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Research Article

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Application of a mixture design to optimize textile azo-dye decolorization using a bacterial consortium

Abstract

Textile wastewaters (TWWs) are characterized by high salinity and alkaline pH. Bioremediation using fungi were proved in many cases as inefficient tools to treat such effluent, giving the relay to haloalkaliphilic bacteria. Here, three extremophilic strains namely *Halomonas desertis* G11, *Kocuria rosea* BU22S and *Microbaterium trichothecenolyticum* TL13 have been selected to conduct textile dye decolorization experiments. The effect of different combinations of these strains was studied by a mixture design (MD) to assess Tubantin Brown GGL (TB GGL) color removal during species growth under optimzed conditions of dye concentration (100 mg/L), pH (9), salinity (5%), inoculum size (5%) and time (10 days). A remarkable decolorization was observed using mono and mixed cultures. Using the NemrodW software, the optimisation calculations were performed to find an optimum mixture proportions for maximum azo dye decolorization. High regression coefficients R2, between the variables and the response indicated excellent evaluation of experimental data by the polynomial regression model. The highest color removal (about 92%) was obtained with binary mixture composed by *H. desertis* G11and *M. trichothecenolyticum* TL13 and it was in close agreement with the estimated response value (93%). This finding shows a biotechnological potential of haloalkaliphilic bacteria in TWWs treatment.

Keywords: Azo dye decolorization, mixture design, consortium, haloalkaliphilic bacteria, textile wastewater

Volume 8 Issue 2 - 2019

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Received: March 12, 2019 | **Published:** April 10, 2019

Introduction

In recent years, the release of textile wastewaters (TWWs), without adequate treatment, into natural ecosystem has become a great threat and one of the main sources of environmental pollution.[1] Apart the high amounts, TWWs have a complex and specific nature.[2] TWWs are highly charged by synthetic dyes. One of the most frequently synthetic dyes used in dyeing units, is azo dyes.[3] More than 10,000 dyes are available commercially and more than 7×10^5 tons of dyestuffs are produced annually.[4] Azo dyes can be grouped as mono-, di-, or tri-azo, according to the number of azo bonds (-N=N-) in their structure.[5] There are about 15% of dyes are lost due to inefficiency dyeing process in wastewater.[6] The release of these dyes in large quantities into surrounding environment have serious concerns in aquatic life and public health especially as they are considered as recalcitrant compounds,[7] stable towards light and temperature and resist to microbial attack.[8,9] Indeed, removal of these toxic compounds remained mandatory. So, different physical and chemical methods have been employed but their continuous application put forth their limitations as they require high energy, time and cost, and generate large amount of sludge and toxic by-products.[10] Bioremediation has been considered as a good alternative process to deal this threat. Different azo dyes decolorizing microorganisms have been reported, including bacteria,[11,12] fungi[13,14] and yeast.[15,16] Since different textile processing steps require an excessive use of salt and sodium hydroxide, TWWs are characterized by alkaline pH and high salinity.[1] Considering the specific nature of TWWs, fungal treatment remained inadequate in TWWs treatment as that are functional at low pH.[17] Researchers have been focused on bacteria dye removal as that it is more adequate to support TWWs characteristics; particularly the focus has been on haloalkaliphilic bacteria. Bioremediation by bacteria can be carried out using two approaches either mono or mixed culture to exploit decolorization potency of each strain and also cooperative interactions between them.[18,19]

In recent years, experimental designs such as mixture designs (MD) have attracted much attention in studies of dye remediation using microbial consortia. MD was used to compare the decolorization percentage of textile dye by single or mixed strains and to examine the interaction among the mixed cultures.[20] With this in view, the current study describes the potential of three haloalkaliphilic bacteria, namely *H. desertis* G11, *M. trichothecenolyticum* TL13 and *K. rosea* BU22S on the decolorization of textile azodye Tubantin Brown GGL (TB GGL). The formulation of mixed cultures for textile azo dye decolorization was optimized using a MD.

