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Full Length Research Paper

Statistical optimization of cultural conditions for decolorization of methylene blue by mono and mixed bacterial culture techniques

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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 methylene blue. *E. coli* was the most potent MB decolorizing and to a lesser extend *P. luteola*. Five different media were tested to elucidate medium formulation in favor of MB decolorization by *E. coli* and *P. luteola*. Ingredients of the basal medium favored the complete decolorization of 50 µg MB/ml after 84 h of fermentation. Time course decolorization of MB by *E. coli* indicated that 75 h of fermentation was satisfactory to decolorize 50 µg MB/ml. It was also able to decolorize different levels of MB up to 150 µg MB/ml after 95 h of fermentation. Bacterial consortium of *E. coli* and *P. luteola* was highly efficient to decolorize MB than monoculture, where the decolorization period reduced by more than 37% and increased decolorization rate (µgMB/h) up to 58%. 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 MB/ml. Under the optimized conditions the decolorization period was reduced by about 31.7% and with increased decolorization rate by 46.4%. Methylene blue can be efficiently decolorized by facultative aerobic bacteria (*E. coli* and *P. luteola*). The decolorization process was markedly influenced by the composition of the fermentation medium and concentration of MB. Mixed culture of *E. coli* and *P. luteola* was highly efficient to decolorize MB than monoculture technique. The cultural conditions were considerably optimized using statistical experimental designs of Plackett-Burman and Box-Behnken.**

Key words: Methylene blue, *Escherichia coli*, *Pseudomonas luteola*, mixed culture, statistical optimization.

INTRODUCTION

Methylene blue (MB), a basic and cationic dye, has been widely used as a colorant, an indicator and an antiseptic agent in clinical therapy (Wainwright and Crossley, 2002; Frankenburg and Baldessarini, 2008). However, disposal of MB-containing waters can cause severe damage to the environment. They not only do serious harm to aquatic species by affecting photosynthesis activity, but also cause cancer and mutation in humans (Aksu and Tezer, 2000; Lucas and Peres, 2006). Many human diseases have been reported to be closely related to MB, such as hemolytic anemia, hyperbilirubinemia and acute renal failure (Albert et al., 2003). It has been reported that even micromolar levels of MB can induce cytotoxicity in SK-N-MC human neuroblastoma and U-373 MG human astrocytoma cells (Lee and Wurster, 1995). Hence, the removal of MB is a very important task in the protection of our environment and health. To date, the most widely used methods for the removal of MB from dye-rich wastewater

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are physicochemical ones, such as photocatalytic degradation (Srinivasan and White, 2007; Zhang et al., 2007; Yao and Wang, 2010; Du et al., 2011), sonochemical degradation (Minero et al., 2005), ultrafitration (Bielska and Szymanowski, 2006) and physical adsorption on activated carbon (Hameed et al., 2007a, b; Ma et al., 2011) or pyrolyzed petrified sediment (Aroguz et al., 2008; Sarioglu and Bisgin, 2010). These methods are attractive for their high efficiency, but are complicated and expensive (Robinson et al., 2001). As a viable alternative, biological processes have received increasing interest owing to their cost, effectiveness, ability to produce less sludge and environmental benignity (Chen et al., 2003), and they have potential to convert or degrade the pollutant into water, carbon dioxide and various salts of inorganic nature (Daneshvar et al., 2007). A wide variety of microorganisms are capable of decolorization of a wide range of dyes including bacteria (mono and mixed cultures), fungi and algae (Chang and Kuo, 2000; Isik and Sponza, 2003; Khehra et al., 2005; Chen et al., 2006; Moosvi et al., 2007; Vilar et al., 2007; Dafale et al., 2008; Kalyani et al., 2008; Kaushik and Malik, 2009; Yu et al., 2009; Aksu et al., 2010; Acemioglu et al., 2010). They can decolorize and even completely mineralize many Azo dyes under certain environmental conditions including medium composition (Pandey et al., 2007; Khalid et al., 2010; Ramya et al., 2010).

Medium optimization using statistical designs was recently used for the decolorization of dyes (Pavan et al., 2005; Chen et al., 2006; El-Sersy, 2007). Therefore, the present work aimed to decolorize MB by bacteria using mono and mixed culture techniques under aerobic conditions. The culture conditions for optimizing bacterial decolorization process of MB were optimized using the experimental designs of Plackett-Burman and Box-Behnken statistical designs.

