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Response surface methodology for optimization of process variable for reactive orange 4 dye discoloration by *Pseudomonas putida* SKG-1 strain and bioreactor trial for its possible use in large-scale bioremediation

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ABSTRACT

In this study, *Pseudomonas putida* SKG-1 isolate employed earlier for pulp-/paper-mill effluent discoloration, was used for bioremediation of reactive orange 4 azo dye under varied cultural and nutritional conditions. The optimization through one-factor-at-a-time approach revealed maximum growth (A_{620} 1.31) and dye discoloration (95.2%) at optimum temperature 35˚C, pH 8.0, inoculum dose 5.0%, sucrose 0.7%, peptone 0.25%, and 50 mg reactive orange 4 dye L^{-1} within 72 h of incubation. Under response surface methodology (RSM; using Box–Behnken design) approach, the dye discoloration enhanced to 97.8% at reactive orange 4 concentration of 50 mg L^{-1}, sucrose 0.7%, and peptone 0.28% during 72 h of incubation. In bioreactor trial, the maximum dye discoloration (98% within 60 h) was achieved in 12 h advance compared to RSMs trial.

Keywords: Azo dye; Discoloration; *Pseudomonas putida*; Reactive orange 4; RSM

1. Introduction

Azo dyes are used by a number of food, pharmaceutical, plastic, pesticide, paper and printing, leather, cosmetic, and textile industries. Over 100,000 different commercial dyes and pigments exist, and more than 7×10^5 tons are produced annually worldwide [1]. These dyes include acidic, basic, reactive, disperse, azo, anthraquinone-based, and metal complex dyes, all of which are able to absorb light in the visible region of spectrum. In textile industries, ~10–15% of the dye gets lost in the effluent during the dyeing process. Many dyes are visible in water at concentration as low as 1 mg L^{-1}. Therefore, textile water containing 10–200 mg dye L^{-1} is highly colored and esthetically unacceptable. The presence of dyes along with heavy metals causes severe damage to aquatic biology due to reduced water transparency and gas solubility in lakes, rivers, and other water bodies. This adversely affects the aquatic plant photosynthesis which ultimately reduces the dissolved oxygen content of water. Therefore, treatment of textile effluent prior to discharge in nearby water bodies is necessary. Some physicochemical methods are effective, but have certain limitations such as excessive use of chemicals, accumulation of sludge which poses secondary disposal problem, costly treatment plants, energy intensive processes, expensive operational costs, etc. [2]. Thus, it becomes imperative to look for an alternative technology for textile effluent treatment, which is not only cost-effective, but also eco-friendly.

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Over the last few decades, microbially mediated discoloration/detoxification technologies are being valued over physicochemical ones. Many researchers have studied live microbial systems for remediation of azo dyes from the simulated synthetic minimal salt media as well as the real textile wastewaters [3,4]. The biological techniques include biosorption and biodegradation in aerobic, anaerobic, or sequentially combined anaerobic/aerobic treatment systems employing bacteria, fungi, yeasts, algae, and plants. However, the bacterial treatment process is faster than other biological systems [5]. During the discoloration process by fungi, algae, and plants, adsorption rather than degradation plays a major role; as a result, the dyes remain in the environment. On the other hand, bacteria can degrade many reactive dyes under certain specific optimal culture conditions [6,7]. Even better, the intermediate products of anaerobic metabolism, such as aromatic amines, can be mineralized by the hydroxylase and oxygenase enzymes produced by aerobic bacteria [8]. Under anoxic conditions, many bacteria reduce the highly electrophilic azo bond (–N=N–) in the dye molecule to produce colorless aromatic amines. The resultant amines remain resistant to further anaerobic degradation, and can be toxic and/or mutagenic to animals. However, these amines can be easily degraded, and finally mineralized to nontoxic products by aerobic bacteria [9]. Therefore, if a sequential anaerobic/aerobic system, preferably by facultative anaerobic bacterium, is employed for textile wastewater treatment, the effluent is decolorized anaerobically and the amines can be mineralized aerobically by a hydroxylation pathway involving ring opening mechanism [10].

The textile effluents are highly complex containing variety of dyes, natural impurities released from the fibers and various chemicals such as dispersants, leveling agents, acids, alkalis, salts, and heavy metals used for textile processing. Generally, the textile effluent is highly dark colored, and associated with high biological oxygen demand, chemical oxygen demand, conductivity, and alkalinity. Further, the effluent is deficient in nutrients such as easily metabolizable carbon/energy and nitrogen sources. The different microbes employed in the bioremediation process of textile dyes have specific requirements of physical and nutritional parameters.

The present study was, therefore, an attempt at optimization of cultural (pH, temperature, dose of inoculum, incubation time, and dye concentration) and nutritional (supplementation of various sugars and organic/inorganic nitrogen sources) factors using conventional (one-factor-at-a-time) and statistical response surface methodologies (RSMs) for discoloration of widely used reactive orange 4 dye (Fig. 1) by a facultative anaerobic *Pseudomonas putida* SKG-1 isolate, which was earlier employed in our previous study for the bioremediation of gunny bag-/bagasse-based pulp-/paper-mill effluent. This study was performed in dye simulated synthetic minimal salt medium, hereafter designated as MSM.

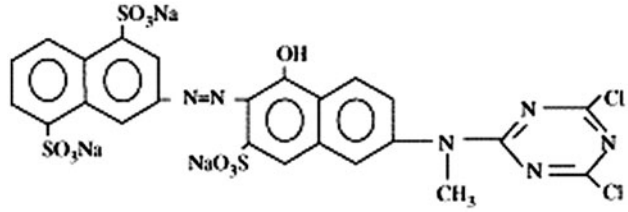

Fig. 1. Chemical structure of reactive orange 4.

