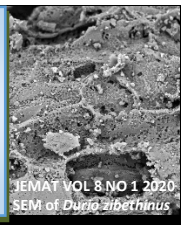

Biebrich Scarlet Biodegradation Optimization Using Response Surface Methodology

Siti Ruqaiyah Mohd Yusoff[1], Ain Aqilah Basirun[1] and Mohd Izuan Effendi Halmi[2]*

[1]Department of Biochemistry, Faculty of Biotechnology and Bio-molecular Sciences, Universiti Putra Malaysia, 43400 UPM Serdang, Selangor, Malaysia.
[2]Department of Land Management, Faculty of Agriculture, University Putra Malaysia, 43400 UPM Serdang, Selangor, Malaysia.

*Corresponding author:
Mohd Izuan Effendi Halmi
Department of Land Management,
Faculty of Agriculture,
University Putra Malaysia,
43400 UPM Serdang, Selangor,
Malaysia
Email: m_izuaneffendi@upm.edu.my

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ABSTRACT

The study was carried out to isolate bacterial strain potential for decolourizing the azo- dye Biebrich Scarlet and to optimize the dye decolourisation using Response Surface Methodology (RSM). From azo- dye screening, five isolates were capable of decolourising Biebrich scarlet and out of these, isolate 33 shows the highest decolourisation rate of $4.9x10^{-6}$ $mgml^{-1}min^{-1}$ compared to the other four. Response Surface Method (RSM) which includes Central Composite design (CCD) was employed following a screening procedure as such the Plackett- Burman design to attain optimal condition in order to achieve full azo- dye Biebrich scarlet decolourisation. Seven variables; pH, temperature (°C), ammonium acetate (%), glucose (%), salinity (%), yeast (%) and dye concentration (ppm) were studied. From Plackett- Burman design, four of these variables were chosen for further optimisation. Out of 30 individual runs of CCD, three variables; dye concentration, pH and yeast excluding ammonium acetate showed significant model terms (P-value < 0.05). Morphological identification was carried out on isolate 33 and results predicted that the isolate was a Gram-negative bacterium with catalase and cytochrome oxidase enzymes. This isolate gave a gamma hemolysis on blood agar medium and it decolourises Biebrich scarlet better in a minimal oxygen condition. The optimum ranges expected for a maximum dye decolourisation from this study were ammonium acetate (%) of 0.310.60; dye decolourisation (ppm) of about 112.16 or less; pH of 6.62-7.38 and yeast (%) of 0.23- 0.37. The significance of this study is to isolate, screen and identify the best isolated bacterial strain capable of decolourising Biebrich scarlet azo- dye, to study on the factors promoting the growth and the decolourisation of azo- dye by the isolated bacterium through means of RSM and to estimate the optimum condition in the factors which has a significant effect on the decolourisation percentage.

INTRODUCTION

Synthetic dyes consist of azo compounds and are the most chosen class in the industries of fabric, food and beverages, cosmetics and paper printing. Rapid industrialization as well as the ease of application of these synthetic dyes in the industries causes an increment in the annual consumption of dyes which thus results in the increase amount of colour effluents into water bodies and thus, adding up to the problem of removing the pollutants. In other hand, wastewater consisting of high dye effluent may cause water deterioration, as such loss of its appeal and attractiveness, depletion in the gas solubility as well as the reduction in the rate of photosynthesis by aquatic plants in conjunction to light penetration due to less water transparency [1].

Azo dyes are synthetic dyes and are the most chosen class in the industries of textile, cosmetic, printing and et cetera [2]. It had been mentioned that the annual consumption of dyes in the field of textile industry alone is more than 107 kg per year, world widely [3]. According to John [4], this is due to the unbearable ease of application in the industries especially in fast colouring and the ability of modifying the structure of azo dyes to meet the

assortment colour binding of natural and synthetic fibers [5]. There are many methods applied in the removal of dyes from the effluent which are divided into three categories namely the chemical, physical and biological method [6]. However, biological treatment has been the most preferred method compared to chemical and physical method in a way that it is more economical. Many bacterial species have been reported capable of decolourising and degrading Biebrich Scarlet azo-dye. *Enterobacteriaceae* sp., *Halomonas* sp., *Anaerovorax* sp., *Clostridiales* sp., *Serratia* sp., *Streptomyces* sp. and *Rhodococcus* sp. [7–10] which prefer higher decolourisation in anoxic condition.

Response surface methodology (RSM) is employed after a screening study to small scale and to study the region of interest of the factors reported by the previous studies [11]. RSM is applied to identify the interactions between operational variables or parameters at various concentrations. In this methodology, Plackett- Burman design is used to screen the different parameters that may produce a significant effect on the decolourisation of dye Biebrich scarlet. Significant parameters are then further optimized using Central Composite design (CCD). Data analysis will be done by using the Design- Expert 6.0.8 Portable version of RSM for regression analysis of the experimental data acquired from the designs [12].

MATERIALS AND METHODS

Sampling of azo-dye decolourising bacteria

Samples were taken from Malaysian local sources polluted with dyes in the nature of liquid, sludge or soil. The samples were taken about 5-7 cm depth from top of respective sources and kept in polycarbonated- screw cap tubes.

