Pol. J. Environ. Stud. Vol. 22, No. 5 (2013), 1297-1306

Original Research

Decolorization of Crystal Violet by Mono and Mixed Bacterial Culture Techniques Using Optimized Culture Conditions

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Received: 4 February 2013
Accepted: 12 May 2013

Abstract

Acinetobacter baumannii, *Corynebacterium* sp., *Cytophaga columnaris*, *Escherichia coli*, *Pseudomonas fluorescens*, and *P. luteola* bacteria isolated from the sewage disposal lake in Jeddah, Saudi Arabia, can decolorize crystal violet (CV). *P. fluorescens* was the most potent CV decolorizer, and *Corynebacterium* sp. was also able to perform this function. Five different media were tested to determine which medium formulation favoured CV decolorization by *P. fluorescens* and *Corynebacterium* sp. The basal medium favoured the highest decolorization percentage of 50 µg CV/ml after 72 h of incubation. *P. fluorescens* was sufficient to decolorize concentrations of CV up to 150 µg/ml after 92 h of incubation. A mixed bacterial culture of *P. fluorescens* and *Corynebacterium* sp. more fully decolorized CV than did a single; the decolorization period for the mixed culture was reduced by more than 37% and the decolorization rate (µg/h) increased by up to 59%. Two-phase multifactorial optimization statistical analysis (Plackett-Burman and Box-Behnken) were carried out to optimize culture conditions in order to increase the ability of a mixed culture to decolorize 150 µg CV/ml. Under the optimized conditions the decolorization period was reduced by more than 22% and the decolorization rate was increased by more than 48%.

Crystal violet can be efficiently decolorized by *P. fluorescens* and *Corynebacterium* sp. The decolorization process is markedly influenced by the composition of the cultivation medium and the concentration of CV. A mixed culture of *P. fluorescens* and *Corynebacterium* sp. was much more efficient at decolorizing CV than was a monoculture. The culture conditions were considerably optimized using Plackett-Burman and Box-Behnken statistical experimental designs.

Keywords: crystal violet, *P. fluorescens* and *Corynebacterium* sp., mixed culture, statistical optimization

Introduction

Of the many classes of synthetic dyes used in the textile and dyeing industries, triphenylmethane dyes are the most versatile and play a predominant role in various industrial applications [1]. The triphenylmethane dye crystal violet has been used extensively in human and veterinary medicine as a biological stain and as a textile dye in the textile processing industry. Crystal violet has been classified as a recalcitrant dye and remains in the environment for long periods of time. It is toxic to aquatic and terrestrial life [1, 2]. Research on crystal violet *in vitro* has shown that this dye is a mitotic poisoning agent. In addition, *in vivo* studies have proven that it should be regarded as a biohazard substance.

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It is a potent clastogenes, meaning it can promote tumor growth in some species of fish, and it is also potent carcinogen [2, 3].

Currently, various chemical and physical treatment methods, including adsorption, chemical precipitation and flocculation, oxidation, electrolysis, reduction, electrochemical treatment, and ion-pair extraction are used to remove this dye from the sewage [1, 3-5]. These methods are attractive because of their efficiency but are complicated and expensive [6]. As a viable alternative, biological processes have received increasing attention due to their cost, effectiveness, ability to produce less sludge, and environmental harmlessness [7]; these processes can convert or degrade this pollutant into water, carbon dioxide and various salts of inorganic nature [8]. A wide variety of microorganisms are able to decolorize a wide range of dyes, including bacteria (single and mixed cultures), fungi, and algae [9-21]. They can decolorize and even completely mineralize many dyes under certain conditions and the medium composition can influence this process [22-24].

Medium optimization using a statistical analysis was recently used for the decolorization of dyes [12, 21, 25, 26]. Therefore, the present work aimed to maximize decolorization of CV by bacteria using single and mixed culture techniques under aerobic conditions. The culture conditions for optimizing the bacterial CV decolorization process were optimized using the Plackett-Burman and Box-Behnken statistical designs.