2. Materials and methods

2.1. Bacterial culture

P. putida strain SKG-1 (MTCC 10510) used in the present study was previously isolated in our laboratory from dairy sludge [11]. The pure culture was preserved and maintained at 4°C on glucose yeast extract (GYE) agar slants containing ($g\,L^{-1}$): glucose 5.0, yeast extract 5.0, peptone 5.0, and agar 20.0.

2.2. Inoculum preparation

The bacterial inoculum was prepared in above-mentioned GYE broth (pH 8.0). The sterilized medium (100 mL) was inoculated with a loopful of *P. putida* culture and incubated at 30°C in an incubator shaker (150 rpm) for 24 h.

2.3. Culture conditions

The dye discoloration trials were performed in MSM (pH 8.0) containing ($g\,L^{-1}$): K_2HPO_4, 1.0; $CaCl_2$, 0.02; $FeCl_3$, 0.05; and $MgSO_4$, 0.02 with carbon/nitrogen sources as per the treatment and amended with reactive orange 4 dye at 50 $mg\,L^{-1}$ concentration. The medium was inoculated with *P. putida* SKG-1 [Absorbance (A_{620}) 1.27] at 4.0% (v/v) containing 3.2×10^6 colony forming units (cfu) mL^{-1}, and incubated at 30°C for 120 h. The samples were drawn periodically at 24 h intervals, and analyzed spectrophotometrically (UV–vis spectrophotometer 117, Systronics) for bacterial growth and dye discoloration.

2.4. Effect of dye concentration

To determine the effect of varied initial dye concentrations on discoloration and growth response

of *P. putida*, a wide range (50–200 mg L^{-1}) of reactive orange 4 dyes were taken in MSM and incubated for 120 h under static culture condition.

2.5. Effect of inoculum size

The MSM (pH 8.0) containing optimized (as above) dye concentration was inoculated with 1.0–6.0% (v/v) exponentially growing culture (A_{620} 1.27) containing 3.2×10^6 cfu mL^{-1} and incubated for 96 h under no shaking condition.

2.6. Effect of carbon and nitrogen sources

The effect of nutritional parameters such as carbon (viz. glucose, sucrose, and starch) at 1.0% (w/v) and nitrogen sources (viz. ammonium sulfate, ammonium chloride, ammonium nitrate, peptone, and urea) at 0.2% (w/v) were evaluated under above optimized conditions so as to find the most suitable carbon and nitrogen source. Thereafter, the optimum concentration of best carbon (at 0.5, 0.7, 1.0, 1.2, and 1.5%, w/v) and nitrogen source (at 0.1, 0.15, 0.2, 0.25, and 0.3%, w/v) for bacterial growth response and dye discoloration were determined.

2.7. Combined effect of temperature and initial pH

The pH of MSM was adjusted in the range of 7.0–9.0 using 0.1 N HCl or 0.1 N NaOH, prior to sterilization. The sterilized screw capped tubes were then inoculated with above optimized dose of inoculum and incubated at 28–37˚C for 96 h in an incubator under static condition.

2.8. Statistical optimization for dye discoloration using Box–Behnken design

In the present study, Box–Behnken design was applied using Design-Expert software. The three-level design was operated for three variables, viz. dye concentration [(A), mg L^{-1}], sucrose (B), and peptone [(C), %, w/v]. These variables were chosen, as the critical factors, based on the findings from experiments performed employing one-factor-at-a-time approach. The statistical significance of model equation and model terms was evaluated by *F* test and analysis of variance (ANOVA). Quality of the quadratic model equation was expressed by determination coefficient R^2 and adjusted R^2. Optimal values were obtained by solving the regression equation. The 3D response surface plots were used for analyzing the interactive effect of each variable.

The effect of each parameter on reactive orange 4 dye discoloration was studied at three different levels (−1, 0, and +1) with minimum, central, and maximum values (Table 1). Seventeen experimental setups were obtained (Table 2). A second-order polynomial equation (Eq. (1)) was used for the analysis of dye discoloration, and the data were fitted in the equation by multiple regression procedure. This resulted in an empirical model. The model equation for analysis is as under:

$$Y = \beta_o + \sum \beta_n X_n + \sum \beta_{nn} X_n^2 + \sum \beta_{nm} X_n X_m \quad (1)$$

where Y is the predicted response, β_o offset term, β_n liner coefficient, β_{nn} squared coefficient, β_{nm} interaction coefficient, X_n nth independent variable, X_n^2 squared effect, and X_nX_m interaction effects. The predicted values for dye discoloration were obtained by applying quadratic model (Design-Expert software). ANOVA was used to analyze the responses under different combinations as defined by the design (Table 3).

2.9. Bench-scale bioreactor level dye discoloration

Under RSM optimized cultural conditions [w/v, reactive orange 4 concentration (50 mg L^{-1}), sucrose (0.7%), and peptone (0.28%)], the dye discoloration was performed in a stirred tank bioreactor (Bioflo 110, New Brunswick Scientific Co., Inc., Edison, NJ, USA) of 3 liter capacity by *P. putida* SKG-1 isolate under static culture conditions. The fermentor was equipped with direct drive dual Rushton style impeller, PID temperature, agitation control, probes, and controller of pH and DO. The MSM (3.0 L) was inoculated (at optimized 5.0%, v/v) with *P. putida* culture, and fermentor operated without aeration and agitation. The samples (5.0 mL each) were drawn periodically at 12 h intervals up to 72 h. The bacterial growth and dye discoloration were assessed as per the analytical determinations.

