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Optimization of physicochemical parameters for phenol biodegradation by *Candida tropicalis* PHB5 using Taguchi Methodology

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ABSTRACT

The Taguchi orthogonal array (OA) design of experiments methodology, a quality optimization tool, was used to improve the phenol biodegradation potential of the yeast *Candida tropicalis* PHB5. At three levels, an OA was selected to analyze the effects of the different physicochemical process factors. Experiments were undertaken to confirm the effectiveness of this method and the main factors affecting the growth of *C. tropicalis* on phenol and its subsequent degradation were found, together with the optimal factor levels. Predicted results showed that biomass yield could be increased from 1,051.96 to 2,495.74 mg/l and the subsequent amount of phenol degraded could be increased from 879.42 to 2,386.43 mg/l. Based on Taguchi methodology, an overall enhancement of growth by 137.24% and phenol degradation by 171.49% could be attained. Validation experiments showed that the growth and phenol biodegradation was significantly improved by up to 132.4% and 165.64%, respectively.

Keywords: Optimization; Degradation; Phenol; *Candida tropicalis*; Taguchi DOE methodology

1. Introduction

Phenol and phenolic compounds are ubiquitous pollutants which come to the natural water resources from the effluents of a variety of chemical industries like petrochemical industries and petroleum refineries, pharmaceuticals, coking operations, coal refining, resin industries, ceramic plants, and steel plants [1,2]. Because of their toxicity to aquatic life, plant, and many other organisms as well as to human, hence, removal of such pollutants is of practical importance [3,4].

Although chemical and photochemical oxidation method have been employed in recent time [5], biological treatment of phenol containing wastewater is a practical and convenient way to treat this kind of effluents compared with chemical (no need to add chemicals) and physical methods and has proven to be environmental friendly and cost-effective; because various populations of microorganisms are able to degrade organic compounds and most of effluents can

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be biologically degraded [6,7]. Several immobilization techniques have been employed including activated carbon-filled polyethersulfone composite hollow-fiber membranes to enhance the phenol tolerance and cometabolism of phenol and other phenolic compounds by degrading microorganisms [8,9].

Different species of bacteria, such as *Alcaligenes* [10], *Bacillus* [11], *Acinetobacter, Sphingomonas* [12], *Pseudomonas* [13], *Brevibacillus* [14], *Corynebacterium* [15] have been reported to be capable of degradation of phenol. In addition, several fungal species, having potential for degrading high concentration of phenol, have also been accounted for. These include species of *Paecilomyces* [16], *Rodotorula*, *Trichosporon* [17], etc. This study describes the efficiency of *Candida tropicalis* PHB5 yeast in phenol degradation, that is, this yeast strain can deal with higher phenol concentrations than most of other microorganisms can. Because each microorganism has its own individual physicochemical requirements for growth, good knowledge of how the different influencing physicochemical parameters contribute to the effective removal of phenol from wastewater is prerequisite [18]. Therefore, it was considered worthwhile to study the effect of different media components and to consider various environmental factors that have influence on phenol biodegradation. Optimization of factors affecting growth and phenol biodegradation by microorganisms is a complex process with a number of interactive parameters. However, practically to optimize all the parameters and to establish the best possible conditions by interrelating all the parameters, numerous experiments have to be carried out with all possible parameter combinations which may not be practically and/or economically feasible if the number of factors is high [19]. Taguchi design of experiment (DOE) method was not considered a usual way for optimizing biotechnological processes. In this regard, other methods such as response surface and Plakett–Burman have been preferred because researchers are more familiar with them [20,21]. The Taguchi approach possesses its own limitations for the optimization at specific levels and parameter values spectrum, one may be unable to find if the mid-range combination parameters values exist which might provide the performance in much more better way [22]. Nevertheless, it has the ability to include categorical factors (e.g., nitrogen source) along with continuous ones (e.g., temperature), thereby allowing one to conduct lesser experiments to optimize a particular process, and thus, this method can be adopted in a simplified manner [23].