Materials and Methods

Bacterial Isolates

The bacteria that were tested for crystal violet (CV) decolorization were isolated locally from the sewage wastewater and sediment habitat of the sewage disposal lake in Jeddah, Saudi Arabia, using an enrichment procedure adapted from Banat et al. [27]. Both liquid and solid minimal media were modified from Wong and Yuen [28], and contained the following (g/l): glucose, 3.0; $(NH_4)_2SO_4$, 1.0; K_2HPO_4, 7.0; $MgSO_4{\cdot}7H_2O$, 0.1 and NaCl, 5.0. The media were fortified with 50 µg CV/ml and used for isolation CV decolorizing bacteria. The purified bacteria were identified as *Acinetobacter baumannii*, *Corynebacterium* sp., *Cytophaga columnaris*, *Escherichia coli*, *Pseudomonas fluorescens*, and *Pseudomonas luteola* according to Bergy's Mannual of Systematic Bacteriology [29]. They were maintained on nutrient agar slants with monthly transfers.

Chemicals

The heterocyclic CV dye ($C_{25}N_3H_{30}Cl$) was of pure grade purchased from Merck Chemical Company. The media ingredients were all of analytical grade and were obtained from recognized chemical suppliers.

Inoculum and Cultivation

Seed cultures were prepared by inoculating Laury Broth (LB) medium containing (g/l): peptone, 1.0; yeast extract, 5.0; and NaCl, 5, with a loop-full of bacteria from a single colony and were shaken for 24 h at 120 rpm at 30ºC and pH 7. Thereafter, a 2 ml inoculum of an absorbance (A_{550}) at 550 nm was used to inoculate 50 ml aliquots of the basal medium, which contained (g/l): glucose, 3.0; yeast extract, 2.0; $(NH_4)_2SO_4$, 2.0; K_2HPO_4, 6.0; KH_2PO_4, 1.0; $MgSO_4{\cdot}7H_2O$, 0.1; NaCl, 5.0; $FeSO_4{\cdot}5H_2O$, 0.001; $MnSO_4{\cdot}H_2O$, 0.01 [30] and was dispensed in 250 ml Erlenmeyer flasks. Stock solutions of these media were sterilized separately by autoclaving at 121ºC for 15 minutes. A stock solution of CV was prepared by dissolving 30 mg in 100 ml of solvent (water: ethanol, 9:1), sterilized by 0.22 µm Syringe Filter, and added separately at a concentration of 50 µg/ml (unless otherwise stated). The flasks were shaken (150 rpm) at 37ºC for the given period. The basal medium was modified according to the statistical experimental designs.

Decolorization Assay

Bacterial growth at the end of the fermentation period was isolated by centrifugation at 10,000 rpm for 15 min to obtain clear supernatant. The CV concentration in the cleared supernatant was determined spectrophotometrically at 595 nm [31]. Medium lacking CV was used as a control. The following parameters were calculated:

Decolorization % = *Decolorized CV* (µg/ml)/ *Initial CV* (µg/ml) ×100

Rate of decolorization (µg/h) = *Decolorized CV* (µg)/ *Fermentation period* (h)

Decolorization efficiency % = *Rate of test decolorization* (µg/h)/ *Highest decolorization rate* (µg/h) ×100

Decolorization of CV by the Bacterial Isolates

The six bacterial isolates were cultivated in 50 ml aliquots of the basal medium fortified with 50 µg CV/ml for 48 h under shaking conditions (150 rpm) at 37ºC. Thereafter, the residual CV was estimated to determine the most efficient CV decolorizing bacterium.

Effect of Different Cultivation Media

The quantity and quality of ingredients of the cultivation medium that favoured decolorization of 50 µg CV/ml by the most active tested bacteria (*P. fluorescens* and *Corynebacterium* sp.) were determined using five different media (including the basal medium as No. 5), as follows (g/l):

1) Starch, 1.3; $(NH_4)NO_3$, 1.0; K_2HPO_4, 1.0; KH_2PO_4, 1.0; $MgSO_4{\cdot}7H_2O$, 0.2; $FeCl_3$, 0.05; NaCl, 4.0; $CaCl_2$, 0.02 [32]

2) Glucose, 1.0; yeast extract, 0.5; K_2HPO_4, 1.0; $(NH_4)NO_3$, 1.0; $MgSO_4{\cdot}7H_2O$, 0.2; $FeCl_3$, 0.05; $CaCl_2$, 0.02 [13]
3) Glucose, 2.0; peptone, 0.5; KH_2PO_4, 0.1; $MgSO_4{\cdot}7H_2O$, 0.05 [33]
4) Glucose, 2.0; yeast extract, 2.0; K_2HPO_4, 0.1; $MgSO_4{\cdot}7H_2O$, 0.5; KCl, 0.5 [34]
5) Basal medium [31].

After an incubation period of 72 h, the residual CV was estimated.

