Full Length Research Paper

Optimization of methyl orange decolorization by mono and mixed bacterial culture techniques using statistical designs

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Acinetobacter baumannii, *Corynebacterium* sp., *Cytophaga columnaris*, *Escherichia coli*, *Pseudomonas fluorescence* and *Pseudomonas luteola* were locally isolated bacteria from sewage Disposal Lake at Jeddah, Saudi Arabia and they can decolorize methyl orange (MO), except *P. luteola*. *P. fluorescence* was the most potent MO decolorizing and to a lesser extends *A. baumannii*. Five different media were tested to elucidate medium formulation in favor MO decolorization by *P. fluorescence* and *A. baumannii*. Ingredients of the basal medium favored the highest decolorization percentage of 50 µg MO/ml after 61 h of fermentation. *P. fluorescence* was satisfactory able to decolorize different levels of MO up to 150 µg/ml after 57 h of fermentation. Bacterial consortium of *P. fluorescence* and *A. baumannii* was highly efficient to decolorize MO than monoculture, where the decolorization period reduced by about 19% and increased decolorization rate (µg/h) by 19%. Statistical designs of two phase multifactorial optimization (Plackett-Burman and Box-Behnken) were carried out to optimize cultural conditions to increase the efficiency of mixed culture to decolorize 150 µg MO/ml. Under the optimized conditions the decolorization period was reduced by more than 31% and with increased decolorization rate by more than 45%. Methyl orange can be efficiently decolorized by *P. fluorescence* and *A. baumannii*. The decolorization process was markedly influenced by the composition of the fermentation medium and concentration of MO. Mixed culture of *P. fluorescence* and *A. baumannii* was highly efficient to decolorize MO than monoculture technique. The cultural conditions were considerably optimized using statistical experimental designs of Plackett-Burman and Box-Behnken.

Key words: Methyl orange, *Pseudomonas fluorescence* and *Acinetobacter baumannii*, mixed culture, statistical optimization.

INTRODUCTION

Synthetic dyes have increasingly been used in the textile and dyeing industries because of their ease and cost-effectiveness in synthesis, firmness, high stability to light, temperature, detergent and microbial attack and variety in color compared with natural dyes (Couto, 2009). This has resulted in the discharge of highly colored effluents that affect water transparency and gas solubility in water bodies (Banat et al., 1996). In addition, many dyes are believed to be toxic, carcinogenic or to be prepared from known carcinogens such as benzidine or other aromatic

compounds that might be formed as a result of microbial metabolism (Novotny et al., 2006; Kariminiaae-Hamedaani et al., 2007). Methyl orange (dimethylamino-azobenzenesulfonate) is an intensely colored azo dye compound used in dyeing and printing textiles and used as indicator. It is toxic, mutagenic, and carcinogenic azo dye (Chen et al., 2011). Therefore, treatment of industrial effluents containing azo dyes and their metabolites is necessary prior to their final discharge to the environment. Various physical/ chemical methods have been used for the removal of dyes from wastewaters (Vandevivere et al., 1998; dos Santos et al., 2007; Saratale et al., 2011). These methods have some drawbacks, such as being economically unfeasible;

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unable to completely remove the recalcitrant azo dyes and/ or their organic metabolites (Anjaneyulu et al., 2005); generating a significant amount of sludge that may cause secondary pollution problems; substantially increasing the cost of these treatment methods; and involving complicated procedures (Forgacs et al., 2004; Eichlerova et al., 2007). Microbial and enzymatic decolorization and degradation of azo dyes have significant potential to address this problem due to their environmentally-friendly, inexpensive nature, and also they do not produce large quantities of sludge (Saratale et al., 2011). Many bacterial and fungal species have been reported for treatment of dye effluents and dyes (Blanquez et al., 2004). Most studies on azo dye biodegradation have focused on bacteria where it is more efficient (Stolz, 2001); fast growth rate and high hydraulic retention time (Saratale et al., 2011). Mixed microbial cultures were reported as an effective dye decolorizing tool than pure cultures (Asgher et al., 2007; Jadhav et al., 2010; Phugare et al., 2011; Ghanem et al., 2011).

The statistical designs, such as the Plackett-Burman design and response surface method (Box-Behnken), are new effective methods for optimization of the operational parameters (Wang and Liu, 2008). The Plackett-Burman design is usually used to evaluate the relative importance of the test parameters (Levin et al., 2005). Response surface methods (Box-Behnken) are often being applied to explore the relationship between a response and a set of design variables (Ferreira et al., 2007). Compare to the traditional" one-factor-at-a-time approach", the Plackett-Burman and Box-Behnken methods are more effective methods to investigate the decolorization of the dyes (Du et al., 2010). Therefore, the present work aimed to decolorize methyl orange by bacteria using mono and mixed culture techniques under aerobic conditions. The culture conditions for optimizing decolorization process of MO were optimized using the experimental designs of Plackett-Burman and Box-Behnken statistical designs.