Bacterial isolation

Cultivation of samples was done in mineral salt medium (MS medium) (g/L) prepared by adding K2HPO4, 0.4 g; KH2PO4, 0.2 g; NaCl, 0.1 g; MgSO4, 0.1 g; MnSO4. H2O, 0.01 g; Fe2 (SO4)3.H2O, 0.01g; Na2MoO4. 2H2O, 0.01 g; (NH4)2SO4, 0.4 g; ph 7; yeast, 1 g into distilled water; and glucose solution, 10 g autoclaved separately at 121°C for 15 min [13]. The cultures were incubated at room temperature (25°C) with shaking at 150 rpm on orbital shaker. Isolation of bacteria capable of decolourising Biebrich scarlet azo- dye was done through preliminary screening with (working medium) MS medium incorporated with 50 mg/L of azo- dye by adding 100 µL of the azo- dye from its stock of 5000 mg/L and 1 mL of bacterial sample (OD 600= 1.0) to make up a final volume working medium of 10 mL in universal bottles.

The control was prepared the same else than the replacement of 1 mL MS medium instead of the bacterial sample. The decolourisation was determined by observation and the samples showing positive results were streaked onto agar plates and incubated for 24 hours at 30 ^{0}C for bacterial isolation [14]. Isolated bacteria were grouped according to similarities in morphological and biochemical test results and undergone secondary screening for isolation of bacterial of interest.

Determination of dye decolourisation

The absorbance reading for the samples and controls were taken once the medium samples decolourised. Decolourisation study was done photometrically at 506 nm [15] whereby the samples were centrifuged using micro- centrifuge (Sigma-Aldrich, USA) at 10, 000 g at room temperature for 5 min.

Supernatant was later taken for absorbance reading to determine the decolourised percentage of azo- dye Biebrich scarlet in which was calculated using formula [16]:

$$Decolourization\ (\%) = \frac{Initial\ absorbance - Final\ absorbance}{Initial\ absorbance}\ x\ 100$$

Identification of selected azo- dye decolourising bacterium

Identification was done from several biochemical tests as such the gram staining, catalase and oxidase test, blood lysis test as well as the bacterial growth curve.

Effect of agitation in azo-dye decolourisation

The samples and controls for this study were prepared in working medium as described in the bacterial isolation from which were incubated at room temperature in agitated and static condition. The decolourisation study was carried out as that in the determination of dye decolourisation.

Optimisation of medium components

Screening with various nitrogen and carbon sources as well as different yeast amounts were done in the first hand for this study to determine the best sources and yeast amounts shall be used in next process of optimisation using Plackett- Burman design and RSM. The several nitrogen sources tested were urea, ammonium sulphate, aspartate, ammonium acetate, glutamic acid, alanine, potassium nitrate, ammonium chloride and sample with no nitrogen source.

The carbon sources tested were glucose, galactose, fructose, lactose, sucrose, maltose, dextrin, arabinose and sample with no carbon source. Yeast amount (%) tested at 0.1, 0.001 and no yeast presence. Optimisation using Plackett- Burman was to screen out significant variables affecting the decolourisation of Biebrich scarlet. The variables studied in Plackett- Burman experiment were nitrogen and carbon sources (%), yeast extract (%), salinity (%), pH, temperature (^{0}C), dye concentration (ppm) were tested at varied combination in two levels of values at n=2 with individual controls for each set. Significant variables from Placket- Burman design were further optimised via Central composite design (RSM) at n=2 with individual controls for each set.

RESULTS AND DISCUSSION

Bacterial isolation

From screening of bacterial isolates, five isolates from a total of 36, showed the ability to reduce Biebrich scarlet and were named as isolate 8, 18, 26, 33, and 35. These isolates undergone second screening from which isolate 33 was chosen for further experiment since it displayed the highest decolourisation of 50 mg/L Biebrich scarlet with rate ($mgmL^{-1}min^{-1}$) of $4.9x10^{-6}$ followed by isolate 26, 8, 35 and 18 with $4.18x10^{-6}$, $4.07x10^{-6}$, $3.6x10^{-6}$, and $6.5x10^{-7}$ respectively.

Identification of selected azo- dye decolourising bacterium

Isolate 33 showed active growth at OD 600= 0.6- 1.8. This isolate was a negative bacterium with positive results for both catalase and oxidase test. When isolate 33 was spread onto blood agar plate, no clear spot was seen showing a gamma hemolysis.

Effect of agitation in azo-dye decolourisation

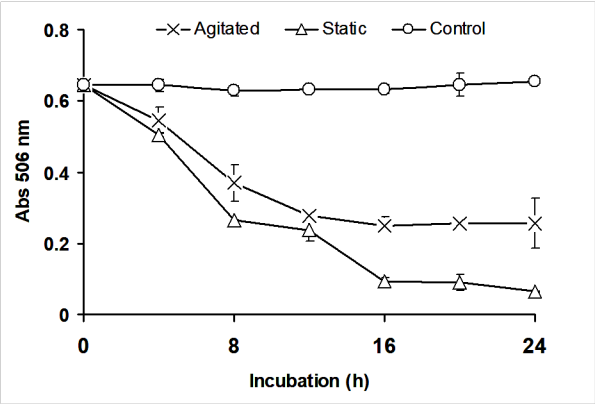

Fig. 1. Shows the effect of agitation and static condition on the percentage of Biebrich scarlet (50 ppm) decolourisation by isolate 33 in static condition and agitated condition (n=2) within the incubation time of 24 hours. Temperature= 27^{0}C, pH= 7.0, agitation by 150 rpm.