Decolorization of Different Concentrations of CV

The decolorization of different concentrations of CV (50, 75, 100, 125, and 150 µg/ml) were determined using the best medium (No. 5) and the most efficient bacterium (*P. fluorescens*). The incubation period for each CV concentration was extended until complete decolorization was achieved.

Decolorization of CV by a Bacterial Consortium

A bacterial culture of the most potent bacterium (*P. fluorescens*) for CV decolorization and *Corynebacterium* sp. (the second most efficient) was inoculated (2 ml/ flask, 1:1) into a 250 ml Erlenmeyer flask containing 50 ml aliquots of the best medium (No. 5) supplemented with 150 µg CV/ml. At the end of the incubation period required for complete decolorization of CV, the residual CV was calculated.

Each experiment was carried out in triplicate and the obtained results are the arithmetic mean. The initial pH value in all experiments was adjusted to 7 and the final pH values ranged from 6.2 to 7.2.

Statistical Optimization

Plackett-Burman Design

The Plackett-Burman experimental design, a fractional factorial design, [35] was used to assess the relative importance of various environmental factors on CV decolorization by a mixed culture. Eleven independent variables were screened in fourteen combinations organized according to the Plackett-Burman design matrix (Table 3) for each variable; a high (+) and a low (-) level were tested. All trials were performed in duplicate and the averages of the decolorization results observed were treated as responses. The main effect of each variable (Table 2) was determined using the following equation:

$$E_{xi} = (\Sigma M_{i+} - \Sigma M_{i-})/N$$

...where E_{xi} is the variable main effect, M_{i+} and M_{i-} are the CV decolorization percentages in trials where the independent variable (xi) was present in high and low concentrations, respectively, and N is the number of trials divided by 2. A main effect figure with a positive sign indicates that the high concentration of this variable is closer to optimum level, and a negative sign indicates that the low concentration of this variable is closer to the optimum level. Using Microsoft Excel, statistical *t*-values for equal unpaired samples (Table 2) were calculated to determine variable significance.

Box-Behnken Design

We used a central composite design [36]. In this model, the most significant independent variables, designated (A), (B), and (C) were included and each of them was examined at three different levels, low (-), high (+) or basal (0). According to the applied design, nine dye treatment combinations were tested. For predicting the optimal level, the following second order polynomial model was fitted to correlate the relationship between independent variables and the response:

$$Y=b_0+b_1A+b_2B+b_3C+b_{12}AB+b_{13}AC+b_{23}BC+ b_{11}A^2+b_{22}B^2+b_{33}C^2$$

...where Y is the dependent variable (CV decolorization %); A, B, and C are the levels of the independent variables; b_0 is the regression coefficient at the centre point; b_1, b_2 and b_3 are linear coefficients; b_{12}, b_{13}, and b_{23} are the second order interaction coefficients; and b_{11}, b_{22}, and b_{33} are quadratic coefficients. The values of the coefficients were calculated using Microcal Origin 4.1 software and the optimum concentrations were predicted using Microsoft Excel 2000. The quality of the fit of the polynomial model equation was expressed using the coefficient of determination, R^2. The optimal CV decolorization value was estimated using the solver function of Microsoft Excel. Three-dimensional graphical representations were also constructed using statistic 6.1 software to reflect the effects as well as the interactions of independent variables in these tests.

Results and Discussion

Decolorization of CV by Bacterial Isolates

The preliminary selection of CV decolorizing bacteria was based on the decolorization of CV on minimal medium plates. The chosen bacterial isolates were able to form clear zones when grown on solid medium containing CV without any visible sorption of CV to the biomass. This suggests that decolorization was achieved by a degradative process by secreting dye hydrolyzing enzymes [21, 30]. The six bacterial isolates (*Acinetobacter baumannii*, *Corynebacterium* sp., *Cytophaga columnaris*, *Escherichia coli*, *Pseudomonas fluorescens*, and *Pseudomonas luteola*) showed variations in the decolorization percentages of CV (50 µg/ml) in liquid medium (Table 1). This reflects differences in enzymatic dye reduction activities [21, 30, 37]. *P. fluorescens* was the most active CV decolorizer (90.54%) followed by *Corynebacterium* sp. (82.43%), while the rest of the tested bacteria displayed lower CV decolorizing activities after 48 h of incubation. It was reported that bacterial species of *Pseudomonas* and *Corynebacterium* can be used successfully for dye decolorization [16, 21, 31, 38, 39]. The previous results indicated that the decolorization of dyes depends

Table 1. Efficiency of the tested bacteria at decolorization 50 µg CV /ml within 48 h of incubation.