There are several articles that report the use of response surface methodology for the optimization of influencing factors for phenol degradation [18,24]. However, to the best of our information Taguchi orthogonal array (OA), which is one of the factorial-based designs facilitating the study of a system by a set of independent variables over a specific level [25], has not been implemented for the evaluation of the effects of different physicochemical factors and their optimization for the enhancement of biodegradation of phenol. This approach allows statistically and ideally similar information to be gained with fewer well-defined experimental sets [26] and facilitates to identify the influence of individual factors, establishing the relationship between factors and operational conditions. Taguchi method recommends the use of the *S*/*N* ratio to measure the quality characteristics. Based on the analysis of the *S*/*N* ratio, the optimal levels of the process factors are determined [27]. Furthermore, analysis of variance (ANOVA) is performed to evaluate statistically significant process factors. Finally, an experiment is conducted to verify the optimal process factors obtained from the Taguchi method [28].

This article presents procedural application of Taguchi methodology for optimization and to study the effects of different proportions of nitrogen source, ions, growth temperature, initial pH, agitation, inoculum size, and incubation time, each in three levels on the biodegradation of phenol. The experiments were designed for 10 factors at three levels with OA layout of L_{27} (3^{10}). The results of experiments designed were validated by experiments performed for obtaining maximum levels of degradation of phenol by the yeast *C. tropicalis* PHB5. We found that the optimization of physicochemical parameters by Taguchi DOE methodology enabled phenol biodegradation to be achieved at maximum concentration of 2,400 mg/l, which is fairly higher than the previous report [2,29].

2. Materials and methods

2.1. Microorganism

C. tropicalis PHB5 yeast was isolated from an effluent of steel plant wastewater in Durgapur, India. Identification of the organism was done by 18s rDNA analysis (NCBI GenBank Accession number: JN542555). The microorganism was maintained at 4˚C in inorganic media providing phenol (at concentration 1,000 mg/l) as sole source of carbon and energy.

2.2. Chemicals and analysis

Chemicals used were of analytical and HPLC grade purchased from Sigma Aldrich (USA), Himedia (India), and Merck (India). Water used for the HPLC

analysis was prepared using Ultrapure Water System (Arium^{R}, 611UF, Sartorius, Germany). The statistical software package, Qualitek-4 software (Nutek Inc., MI, USA) was used to analyze the experimental design and the analysis of the experimental data. Qualitek-4 is user-friendly windows-based software that enables to use L-4 to L-64 arrays along with selection of 2 to 63 factors with two, three, and four levels to each factor.

2.3. Taguchi methodology

Taguchi method involves the establishment of different experimental situations through OAs to reduce experimental errors and to enhance the efficiency and reproducibility of experiments. Robust design has been considered in this study because it helps to minimize the effect of noise factor in the process of optimization and leads to a dynamic or robust experimental design [30]. There are four phase for Taguchi methodology study, they are planning, conducting, analysis, and validation and have been schematically represented in Fig. 1. Each phase is separated with distinct objective and is interconnected in sequence wise to achieve the overall optimization process [19].

2.3.1. DOEs (Phase 1)

In the first step of experiments, the various factors to be optimized that have significant effect on the biodegradation of phenol were determined. All the variables were investigated within the feasible range so that the variation inherent in the process does not