The percentage of decolourisation of the azo- dye was greatly achieved in static condition with a final decolourisation percentage of 90.13 % compared to the final decolourisation of 60.83 % achieved in agitated condition. Static condition was preferred but this experiment was carried out not entirely in anaerobic condition. This is because, there was still air space left in the universal bottle, which contain oxygen and may cause the azo-dye decolourisation be less susceptible resulting in lower decolourisation percentage obtained in this condition. This shall be explained by the structure of the azo- dye Biebrich scarlet itself since it has both sulfo and azo groups. The sulfo group has the electron withdrawing ability, which leads to electron deficiencies in the dye molecules, causing the compound to be less susceptible to oxidative decolourisation [17]. Anaerobic condition offers a reductive cleavage taking place on the azo- dye bond which means, it gets rid of the dye colour but, the free aromatic amines resulting from the azo-bonds' cleavage are not mineralized or broken down and hence led to the accumulation of toxic intermediates.

Carbon, nitrogen and yeast preliminary screening

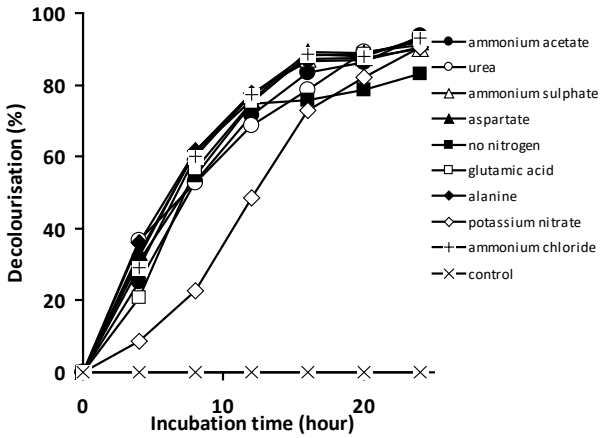

Fig. 2. Shows the decolourisation percentage of different nitrogen sources by isolate 33 with standard error of the mean n= 3.

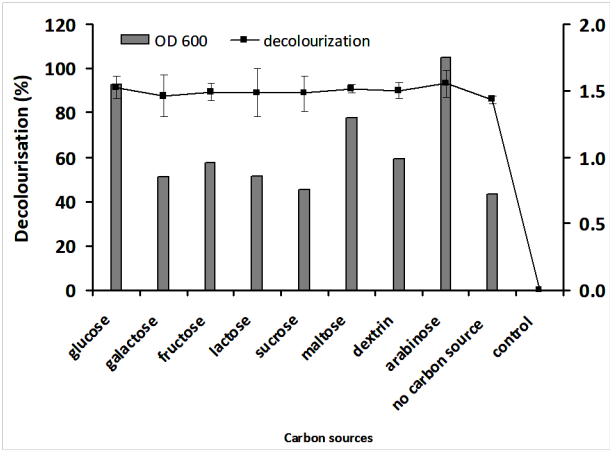

Fig. 3. Shows the decolourisation percentage of different carbon sources by isolate 33 at 50 ppm initial dye concentration with standard error of the mean n= 3.

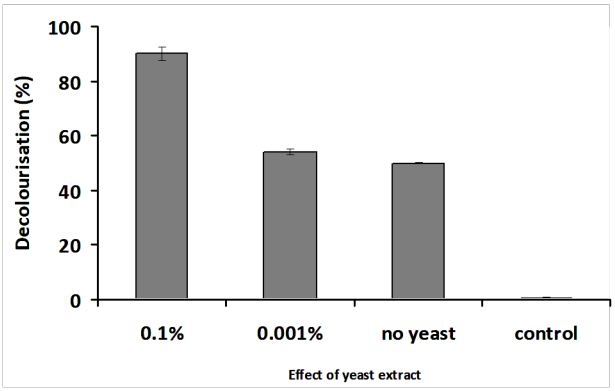

Fig. 4. Shows the effect of yeast on Biebrich scarlet decolourisation by isolate 33 at 50 ppm initial dye concentration with standard error of the mean n= 3.

The result plotted in **Fig. 2**, showed no significant difference (P- value > 0.05) between medium incorporated with nitrogen sources and the one with no nitrogen inclusion. The nitrogen concentration tested in this experiment was at 0.04% at 50 ppm initial dye concentration. It could be possible that, upon increasing the nitrogen concentration, the rate of decolourisation could be higher and the difference shall be more distinct. Result has been reported by [18] for ammonium sulphate as the best nitrogen source on the decolourisation of 50 ppm (a diazo- dye) dye by *Yarrowia lipolytica* in which the best decolourisation was obtained at 1-1.5g L^{-1} ammonium sulfate concentrations. In contrast, [19], stated that peptone results the best decolourisation of 100 ppm acid orange 7 (a monoazo- dye) by *Burkholderia cepacia*-TM5 at peptone concentration of 0.5gL^{-1} within 24 hours of incubation time. Decolourisation does occur without nitrogen in sample medium since it happened due to the cleavage and utilization of azo- nitrogen presents in azo- dyes [20], but, reports also have been issued on higher decolourisation percentage achieved when nitrogen was included in the medium run.

Fig. 3 showed no significant difference (P- value > 0.05) among the carbon sources samples as well as on no carbon inclusion sample. The carbon concentration included in the medium was $1gL^{-1}$ and experimented at 50 ppm initial dye concentration. It had been reported to enhance the decolourisation ability of methyl violet (a diazo-dye) by *Aspergillus sp.* [21]. Meanwhile, glucose has been widely chosen as the best carbon source which promotes decolourisation in many studies due to its readily usable structure and abundance. [18] from their study showed that glucose efficiently decolourised 50 ppm Reactive Black 5at 5-7.5g L^{-1} glucose concentration within 24 hours of incubation time. Glucose was hypothesised be the source of reducing equivalent to support the decolourisation [17]. In contrast, starch of concentration 0.5 gL^{-1} has been reported to enhance the decolourisation of 100 ppm direct blue 75 (a triazo-dye) by *Comamonas acidovorans*-TM1 [19].