Bacterium	Final pH	Residual CV (µg/ml)	Decolorized CV (µg/ml)	Decolorization (%)	Rate of decolorization (µg/h)	Decolorization efficiency (%)
Pseudomonas luteola	6.88	25.68	24.32	48.64	0.51	56.04
P. fluorescens	6.88	4.73	45.27	90.54	0.91	100
Cytophaga columnaris	6.88	10.81	39.19	78.38	0.82	90.11
Escherichia coli	6.88	24.90	25.10	50.20	0.52	57.14
Acinetobacter baumannii	6.88	21.62	28.38	56.76	0.59	64.84
Corynebacterium sp.	6.88	8.78	41.22	82.43	0.86	91.51

Decolorization % = Decolorized CV (µg/ml)/Initial CV (µg/ml) ×100
Rate of decolorization (µg/h) = Decolorized CV (µg)/Incubation period (h)
Decolorization efficiency % = Rate of test decolorization (µg/h)/Highest decolorization rate (µg/h) ×100

Table 2. Independent variables affecting crystal violet decolorization and their levels in the Plackett-Burman experiment.

Factor	Symbol	Level			Main effect	*t*-value (at 5% significant)
		-1	0	1		
Glucose	G	1.0	3.0	5.0	-61.74	-9.65
Yeast extract	YE	1.0	2.0	3.0	5.29	0.83
$(NH_4)_2SO_4$	NH	1.0	2.0	3.0	-3.14	-0.49
K_2HPO_4	K_2	3.0	6.0	9.0	9.41	1.47
KH_2PO_4	KH	0.5	1.0	1.5	3.13	0.49
$MgSO_4{\cdot}7H_2O$	Mg	0.05	0.1	0.15	-2.78	-0.43
NaCl	Na	2.5	5.0	7.5	1.52	0.24
$FeSO_4{\cdot}5H_2O$	Fe	0.0	0.001	0.002	10.30	1.61
$MnSO_4{\cdot}H_2O$	Mn	0.05	0.1	0.15	-16.22	-2.53
ml medium/flask	Ml	25	50	75	-22.85	-3.57
Inoculum (*P. fluorescens* and *Corynebacterium* sp.)	In	1.5:0.5	01:01	0.5:1.5	-12.99	-2.03

on the bacterial genus and species, and may depend on its strain. Previous work [21], on the same bacterial species using the same culture conditions, indicated that *P. fluorescens* and *Corynebacterium* sp. poorly decolorized methylene blue, while *E. coli* and *P. luteola* were the most active. However, in the present work *E. coli* and *P. luteola* were the least active CV decolorizing bacteria (24.32 and 25.10%, respectively). This indicates that decolorization of dyes depends on the decolorizing organism and on the type of dye.

Effect of Different Cultivation Media on CV Decolorization by *P. fluorescens* and *Corynebacterium* sp.

Our results (Fig. 1 a, b) indicated that the formulation of medium No. 5 contained the necessary ingredients favor the production and/or activity of the enzymes responsible for 93.24% decolorization by *P. fluorescens* and 83.78% decolorization by *Corynebacterium* sp. of 50 µg CV/ml medium after 72 h of incubation. In accordance with this finding, Ghanem et al. [21] found that the same medium was the best for decolorization of methylene blue by *E. coli* and *P. luteola*. This indicates that this medium is a good medium for dye decolorization and suggests that the decolorization of dyes depends mainly on the dye structure and the test organism. The importance of the environmental conditions, including the components of the cultivation medium, in the decolorization process and even complete mineralization of dyes by microorganisms is clearly shown [21-24].

Decolorization of Different Concentrations of CV by *P. fluorescens*

The ability of *P. fluorescens* to decolorize different concentrations of CV (50, 75, 100, 125, and 150 µg/ml) indi-

Table 3. Plackett - Burman experimental design for 11 factors.