MATERIALS AND METHODS

Bacterial isolates

The tested bacteria for methylene blue (MB) 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; $MgSO_4.7H_2O$, 0.1 and NaCl, 5.0 fortified with 50 µg MB/ml, was used to isolate MB decolorizing bacteria. The purified bacteria were identified according to Bergy,s Mannual of Systematic Bacteriology (Brenner et al., 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

The heterocyclic MB dye ($C_{16}H_{18}$ Cl N_3 S. H_2O) 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 MB 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 shaked (150 rpm) at 37°C for the request period (at which MB completely decolorized). 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. MB concentration was determined in the clear supernatant at 670 nm (Alam et al., 2009). Medium lacking MB was used as a control. The following parameters were calculated:

1. Decolorization% = Decolorized MB (µg/ml)/Initial MB (µg/ml) x 100
2. Rate of decolorization (µg/h) = Decolorized MB (µg)/fermentation period (h)
3. Decolorization efficiency% = Rate of test decolorization (µg/h)/highest decolorization rate (µg/h) x 100

Decolorization of MB by the bacterial isolates

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

Effect of different fermentation media

The quantity and quality of ingredients of the fermentation medium in favor of MB decolorization by the most active test bacteria (*E. coli* and *P. luteola*), 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 84 h, at which 50 µg MB/ml was completely decolorized, the residual MB was estimated.

Time course decolorization of MB by *E. coli*

In order to minimize the fermentation period required for complete decolorization of 50 µg MB /ml by *E. coli* (the most efficient). The

bacterium was inoculated into 50 ml aliquots of medium no. 5 (the best medium), incubated under shake conditions (150 rpm) at 37°C for different incubation periods (until complete MB decolorization), thereafter the residual MB was estimated.

Decolorization of different levels of MB

The decolorization of different levels of MB (50, 75, 100, 125 and 150 µg/ml) were elucidated using the best medium (no. 5) and the most efficient bacterium (*E. coli*). The incubation period for each MB level was extended until its complete decolorization.

Decolorization of MB by bacterial consortium

Bacterial consortium of the most potent bacterium (*E. coli*) for MB decolorization and *P. luteola* (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 µgMB/ml. At the end of the fermentation period required for complete decolorization of MB, the residual MB 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 6.85 and 7.2.

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 MB 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 duplicates 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 MB 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 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 (MB 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 MB decolorization was estimated using the solver function of Microsoft excel tool. Three-dimensional graphical representations were also constructed using statistica 6.1 software to reflect the effects as well as the interactions of independent variables on the objective.

RESULTS AND DISCUSSION

Decolorization of MB by bacterial isolates

Preliminary selection of MB decolorizing bacteria was based on the decolorization of MB on minimal medium plates. The bacterial isolates were able to form clear zones when grown on solid medium containing MB without any visible sorption of MB to the biomass. This suggests that, decolorization was achieved by a degradative process by secreting azoreductases extracellulary (El-Sesy, 2001). The six bacterial isolates (*A. baumannii, Corynebacterium* sp., *C. columnaris*, *E. coli*, *P. fluorescence* and *P. luteola*), shown variations in the decolorization percentages of MB (50 µg/ml) in liquid medium (Table 1). This reflects the differences in enzymatic Azo dye reduction activities (Rafii et al., 1990; El-Sersy, 2001). *E. coli* was the most active MB decolorizing (51.28%) followed by *P. luteola* (49.74%), while the rest tested bacteria were with lower MB decolorizing activities (38.46 to 44.1%), after 48 h of fermentation. It was reported that *E. coli* produces enzymes catalyzing Azo bond reduction and it is used successfully as a model system for bacterial decolorization of an Azo dye (Chang and Kuo, 2000; Naknishi et al., 2001). On the other hand, *P. lute*ola was one of the most potent Azo dyes decolorizing bacterium (Pandy et al., 2007; Hsueh and Chen, 2008).