Table 1
Experimental range and the levels of three independent variables employed in RSM in terms of actual and coded factors

	Levels		
Variables	–1	0	+1
Dye concentration (mg L^{-1})	50	60	70
Sucrose (%, w/v)	0.6	0.7	0.8
Peptone (%, w/v)	0.22	0.25	0.28

Table 2
Experimental designs used in RSM studies by using four independent variables showing observed and predicted values of reactive orange dye decolorization

Standard order	Factor A (sucrose, %, w/v)	Factor B (peptone, %, w/v)	Factor C (dye level, mg L^{-1})	Dye discoloration (%)	
				Actual response	Predicted response
1	0.60	0.22	60	84.50	84.36
2	0.80	0.22	60	88.20	87.84
3	0.60	0.28	60	87.00	87.36
4	0.80	0.28	60	91.40	91.54
5	0.60	0.25	50	93.50	93.30
6	0.80	0.25	50	96.20	96.22
7	0.60	0.25	70	80.60	80.57
8	0.80	0.25	70	85.10	85.30
9	0.70	0.22	50	93.50	93.84
10	0.70	0.28	50	97.80	97.64
11	0.70	0.22	70	82.30	82.46
12	0.70	0.28	70	85.70	85.36
13	0.70	0.25	60	87.10	87.42
14	0.70	0.25	60	87.40	87.42
15	0.70	0.25	60	87.80	87.42
16	0.70	0.25	60	87.50	87.42
17	0.70	0.25	60	87.30	87.42

Table 3
ANOVA for response surface quadratic model for dye discoloration

Source	Sum of squares	Df	Mean square	*F*-value	*p*-value
Model	347.80	9	38.64	290.71	<0.0001
A-Sucrose	29.26	1	29.26	220.13	<0.0001
B-Peptone	22.44	1	22.44	168.85	<0.0001
C-Dye level	279.66	1	279.66	2,103.85	<0.0001
AB	0.12	1	0.12	0.92	0.3690
AC	0.81	1	0.81	6.09	0.0429
BC	0.20	1	0.20	1.52	0.2569
A^2	0.40	1	0.40	3.04	0.1246
B^2	1.86	1	1.86	14.01	0.0072
C^2	12.75	1	12.75	95.90	<0.0001
Residual	0.93	7	0.13		
Lack of fit	0.66	3	0.22	3.30	
Pure error	0.27	4	0.067		
Cor total	348.73	16			
Standard deviation		0.36	*R*-squared		0.9973
Mean		88.41	Adjusted *R*-squared		0.9939
Coefficient of variation (C.V.%)		0.41	Predicted *R*-squared		0.9684
PRESS		11.02	Adequate precision		61.018

3. Analytical determinations

3.1. Bacterial growth

The growth of SKG-1 strain was determined at every 24 h interval in dye containing synthetic medium. For bacterial growth measurement, the control and experimental samples (1.0 mL each) were centrifuged at 10,000 rpm (4°C) for 10 min and supernatant decanted. The bacterial pellets were washed with sterilized deionized water to remove the color present on

bacterial cell surface and centrifuged as above. The washed pellets were resuspended in deionized water (1.0 mL each) and used for bacterial growth determination spectrophotometrically at 620 nm.

3.2. Dye discoloration assay

In order to determine the extent of dye discoloration, the samples drawn at every 24 interval during 96 h incubation were centrifuged at 10,000 rpm for 10 min in refrigerated centrifuge (4°C). The supernatant was analyzed spectrophotometrically at 482 nm [12] against uninoculated dye medium for dye discoloration. The extent of dye discoloration was calculated from the difference between initial (at 0 h) and final (at different incubation times) absorbance using Eq. (2) as under:

$$\text{Decolorization}\,(\%) = \frac{\text{Initial absorbance} - \text{Final absorbance}}{\text{Initial absorbance}} \times 100 \quad (2)$$

3.3. Statistical analyses

All experiments were performed in triplicate. The standard deviation was calculated using Microsoft Excel program, and results are presented as mean ± SD values.

4. Results and discussion

In biological treatment processes, various operational parameters such as the level of oxygen, dye concentration, inoculum size, supplementation of different carbon and nitrogen sources, pH, and temperature directly influence the bacterial discoloration performance of azo dyes. Thus, to make the process more efficient, faster, and practically applicable, the effect of each factor on the bacterial discoloration of azo dyes should be studied.

4.1. Effect of dye concentration

The type and concentration of dyestuff can influence the efficiency of its removal through a combination of factors including the toxicity of dye at higher concentrations, and ability of enzyme(s) to recognize the substrate efficiently at very low concentrations that may be present in some wastewaters. Fig. 2 depicts a direct correlation between growth response of *P. putida* and the extent of reactive orange 4 dye discoloration. The results further reveal that increasing concentration of dye (50–200 mg L^{-1}) was inhibitory

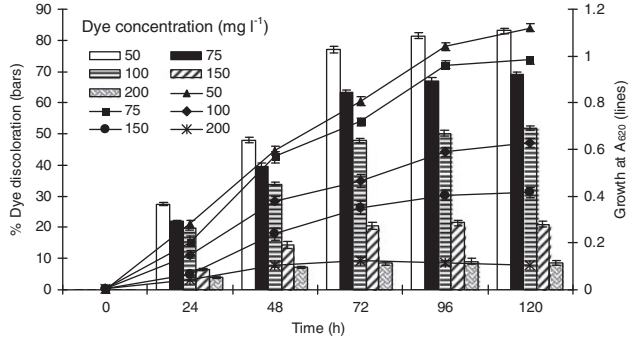

Fig. 2. Effect of reactive orange 4 concentration (50–200 mg L^{-1}) on dye decolorization and growth response of *P. putida* at unoptimized pH 8.0, 30°C, and 4.0% (v/v) inoculum under static culture conditions (*Error bars* depict standard deviation).

for the growth as well as the extent of dye discoloration at every time of incubation (24–120 h) under study. The decline in discoloration efficiency at higher dye concentration may be due to the toxicity of sulfonic acid (SO_3H) groups present on reactive orange 4 which may exert inhibitory effect on the growth of SKG-1 strain.