Trial	Independent variables											Decolorization (%)	Rate of decolorization (µg/h)	Decolorization efficiency (%)
	G	YE	NH	K_2	KH	Mg	Na	Fe	Mn	Ml	In			
1	+	-	+	-	-	-	+	+	+	-	+	17.74	0.68	17.66
2	+	+	-	+	-	-	-	+	+	+	-	24.19	0.93	24.15
3	-	+	+	-	+	-	-	-	+	+	+	53.22	2.05	53.25
4	+	-	+	+	-	+	-	-	-	+	+	6.91	0.27	7.01
5	+	+	-	+	+	-	+	-	-	-	+	44.62	1.72	44.68
6	+	+	+	-	+	+	-	+	-	-	-	51.07	1.96	50.91
7	-	+	+	+	-	+	+	-	+	-	-	94.09	3.62	94.03
8	-	-	+	+	+	-	+	+	-	+	-	98.38	3.78	98.18
9	-	-	-	+	+	+	-	+	+	-	+	90.86	3.49	90.65
10	+	-	-	-	+	+	+	-	+	+	-	1.07	0.04	1.04
11	-	+	-	-	-	+	+	+	-	+	+	78.49	3.02	78.44
12	-	-	-	-	-	-	-	-	-	-	-	100	3.85	100
13	0	0	0	0	0	0	0	0	0	0	0	66.51	2.56	66.49
14	+	+	+	+	+	+	+	+	+	+	+	54.30	2.09	54.29

Table 4. Box-Behnken design for the three most significant variables and levels that affected crystal violet decolorization by a mixed culture of *P. fluorescens* and *Corynebacterium* sp. after 38 h of incubation.

Trial	Variable			Decolorization (%)	Rate of decolorization (µg/h)	Decolorization efficiency (%)
	A (glucose)	B ($MnSO_4 \cdot H_2O$)	C (ml medium/flask)			
1	0.0 (-)	0.0 (-)	10 (-)	84.90	3.36	84.81
2	0.0 (-)	0.05 (0)	40 (+)	90.91	3.48	88.12
3	0.0 (-)	0.1 (+)	25 (0)	89.88	3.64	92.15
4	1.0 (0)	0.0 (-)	40 (+)	92.15	3.80	96.20
5	1.0 (0)	0.05 (0)	25 (0)	96.23	3.89	98.48
6	1.0 (0)	0.1 (+)	10 (-)	98.61	3.86	97.72
7	2.0 (+)	0.0 (-)	25 (0)	97.79	3.80	96.16
8	2.0 (+)	0.05 (0)	10 (-)	97.62	3.85	96.47
9	2.0 (+)	0.1 (+)	40 (+)	100	3.95	100

cated that the required incubation periods for complete decolorization were 75, 79, 83, 88, and 92 h, respectively. These results indicate that as the CV concentration increased by 300% (50-150 µg/ml), the incubation period for complete decolorization increased by only 22.67%, and the rate of decolorization (µg/h) showed a 143.3% increase. These results indicate that a substrate (CV) inhibition effect on *P. fluorescens* may occur at a dye concentration higher than 150 µg/ml (Fig. 2). Because the maximal decolorization rate (µg CV/h) occurred at a relatively high dye concentration, *P. fluorescens* seems to be suitable for decolorization of an environment with a high dye load. Similar findings that increases in the incubation period with elevated dye concentrations were usually accompanied by elevated decolorization rates have been reported [9, 10, 16, 21, 32, 40].

Decolorization of CV by Bacterial Consortium of *P. fluorescens* and *Corynebacterium* sp.

To increase the rate of CV decolorization, a mixed culture of the most potent decolorizing bacteria (*P. fluorescens* and *Corynebacterium* sp.) was used to decolorize 150 µg CV/ml. The results (Fig. 3) indicated that the decolorization process was considerably enhanced, as 150 µg of CV/ml was completely decolorized after only 58 h of incubation as opposed to 92 h by a single culture of *P. fluorescens*. This indicates a 37% decrease in the incubation period and a 59% increase in the decolorization rate (µg/h), indicating the economy and the efficiency of using a mixed culture rather than a single culture for the decolorization of CV. The importance and efficiency of a bacterial consortium to decolorize dyes instead of a single culture technique has been previously reported [10-13, 15, 21, 32, 40-43].