MATERIALS AND METHODS

Bacterial isolates

The tested bacteria for methyl orange decolorization were locally isolated from sewage wastewater and sediment habitat of sewage Disposal Lake of Jeddah, Saudi Arabia using the enrichment procedure that was adopted after Banat et al. (1997). Both liquid and solid minimal medium of modified Wong and Yuen, 1996 (g/L): Glucose, 3.0; $(NH_4)_2SO_4$, 1.0; K_2HPO_4, 7.0; $MgSO_4.7H2O$, 0.1 and NaCl, 5.0 fortified with 50 µg MO/ml, was used to isolate MO decolorizing bacteria. The purified bacteria were identified according to Bergy's Mannual of Systematic Bacteriology (2005) as *Acinetobacter baumannii, Corynebacterium* sp., *Cytophaga columnaris*, *Escherichia coli*, *Pseudomonas fluorescence*, and *Pseudomonas luteola*. They were maintained on nutrient agar slants with monthly transfers.

Chemicals

Methyl orange azo dye, dimethylamino-azobenzensulfonate

$(C_{14}H_{14}N_3NaO_3S)$ was of pure grade purchased from Merck Chemical Company. Ingredients of media were all of analytical grade, obtained from recognized chemical suppliers.

Inoculum and cultivation

Seed cultures were prepared by inoculating Laury Broth (LB) medium contained (g/L): Peptone, 1.0; yeast extract, 5.0; and NaCl, 5, by a loop full bacterium from single colony and shaken for 24 h at 120 rpm at 30°C, pH 7. Thereafter, 2 ml inoculum of an absorbance (A_{550}) 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; $MgSO_4.7H_2O$, 0.1; NaCl, 5.0; $FeSO_4.5H_2O$, 0.001; $MnSO_4. H_2O$, 0.01 (El-Sersy, 2001) and dispensed in 250 ml Erlenmeyer flasks. Stock solutions of these media were separately sterilized by autoclaving at 121°C for 15 min. A stock solution of MO was prepared by dissolving 30 mg in 100 ml solvent (water: ethanol, 9:1), sterilized by bacterial filter, and added separately at 50 µg/ml medium level (unless otherwise stated). The flasks were shaking (150 rpm) at 37°C for the request period. The last mentioned medium (basal) was modified according to the statistical experimental designs.

Decolorization assay

The bacterial growth at the end of the fermentation period was separated by centrifugation at 10,000 rpm for 15 min. MO concentration was determined in the clear supernatant at 464 nm (Chen et al., 2011). Medium lacking MO was used as a control. The following parameters were calculated:

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

Decolorization of MO by the bacterial isolates

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

Effect of different fermentation media

The quantity and quality of ingredients of the fermentation medium in favor MO decolorization by the most active test bacteria (*P. fluorescence* and *A. baumannii*), were tested 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; $MgSO_4.7H_2O$, 0.2; $FeCl_3$, 0.05; NaCl, 4.0; $CaCl_2$, 0.02 (Tony et al., 2009); 2) Glucose, 1.0; yeast extract, 0.5; K_2HPO_4, 1.0; $(NH_4)NO_3$, 1.0; $MgSO_4.7H_2O$, 0.2; $FeCl_3$, 0.05; $CaCl_2$, 0.02 (Moosvi et al., 2007); 3) Glucose, 2.0; peptone, 0.5; KH_2PO_4, 0.1; $MgSO_4.7H_2O$, 0.05 (Pioo et al., 2003); 4) Glucose, 2.0; yeast extract, 2.0; K_2HPO_4, 0.1; $MgSO_4.7H_2O$, 0.5; KCl, 0.5 (Mohana et al., 2005); 5) Basal medium (El-Sersy, 2001). After fermentation period of 61 h, at which MO is completely decolorized, the residual MO was estimated.

Decolorization of different concentrations of MO

The decolorization of different levels of MO (50, 75, 100, 125, and

150 µg/ml) were elucidated using the best medium (no.5) and the most efficient bacterium (*P. fluorescence*). The incubation period for each MO level was extended until its complete decolorization.

Decolorization of MO by bacterial consortium

Bacterial consortium of the most potent bacterium (*P. fluorescence*) for MO decolorization and *A. baumannii* (the second efficient) was inoculated (2 ml/ flask, 1:1) into 250 ml Erlenmeyer flask containing 50 ml aliquots of the best medium (no. 5) fortified by 150 µg MO/ml. At the end of the fermentation period required for complete decolorization of MO, the residual MO was calculated.

Each experiment was carried out in triplicate and the obtained results are the arithmetic mean. The initial pH value of all experiments was adjusted at pH 7 and the final pHs were ranged between 6.92 and 7.20.

Statistical optimization

Plackett-Burman design

The Plackett-Burman experimental design, a fractional factorial design, (Plackett and Burman, 1946) was used to reflect the relative importance of various environmental factors on MO decolorization by 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 (+) as well as low (-) level was tested. All trials were performed in duplicate and the averages of decolorization observation results were treated as responses. The main effect of each variable (Table 2) was determined with 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 MO decolorization percentages in trials where the independent variable (xi) was present in high and low concentrations, respectively, and N is the number of trails divided by 2.

The main effect of figure with a positive sign indicates that the high concentration of this variable is nearer to optimum and a negative sign indicates that the low concentration of this variable is nearer to optimum. Using Microsoft Excel, statistical *t*-values for equal unpaired samples (Table 2) were calculated for determination of variable significance.