Effect of different fermentation media on MB decolorization activity by *E. coli* and *P. luteola*

The results (Figures 1a and b) indicated that formulation of medium no.5 fortified *E. coli* with the necessary ingredients (qualitatively and/or quantitatively) in favor, the production and/or activity of the enzymes responsible for complete decolorization of 50 µgMB/ml medium after 84 h of fermentation. Under the same conditions *P. luteola* can decolorize 98% of MB. However, the other tested media gave less decolorization percentages ranged between 29.1 and 77% for both bacteria. The importance of the components of the fermentation medium in decolorization process of Azo dyes by

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

Bacterium	Final pH	Residual MB (µg/ml)	Decolorized MB (µg/ml)	Decolorization (%)	Rate of decolorization (µg/h)	Decolorization efficiency (%)
Pseudomonas luteola	6.85	25.13	24.87	49.74	0.52	98.11
Pseudomonas fluorescence	6.85	29.74	20.26	40.51	0.42	79.25
Cytophaga columnaris	6.85	30.26	19.74	39.48	0.41	77.36
Escherichia coli	6.85	24.36	25.64	51.28	0.53	100
Acinetobacter baumannii	6.85	30.77	19.23	38.46	0.40	75.47
Corynebacterium sp.	6.85	27.95	22.05	44.10	0.46	86.79

Decolorization% = decolorized MB (µg/ml)/initial MB (µg/ml) x 100. Rate of decolorization (µg/h) = decolorized MB (µg)/fermentation period (h). Decolorization efficiency% = rate of test decolorization (µg/h)/highest decolorization rate (µg/h) x 100.

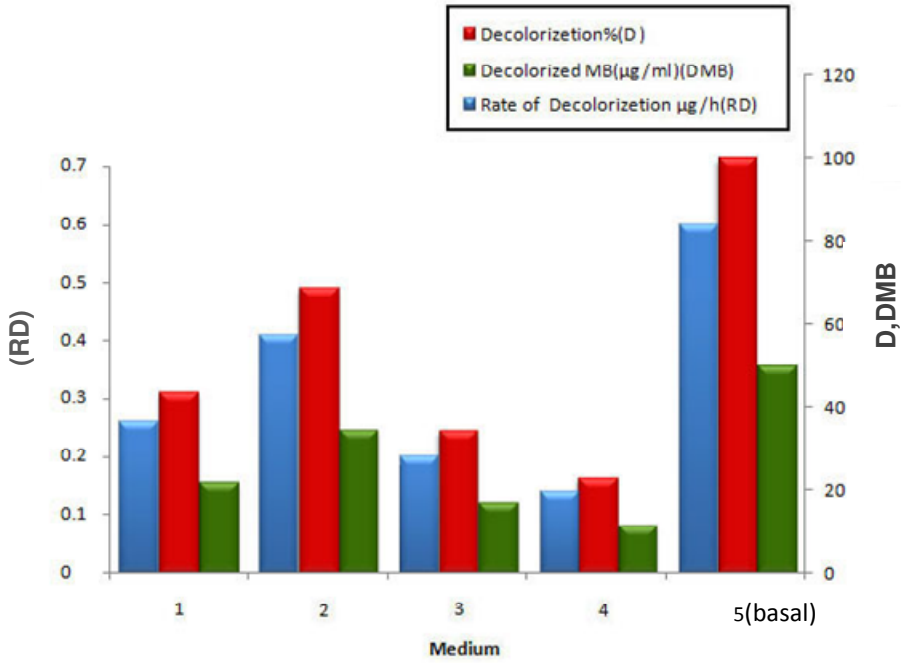

Figure 1a. Effect of different fermentation media on the efficiency of *E. coli* to decolorize 50 µg/ml MB within 84 h of fermentation.