At 50–100 mg dye L^{-1} MSM, the growth and discoloration increased with time throughout the incubation period up to 120 h. However, at 150 and 200 mg dye L^{-1}, both the determinations increased in harmony with time up to 96 h, followed by a slight decrease at 120 h incubation. Maximum growth (A_{620} 1.12) and dye discoloration (83.0%) were evident at 50 mg dye L^{-1} within 120 h incubation (Fig. 2), and therefore, selected for further studies.

Khehra et al. [13] and Kalme et al. [14] reported that dye discoloration can be strongly inhibited when a high concentration of dyestuff was used to examine the toxic effect of dye on the degrading micro-organisms. In consonance with our findings, Wang et al. [15] observed that the discoloration of reactive red 180 by *Citrobacter* sp. CK3 decreased with increase in the initial dye concentration. The time required for discoloration proportionately increased with increase in dye concentration [9]. The authors observed 90.0% discoloration of acid orange 10 within 16, 20, and 36 h for the concentrations of 100, 250, and 500 mg dye L^{-1}, respectively, by *P. putida* MTCC 102. The survey of literature suggests that increasing dye concentration decreases the discoloration rate probably due to the toxic effect of dyes against individual bacteria, inadequate biomass concentration (or improper cell to dye ratio), and/or blockage of azoreductase active sites by dye molecules [16–18]. Further, the reactive azo dyes

with SO_3H groups inhibit the growth of micro-organisms at higher dye concentrations [19,20].

4.2. Effect of inoculum size

The results in Fig. 3 illustrate that at every dose of inoculum (1.0–6.0%, v/v) under study, the growth response and dye discoloration increased with time during 24–96 h incubation. When the inoculum size was increased from 1.0 to 5.0% (v/v), the extent of growth and discoloration increased in synchrony with each other at every time up to 96 h incubation. Further increase in inoculum size from 5.0 to 6.0% decreased the extent of dye discoloration and growth response during 72–120 h incubation. However, both the determinations decreased with nearly all inoculum sizes during 96–120 h incubation. Maximum growth (A_{620} 1.16) and dye discoloration (87.5%) were attained with 5.0% (v/v) inoculum within 96 h incubation (Fig. 3). Therefore, 5.0% (v/v) dose of *P. putida* inoculum was chosen optimum for further dye discoloration studies. Contrary to our findings, Bayoumi et al. [21] could not find any distinct relationship between inoculum size of *Comamonas acidovorans*-TM1 and *Burkholderia cepacia*-TM5 and textile azo dyes (acid orange 7 and direct blue 75) discoloration. Whereas, Ponraj et al. [22] reported 4.0, 6.0, 6.0, and 10.0% (v/v) inocula optimum for 86.72, 67.19, 53.91, and 50.0% discoloration of reactive orange 3R dye by species of *Bacillus*, *Klebsiella*, *Salmonella*, and *Pseudomonas*, respectively.

4.3. Effect of carbon and nitrogen supplements

Micro-organisms require carbon (C) and nitrogen (N) sources in certain proportions (ratio) for their proper growth and metabolism. Figs. 4(a) and 5(a) depict the influence of carbon (glucose, sucrose, and starch) co-substrate and different organic/inorganic nitrogen sources (yeast extract, ammonium sulfate, ammonium nitrate, ammonium chloride, peptone, and urea) on the growth of SKG-1 strain and dye discoloration under optimized 50 mg reactive orange 4 L^{-1} MSM, 5.0% inoculum at unoptimized pH 8.0 and 30˚C temperature under still culture conditions during 96 h incubation.

Maximum growth (A_{620} 1.29) and dye discoloration (90.2%) were evident with sucrose as a co-substrate at 1.0% (w/v) level during 96 h incubation (Fig. 4(a)). Further, the effect of varied sucrose concentrations (0.5–1.5%, w/v) revealed maximum 91.3% dye discoloration and growth (A_{620} 1.29) at 0.7% sucrose concentration (Fig. 4(b)).

The extent of dye discoloration and growth response in the presence of glucose as a co-substrate

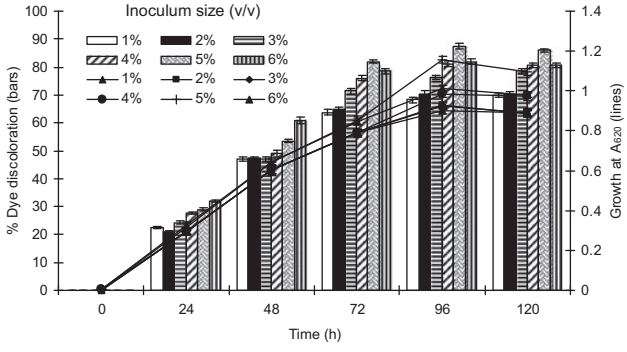

Fig. 3. Effect of inoculum size (1.0–6.0%, v/v) on dye decolorization and growth response of *P. putida* at unoptimized pH 8.0, 30˚C, and optimized 50 mg reactive orange 4 dye L^{-1} concentration under static culture conditions (*Error bars* depict standard deviation).