Box-Behnken design

It is a central composite design (Box and Behnken, 1960). 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 executed. For predicting the optimal point, the following second order polynomial model was fitted to correlate relationship between independent variables and 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 (MO decolorization %), A, B and C are the levels of the independent variables; b_0 is regression coefficient at the center 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 by coefficient of determination, R^2. The optimal value of MO decolorization was estimated using the solver function of Microsoft Excel tool. Three-dimensional graphical representations were also constructed using statistical 6.1 software to reflect the effects as well as the interactions of independent variables on the objective.

RESULTS AND DISCUSSION

Decolorization of MO by bacterial isolates

Preliminary selection of MO decolorizing bacteria was based on the decolorization of MO on minimal medium plates. The bacterial isolates were able to form clear zones when grown on solid medium containing MO without any visible sorption of MO to the biomass. This suggests that, decolorization was achieved by a degradative process by secreting azoreductases extracellular (El-Sersy, 2001; Ghanem et al., 2011). The six bacterial isolates (*A. baumannii, Corynebacterium* sp., *C. columnaris*, *E. coli*, *P. fluorescence* and *P. luteola*), shown variations in the decolorization percentages of MO (50 µg/ml) in liquid medium (Table 1), except *P. luteola* that failed to decolorize MO. This reflects the differences in enzymatic azo dye reduction activities (Rafii et al., 1990; El-Sersy, 2001; Ghanem et al., 2011). *P. fluorescence* was the most active MO decolorizing (80.27%) followed by *A. baumannii* (76.73%), while *Corynebacterium* sp. was poorly MO decolorizing (20.16%), after 48h of fermentation. The efficiency of the species of *Pseudomonas* to decolorize azo dyes was reported (Du et al., 2010; Kadam et al., 2011; Phugare et al., 2011). The previous results indicated that the decolorization of azo dyes depends on the genus, species and may on the strain of the azo dye decolorizing bacterium, as well as on the dye structure. Where in a previous work (Ghanem et al., 2011), on the same bacterial species and under the same culture conditions, *E. coli* and *P. luteola* were the most active methylene blue decolorizing. Where, *P. fluorescence* and *Corynebacterium* sp. were the most active for decolorizing crystal violet. On the other hand, *P. luteola* failed to decolorize MO. However, *P. fluorescence* and *A. baumannii* (in the present work) were the most active MO decolorizing bacteria.

Effect of different fermentation media on MO decolorization activity by *P. fluorescence* and *A. baumannii*

The results (Figures 1a and b) indicated that formulation of medium no.5 fortified the two bacteria with the necessary ingredients (qualitatively and/or quantitatively) in favor the production and/or activity of the enzymes responsible for 100% decolorization by *P. fluorescence*

Table 1. Efficiency of the tested bacteria to decolorize 50 µg/ml MO within 48 h of fermentation.

Bacterium	Final pH	Residual MO (μ g/ml)	Decolorized MO (µg/ml)	Decolorization (%)	Rate of decolorization (µg/h)	Decolorization efficiency (%)
Pseudomonas fluorescence	6.99	9.86	40.14	80.27	0.84	100
Cytophaga columnaris	6.94	13.85	36.15	72.3	0.75	89.29
Escherichia coli	6.93	17.95	32.05	64,10	0.76	79.76
Acinetobacter baumannii	7.01	11.64	38.36	76.73	0.8	95.24
Corynebacterium sp.	6.96	39.92	10.08	20.16	0.27	32.14

Decolorization % = Decolorized MO (μ g/ml) / Initial MO (μ g/ml) ×100; Rate of decolorization (μ g/h) = Decolorized MO (μ g) / Fermentation period (h); Decolorization efficiency % = Rate of test decolorization(μ g/h) / Highest decolorization rate(μ g/h) ×100.

and 95.60% decolorization by *A. baumannii* of 50 µg MO/ml medium after 61 h of fermentation. In accordance with this finding Ghanem et al (2011) found that the same medium was the best for decolorization of methylene blue but with *E. coli* and *P. luteola*. This indicates that this medium is a best medium that can be used in azo dye decolorization and assesses that decolorization of azo dyes depends mainly in the dye structure and test organism, as well as on the fermentation medium. The importance of the environmental conditions including the components of the fermentation medium in decolorization process and even complete mineralization of azo dyes by microorganisms was clearly improved (Pandey et al., 2007; Khalid et al., 2010; Ramya et al., 2010; Ghanem et al., 2011).

Decolorization of different concentrations of MO by *P. fluorescence*

The efficiency of *P. fluorescence* to decolorize different concentrations of MO (50, 75, 100, 125, and 150 µg/ml) indicated that the required fermentation periods for complete decolorization

were 46, 49, 51, 54, and 57 h, respectively. These indicate that as the MO level increased 300% (50 to 150 µg/ml), the fermentation period for complete decolorization increased by only 23.91% and the rate of decolorization (µg/h) showed 141.3% increase. These indicate that a substrate (MO) inhibition effect on *P. fluorescence* may occur at a dye concentration higher than 150 µg/ml (Figure 2). Since the maximal decolorization rate (µg MO/h) took place at a relatively high dye concentration, *P. fluorescence* strain seems to be suitable for decolorization of an environment with high azo dye load. Similar findings concerned with increasing the fermentation period with elevated azo dye level that usually accompanied by elevated decolorization rate were reported (Chang and Kuo, 2000; Isik and Sponsa, 2003; Kalyani et al., 2008; Tony et al., 2009; Ghanem et al., 2011). The present work indicated that *P. fluorescence* is more efficient to decolorize MO (150 µg/ml, after 57 h of fermentation) than its efficiency to decolorize crystal violet (150 µg/ml, after 92 h of fermentation), under the same culture conditions (work under consideration for the author). These indicated that *P. fluorescence* can be used satisfactory in decolorizing different types of azo

dyes.