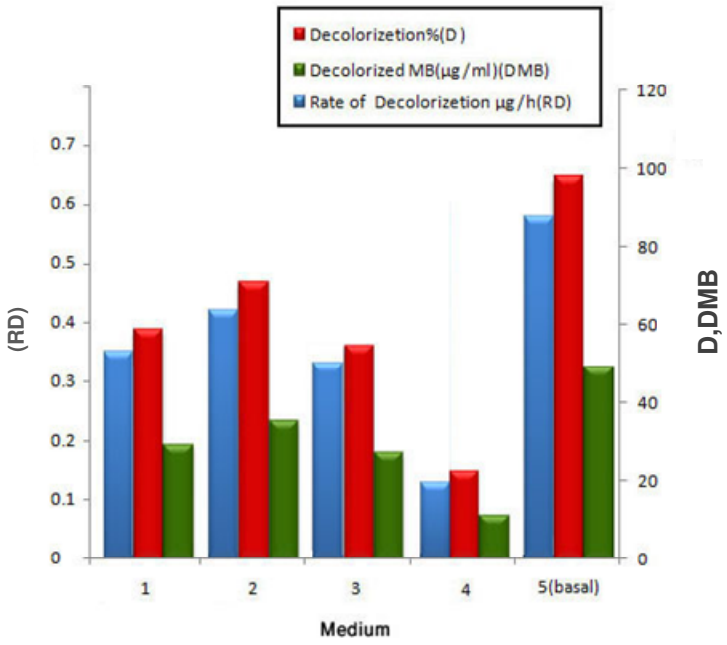

Figure 1b. Effect of different fermentation media on the efficiency of *P. luteola* to decolorize 50 µg/ml MB within 84 h of fermentation.

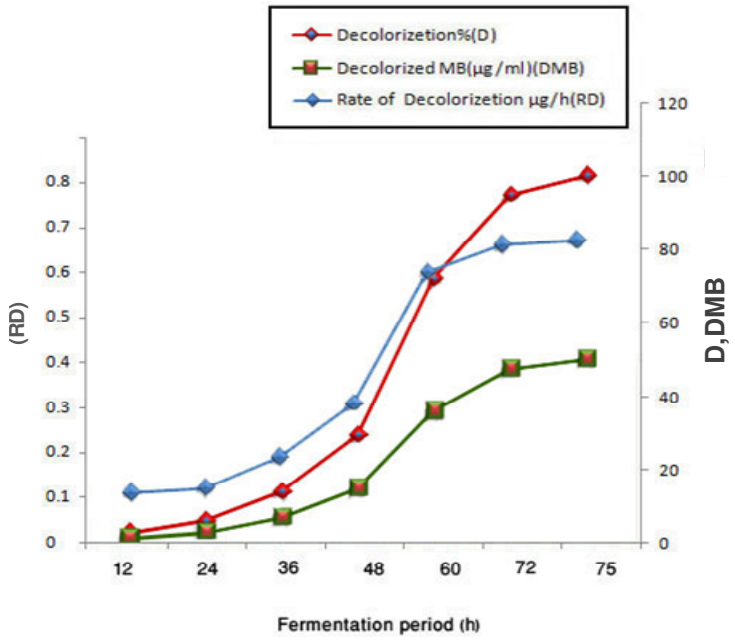

Figure 2. Decolorization of 50 µg/ml MB by *E. coli* at different fermentation periods.

microorganisms was clearly improved (Khehra et al., 2005; Moosvi et al., 2007; Kaushik and Malik, 2009). It was also reported that environmental conditions including medium composition have important role in decolorization and even complete mineralization of Azo dyes by microorganisms (Pandy et al., 2007; Khalid et al., 2010; Ramya et al., 2010).