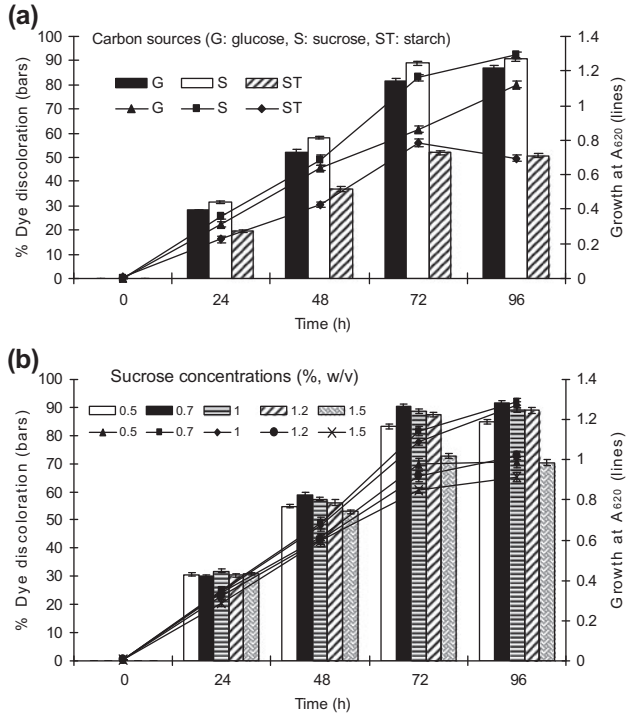

Fig. 4. (a) Effect of carbon sources (glucose, sucrose and starch at 1.0%, w/v) on reactive orange 4 dye (at optimized initial 50 mg L^{-1}) decolorization and growth of SKG-1 strain at unoptimized pH 8.0, 30˚C, and optimized 5.0% (v/v) inoculum under static culture conditions (*Error bars* depict standard deviation) and (b) Effect of varied sucrose concentrations (0.5–1.5%, w/v) on reactive orange 4 dye (at optimized initial 50 mg L^{-1}) decolorization and growth of SKG-1 strain at unoptimized pH 8.0, 30˚C, and optimized 5.0% (v/v) inoculum under static culture conditions (*Error bars* depict standard deviation).

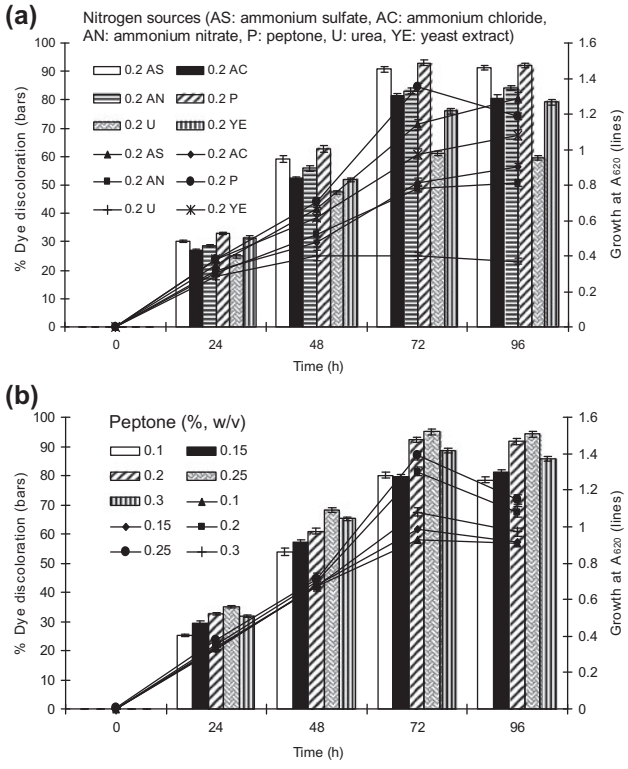

Fig. 5. (a) Effect of nitrogen sources (at 0.2%, w/v) on reactive orange 4 dye decolorization and growth of *P. putida* at optimized 50 mg dye L^{-1} concentration, 5.0% (v/v) inoculum, 0.7% (w/v) sucrose and unoptimized pH 8.0, and 30°C temperature under static culture conditions (*Error bars* depict standard deviation) and (b) Effect of best nitrogen source (peptone) concentration (0.1–0.3%, w/v) on reactive orange 4 decolorization and growth of *P. putida* at optimized 50 mg dye L^{-1} concentration, 5.0% (v/v) inoculum, 0.7% (w/v) sucrose, unoptimized pH 8.0, and 30°C under static culture conditions (*Error bars* depict standard deviation).

was slightly less than sucrose, and minimum response for both the determinations was observed with starch as a carbon source (Fig. 4(a)). Similar to our findings, Ponraj et al. [22] reported sucrose (at 1.0%, w/v) as the best co-substrate for maximum 87.8% discoloration of orange 3 K dye by *Bacillus* sp. Conversely, Alalewi and Jiang [23] reported starch as the best carbon co-substrate for acid orange 7 and direct blue 75 discoloration by *C. acidovorans* and *B. cepacia* isolates. On the other hand, many researchers reported glucose as the best co-substrate for discoloration of reactive azo dyes by *Pseudomonas luteola* [24], remazol black B by halotolerant/halophilic bacteria [6], and reactive red 180 by *Citrobacter* sp. [15]. It is evident from the foregoing that the preference of carbon/energy co-substrate is variable for different micro-organisms. The increase in dye discoloration after supplementation of carbon source is attributed to the fact that dyes are deficient in carbon content, and biodegradation without any extra carbon/energy source is difficult [25].