**Decolorization of MO by bacterial consortium of *P. fluorescence* and *A. baumannii***

In order to increase the rate of MO decolorization, a consortium of the most potent decolorizing bacteria (*P. fluorescence* and *A. baumannii*) was used to decolorize 150 µg MO/ml. The results (Figure 3) indicated that the decolorization process was considerably enhanced, where 150 µg MO/ml was completely decolorized after only 48 h of fermentation instead of 57 h by monoculture of *P. fluorescence.* This indicates about 19% save in fermentation period and 19% increase in decolorization rate (µg/h) that proves the economy and the efficiency of mixed culture than monoculture in decolorization of MO. The importance and the efficiency of bacterial consortium to decolorize azo dyes than monoculture technique was reported (Isik and Sponza, 2003; Khehra et al., 2005; Chen et al., 2006; Moosvi et al., 2007; Dafale et al., 2008; Sharma et al., 2009; Tony et al., 2009; Chaube et al., 2010; Sugumar and Sadanandan, 2010;

Table 2. Factors examined as independent variables affecting methyl orange decolorization and their levels in the Plackatt - Burman experiment.

Factor	Symbol	Level			Main effect	t-value (at 5% significant)
		-1	0	+1		
Glucose	G	1.0	3.0	5.0	-66.28	-4.10
Yeast extract	YE	1.0	2.0	3.0	8.81	0.55
$(NH_4)_2SO_4$	NH	1.0	2.0	3.0	-3.07	-0.19
K_2HPO_4	K_2	3.0	6.0	9.0	4.98	0.31
KH_2PO_4	KH	0.5	1.0	1.5	3.06	0.19
$MgSO_4.7H_2O$	Mg	0.05	0.1	0.15	-4.21	-0.26
NaCl	Na	2.5	5.0	7.5	-0.38	-0.02
$FeSO_4.5H_2O$	Fe	0.0	0.001	0.002	0.77	0.05
$MnSO_4.H_2O$	Mn	0.05	0.1	0.15	-9.58	-0.59
ml medium / flask	Ml	25	50	75	-14.56	-0.90
Inoculum (*E. coli* and *P. luteola)*	In	1.5:0.5	1:1	0.5:1.5	-0.77	-0.05