Time course decolorization of MB by *E. coli*

The results (Figure 2) showed that *E. coli* with high

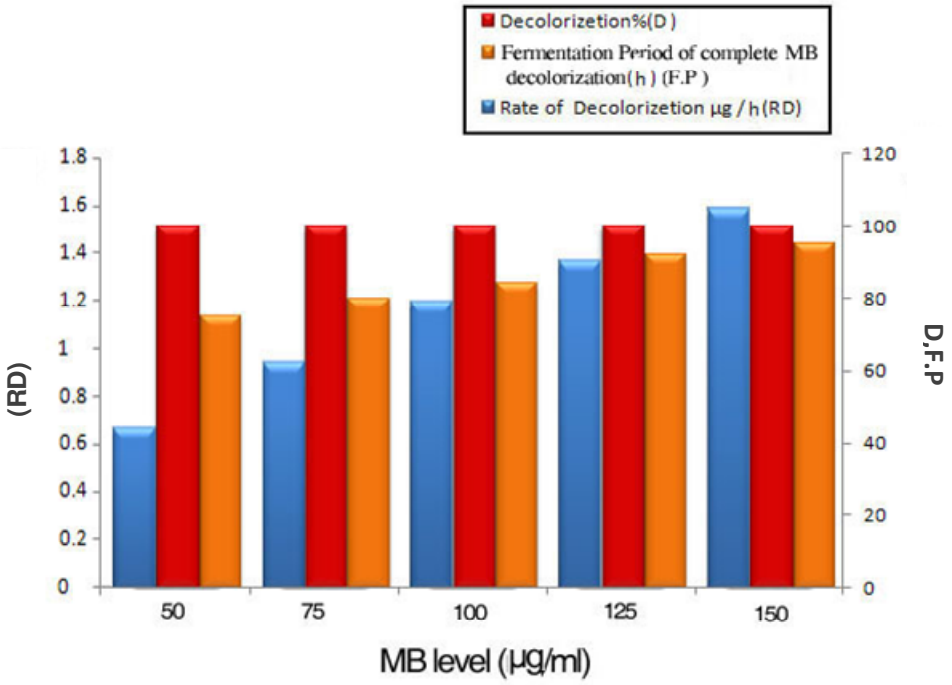

Figure 3. Decolorization of different levels of MB-decolorization% (D), and rate of decolorization (µg/h) (RD)-at fermentation periods (h) of complete decolorization of each MB level (F.P).

potency to produce MB decolorizing enzymes, which increased regularly with the extension of the fermentation period and 75 h of incubation, was satisfactory for complete decolorization of 50 µgMB/ml.

Decolorization of different concentrations of MB by *E. coli*

The efficiency of *E. coli* to decolorize different concentrations of MB (50 to 150 µg/ml) showed that about 26% decrease of MB decolorization as the MB concentrations increased from 50 to 150 µg/ml and complete decolorization of 150 µg MB/ml takes place at 95 h of fermentation (Figure 3). This finding was reported by many workers for Azo dye decolorization by bacteria (Chang and Kuo, 2000; Kalyani et al., 2008; Tony et al., 2009). The results showed that the rate of MB decolorization (µg/h) increased along with increasing MB concentration and reached a maximum value of 1.47 µg/h at 150 µg MB/ml. These indicate that a substrate (MB) inhibition effect on *E. coli* may occur at a dye concentration higher than 150 µg/ml. Since the maximal decolorization rate (µg MB/h) took place at a relatively high dye concentration. *E. coli* strain seems to be suitable for decolorization of an environment with high Azo dye load. Similar findings were reported for decolorization of Reactive Red22 by *E. coli* NO3 (Chang and Kuo, 2000; Isik and Sponsa, 2003).

Decolorization of MB by bacterial consortium of *E. coli* and *P. luteola*

In order to increase the rate of MB decolorization, a consortium of the most potent decolorizing facultative aerobic bacteria (*E. coli* and *P. luteola*) was used to decolorize 150 µg MB/ml, under aerobic conditions. The results (Figure 4) indicated that about 37% reduction of fermentation period (from 95 to 60 h) for complete decolorization of MB by the mixed culture technique as that monoculture of *E. coli*. This finding assessed the efficiency of bacterial consortium to decolorize different Azo dyes than monoculture. This indicates that in this model of binary biosystem the presence of *P. luteola* significantly enhanced decolorization performance of *E. coli*. 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).

Optimization of culture conditions affecting MB decolorization using Plackett-Burman statistical design

For elucidation of the culture conditions affecting MB decolorization, the independent variables examined in the