Methods

Dye, media and chemicals

TB GGL used in this study was supplied from a textile factory in Nabeul, Tunisia and was of commercial quality. Stock dye solution was prepared at concentration of 10.000 mg/L (w/v) autoclaved at 121°C for 15 min and stored at 4°C. Maximum absorbance λ max of dye was determined in diluted dye aqueous solutions by using a scanning UV-Vis spectrophotometer. Nutrient Broth (NB) and nutrient agar were used in all the experiments. The composition of NB was: peptone, 5 gL^{-1} and beef extract, 3 gL^{-1}. All chemicals used in this study were of the highest purity available and of analytical grade.

Bacterial strains and culture conditions decolorization experiments

Bacterial strains used in this study were provided from the

collection of Laboratory of Biotechnology and Valorization of Bio-Geo-Resources (BVBGR) (LR11ES31, University of Manouba). These plant growth-promoting, bioremediating strains were previously isolated from extreme environmental sites, and taxonomically identified by 16S rRNA gene sequence analysis and stored in cryovials with 20% (v/v) glycerol solution at -80°C until use.[21,22] The strains were reactivated on Nutrient Agar plates at 30°C for 48 hr. An antagonism test was carrying out to prove the synergism between strains to facilitate the development of consortium.[23] The acclimatization was performed by gradually exposing each strain to higher concentrations of TB GGL.[24] Through preliminary studies and a literature review, optimal conditions were fixed at 30°C, pH 9, salinity 5%, inoculum size 5% and dye concentration 100 mg/L under static conditions. For dye decolorization experiment, flasks (125 ml) containing 50 ml of sterilized NB amended with the appropriate dye in order to obtain the final desired concentration, were inoculated from fresh liquid culture of each strain (O.D. 600 nm ≈ 0.6) and incubated at 30 °C under static conditions. Abiotic control was maintained without inoculation. After 10 days of incubation, the culture was centrifuged at 10000 rpm during 15 min at 4°C to separate the bacterial cell mass. The decolorization was recorded by measuring the decrease in maximum absorbance of the dye using UV-Vis spectrophotometer (Shimadzu UV-1800 PC model Kyoto, Japan). Dye decolorization (%) was calculated in the maximum absorbance of TB GGL (λ max = 430 nm) according to the formula given by Chen at al.,[25] as follows:

Decolorization (%) = [(initial absorbance of TB - observed absorbance) / initial absorbance] × 100

Optimization of decolorization by bacterial consortium using a mixture design

MD is a statistical technique used to study the formulation of experiments such as food, pesticides, chemicals, fertilizer and other products. It can estimate the relationship between formulation and performance through the regression analysis in reducer experimental time.[26,27] The application of MD techniques in dye removal processes could result in improved decolorization, reduced process variability, closer confirmation of the output response to nominal and targeted requirements, as well as lesser development time and overall costs.[18] Generally, the MD is used to model the relationship between the proportion of different variables and responses and also to optimize the consortium formula through the regression analysis.[20,28] In the case of a mixture containing three strains, the factorial space constituted by all the possible fractions of the strains is a triangle whose vertices correspond to pure strains. In the present research, *H. desertis* G11, *M. trichotecenolyticum* TL13 and *K. rosea* BU22S were selected to be used as monoculture, binary and ternary mixtures, in varying proportions ranging from 0 to 100%, as shown on Table 1. Decolorization experiments were conducted according to the ratio given by the experimental design, and 5% of the formulated consortium was inoculated into the nutrient broth containing NaCl 5 % and dye 100 mg/L at pH 9, 30°C for 24 h under static conditions. Each experiment was replicated twice in order to estimate the variance of the experimental error. Following the experimentation, the MD data were used to fit the empirical model and to test its adequacy. This latter was used to plot the contours of the predicted responses and to determine the optimal settings of the component proportions.