Among various organic and inorganic nitrogen supplements under study, all sources were variedly effective in dye discoloration; however, the former were better than the latter sources. The extent of growth (A_{620}1.39) and dye discoloration of 93.0% were maximum in the presence of peptone at 0.2% (w/v) level within 72 h incubation (Fig. 5(a)). Further increase in incubation time from 72 to 96 h did not significantly affect both the determinations. Other nitrogen sources employed in the present study may be arranged in the following decreasing order based on their efficiencies towards dye discoloration at 72 h incubation: ammonium sulfate (90.7%) > ammonium nitrate (83.0%) > ammonium chloride (81.2) > yeast extract (76.0%) > urea (61.0%). Further, the dye discoloration tested at varied peptone concentrations (0.1–0.3%, w/v) revealed maximum dye discoloration (95.0%) and growth (A_{620} 1.39) with 0.25% peptone at 72 h incubation.

Similar to our results, other researchers [21,26] also reported peptone as the best nitrogen source for dye discoloration by different bacterial strains. Whereas, Kapdan et al. [27] supplemented yeast extract as a dual carbon and nitrogen source for discoloration of various dyes by mixed bacterial population. Contrary to our findings, Mendez-Paz et al. [28] used inorganic nitrogen source (NH_4Cl) as the best supplement for anaerobic discoloration of azo dye acid orange 7.

4.4. Combined effect of pH and temperature

The growth behavior of different bacterial strains varies with variation in pH and temperature for discoloration of various textile dyes. The pH tolerance of decolorizing bacteria is quite important because reactive azo dyes bind to cotton fibers by addition or substitution mechanism under alkaline condition [29]. In micro-organisms, the cells also respond to temperature changes by adaptation via biochemical or enzymatic mechanisms. Consequently, temperature is also a factor of paramount importance for all processes associated with microbial vitality, including the remediation process. Some studies dealing with the activation energy of microbial discoloration of azo dyes have been undertaken [30], in which narrow temperature ranges were determined as being necessary for the discoloration of azo dyes by extremely complex consortia of micro-organisms.

Table 4 illustrates the capability of SKG-1 strain to grow and decolorize reactive orange 4 dye at 28–37˚C and pH range of 7.0–9.0. The increase in pH of medium from 7.0 to 8.0 resulted in increased growth and dye discoloration in harmony with time during 24–72 h, irrespective of incubation temperature employed. However, the increased pH from 8.0 to 9.0 caused decrease in both the determinations. At every pH and temperature under study, the growth and dye discoloration increased with time during 24–72 h incubation. Maximum growth response (A_{620} 1.31) and highest degree of dye discoloration (95.2%) occurred at optimum pH 8.0 and 35˚C within 72 h incubation. With increase in temperature from 28 to 35˚C, both the determinations increased with time, irrespective of initial pH of the medium in the range of 7.0–9.0. The discoloration rate increased marginally (93.1–95.2%) up to the optimal temperature (30–35˚C), and afterwards (at 37˚C) there was reduction (by 4.8%) in the extent of discoloration. This decline at slightly higher temperature can be attributed to reduced cell viability or slight denaturation of an azo reductase enzyme [18]. The results further reveal that any deviation in pH and/or temperature from optimum decreased the extent of dye discoloration and growth response of SKG-1 strain.

The pH and temperature have some effect on the efficiency of dye discoloration, and that optimal conditions vary between pH 7.0–9.0 and 28–37˚C, respectively [6,22,26]. Majority of azo dye reducing bacterial species perform best discoloration at or near neutral pH [14,31,32]. Jadhav et al. [33] showed that *Pseudomonas aeruginosa* BCH decolorized remazol red maximally (97.0%) at pH 7.0, while at pH 9.0 only 20.0% discoloration was evident. Likewise, Patel et al. [34] noted highest discoloration rate of acid maroon V by consortium EDPA at pH 7.0 and 8.0 and temperature 30–37˚C. Tripathi and Srivastava [9] reported pH 7.0 and temperature 37˚C optimum for maximum discoloration of acid orange 10 by *P. putida* MTCC 102.

The rate of color removal is higher at the optimum pH, and tends to decrease rapidly at strongly acidic or alkaline pH. It is thought that the effect of pH may be related to the transport of dye molecules across the cell membrane, which is considered as the rate limiting step for dye discoloration [35,36].

4.5. Statistical optimization for reactive orange 4 dye discoloration

This is the first report on statistical optimization for reactive orange 4 dye discoloration by strain. Interactive effects of the important conventionally optimized factors, initial concentration of dye, carbon (sucrose) and nitrogen (peptone) sources, were examined by RSM using Box–Behnken design. ANOVA yielded the following regression equation (Eq. (3)) in terms of dye discoloration (Y) as a function of initial concentration of dye (A), carbon (sucrose, B), and nitrogen (peptone, C):

$$Y = 87.42 + 1.91 \times A + 1.68 \times B - 5.91 \times C + 0.17 \times AB + 0.45 \times AC - 0.22 \times BC - 0.31 \times A^2 + 0.66 \times B^2 + 1.74 \times C^2 \quad (3)$$

Table 2 shows the predicted responses of Box–Behnken design on the basis of above polynomial equation (Eq. (3)). This regression equation was assessed statistically for ANOVA, and the results are presented in Table 3. ANOVA of regression model for dye discoloration demonstrated the correlation coefficient (R^2) 0.9973, which means 99.73% variability in the response could be explained by this model. The R^2 value is always between 0 and 1.0. The model is stronger and predicts better response when R^2 value is closer to 1.0 [37]. The value of adjusted correlation coefficient (adjusted R^2) is 0.9939. This higher value of adjusted R^2 indicates greater significance of the model.