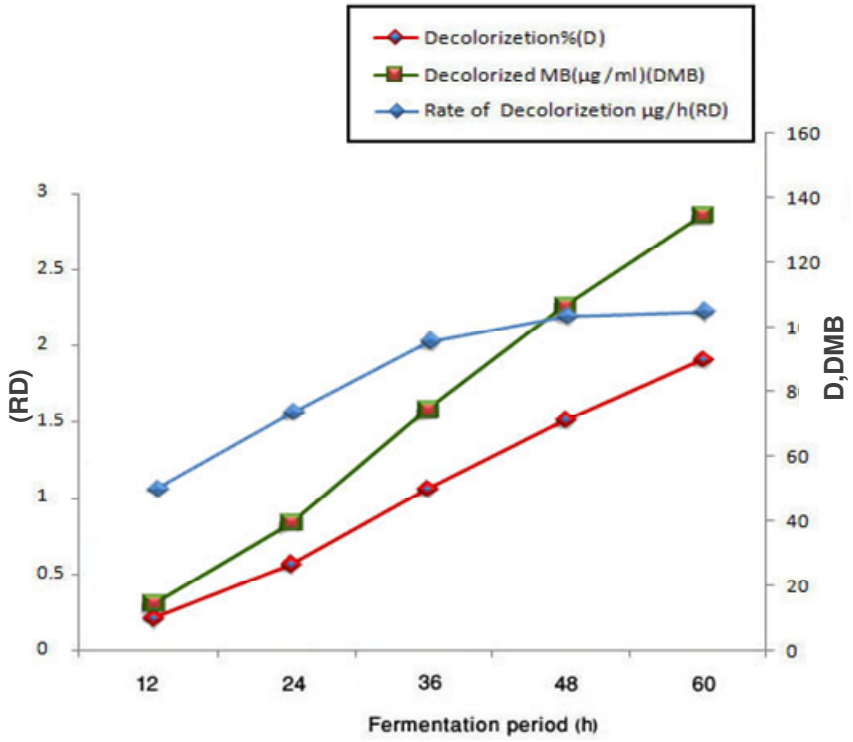

Figure 4. Decolorization of 150 µg/ml MB by mixed culture of *E. coli* and *P. luteola* at different incubation periods.

Table 2. Factors examined as independent variables affecting methylene blue 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	-40.59	-8.19
Yeast extract	YE	1.0	2.0	3.0	-7.75	-1.56
$(NH_4)_2SO_4$	NH	1.0	2.0	3.0	-2.00	-0.40
K_2HPO_4	K_2	3.0	6.0	9.0	14.40	2.91
KH_2PO_4	KH	0.5	1.0	1.5	-8.95	-1.81
$MgSO_4.7H_2O$	Mg	0.05	0.1	0.15	-9.64	-1.95
NaCl	Na	2.5	5.0	7.5	-1.17	-0.24
$FeSO_4.5H_2O$	Fe	0.0	0.001	0.002	10.50	2.12
$MnSO_4.H_2O$	Mn	0.05	0.1	0.15	-3.96	-0.80
ml medium/flask	Ml	25	50	75	-16.06	-3.24
Inoculum (*E. coli* and *P. luteola*)	In	1.5:0.5	1:1	0.5:1.5	-9.21	-1.86

Plackett-Burman experiment and their settings are shown in Table 3. The main effect of each variable was calculated according to MB decolorization% results and the results presented (Table 2) revealed that the most significant three factors which were more efficient were glucose, K_2HPO_4, and volume of medium/flask (aeration). While $FeSO_4.5H_2O$ showed positive non-significant effect on MB decolorization. However, the rest examined factors were negative. According to these results it can be predicted that the near optimum medium for decolorization of 150 µgMB/ml by bacterial consortium is (g/l): glucose, 1.0; yeast extract, 1.0; $(NH_4)_2SO_4$, 0.1; K_2HPO_4, 3.0; KH_2PO_4, 0.5; $MgSO_4.7H_2O$, 0.05; NaCl, 2.5; $MnSO_4.H_2O$, 0.05; 25 ml medium/250 ml Erlenmeyer flask and inoculum size of 1 ml (3:1, *E. coli*: *P. luteola*). Under these conditions 41 h of fermentation were enough for complete decolorization (100%) of MB with high decolorization rate of 3.66 µg/h, that is, Plackett-Burman

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	+	-	+	-	-	-	+	+	+	-	+	53.57	1.96	53.57
2	+	+	-	+	-	-	-	+	+	+	-	56.54	2.07	65.54
3	-	+	+	-	+	-	-	-	+	+	+	52.08	1.91	52.08
4	+	-	+	+	-	+	-	-	-	+	+	36.90	1.35	36.90
5	+	+	-	+	+	-	+	-	-	-	+	46.73	1.71	46.73
6	+	+	+	-	+	+	-	+	-	-	-	41.57	1.52	41.57
7	-	+	+	+	-	+	+	-	+	-	-	89.88	3.29	89.88
8	-	-	+	+	+	-	+	+	-	+	-	93.15	3.41	93.15
9	-	-	-	+	+	+	-	+	+	-	+	96.72	3.54	96.72
10	+	-	-	-	+	+	+	-	+	+	-	19.64	0.72	19.64
11	-	+	-	-	-	+	+	+	-	+	+	66.67	2.44	66.67
12	-	-	-	-	-	-	-	-	-	-	-	100	3.66	100
13	0	0	0	0	0	0	0	0	0	0	0	71.72	2.62	71.72
14	+	+	+	+	+	+	+	+	+	+	+	61.31	2.24	61.31