Optimization of Culture Conditions Affecting CV Decolorization Using Plackett-Burman Statistical Design

For elucidation of the culture conditions affecting CV decolorization, the independent variables examined in the Plackett-Burman experiment and their settings are shown in Table 3. The main effect of each variable was calculated according to the CV decolorization percentage results. The results presented (Table 2) revealed that the three most significant factors determining the efficiency of the decolorization process were glucose concentration, the volume of the medium/flask (aeration), and $MnSO_4 \cdot H_2O$, with negative main effects of -61.74, -22.85, and -16.22, respectively. This reflects the importance of glucose as a carbon and energy source. Within the range of its tested concentrations (1-5 g/l), the lower glucose concentration led to increased CV decolorization. This color removal ability under relatively low carbon (glucose) source concentrations has been reported for degradation and decolorization of dyes either by single or mixed bacterial cultures [10, 11, 13, 16, 42]. $MnSO_4 \cdot H_2O$ has a negative main effect within its tested concentrations (0.05-0.15 g/l). This indicated that Mn ions may inhibit the production and/or activity of CV decolorizing enzymes. On the other hand, the volume of the medium/250 ml Erlenmeyer flask (aeration) has a negative main effect of -22.85. This means that the air content (225 ml) remaining in the flask (250 ml flask volume minus 25 ml of medium) combined with the shaking rate of 150 rpm pro-

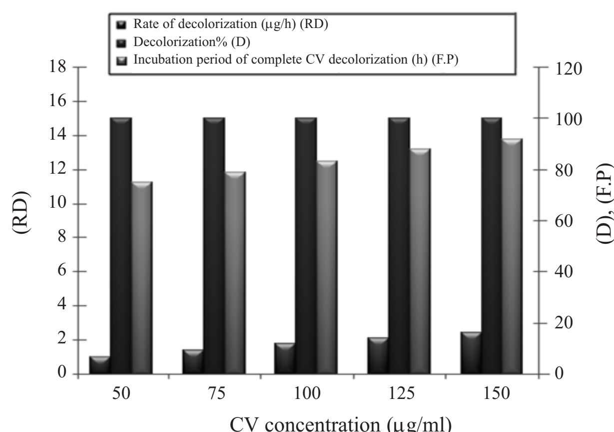

Fig. 2. Decolorization of different concentrations of CV (µg/ml), rate of decolorization (µg/h) (RD), and decolorization % (D) at incubation periods of complete decolorization of each CV level (h) (F.P) by P. *fluorescens*.

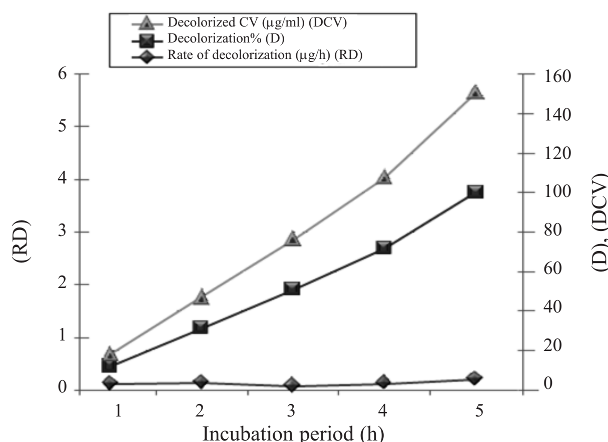

Fig. 3. Effect of incubation period (h) on the decolorization of 150 µg CV/ml, decolorized CV µg/ml (DCV), decolorization % (D), and rate of decolorization µg/h (RD), by a mixed culture of *P. fluorescens* and *Corynebacterium* sp.

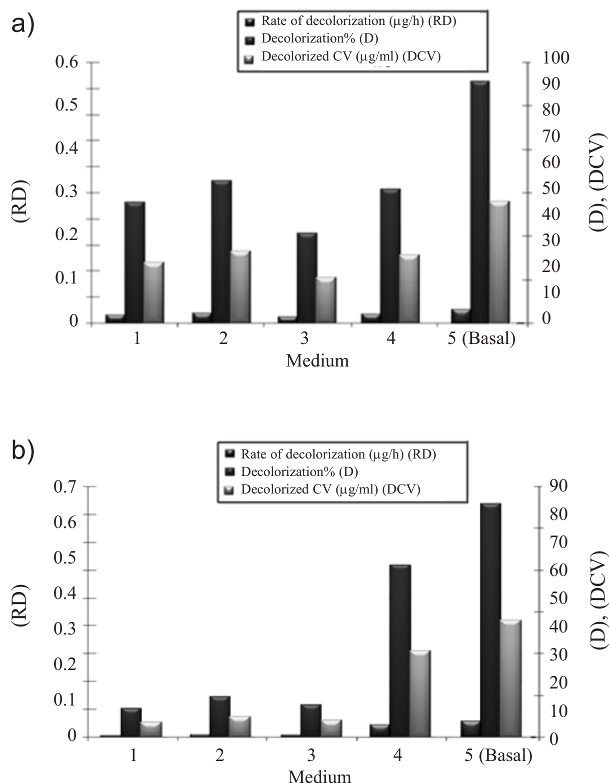

Fig. 1. Effect of different cultivation media on decolorization of 50 µg CV/ml, rate of decolorization µg/h (RD), decolorization % (D), and decolorized CV µg/ml (DCV) within 72 h of incubation by a) *P. fluorescens*, b) *Corynebacterium* sp.