According to **Fig. 4**, yeast amount (%) of 0.1% shows the highest decolourisation with 90.31%, followed by 0.001% and no yeast presence with 54.10% and 49.92% dye decolourisation showing a distinct difference (P- value < 0.05). Yeast was included in the experiment to stimulate higher decolourisation of azo- dye due to the azo- bond cleavage which occurs extracellularly. The mechanism of azo reduction includes redox mediators which function in the transports electrons between the dye (located in the extracellular of bacterial cells) and the intracellular reductase [22]. According to [17], the yeast extract was used to supply redox mediator which in return supports the growth of bacteria by converting nitrogen to glutamate and glutamine amino acids for these two amino acids act as nitrogen donor, still, the nitrogen catabolic repression is regulated by ammonia and glutamine [23].

Decolourisation of azo- dye Biebrich scarlet was due to the cleavage of the chromophoric group (azo- group) which later results in the release of free aromatic amines as metabolic intermediates. The cleaving of this chromophoric group is a reduction process since electron shall be obtained from the oxidation of reduced electron carrier (NADH) [9] provided by the bacterial isolate. Hypothetically, the presence of nitrogen, carbon and yeast in the working solution (medium) could have promote the increase of bacterial biomass thus helps to encourage a higher dye decolourisation, since the enzymes production which breaks down the azo- group would be increased as well. If the study is to be repeated in future, experiment on initial screening for best carbon source, nitrogen source and yeast extract shall be done in varied sources, varied time incubation as well as in varied concentration to further support the result in CCD.

Plackett- Burman design

Twelve sets of experiments were conducted with individual controls. Maximum decolourisation of 95.36% was achieved from the factor combination of pH= 6.00, temperature= 27.00 (°C), ammonium acetate= 0.00 (%), glucose= 5.00 (%), salinity= 1.00 (%), yeast= 0.50 (%) and dye concentration= 50.00(ppm). The P- value for the model used was lower than 0.05 ($\alpha = 0.05$, or 95% confidence) (Ahmad and Mohammed, 2010) indicating that the model was statistically significant. Values greater than 0.1000 indicate the model terms are not significant thus, the insignificant model factor term (not counting those required to support hierarchy), could be removed to improve the model [12]. The coefficient of determination, r^2 of the model was 0.9308, which meant, the model could explain about 93% of the combined variables' interaction. From data analysis of variance (ANOVA) for Plackett- Burman design, significant variables which may affect the colour reduction of Biebrich scalet included the pH, ammonium acetate (%), yeast (%) and dye concentration (ppm) and were further optimised using CCD.

Central Composite design

Table 1: The assigned values of variables in CCD for four significant variables and the response by isolate 33.

Run	Ammonium acetate (%)	Dye concentration (ppm)	pH	Yeast (%)	Decolourisation (%)	
					Experimented	Predicted
1	0.00	50.00	8.00	0.50	97.856	95.49
2	1.00	50.00	8.00	0.50	92.839	95.02
3	0.00	250.00	8.00	0.10	63.196	67.87
4	0.00	250.00	6.00	0.10	60.635	60.63
5	0.00	50.00	6.00	0.10	88.194	90.77
6	1.00	250.00	8.00	0.50	95.070	93.87
7	0.50	150.00	7.00	0.30	96.696	96.65
8	0.50	150.00	7.00	0.30	96.696	96.65
9	0.50	150.00	7.00	0.30	96.696	96.65
10	1.00	250.00	8.00	0.10	79.687	78.21
11	0.50	150.00	7.00	0.30	96.696	96.65
12	1.00	250.00	6.00	0.10	60.470	64.21
13	0.50	50.00	7.00	0.30	94.765	95.49
14	1.00	50.00	6.00	0.10	92.965	91.07
15	1.00	250.00	6.00	0.50	68.510	72.81
16	0.00	250.00	6.00	0.50	78.333	76.76
17	0.50	150.00	5.00	0.30	81.131	79.16
18	0.50	150.00	7.00	0.70	95.845	94.40
19	0.50	150.00	7.00	0.30	96.696	96.65
20	0.00	50.00	8.00	0.10	85.864	83.73
21	1.00	50.00	6.00	0.50	91.550	88.25
22	0.00	250.00	8.00	0.50	86.986	91.05
23	0.00	150.00	7.00	0.30	96.89	94.22
24	0.00	50.00	6.00	0.50	91.83	95.48
25	0.50	350.00	7.00	0.30	56.36	52.19
26	1.00	50.00	8.00	0.10	87.836	90.79
27	0.50	150.00	7.00	0.30	96.696	96.65
28	0.50	150.00	7.00	0.00	85.755	82.03
29	1.50	150.00	7.00	0.30	98.203	97.33
30	0.50	150.00	9.00	0.30	94.756	93.18