The regression model equation of decolorization% was as follows:

$$Y = b_1S_1 + b_2S_2 + b_3S_3 + b_{12}S_1S_2 + b_{13}S_1S_3 + b_{23}S_2S_3$$

Where, Y is the observed response**;** S_1: *H. desertis* G11; S_2: *M. trichothecenolyticum*; S_3: *K. rosea***;** b_1, b_2 and b_3 are the linear coefficients and b_{12}, b_{13} and b_{23} are the interaction coefficients (Table 1).

Table 1 Mixture design matrix, experimental conditions and the corresponding experimental and theoretical responses

N	S1 (%)	S2 (%)	S3 (%)	Measured TB GGL decolorization	Estimated TB GGL decolorization
				(%)	(%)
1	100	0	0	70	68.12
2	100	0	0	67	68.12
3	0	100	0	61	58.62
4	0	100	0	57	58.62
5	0	0	100	76	77.12
6	0	0	100	79	77.12
7	50	50	0	85	93
8	50	50	0	98	93
9	50	0	50	58	60.5
10	50	0	50	60	60.5
11	0	50	50	50	53
12	0	50	50	53	53
13	33.33	33.33	33.33	75	69.12
14	33.33	33.33	33.33	70	69.12

Statistical analysis

The NemrodW software[29] was used to build the experimental design and to conduct all data calculations and processing. The regression model was evaluated by analyzing the values of the regression coefficients, analysis of variance (ANOVA), the *P*-value and the *F*-value. The Student's t-test was applied to determine the significance of each variable.

UV-Vis analysis of products generated after Tubantin Brown GGL treatment

Decolorization percentage of TB GGL after treatment by binary mixture (*H. desertis* G11 and *M. trichothecenolyticum* TL13) was analyzed by UV-Vis spectrophotometer (Shimadzu UV-1800 PC model Kyoto, Japan) by scanning centrifuged cell-free supernatant samples in the range 200–800 nm. The supernatant used was obtained after centrifugation 10000 rpm for 15 min at 4°C.

Results

Model establishment

MD is a design of experiments tool used to determine the optimum combination of constituents that deliver a desired response using a minimum number of runs. In this study, a MD was carried out to evaluate the relationships between the proportion of haloalkaliphilic bacteria namely, *H. desertis* G11, *K. rosea* BU22S and *M. trichothecenolyticum* TL13 and the TB GGL decolorization yield under saline and alkaline conditions (pH 9 and 5% NaCl). 14 experiments were carried out according to the experimental conditions indicated on Table 1. The model coefficients were determined using the last square method and the predicted responses were calculated by Nemrod W software.

The regression model equation was as follows:

$$Y_{(TB\ GGL\ decolorization,\ \%)} = 68.125\ S_1 + 58.625\ S_2 + 77.125\ S_3 + 118.508\ S_1S_2 - 48.492\ S_1S_3 - 59.492\ S_2S_3$$

Where: S_1: *H. desertis* G11; S_2: *M. Trichothecenolyticum* TL13 and S_3: *K. rosea* BU22S

Statistical analysis of the models

The analysis of variance of the developed regression model demonstrated high significance ($P<0.001$) of the model and an insignificant lack of fit (Table 2), indicating that most of the variability in the responses could be explained by the model equation ($R^2 = 0.932$ and $R^2_{adjust} = 0.889$).

The significance of MD model coefficients was determined by Student's *t*-test which illustrates the interaction pattern between the three strains S1, S2 and S3 on TB GGL decolorization. The *t*-values and *p*-values for the linear and interactive model terms were illustrated in Table 3. The larger the magnitude of *t*-value and smaller the *p*-value indicate the high significance of the corresponding model coefficient.[21,28] It can be seen from Table 3 that the linear effect of the parameters S1, S2 and S3 and the bacterial strain interactions S1S2, S1S3 and S2S3 were statistically significant. Furthermore, the variables with the largest significant effect ($p \leq 0.001$) were the linear term of S1, S2 and S3, as well as the squared term of S1S2. It was found from the statistical analysis that the selected haloalkaliphilic bacteria have pronounced effects on TB GGL decolorization, either individually or in consortia (Table 3).