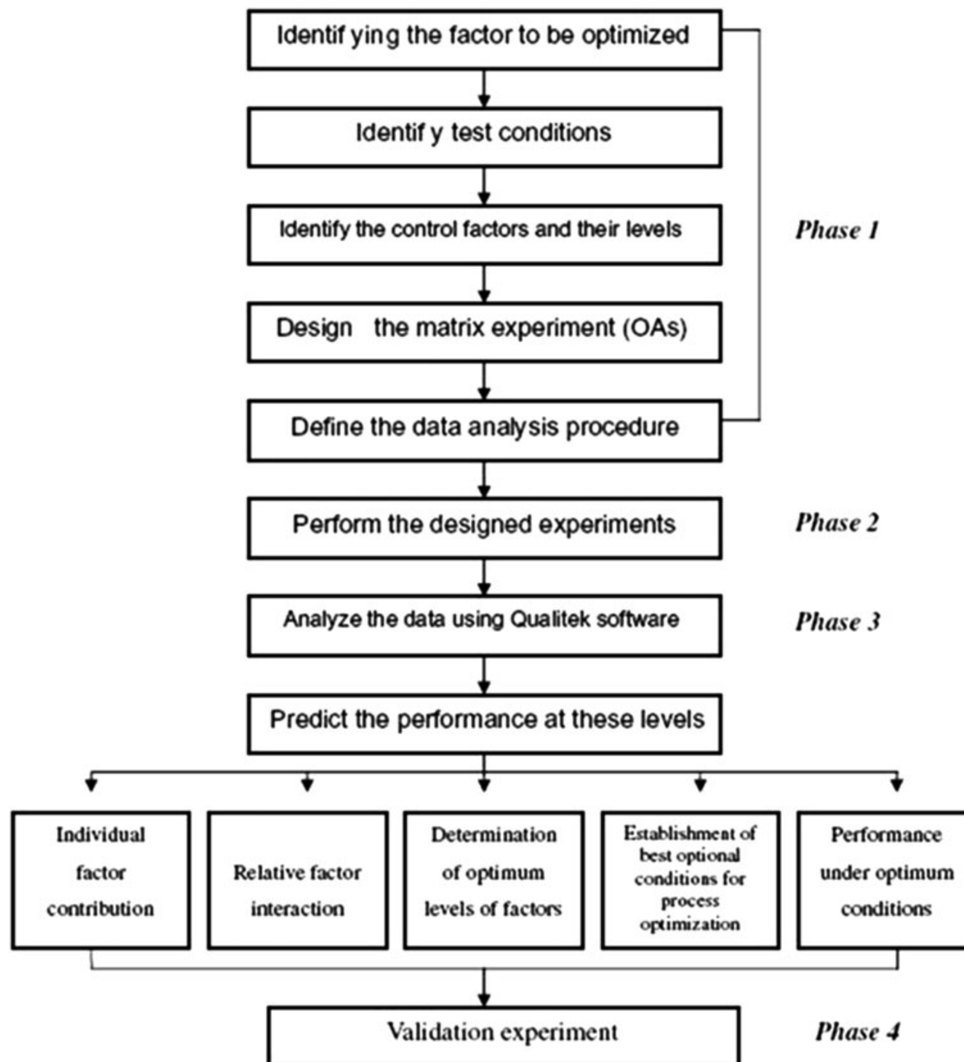

Fig. 1. Schematic representation of the steps involved in the Taguchi DOE methodology [19].

mask the factor effect [19]. In this study, nitrogen source, metal ion source (Mg^{2+}, Ca^{2+}), phosphate, trace elements, inoculum size, pH, temperature, agitation, and incubation time were considered as important factors for growth and phenol degradation by the yeast *C. tropicalis* (Table 1). All the variables were investigated at three widely spaced levels shown in Table 1 decided from previous unreported work. Selection of most suitable nitrogen, phosphate, and metal ion was done by one variable at a time method. In the next step, matrix was designed with the appropriate OAs for the selected parameters and their levels. Taguchi provides many standard OAs and corresponding linear graphs for this purpose [31]. In this study, three levels of 10 factors (Tables 1 and 2) were considered and the size of experimentation was represented by symbolic array of L-27 (which indicated 27 experimental trials).