The results from multiple regression analysis in Table 2 showed that dye concentration (ppm), pH and yeast (%) had a major influence on the percentage of decolourisation of Biebrich scarlet azo- dye which could be reflected from the P- values obtained (P-value < 0.05). multiple correlation coefficient from this experiment displayed r^2 of 0.9597 which is as close to 1 meaning that the predicted and experimented r^2 were in a good correlation. This enables the model to explain up to 95.97% variation of Biebrich scarlet decolourisation by isolate 33. The coefficient of variation (C.V) was 4.09 showing that the degree to which the data are clustered around the distribution mean was narrowed, giving a more precise statistical analysis of data. Upon subjected to regression analysis, a consequent equation representing Biebrich scarlet decolourisation was achieved, where A, B, C and D were the coded levels of ammonium acetate, dye concentration, pH and yeast:

$$\% \text{ Decolourisation} = 96.65 + 0.78\,A - 7.82\,B + 3.50\,C + 5.09\,D - 0.22\,A^2 - 7.20\,B^2 - 2.62\,C^2 - 3.11\,D^2 + 0.82\,AB + 1.69\,AC - 1.88\,AD + 3.57\,BC + 2.86\,BD + 1.76\,CD$$

From the equation, not only the sole model terms contributed to the percentage of Biebrich scarlet decolourisation but also, the combination effect of all the variables studied [24]. This could be shown from the presence of second- order effect (B^2, C^2, D^2) as well as two- level interaction effect of the variables (BC and BD) which is why, RSM is desirable in nowadays optimisation study since, it tells the interaction that occur in between the subjected variables.

Table 2: Regression analysis of Central Composite design data for Biebrich scarlet decolourisation by isolate 33. Observed r^2 was 0.9597, C.V 2 was 4.09.

Term	Coefficient	SE coefficient	Student's t	P value
Ammonium acetate	0.78	0.72	1.0833	0.3002
Dye concentration	-7.82	0.82	-9.5366	< 0.0001
pH	3.50	0.72	4.8611	0.0002
Yeast	5.09	0.77	6.6104	< 0.0001
Ammonium acetate x ammonium acetate	-0.22	0.67	-0.3284	0.7464
Dye concentration x dye concentration	-7.20	0.88	-8.1818	< 0.0001
pH x pH	-2.62	0.67	-3.9104	0.0014
Yeast x yeast	-3.11	0.80	-3.8875	0.0015
Ammonium acetate x dye concentration	0.82	0.89	0.9213	0.3693
Ammonium acetate x pH	1.69	0.89	1.8989	0.0763
Ammonium acetate x yeast	-1.88	0.89	-2.1124	0.0511
Dye concentration x pH	3.57	0.89	4.0112	0.0011
Dye concentration x yeast	2.86	0.89	3.2135	0.0057
pH x yeast	1.76	0.89	1.9775	0.0656

The interaction effects and the optimum variable conditions are shown in the response surface contour plots with respect to simultaneous change in two variables as in **Figs. 6a until 6f**.

Effect of variables

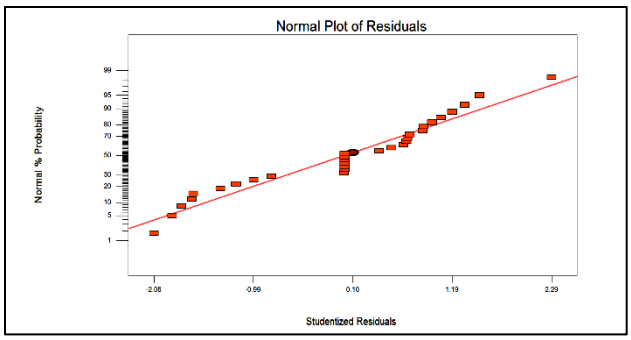

Fig. 5. Normal probability plot of residuals shows a close distribution of residuals. The residuals distributed are approximate to linear.

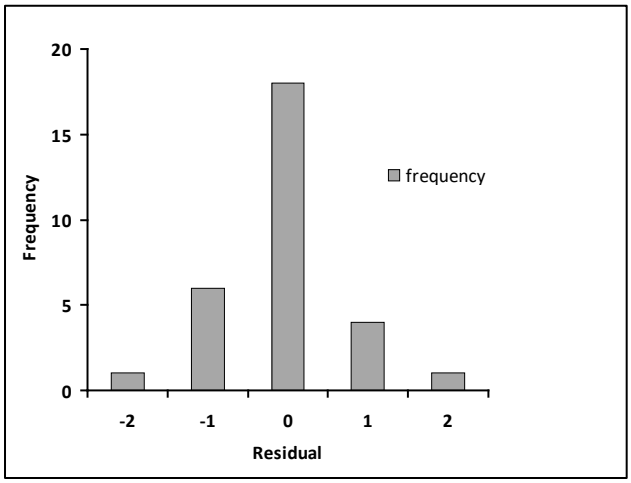

Fig. 6. Histogram of the residuals shows the distribution of the residuals for all 30 runs or observations. The histogram shows an almost symmetrical histogram.

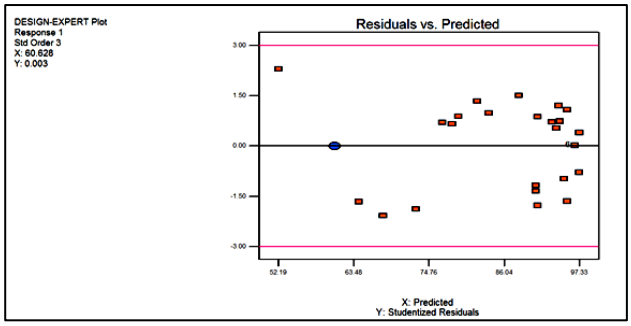

Fig. 7. The plot of residuals versus the predicted response for decolourisation percentage shows that the residuals are scattered randomly about zero.