The adequate precision values of 61.018 measure signal-to-noise ratio, and a ratio >4.0 is desirable. In this case, higher ratio indicates an adequate signal, and also proves that model can be used to navigate the design space. The *F*-value of 220.132 (Table 3) implies that the model is significant. From ANOVA analysis, lower value of the coefficient of variation (C.V. 0.41% for dye discoloration) indicates a better precision and reliability of the experiments performed. The C.V. as the ratio of the standard error of estimated to the mean value of the observed response (as a percentage) is a measure of reproducibility of the model. As a general rule, a model can be considered reasonably reproducible if its C.V. is not greater than 10% [38].

Response surface curves for the variation in dye discoloration were constructed, and are depicted in Fig. 6. In each set, two variables varied within their experimental range, while third variable remained constant at zero level. Fig. 6(a) depicts reactive orange 4 dye discoloration with respect to peptone vs. sucrose concentration.

The flattened curve indicates that dye discoloration was not significantly affected with increased or decreased levels of carbon (sucrose) and nitrogen (peptone) sources when dye concentration was kept constant. However, Fig. 6(b) depicts that increased reactive orange 4 concentration was inhibitory for dye

Table 4
Combined effect of pH and temperature on reactive orange discoloration and growth response of SKG-1 strain

Temperature (°C)	Incubation time (h)																			
	0					24					48					72				
	Initial pH																			
	7.0	7.5	8.0	8.5	9.0	7.0	7.5	8.0	8.5	9.0	7.0	7.5	8.0	8.5	9.0	7.0	7.5	8.0	8.5	9.0
Growth (A_{620})																				
28	0.002	0.003	0.002	0.004	0.003	0.237	0.287	0.302	0.225	0.157	0.502	0.598	0.690	0.512	0.439	0.795	0.856	0.982	0.813	0.772
30	0.004	0.002	0.003	0.003	0.004	0.253	0.275	0.375	0.265	0.173	0.613	0.650	0.722	0.645	0.502	0.815	0.872	1.350	0.879	0.823
32	0.003	0.004	0.002	0.004	0.002	0.262	0.292	0.388	0.279	0.195	0.642	0.685	0.740	0.658	0.541	0.837	0.912	1.227	0.931	0.815
35	0.004	0.002	0.004	0.003	0.003	0.279	0.305	0.410	0.285	0.207	0.665	0.712	0.775	0.679	0.595	0.865	0.970	1.315	0.985	0.869
37	0.002	0.003	0.003	0.003	0.003	0.260	0.288	0.392	0.237	0.168	0.608	0.619	0.683	0.585	0.522	0.790	0.887	1.128	0.930	0.791
Decolorization (%)																				
28	0	0	0	0	0	20.2	23.5	29.1	18.5	13.9	45.9	56.1	62.5	48.2	39.7	65.3	72.8	90.7	61.5	54.0
30	0	0	0	0	0	23.0	26.9	34.5	29.2	21.0	52.5	57.9	66.8	51.7	42.8	71.5	76.1	93.1	67.8	57.2
32	0	0	0	0	0	25.7	32.1	37.0	31.8	26.5	55.8	60.4	67.1	53.9	47.1	73.0	79.5	93.9	69.2	61.5
35	0	0	0	0	0	26.1	34.2	39.2	34.5	25.8	57.1	62.5	69.3	57.0	50.5	75.2	83.7	95.2	73.9	66.1
37	0	0	0	0	0	21.5	30.8	30.7	33.1	24.2	51.9	58.0	61.9	54.1	39.5	74.0	80.2	90.4	71.5	62.9