vided the necessary oxygen (air) needed for aerobic respiration for the tested bacteria (*P. fluorescens* and *Corynebacterium* sp.) to release energy (ATP), which is needed for the decolorization process, at a low glucose level (1.0 g/l) and higher phosphate levels (K_2HPO_4, 9.0 and K_2HPO_4, 1.5 g/l).

According to the results of the Plackett-Burman design, a near optimum medium for decolorization of 150 μg CV/ml by the tested bacterial consortium can be predicted (g/l): glucose, 1.0; yeast extract, 3.0; $(NH_4)_2SO_4$, 0.1; K_2HPO_4, 9.0; KH_2PO_4, 1.5; $MgSO_4{\cdot}7H_2O$, 0.05; NaCl, 7.5; $FeSO_4{\cdot}5H_2O$, 0.002; $MnSO_4{\cdot}H_2O$, 0.05; 25 ml medium/250 ml Erlenmeyer flask and inoculum size of 2 ml (3:1, *P. fluorescens*: *Corynebacterium* sp.). Under these conditions, a 38.37 h incubation period was enough for complete decolorization (100%) of CV with a high decolorization rate of 3.85 μg/h, i.e., Plackett-Burman optimization reduced the incubation period by more than 22% and increased the decolorization rate by more than 48%, compared to the basal non-optimized conditions. In a confirmatory experiment, the incubation period was found to be 39 h under the same decolorization parameters. This indicated that 98.59% of the predicted period was reached.

Optimization of CV Decolorization Factors by Box-Behnken Design

In this second optimization step the levels of the three significant independent variables glucose (A), ml medium/flask (B) and $MnSO_4{\cdot}H_2O$ (C) were further investigated at three different levels (Table 4). Near optimum levels of the other factors, as suggested by the Plackett-Burman experimental results, were used in all trials. All trials were performed in duplicate and the average of observations (CV

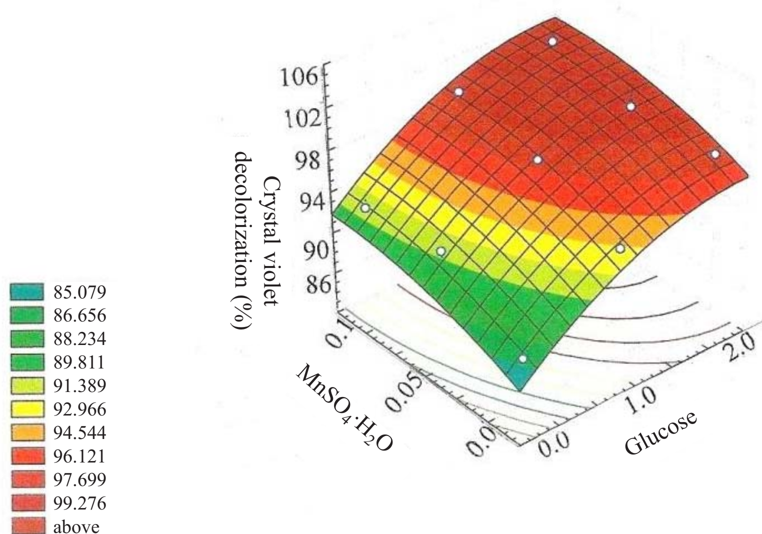

Fig. 4. The interaction of glucose with $MnSO_4.H_2O$.