2.3.2. *Growth and phenol biodegradation by C. tropicalis* with selected factors and levels (Phase 2)

Fresh seed culture ($OD_{550} \approx 0.2$) was inoculated in 50 ml inorganic media in 250-ml Erlenmeyer flask, containing 2,400 mg/l of phenol, and other ingredients opted for optimization; (in g/l) nitrogen source (NH_4NO_3: 0.4, 0.5, and 0.6), magnesium ion ($MgSO_4$: 0.1, 0.2, and 0.3), calcium ion ($CaCl_2$: 0.01, 0.02, and 0.03), phosphate ion (K_2HPO_4:KH_2PO_4 = 1:1; 0.4, 0.5, and 0.6), trace element solution (Table 1) (1X, 2X, and 3X). Biodegradation studies were carried out at different inoculum percentage (1, 2, and 3% v/v), temperatures (28, 30, and 32 degree Celsius), pH (5.0, 6.0, and 7.0), agitation (120, 150, and 180 rpm) and incubation time (40, 48, and 56 h). The culture was centrifuged at 15,000×*g* for 10 min at 4 degree Celsius. The cell pellet was used for growth study, and the supernatant was used for the determination of residual phenol concentration. For dry cell weight estimation, the cell pellet was washed twice with distilled water and then dried in a hot air oven. Residual phenol concentration was determined using HPLC. All experiments were carried out in triplicate. For sample preparation, the supernatant was filtered through 0.45-μm nylon membrane filters (Milipore). After appropriate dilutions with deionized water, samples were analyzed by HPLC system (Waters™ 600) equipped with UV/Visible Detector and a C_{18} hypersil column (4.6 mm × 250 mm; 5 μm particle size; Waters, USA). Mobile phase used was acetonitrile-water (70:30 v/v); at a flow rate of 1 ml/min. Temperature of the column oven was maintained at 30˚C. The sample (20 μl) was injected and analyzed at 270 nm using the UV/visible detector.

2.3.3. *Data analysis and prediction of performance (Phase 3)*

The data obtained from the experiments was processed using Qualitek-4 software (Nutek Inc., MI, USA) to evaluate the influence of individual factors, multiple interaction of the selected factors, determination of optimum conditions and the process performance on growth of the organism and phenol degradation. In this study, *S/N* analysis was employed with bigger-is-better performance characteristics for all the experimental cases to estimate the performance (growth and phenol removal) at the optimum conditions. In the Taguchi method, the term "signal" represents the desirable value (mean) and the term "noise" represents the undesirable value (SD) for the output characteristic [32]. Therefore, the signal-to-noise (*S/N*)

Table 1
Selected factors and their assigned levels taken under investigation

Serial No.	Factor Codes	Factor	Level-1	Level-2	Level-3
1	A	NH_4NO_3 (g/l)	0.4	0.5	0.6
2	B	$MgSO_4,7H_2O$ (g/l)	0.1	0.2	0.3
3	C	$CaCl_2,2H_2O$ (g/l)	0.01	0.02	0.03
4	D	Phosphate ion[a] (g/l)	0.4	0.5	0.6
5	E	Trace Element Solution[b] (% v/v)	1	2	3
6	F	Inoculum size(% v/v)	1	2	3
7	G	Temperature(°C)	28	30	32
8	H	pH	5	6	7
9	I	Incubation time(Hours)	40	48	56
10	J	Agitation (RPM)	120	150	180

[a]phosphate ion contains 1:1 proportion of K_2HPO_4 and KH_2PO_4; [b]Trace element solution contains 0.3 g/l $FeSO_4,7H_2O$; 0.05 g/l $MnSO_4,H_2O$; 0.1 g/l $CoCl_2,6H_2O$; 0.034 g/l $Na_2MoO_4,2H_2O$; 0.04 g/l $ZnSO_4$; and 0.05 g/l $CuSO_4,5H_2O$.