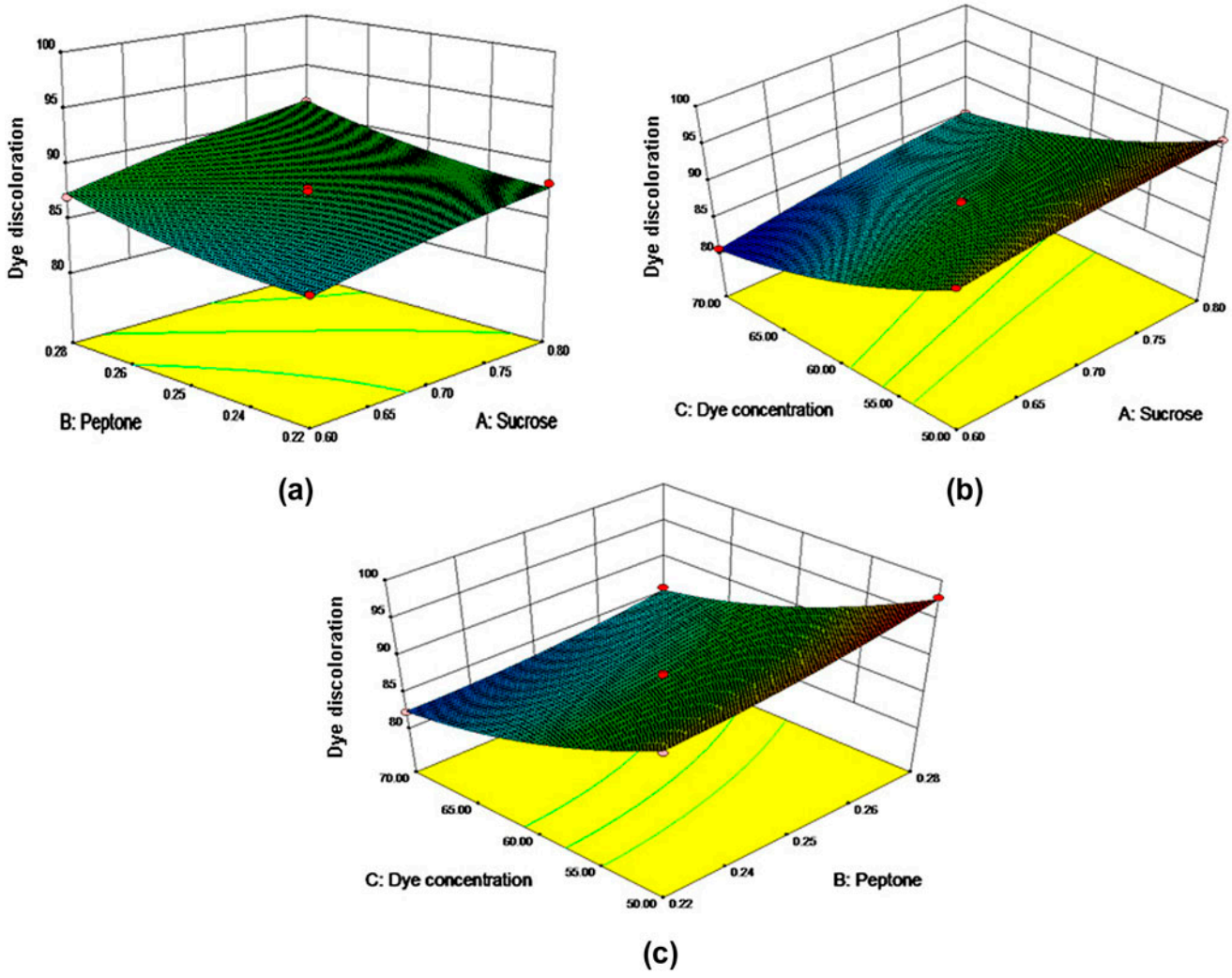

Fig. 6. Response surface curves showing interactive effects of (a) peptone and sucrose, (b) dye concentration and sucrose, (c) dye concentration and peptone, on reactive orange 4 decolorization by *P. putida* SKG-1.

discoloration when peptone was at constant concentration. Fig. 6(c) shows the maximum dye discoloration (97.8%) with optimal 0.28% (w/v) peptone, 0.7% sucrose, and 50 mg reactive orange 4 L^{-1} concentration during 72 h incubation. This accorded a run number of 10, which is considered as the optimal condition of test variables (Table 2). The results further reveal 97.8% dye discoloration at reactive orange 4 concentration 50 mg L^{-1} (−1 in coded unit), sucrose 0.7% (0 in coded unit), and peptone 0.28% (+1 in coded unit). The above optimized results (Table 2) concerning three variables were repeated once again for final verification, and confirmed 97.8% reactive orange 4 dye discoloration at 72 h incubation.

With RSM, the interactions of possible influencing parameters on treatment ability can be evaluated with a limited number of experiments. Whereas in conventional approach, optimization is usually carried out by varying one-factor-at-a-time, while keeping all other factors constant under specific set of conditions. Therefore, it is not only time-consuming, but also usually incapable of reaching the true optimum due to ignoring the eventual interactions among variables.

4.6. Dye discoloration at bench-scale bioreactor level

Fig. 7 depicts maximum bacterial growth (A_{620} 1.38) and dye discoloration (98.0%) within 60 h under RSM optimized conditions. The results reveal that the maximum reactive orange 4 discoloration was achieved in 12 h advance (98.0% within 60 h) compared to screw capped tubes level trial using

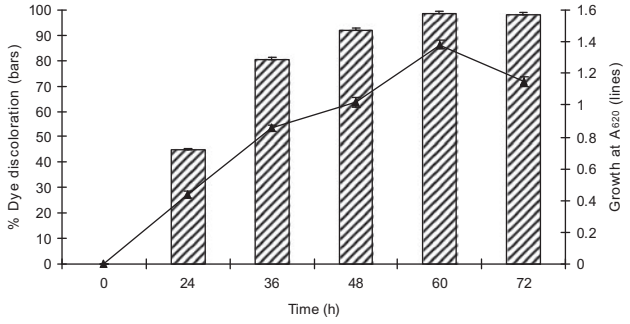

Fig. 7. Bioreactor trial for reactive orange 4 dye discoloration under optimized conditions (0.28% (w/v) peptone, 0.7% sucrose, and 50 mg reactive orange 4 L^{-1} concentration during 72 h incubation) of RSM using Box–Behnken design.

Box–Behnken design (97.8% in 72 h). Therefore, enhanced level of dye discoloration, in terms of time (saving of 12 h), was achieved by *P. putida* at bench-scale bioreactor level.

5. Conclusions

The statistical response surface methodological approach has not been reported earlier for optimization of process variables for discoloration of reactive orange 4 dye by *P. putida* SKG-1 isolate. This strain proved high extent of dye discoloration under specific range of environmental conditions of pH and temperature. Optimization of various process parameters employing Box–Behnken design suggests that dye concentration is the most critical factor for efficient discoloration of reactive orange 4 dye. High level of dye discoloration (98.0%) at bioreactor level occurred in 60 h under optimum cultural and nutritional conditions with a time-wise economy of 12 h. Therefore, under specific range of cultural and nutritional conditions, *P. putida* SKG-1 has better potential for discoloration of azo reactive dye containing textile effluents. However, the potential of this strain needs to be demonstrated for its application in bioremediation of real textile dye effluent, as variety of reactive dyes with various chemical structures present therein may respond differently to the treatment.

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