Effect of dye concentration and ammonium acetate

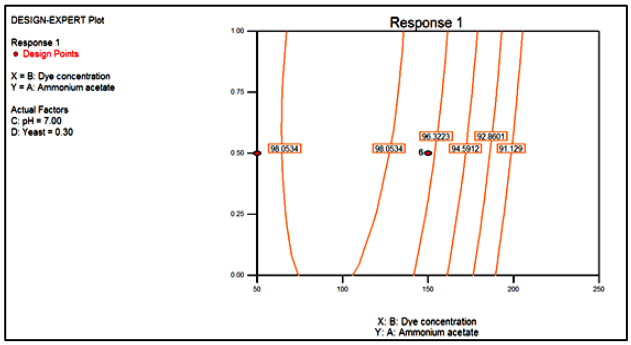

Fig. 8. The contour plot shows effect of dye concentration and ammonium acetate on the decolourisation of Biebrich scarlet by isolate 33. Yeast and pH are kept at a constant value. Response 1 was the decolourisation percentage.

Effect of pH and ammonium acetate

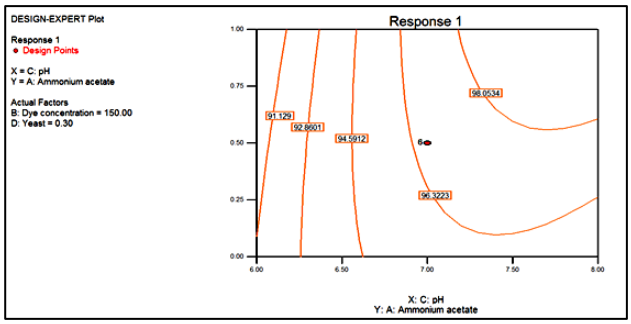

Fig. 9. The contour plot shows effect of pH and ammonium acetate on the decolourisation of Biebrich scarlet by isolate 33. Dye concentration and yeast are kept at a constant value. Response 1 was the decolourisation percentage.

Effect of yeast and ammonium acetate

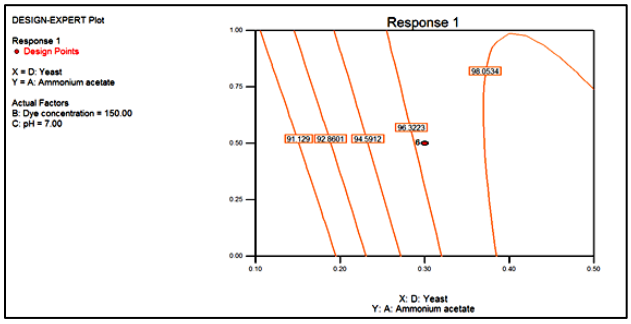

Fig. 10. The contour plot shows effect of yeast and ammonium acetate on the decolourisation of Biebrich scarlet by isolate 33. Dye concentration and pH are kept at a constant value. Response 1 was the decolourisation percentage**.**

Effect of dye concentration and pH

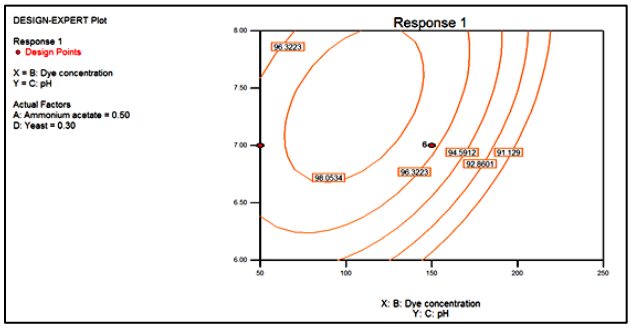

Fig. 11. The contour plot shows effect of dye concentration and pH on the decolourisation of Biebrich scarlet by isolate 33. Ammonium acetate and yeast are kept at a constant value. Response 1 was the decolourisation percentage.

Effect of dye concentration and yeast

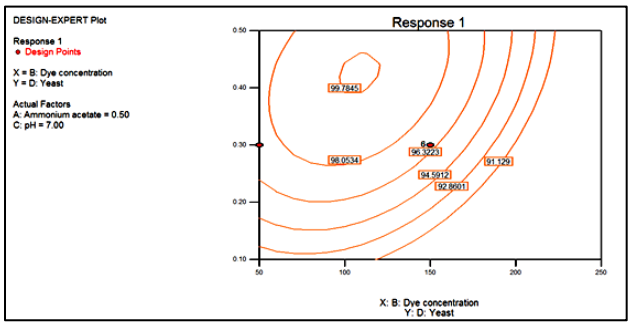

Fig. 12. The contour plot shows effect of dye concentration and yeast on the decolourisation of Biebrich scarlet by isolate 33. Ammonium acetate and pH are kept at a constant value. Response 1 was the decolourisation percentage.

Effect of yeast and pH

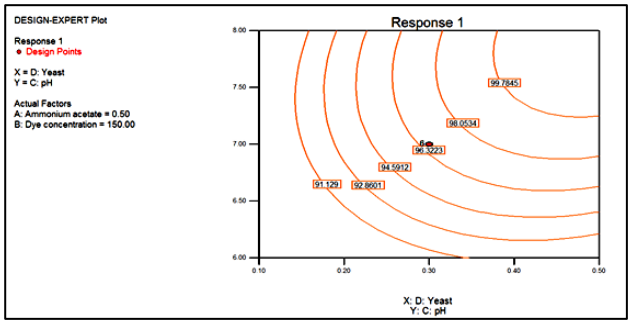

Fig. 13. The contour plot shows effect of yeast and pH on the decolourisation of Biebrich scarlet by isolate 33. Ammonium acetate and dye concentration are kept at a constant value. Response 1 was the decolourisation percentage.