Table 2
L_{27} OA (3^{10}) of designed experiments for 10 factors with actual growth and phenol degradation

Trial No.	A	B	C	D	E	F	G	H	I	J	Biomass (mg/l)			Consumed phenol (mg/l)		
1	1	1	1	1	1	1	1	1	1	1	432.5	450	442.5	368.39	375.87	383.38
2	1	1	1	1	2	2	2	2	2	2	1,127.5	1,175	1,152.5	958.86	978.33	997.89
3	1	1	1	1	3	3	3	3	3	3	912.5	930	8.08	690.11	663.12	676.58
4	1	2	2	2	1	1	1	2	2	2	900	882.5	920	780.98	750.43	765.67
5	1	2	2	2	2	2	2	3	3	3	1,462.5	1,435	1,492.5	1,128.97	1,106.5	1,151.55
6	1	2	2	2	3	3	3	1	1	1	832.5	815	800	578.48	566.96	590.05
7	1	3	3	3	1	1	1	3	3	3	912.5	895	877.5	818.15	801.87	834.51
8	1	3	3	3	2	2	2	1	1	1	1,147.5	1,125	1,102.5	956.46	937.42	975.59
9	1	3	3	3	3	3	3	2	2	2	1,560	1,530	1,500	1,299.6	1,273.74	1,325.59
10	2	1	2	3	1	2	3	1	2	3	1,187.5	1,162.5	1,140	988.32	968.65	1,008.09
11	2	1	2	3	2	3	1	2	3	1	1,287.5	1,237.5	1,262.5	1,050.48	1,071.81	1,093.24
12	2	1	2	3	3	1	2	3	1	2	880	897.5	862.5	845.75	862.92	880.18
13	2	2	3	1	1	2	3	2	3	1	1,625	1,657.5	1,592.5	1,380.23	1,352.77	1,407.84
14	2	2	3	1	2	3	1	3	1	2	817.5	850	832.5	708.45	694.35	722.62
15	2	2	3	1	3	1	2	1	2	3	972.5	1,012.5	992.5	843.44	826.66	860.31
16	2	3	1	2	1	2	3	3	1	2	1,280	1,255	1,305	1,087.4	1,065.76	1,109.15
17	2	3	1	2	2	3	1	1	2	3	892.5	875	910	772.88	742.65	757.73
18	2	3	1	2	3	1	2	2	3	1	1,650	1,617.5	1,682.5	1,402.16	1,430.21	1,374.26
19	3	1	3	2	1	3	2	1	3	2	1,370	1,342.5	1,397.5	1,106.64	1,128.78	1,084.62
20	3	1	3	2	2	1	3	2	1	3	812.5	795	827.5	575.85	587.37	564.39
21	3	1	3	2	3	2	1	3	2	1	960	942.5	980	702.28	716.33	688.31
22	3	2	1	3	1	3	2	2	1	3	1,007.5	1,050	1,030	874.22	856.83	891.71
23	3	2	1	3	2	1	3	3	2	1	870	887.5	852.5	853.5	836.51	870.57
24	3	2	1	3	3	2	1	1	3	2	1,480	1,507.5	1,450	1,231.65	1,281.79	1,256.66
25	3	3	2	1	1	3	2	3	2	1	995	1,015	975	828.23	861.94	845.04
26	3	3	2	1	2	1	3	1	3	2	1,237.5	1,262.5	1,212.5	1,030.83	1,072.8	1,051.76
27	3	3	2	1	3	2	1	2	1	3	902.5	920	885	751.38	781.97	766.64

ratio is the ratio of the mean to the SD. Taguchi uses the *S/N* ratio to measure the quality characteristic deviating from the desired value. A loss function $[L(y)]$ is developed for the deviation [25] as represented by $L(y) = k \times (y - m)^2$, where k denotes the proportionality constant, m represents the target value, and y is the experimental value obtained for each trail. In case of bigger and better quality characteristics the loss function can be written as $L(y) = k \times (1/y^2)$ and the expected loss function can be represented by