Table 3. Plackatt - 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	+	-	+	-	-	-	+	+	+	-	+	20.69	0.94	20.69
2	+	+	-	+	-	-	-	+	+	+	-	24.14	1.10	24.14
3	-	+	+	-	+	-	-	-	+	+	+	83.90	3.81	83.90
4	+	-	+	+	-	+	-	-	-	+	+	16.09	0.73	16.09
5	+	+	-	+	+	-	+	-	-	-	+	49.42	2.25	49.42
6	+	+	+	-	+	+	-	+	-	-	-	39.08	1.78	39.08
7	-	+	+	+	-	+	+	-	+	-	-	96.55	4.39	96.55
8	-	-	+	+	+	-	+	+	-	+	-	90.80	4.13	90.80
9	-	-	-	+	+	+	-	+	+	-	+	94.25	4.28	94.25
10	+	-	-	-	+	+	+	-	+	+	-	8.05	0.37	8.05
11	-	+	-	-	-	+	+	+	-	+	+	89.65	4.08	89.65
12	-	-	-	-	-	-	-	-	-	-	-	100	4.55	100
13	0	0	0	0	0	0	0	0	0	0	0	72.16	3.28	72.16
14	+	+	+	+	+	+	+	+	+	+	+	61.31	2.87	63.21
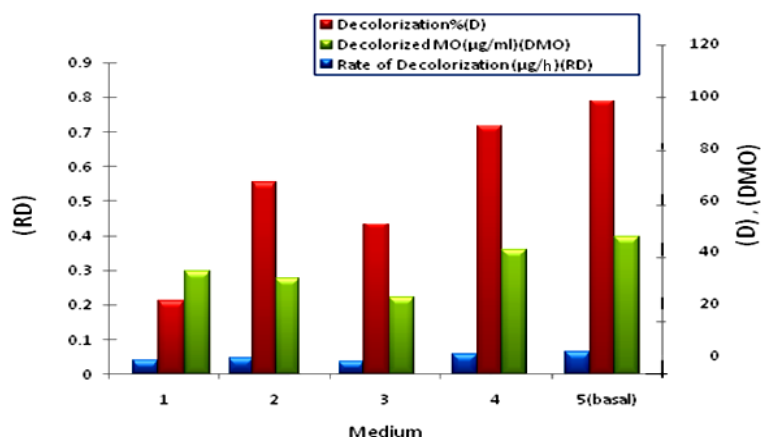
Figure 1a. Effect of different fermentation media on the efficiency of *P. fluorescence* to decolorize 50 µg/ml MO within 61 h of fermentation.
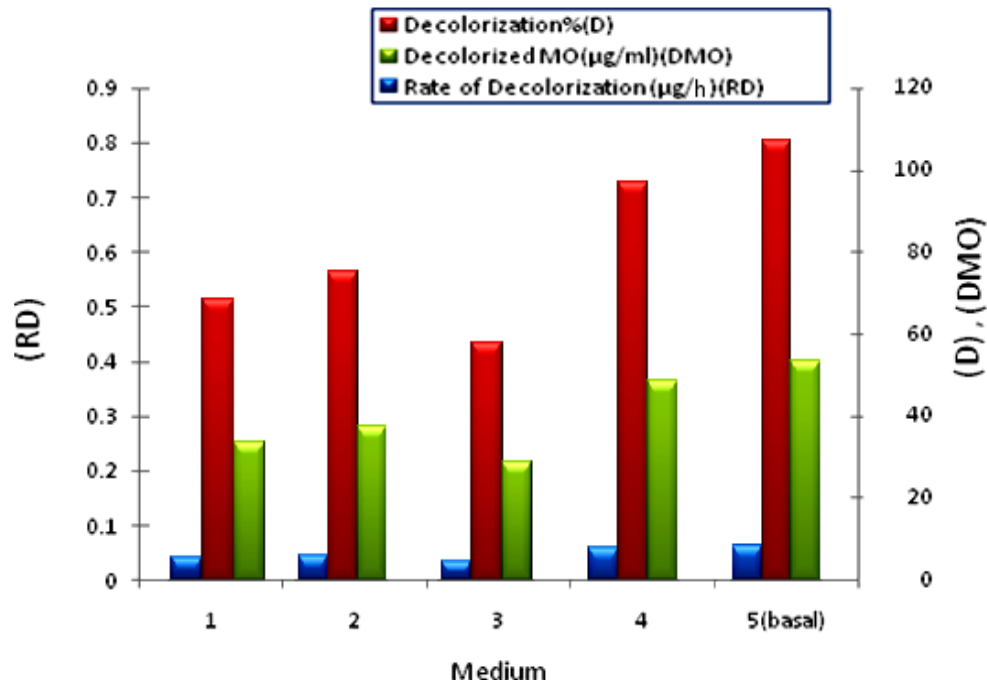
Figure 1b. Effect of different fermentation media on the efficiency of *A. baumannii* to decolorize 50 µg/ml MO within 61 h of fermentation.
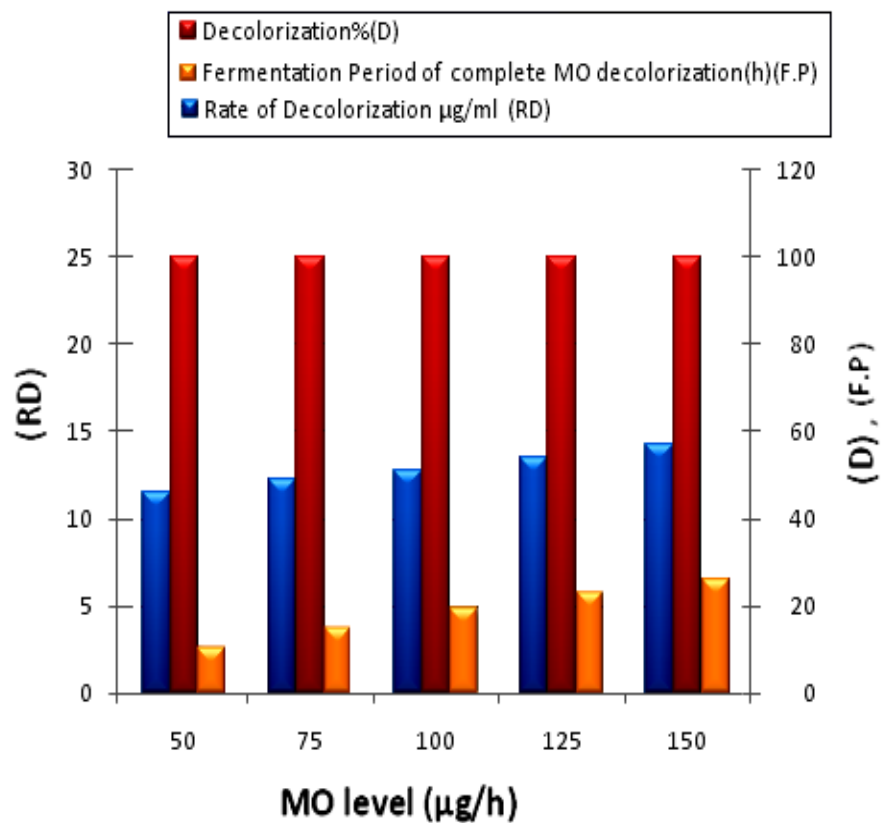