Fig. 5 showed that the residuals are approximately linear implying that the errors were distributed normally. This normal distribution of errors could be supported by the histogram of residuals (**Fig. 6**) whereby the errors were normally distributed within zero mean. Figure 1c displays scattered residuals about zero. This means that the errors had a constant variance [25].

Figs. 8 to **13** show two- dimensional contour plots of percentage decolourisation of Biebrich scarlet by isolate 33. Each contour plots are of pair factors value, whilst keeping the other two factors at fixed value. Response surface method is applied to enable the lookout for the optimum condition which promotes a maximum response as in this case, the decolourisation percentage.

Fig. 8 exhibits the interaction between dye concentration and ammonium acetate and their combination effect on the decolourisation of Biebrich scarlet by isolate 33. From the contour plot, we could understand that, further increase in dye concentration above 100 ppm has negative effect on the azo- dye decolourisation percentage, while ammonium acetate has only a slight effect in promoting the decolourisation. **Fig. 9** illustrates the interaction between ammonium acetate and pH and their combination effect upon the decolourisation of Biebrich scarlet by isolate 33 by which we could deduce that increase in pH results in a higher decolourisation percentage. However, ammonium acetate showed a slight effect in the response.
On the other hand, **Fig. 10** exhibits the interactive effect between ammonium acetate and yeast on azo- dye decolourisation. From the contour plot, we could infer that the dye decolourisation increased as the yeast amount (%) and ammonium acetate (%) were increased. **Fig. 11** depicts the interaction between dye concentration and pH level. Based from this contour plot, we can say that dye concentration of higher than 100 ppm has a detrimental effect on the dye response. Then again, dye decolourisation increased as the pH was increased from 6 to around 7.3, but further increase caused reduction in dye response. This way of interaction also has been reported from which the study carried out by [26] showed that a maximum decolourisation percentage was achieved at pH of 6.9 and at a higher dye concentration of 524 ppm. Here, both of our results will show a decrease in dye decolourisation if there was any further increase or decrease in both of the mentioned variables.

Fig. 12 describes the interactive effect of dye concentration and yeast onto the percentage of dye decolourisation. Decolourisation was favoured when the dye concentration was increased up to 100 ppm and further increase of the latter caused a decrease in dye decolourisation. Nevertheless, an increase in yeast amount encouraged the dye decolourisation. **Fig. 13** tells us the effect of yeast and pH upon the decolourisation of Biebrich scarlet by isolate 33. From the contour plot, we could assume that increase in yeast amount and pH level in the system, promotes a higher percentage of decolourisation.

Optimisation and verification of model.

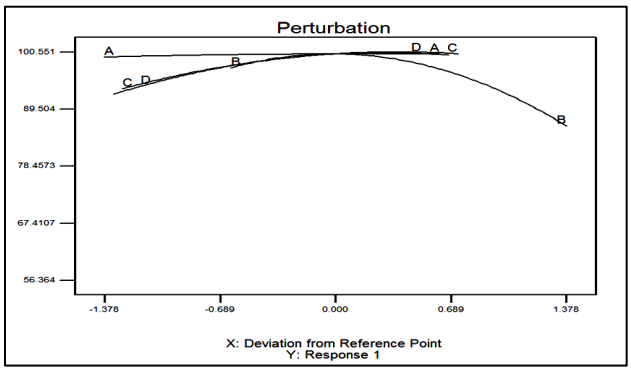

Fig. 14. Shows the perturbation graph. Letter A, B, C and D represent ammonium acetate, dye concentration, pH and yeast respectively.

Perturbation graph was used to find the range of factors which were significantly affecting the dye decolourisation. From this figure, the deviation from the reference point should be closer to 0.00 to indicate the range combinations of factors that could maximise the dye decolourisation are about the efficient level. A narrower deviation was preferred in order to exhibit a precise response. Using perturbation graph (**Fig. 14**), the optimum ranges for maximum dye decolourisation are ammonium acetate (%) of 0.31- 0.60; dye decolourisation (ppm) of about 112.16 or less; pH of 6.62- 7.38 and yeast (%) of 0.23- 0.37 and these values could be used in a repeat test, only that, the result may not be as precise, but about similar.

CONCLUSION

The isolated bacteria capable of reducing azo- dye Biebrich scarlet was an unknown isolate named as isolate 33. The isolate was a gram-negative bacterium capable of producing catalase and cytochrome oxidase enzymes. This isolate gave a gamma hemolysis on blood agar medium and it decolourises Biebrich scarlet better in a minimal oxygen condition (anoxic condition). Optimisation of medium for maximising the dye decolourisation happens in stages for which in this study, three levels were employed which were; the initial important sources, the Plackett-Burman screening and lastly the Central Composite design. The significant factors (P- value < 0.05) affecting Biebrich scarlet decolourisation from these optimisation process are the dye concentration, yeast amount and pH level. From the experiment conducted, the optimum ranges for maximum dye decolourisation shown via the perturbation graph were ammonium acetate (%) of 0.31- 0.60; dye decolourisation (ppm) of about 112.16 or less; pH of 6.62- 7.38 and yeast (%) of 0.23- 0.37