Table 2 Analysis of variance (ANOVA) for Tubantin Brown GGL decolorization by linear, binary and ternary mixtures

Source of variation	Sum of squares	Degrees of freedom	Mean square	Ratio	Significance
Regression	2153.78	5	430.76	21.8492	***
Residuals	157.72	8	19.71		
Validity	37.22	1	37.22	2.16	18.3% (NS)
Error	120.5	7	17.21		
Total	2311.5	13			

***Significant at the level 99.9% ($p \leq 0.001$); NS: non-significant

Table 3 Results of regression analysis of the mixture design

Predictor	Coefficient	F.Inflation	Standard deviation	t-value	Signification
S1	b1 : 68.125	1.6	3.128	21.78	***
S2	b2 : 58.625	1.6	3.128	18.74	***
S3	b3: 77.125	1.6	3.128	24.66	***
S1S2	b12: 118.508	1.57	14.378	8.24	***
S1S3	b13: -48.492	1.57	14.378	-3.37	**
S2S3	b23: -59.492	1.57	14.378	-4.14	**

***Significant at the level 99.9% ($p \leq 0.001$); **Significant at the level 99.0% ($p \leq 0.01$)

Citation: Sghaier I, Ouertani R, Mahjoubi M, et al. Application of a mixture design to optimize textile azo-dye decolorization using a bacterial consortium. *Biom Biostat Int J.* 2019;8(2):58–63. DOI: 10.15406/bbij.2019.08.00272

Graphical analysis of the model

Figure 1 shows the effect of the interaction of *H. desertis* G11, *M. trichothecenolyticum* TL13 and *K. rosea* BU22S on TB GGL decolorization. The mixture surface and contour plots, presented by three and two dimensional graphs using color removal based on the simultaneous variation of strains composition from 0 to 100% for each strain. The mixture surface plot also described the individual and cumulative effect of these three variables (strains S_1, S_2 and S_3) and tested their subsequent effect on the response (color removal) (Figure 1).

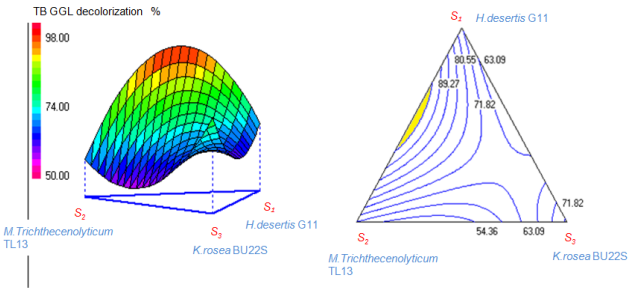

Figure 1 Three-dimensional response surface and mixture contour plots for the effect of the variables (H. desertis G11, M. trichothecenolyticum TL13 and K. rosea BU22S) on decolorization removal of Tubantin Brown GGL textile azo dye.

Validation of the model

In order to validate the model, experiments were carried out under optimal operating conditions (dye concentration 100 mg/L, pH 9, salinity 5%, inoculum size 5%, time 10 days) generated by NemrodW software. Figure 2 presents some of the textile dye decolorization experiments performed under saline and alkaline conditions using mono and mixed cultures. The highest decolorization (about 92%) was obtained with binary mixture (*H. desertis* G11 and *M. trichothecenolyticum* TL13) and it was in close agreement with the estimated response value (93%) (Figure 2).