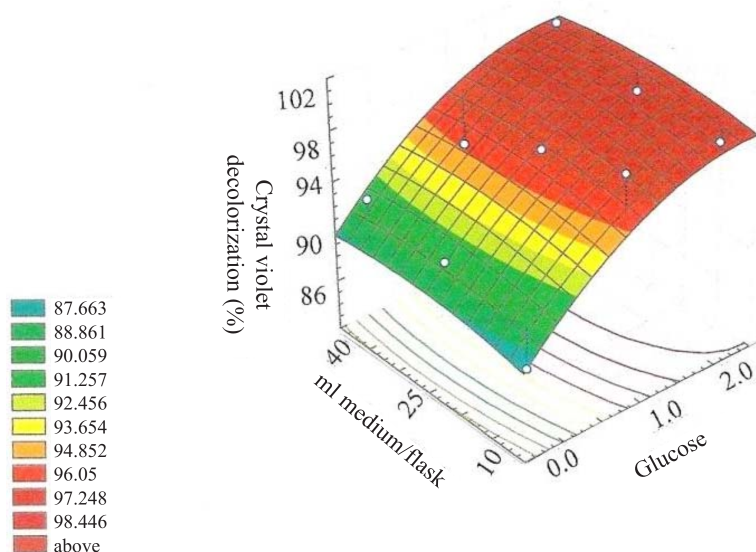

Fig. 5. The interaction of glucose with medium volume (ml)/250 ml Erlenmeyer flask.

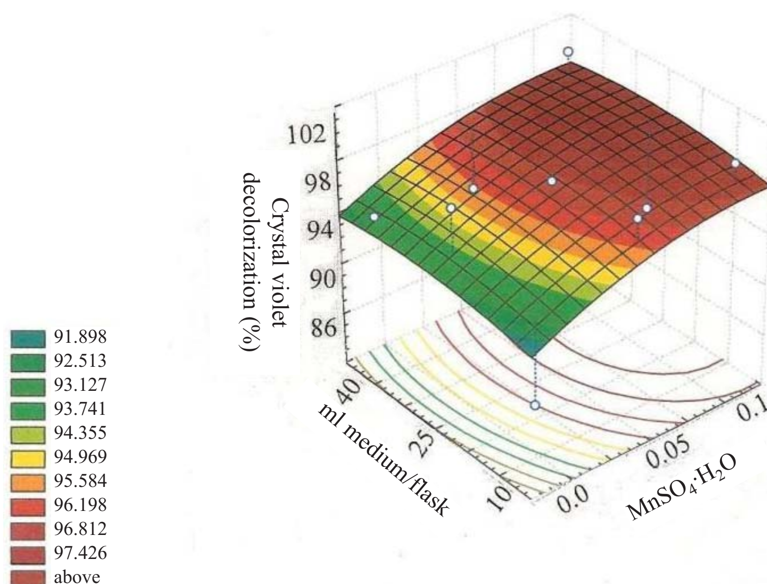

Fig. 6. The interaction of $MnSO_4.H_2O$ with medium volume (ml)/250ml Erlenmeyer flask.

decolorization %) was used. The experimental results, presented in the form of surface plots (Figs. 4-6), showed the relationship and interaction between the independent variables (glucose, ml of medium/flask, and $MnSO_4 \cdot H_2O$) and response (CV decolorization %). The levels of the examined independent variables needed to attain 100% decolorization of 150 μg CV/ml were calculated and tested in a verification experiment. The similarity between the predicted (100% CV degradation) and the observed results (100%), at 38 h of incubation, proves the accuracy of the model and its application validity. The validity of the Box-Behnken design in the decolorization processes of dyes by microorganisms was recorded [12, 21, 25, 41, 44-46].

The results presented here indicated that a consortium of *P. fluorescens* and *Corynebacterium* sp., under statistically optimized conditions, could completely decolorize 150 μg CV/ml after 38 h of incubation with decolorization rate of 3.95 μg/h. This concentration is higher than that recorded by El-Naggar et al. [31] using *P. aeruginosa*, which can only decolorize 50 μg CV/ml with a 1.35 μg/h decolorization rate. This is also more than that recorded by Parshetti et al. [47], who reported that *Agrobacterium radiobacter* can decolorize at most 100 μg CV/ml after 86 h of incubation (decolorization rate equal to 1.6 μg/h).

Conclusion

Crystal violet can be efficiently decolorized by *P. fluorescens* and *Corynebacterium* sp. The decolorization process is markedly influenced by the composition of the cultivation medium and the concentration of CV. A mixed culture of *P. fluorescens* and *Corynebacterium* sp. was more efficient at decolorizing CV than was a single culture technique (150 μg CV/ml was completely decolorized after only 58 h of incubation instead of 92 h by a single culture of *P. fluorescens*). The culture conditions were considerably optimized using the statistical experimental designs of Plackett-Burman and Box-Behnken (150 μg CV/ml after 38 h of fermentation).

Acknowledgements

The authors are thankful to the Ministry of Higher Education, King Abdul-Aziz University, Deanship of Scientific Research, Jeddah, Saudi Arabia for the financial support to carry out this research (Project No. 41/130/431).

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