optimization reduced the fermentation period by about 32% with increased decolorization rate of about 46%, as compared to the basal non-optimized conditions. The data revealed that lower glucose level led to increased MB decolorization. The 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). K_2HPO_4, at its lower value (3 g/l), also is necessary for higher MB decolorization. The lower K_2HPO_4 and 225 ml air (reminder in 250 ml Erlenmeyer flask containing 25 ml medium) are concomitant for aerobic energy release from the lower level of carbon source (glucose), which is needed for decolorization process achieved by the facultative bacterial consortium of *E. coli* and *P. luteola*.

It is known that anaerobic fermentation is more suitable for Azo dye decolorization and aerobic fermentation is required to the hydrolysis of the substituted amines, that is, the experimental facultative bacterial consortium completely decolorize and hydrolyze MB. Some workers used combined anaerobic-aerobic bacterial degradation of dyes (Karatas et al., 2010; Sugumar and Sadanandan, 2010).

Optimization of MB decolorization factors by Box-Behnken design

In this second optimization step the levels of the three significant independent variables glucose (A), K_2HPO_4 (B) and ml medium/flask (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 average of observations (MB decolorization%) were used. The experimental results presented in the form of surface plots (Figures 5, 6 and 7) showed the relationship and interaction between the independent variables (glucose, K_2HPO_4 and ml medium/flask) and response (MB decolorization%). The levels of examined independent variables predicted to attain 100% decolorization of 150 µgMB/ml were calculated and applied in a verification experiment. The great similarity between the predicted (100% MB degradation) and the observed results (99.98%), at 40 h of fermentation, proves the accuracy of the model and its application validity. 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). The results presented indicated that a consortium of

Table 4. Box-Behnken design for the most significant three variables and their levels that affected methylene blue decolorization by a mixed culture of *E. coli* and *P. luteola*.

Trial	variable			Decolorization (%)	Rate of decolorization (µg/h)	Decolorization efficiency (%)
	A (glucose)	B (K_2HPO_4)	C (ml medium/flask)			
1	0.0 (-)	1.0 (-)	10 (-)	90.12	3.38	90.13
2	0.0 (-)	3.0 (0)	40 (+)	90.98	3.41	90.93
3	0.0 (-)	5.0 (+)	25 (0)	92.56	3.47	92.53
4	1.0 (0)	1.0 (-)	40 (+)	95.52	3.58	95.47
5	1.0 (0)	3.0 (0)	25 (0)	98.78	3.70	98.67
6	1.0 (0)	5.0 (+)	10 (-)	94.88	3.56	94.93
7	2.0 (+)	1.0 (-)	25 (0)	98.37	3.69	98.40
8	2.0 (+)	3.0 (0)	10 (-)	98.60	3.70	98.67
9	2.0 (+)	5.0 (+)	40 (+)	100	3.75	100