Figure 2. Decolorization of different levels of MO, decolorization % (D), and rate of decolorization (µg/h) (RD) at fermentation periods (h) of complete decolorization of each MO level (F.P) by *P. fluorescence*.
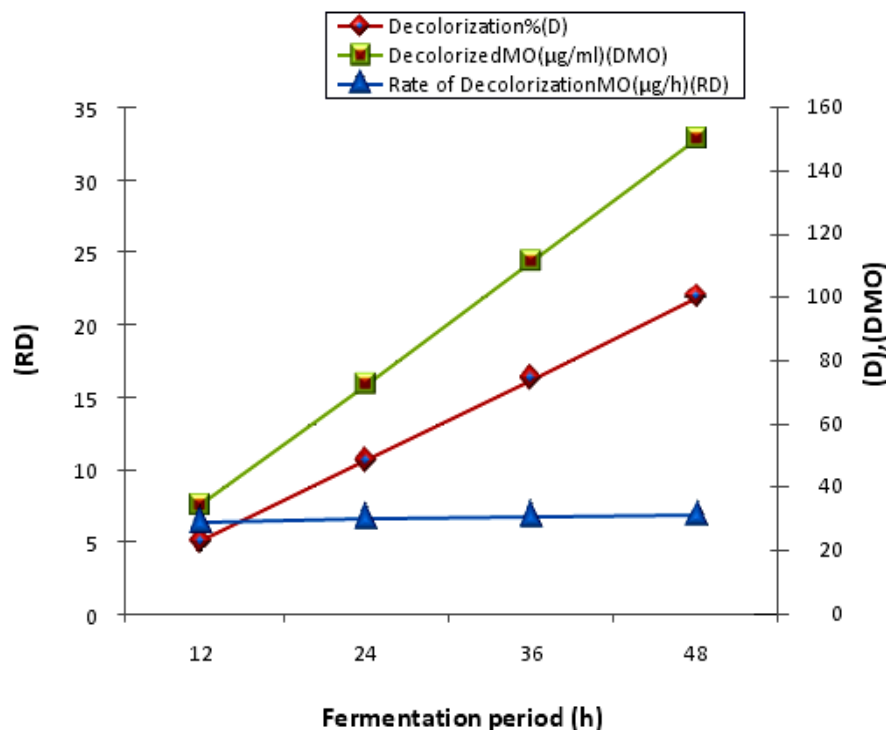
Figure 3. Decolonization of 150 µg/ml MO by mixed culture of *P. fluorescence* and *A. baumannii* at different incubation periods.

Phugare et al., 2011). Ghanem et al. (2011) found that more than 50% decolorization efficiency and about 37% reduction in fermentation period were estimated using mixed culture of *E. coli* and *P. luteola* for decolorization methylene blue than monoculture of *P. luteola.*

Optimization of culture conditions affecting MO decolorization using Plackett-Burman statistical design
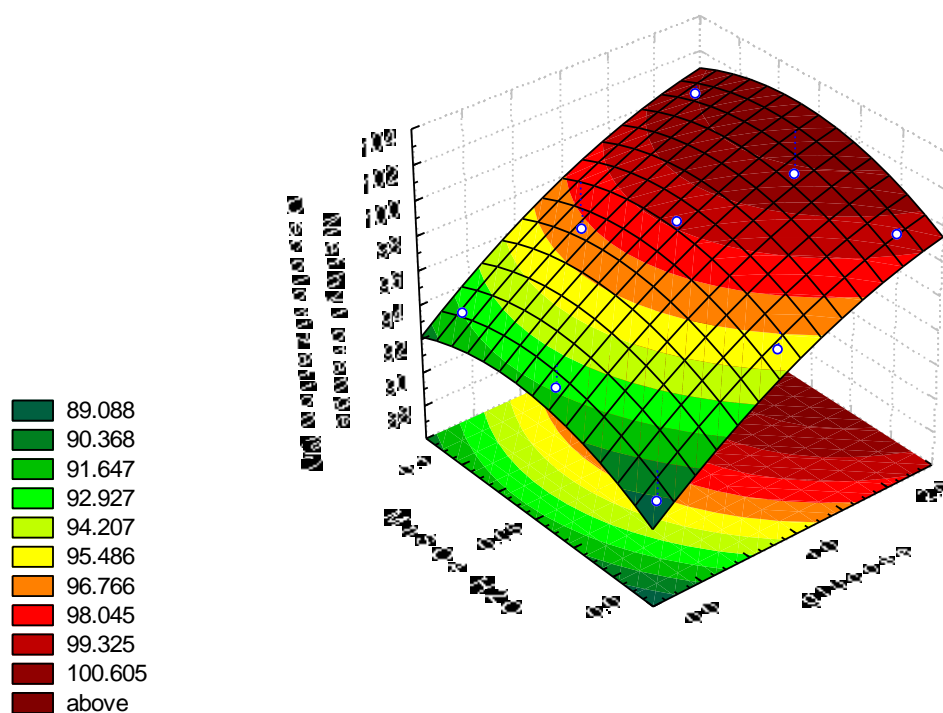
For elucidation of the culture conditions affecting MO decolorization, the independent variables examined in the Plackett-Burman experiment and their settings are shown in Table 3. The main effect of each variable was calculated according to MO decolorization percentage results. The results presented (Table 2) revealed that the most significant three factors which were more efficient in the decolorization process were glucose, volume of medium/flask (aeration), and $MnSO_4.H_2O$, with negative main effects of -66.28, -14.56, and -9.58, respectively. This reflects the importance of glucose as a carbon and energy source. Within its tested concentrations range (1 to 5 g/L), lower glucose concentration led to increased MO decolorization. This color removal ability under relatively low carbon (glucose) source was reported for degradation and decolorization of azo dyes either by mono or mixed bacterial cultures (Isik and Sponza, 2003; Khehra et al., 2005; Moosvi et al., 2007; Kalyani et al.,