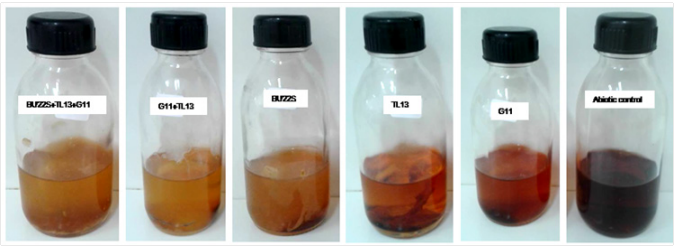

Figure 2 Tubantin Brown GGL decolorization by monoculture, binary and ternary mixtures grown on nutrient broth, 5% NaCl, pH 9, 100 mg/L of dye and inoculated by an inoculum of 5%, at 30°C under static conditions and 10 days of incubation.

UV-Vis spectral analysis of formulated consortium

Figure 3 shows the variation of TB GGL (100 mg/L) in UV-visible. Major peak was observed in UV region near 430 nm, which corresponds to the maximal absorbance of TB GGL. The major peak was disappeared after bacterial treatment, indicating the color removal.[30] The great change occurring in UV-Vis spectra demonstrates that the structure of TB GGL changed evidently after bacterial treatment. The brown color of TB GGL was related to the conjugated structure of azo bonds (chromophore) and amino group. It could be presumed that the azo bonds cleaved during the process. These results indicate that the decolorization process of TB GGL by *H. desertis* G11 is a biodegradation, and also supported the conclusion that decolorization by bacteria is due to biodegradation, rather than inactive surface adsorption.[31]

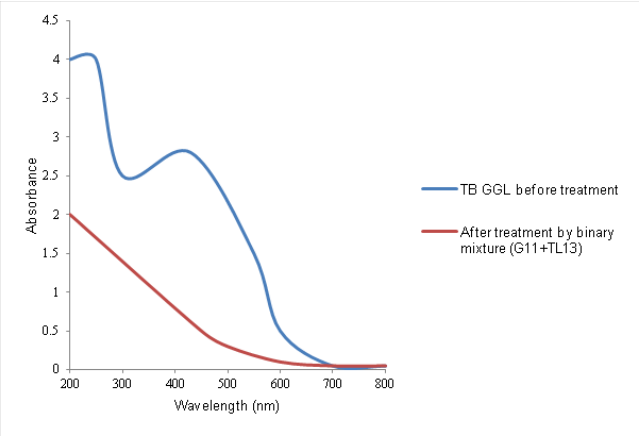

Figure 3 UV-Vis spectrum of Tubantin Brown GGL before and after treatment by formulated consortium.

Discussion and conclusion

Three haloalkaliphilic bacteria, namely *H. desertis* G11, *K. rosea* BU22S and *M. trichothecenolyticum* TL13, were selected for the optimization study based on their high ability to decolorize TB GGL textile azo-dye under alkaline and saline conditions (pH 9 and 5% NaCl). Among these three strains, only *K. rosea* had been previously reported to be useful in bioremediation of textile azo dyes,[32] while no studies were focused on the decolorization potentialities of *H. desertis* G11 and *M. trichothecenolyticum* TL13. These results suggested that these haloalkaliphilic bacteria may have an interesting potential for treating TWWs under harsh industrial conditions. A MD has been conducted in order to examine the interaction between the three strains during the TB GGL decolorization process and to get an optimal combination that had highest decolorization percentage. The NemrodW software was used to select the design region and analyze the experimental data. The MD data were modelled with a polynomial equation to explain the variation of decolorization percentages of TB GGL dye with the change of ratios of *H. desertis* G11, *K. rosea* BU22S and *M. trichothecenolyticum* TL13.