REFERENCES

1. Asad S, Amoozegar MA, Pourbabaee AA, Sarbolouki MN, Dastgheib SMM. Decolorization of textile azo dyes by newly isolated halophilic and halotolerant bacteria. Bioresour Technol. 2007;98(11):2082–8.
2. Pandey A, Singh P, Iyengar L. Bacterial decolorization and degradation of azo dyes. Int Biodeterior Biodegrad. 2007;59(2):73–84.
3. Gong R, Ding Y, Li M, Yang C, Liu H, Sun Y. Utilization of powdered peanut hull as biosorbent for removal of anionic dyes from aqueous solution. Dyes Pigments. 2005;64(3):187–92.
4. John A. Microbial degradation of azo dyes. Biotransformations Microb Degrad Health Risk Compd. 1995;157–76.
5. Sahasrabudhe MM, Saratale RG, Saratale GD, Pathade GR. Decolorization and detoxification of sulfonated toxic diazo dye C.I. Direct Red 81 by *Enterococcus faecalis* YZ 66. J Environ Health Sci Eng [Internet]. 2014 [cited 2019 Dec 16];12. Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4299565/
6. Robinson T, McMullan G, Marchant R, Nigam P. Remediation of dyes in textile effluent: a critical review on current treatment technologies with a proposed alternative. Bioresour Technol. 2001;77(3):247–55.
7. Gondaliya SB, Pathak SJ. Kinetic of azo dyes decolourization by *Enterobacteriaceae* species in the intact cell assay system. J Appl Sci Environ Manag [Internet]. 2007 [cited 2019 Dec 16];11(3). Available from: https://www.ajol.info/index.php/jasem/article/view/55079
8. Guo J, Zhou J, Wang D, Tian C, Wang P, Salah Uddin M, et al. Biocalalyst effects of immobilized anthraquinone on the anaerobic reduction of azo dyes by the salt-tolerant bacteria. Water Res. 2007;41(2):426–32.
9. Syed M, Sim H, Khalid A, Shukor M. A simple method to screen for azo-dye-degrading bacteria. J Env Biol. 2009;30(1):89–92.
10. Chengalroyen MD, Dabbs ER. The microbial degradation of azo dyes: minireview. World J Microbiol Biotechnol. 2013;29(3):389–99.
11. Sakkas VA, Islam MdA, Stalikas C, Albanis TA. Photocatalytic degradation using design of experiments: A review and example of the Congo red degradation. J Hazard Mater. 2010;175(1):33–44.
12. Rodrigues RCLB, Kenealy WR, Dietrich D, Jeffries TW. Response surface methodology (RSM) to evaluate moisture effects on corn stover in recovering xylose by DEO hydrolysis. Bioresour Technol. 2012;108:134–9.
13. Tanaka T, Kawasaki K, Daimon S, Kitagawa W, Yamamoto K, Tamaki H, et al. A hidden pitfall in the preparation of agar media undermines microorganism cultivability. Appl Environ Microbiol. 2014;80(24):7659–66.
14. Khadijah O, Lee K, Abdullah M. Isolation, screening and development of local bacterial consortia with azo dyes decolourising capability. Malays J Microbiol. 2009;5(1):25–32.
15. Singh AD, Sabaratnam V, Abdullah N. Decolourisation of chemically different dyes by enzymes from spent compost of Pleurotus sajor-caju and their kinetics. Afr J Biotechnol. 2010;9(1):41–54.
16. Chen K-C, Wu J-Y, Liou D-J, Hwang S-CJ. Decolorization of the textile dyes by newly isolated bacterial strains. J Biotechnol. 2003;101(1):57–68.
17. Bhatt N, Patel K, Keharia H, Madamwar D. Decolorization of diazo-dye Reactive Blue 172 by *Pseudomonas aeruginosa* NBAR12. J Basic Mircobiol. 2005;45(6):407–18.
18. Doruk AY, Cihangir N. Decolorization of Reactive Black 5 by *Yarrowia lipolytica*. Am J Microbiol Res. 2013;20(1):16–20.
19. Bayoumi R, Musa S, Bahobil A. Biodecolorization and biodegradation of azo dyes by some bacterial isolates. J Appl Env Bio Loc Ical Sci. 2010;1(1):1–25.
20. Rajee O, Patterson J. Decolorization of Azo Dye (Orange MR) by an *Autochthonous bacterium*, *Micrococcus* sp. DBS 2. Indian J Microbiol. 2011;51(2):159–63.
21. Kumar C, Mongolla P, Basha A, Joseph J, Sarma V, Kamal A. Decolorization and biotransformation of triphenylmethane dye, methyl violet, by *Aspergillus* sp. isolated from Ladakh, India. J Microbiol Biotechnol. 2011;21(3):267–73.
22. Ramalho P, Scholze H, Cardoso M, Ramalho M, Oliveira-Campos A. Improved conditions for the aerobic reductive decolourisation of

azo dyes by *Candida zeylanoides*. Enzyme Microb Technol. 2002;31(6):848–54.
23. Ter Schure E, Van Riel N, Verrips C. The role of ammonia metabolism in nitrogen catabolite repression in *Saccharomyces cerevisiae*. FEMS Microbiol Rev. 2000;24(1):67–83.
24. Noordin M, Venkatesh V, Sharif S, Elting S, Abdullah A. Application of response surface methodology in describing the performance of coated carbide tools when turning AISI 1045 steel. J Mater Process Technol. 2004;145(1):46–58.
25. Hasan S, Srivastava P, Talat M. Biosorption of Pb(II) from water using biomass of *Aeromonas hydrophila*: Central composite design for optimization of process variables. J Hazard Mater. 2009;168(3):1155–62.
26. Tripathi A. Biodegradation of orange G by a novel isolated bacterial strain *Bacillus megaterium* ITBHU01 using response surface methodology. Afr J Biotechnol. 2012;11(7):1768–81.