2008; Chaube et al., 2010). $MnSO_4.H_2O$ has a negative main effect within its tested concentrations (0.05 to 0.15 g/L). This indicated that Mn ions may be of inhibitory action on the production and/or activity of MO decolorizing enzymes. On the other hand, volume of medium/250 ml Erlenmeyer flask (aeration) has a negative main effect of -14.56. This means that air content (225 ml) remained in the flask (250 ml flask volume minus 25 ml medium) beside that favored from shaking rate at 150 rpm provided the necessary oxygen (air) needed for aerobic respiration for the test bacteria (*P. fluorescence* and *A. baumannii*) to release energy (ATP), needed for the decolorization process, from low glucose level (1.0g/l) and higher phosphate levels (K_2HPO_4, 9.0 and KH_2PO_4, 1.5 g/L).

According to the results of Plackett-Burman design a near optimum medium for decolorization of 150 µg MO/ml by the bacterial consortium can be predicted (g/L): Glucose, 1.0; yeast extract, 3.0; $(NH_4)_2SO_4$, 1.0; K_2HPO_4, 9.0; KH_2PO_4, 1.5; $MgSO_4.7H_2O$, 0.05; NaCl, 2.5; $FeSO_4.5H_2O$, 0.002; $MnSO_4.H_2O$, 0.05; 25 ml medium/250 ml Erlenmeyer flask and inoculum size of 2 ml (3:1, *P. fluorescence*: *A. baumannii*). Under these conditions 32.27 h of fermentation period were enough for complete decolorization (100%) of MO with high decolorization rate of 4.55 µg/h. That is, Plackett-Burman optimization reduced the fermentation period by more than 31% with increased decolorization rate by more than 45%, as compared to the basal non-optimized

Table 4. Box-Behnken design for the most significant three variables and their levels that affected methyl orange decolorization by a mixed culture of *P. fluorescence* and *A. baumannii*

Trial	Variable			Decolorization (%)	Rate of decolorization (µg/h)	Decolorization efficiency (%)
	A (glucose)	B (MnSO$_4$.H$_2$O)	C (ml medium/flask)			
1	0.0 (-)	0.0 (-)	10 (-)	87.8	4.05	87.66
2	0.0 (-)	0.05 (0)	40 (+)	90.91	4.2	90.91
3	0.0 (-)	0.1 (+)	25 (0)	91.54	4.22	91.34
4	1.0 (0)	0.0 (-)	40 (+)	94.25	4.35	94.16
5	1.0 (0)	0.05 (0)	25 (0)	98.64	4.55	98.48
6	1.0 (0)	0.1 (+)	10 (-)	93.88	4.33	93.72
7	2.0 (+)	0.0 (-)	25 (0)	98.46	4.54	98.27
8	2.0 (+)	0.05 (0)	10 (-)	98.36	4.54	98.27
9	2.0 (+)	0.1 (+)	40 (+)	100	4.62	100

89.088
90.368
91.647
92.927
94.207
95.486
96.766
98.045
99.325
100.605
above

Figure 4. The interaction of glucose with MnSO$_4$.H$_2$O.

conditions. In confirmatory experiment the fermentation period was found to be 33 h to record the same decolorization parameters. This indicated that 98.34% of the predicted period was reached.

Optimization of MO 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$_4$.H$_2$O (C) were further investigated each at three different levels (Table 4). Near optimum levels of the other factors, suggested by the