The obtained special model has correlation coefficients (R^2) for textile dye decolorization near to 1 and were highly significant (P<0.001), indicating that the model predicted values coincided with the experimental values. The 3D surface and contour plots were generated to study the interactions among the three strains and to visualize the combined effect of strains on dye decolorization percentages (Figure 1). The response surface plots showed the decolorization percentages by the strains in different ratios (from 0 to 100%). *H. desertis* G11 and *K. rosea* BU22S were the most potent candidates on TB GGL decolorization based on MD experiments. For binary mixtures, the co-culture (G11 and TL13 strains) has a significant increase on TB GGL decolorization by about 40%. The enhancement of decolorization (%) in the case of binary mixtures could be attributed to the cooperative interactions and the co-metabolic activity of individual strains. Similar decolorizing behaviors have been reported by other researchers.[19,20]

The optimal decolorization zone was highlighted in yellow in the mixture contour plot of Figure 1. Under 50:50% strain ratio, the decolorization percentages were significantly increased compared to those obtained by each strain individually. As reported in this study, comparative decolorization studies between mono and mixed culture prove the significant efficiency of consortia towards pure cultures (Table 4).

Table 4 Comparative decolorization potency between mono and mixed culture

Strains	Dye/concentration	Class	Monoculture	Mixed culture	Reference
Escherichia coli *Enterobacter dissolvens* *Pseudomonas aeruginosa* *Klebsiella oxytoca*	Congo Red/100 ppm	Diazo	97%, 14 days ND 96%, 30 days 92%, 14 days	~94%, 7 days	[33]
Pseudomonas desmolyticum NCIM 2112 *Kocuriarosea* MTCC 1532 *Micrococcusglutamicus*	Acid Blue 15 and Methylene Blue/100 ppm	Triphenylmethane	40%, 96 h	~86%, 96 h	[18]
Acinetobacter baumannii *Corynebacterium sp. Cytophaga columnaris* *Escherichia coli* *Pseudomonas fluorescence* *Pseudomonas luteola*	Methylene Blue/50 ppm	Triphenylmethane	90%,95 h	96%,60 h	[24]
Neisseria sp. *Vibrio* sp. *Bacillus* sp. *Bacillus* sp. *Aeromonas*sp.	Novacron Brilliant Blue/100 ppm	Azo	ND	65%, 6 days	[19]
Halomonas desertis G11 *Microbacterium trichothecenolyticum* TL13 *Kocuriarosea* BU22S	Tubantin Brown GGL/100ppm	Azo	68% 60% 77%, 10 days	92% (binary mixture) 73% (ternary mixture),10 days	Present study

D: No Decolorization

In conclusion, a MD has been successfully applied to decolorize a recalcitrant textile azo dye using an alkali-halotolerant microbial consortium, and to determine optimal mixture compositions that lead to maximum decolorization (Table 3). The interaction effects between the selected bacteria have been evaluated through the establishment of the MD regression model, ANOVA and the response contour and surface plots. The binary *H. desertis* - *M. trichothecenolyticum* mixture culture as well as *H. desertis* G11 monoculture showed great potential as microbial catalysts in view of its high decolorizing ability against textile azo dye. Further biochemical and genomic studies are required to elucidate more details about textile dye degradation by *H. desertis* G11 and *K. rosea* BU22S and their stability against harsh conditions such as high salinity and alkalinity. A pilot-scale decolorization study with textile wastewater will be conducted with this valuable biocatalytic process for a real industrial application.

Author contributions

I.S, W.H, As. Ch, M.N. conceived and performed decolorization experiments; R.O, M.M. and D.E performed bacterial identification and characterization, M.N. and H.C. designed the experiments and interpreted the experimental design software data; H.C, A.J, A.C. and M.N. analyzed and evaluated the results and contributed to paper writing & editing.

Acknowledgments & funding details

The authors acknowledge financial support from the PhosAgro/UNESCO/IUPAC partnership in the ambit of the Green Chemistry for Life grant programme (project N° 45003552865) and the Tunisian Ministry of Higher Education and Scientific Research in the ambit of the laboratory project LR11ES31.

Conflicts of interest

Author declares that there are no conflicts of interest.

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