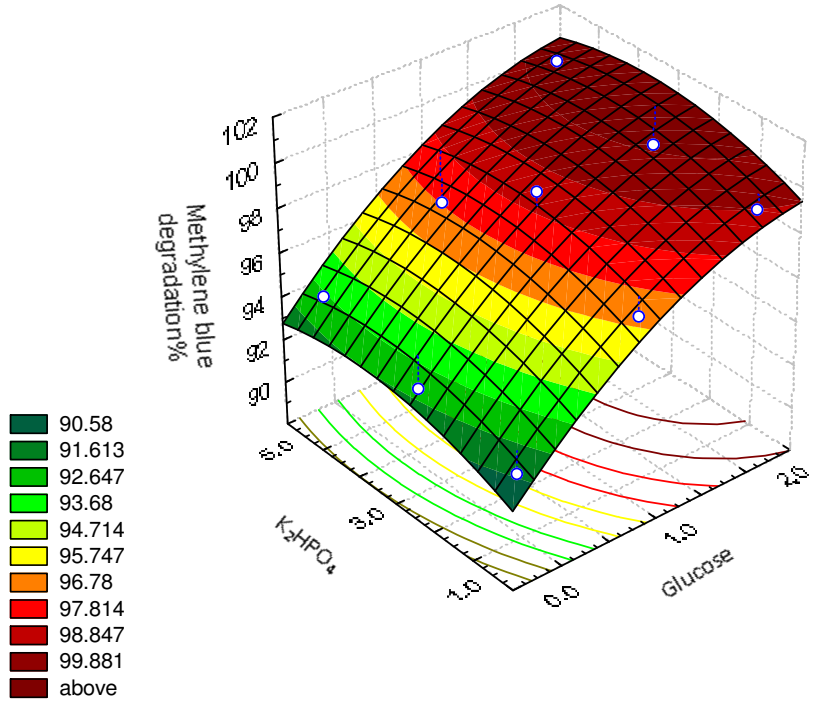

Figure 5. The interaction of glucose with K_2HPO_4 level.

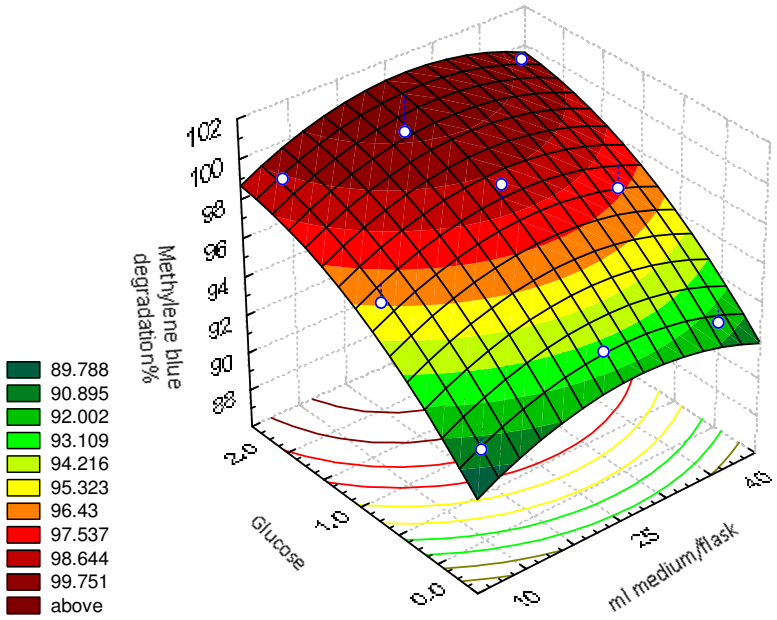

Figure 6. The interaction of glucose with ml medium/flask.

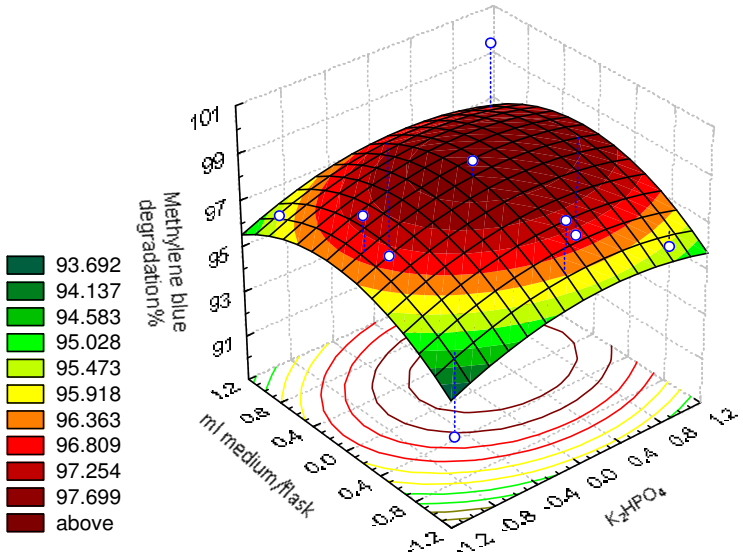

Figure 7. The interaction of K_2HPO_4 level with ml medium/flask.

E. coli and *P. luteola*, under statistically optimized conditions, could completely decolorize 150 µgMB/ml after 40 h of fermentation. This concentration is higher than recorded by Alam et al. (2009) in fugal decolorization of MB (20 µg/ml).

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