Plackett-Burman experimental results were used in all trials. All trials were performed in duplicate and the averages of observations (MO decolorization percentage) were used. The experimental results presented in the form of surface plots (Figures 4, 5 and 6) showed the relationship and interaction between the independent variables (glucose, ml medium/flask and MnSO$_4$.H$_2$O) and response (MO decolorization percentage). The levels of examined independent variables predicted to attain 100% decolorization of 150 µg MO/ml were calculated and applied in a verification experiment. The similarity between the predicted (100% MO degradation) and the observed results (100%), at 31h of fermentation, proves the accuracy of the model and its application validity.
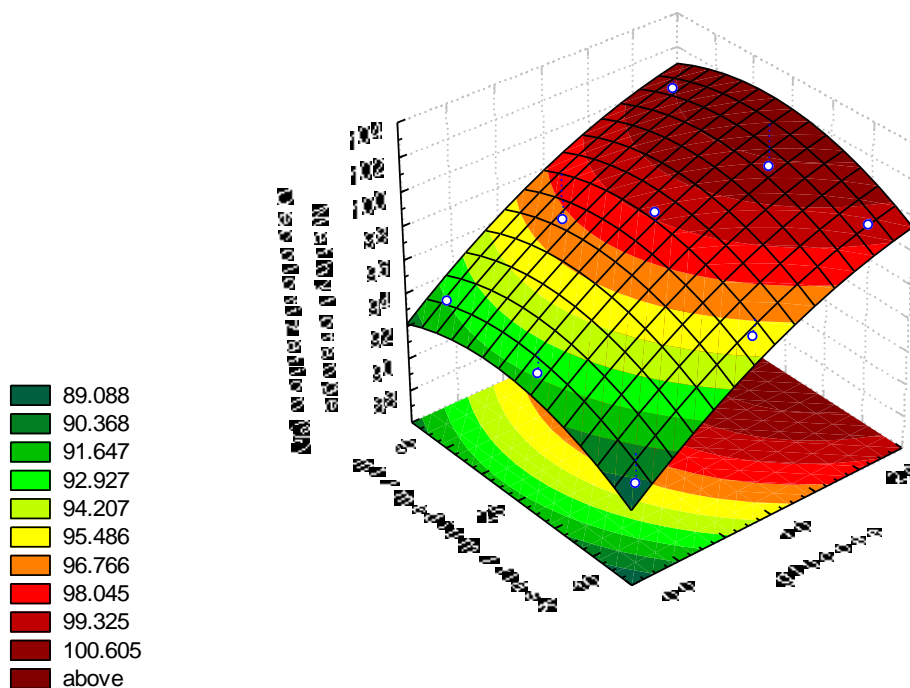
■	89.088
■	90.368
■	91.647
■	92.927
■	94.207
■	95.486
■	96.766
■	98.045
■	99.325
■	100.605
■	above

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

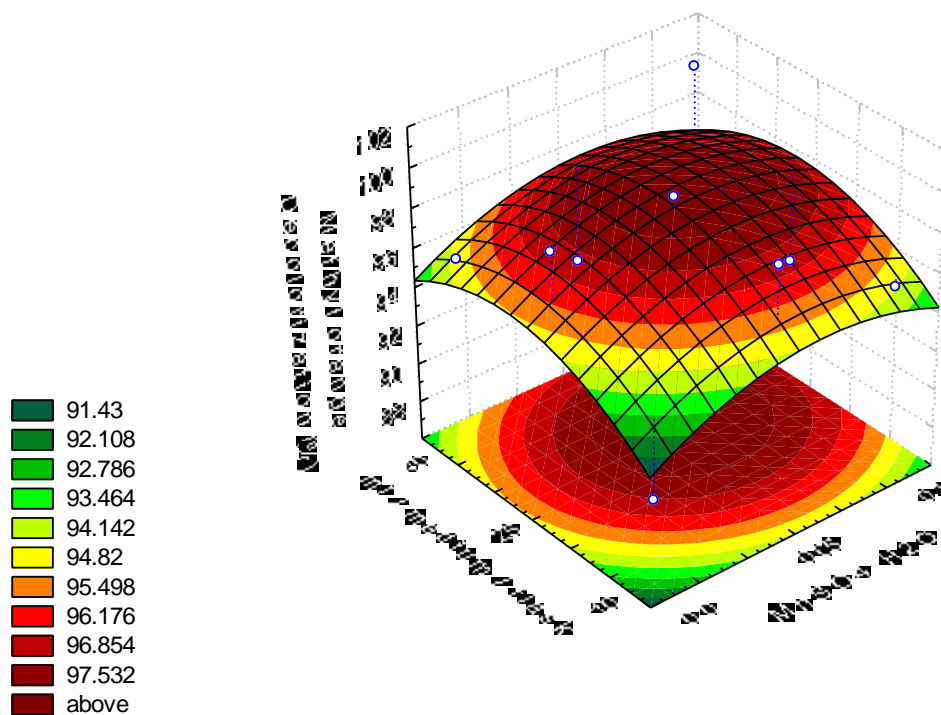|	
■	91.43
■	92.108
■	92.786
■	93.464
■	94.142
■	94.82
■	95.498
■	96.176
■	96.854
■	97.532
■	above

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

Validity of Box-Behnken design in decolorization processes of azo dyes by microorganisms was recorded (Chen et al., 2006; El-Sersy, 2007; Alam et al., 2009; Sharma et al., 2009; Ayed et al., 2010; Du et al., 2010; Ghanem et al., 2011). Under these conditions of Box-Behnken, the fermentation period reduced by more than 6% and the decolorization rate increased by more than 1.5%.

The results presented indicated that a consortium of *P. fluorescence* and *A. baumannii*, under statistically optimized conditions, could completely decolorize 150 µg MO/ml after 31 h of fermentation with decolorization rate of 4.62 µg/h. This concentration is higher than recorded by Parshetti et al. (2010) using *Kocuria rosea* MTCC1532 to decolorize 50mg/l of MO after 72 h of fermentation and that recorded by Ayed et al. (2010) using bacterial consortium of *Sphingomonas paucimobilis*, *Bacillus cereus* ATCC14579, and *B. cereus* ATCC11778 for decolorization of azo dyes including MO after 48 h of fermentation.

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