$$E[L(y)] = kE(1/_{y^2}) \quad (1)$$

where $E(1/y^2)$ can be estimated from a sample of n as

$$\sum_{i=1}^{n}[1/_{y_i^2}]/n \quad (2)$$

Taguchi used the signal-to-noise *(S/N)* ratio as a performance measurement of a dynamic system to evaluate the robustness of the overall process [33]. The mathematical expression for the S/N ratio for the "bigger is better" case for the performance statistics that measure deviation from the target, called as mean square deviation (MSD) was given by

$$Z = -10\log(MSD) = -10\log\sum_{i=1}^{n}[1/_{y_i^2}]/n \quad (3)$$

2.3.4. Validation of the experimental model

In order to validate the methodology, the optimized culture conditions were finally evaluated by performing the biodegradation experiment.

3. Results

Selection of suitable factors at appropriate level is crucial in achievement of biodegradation of phenol at possible highest concentration. The composition of nutrients and their proportions in the medium and other physical factors ensure phenol biodegradation to its peak. So, in this study, a systematic and robust optimization strategy was adopted to find out the optimum critical parameters for enhancement of phenol biodegradation by *C. tropicalis* PHB5.

3.1. Influence of individual factors:

Experiments studied with the designed experimental conditions showed a significant variation in phenol degradation and biomass production. The average effects of each factor, along with the interaction at the assigned levels, on growth and phenol degradation by *C. tropicalis* was represented in Table 2. Results obtained with the designed experimental sets (Table 3) showed that the process efficiency was found to be very much dependent on the selected process conditions. Individually, both inoculum size and agitation showed significant influential effect at level 2 on both growth and phenol biodegradation, whereas the effects of temperature and $MgSO_4,7H_2O$ were higher in level 2 and level 3 on growth and phenol biodegradation respectively. At level 2, major effects were given by the temperature and inoculum size compared to other factors followed by ammonium nitrate, pH, and agitation in their selected range. The magnitude of difference between the average effects $(L_2 - L_1)$ represents the relative influence of the factor or interaction to the variability of results. The larger the difference, the stronger is the influence [31]. It can be seen from Table 3 that among the studied factors, inoculum size showed stronger influence on the growth followed by temperature, incubation time, ammonium nitrate, pH, agitation, phosphate ion and $MgSO_4,7H_2O$ in their selected range. In case of phenol degradation, temperature showed stronger influence followed by inoculum size, ammonium nitrate, incubation time, agitation, pH, $MgSO_4,7H_2O$ and phosphate ion in their selected range. Fig. 2(a) and (b) represents the influence of the selected factors on growth and phenol degradation. The influence of each individual factor on the growth of the yeast and phenol degradation is shown in Table 3. Increase in the concentration of factors such as $MgSO_4,7H_2O$ and phosphate ion resulted in increased degradation of phenol. In the case of growth of *C. tropicalis*, increase in the concentration of $MgSO_4,7H_2O$ and phosphate ion showed remarkable positive impact, whereas increase in the concentration of $CaCl_2,2H_2O$ and trace element solution showed minor positive effect on growth. In the case of the ammonium nitrate, inoculum size, temperature, pH and agitation, the growth and phenol degradation were higher up to level 2, but a subsequent increase in the concentration level (level 3) proved to be repressive on growth and phenol biodegradation. $CaCl_2,2H_2O$ and trace element solution, though, seemed to have minor role in the growth of the organisms, did not show significant effect on the phenol degradation by the organism (Table 3, Fig. 2).

3.2. Influence of factors interaction

The severity indexes (SIs) of the factors interacting at various levels are depicted in Table 4. It was evaluated from Taguchi DOE that represents the influence of two individual factors at various levels of

Table 3
Main effects of the selected factors

Serial No.	Coded factor	Factor	Biomass production (S/N) ratio				Phenol degradation (S/N) ratio			
			Level 1	Level 2	Level 3	L2-L1	Level 1	Level 2	Level 3	L2-L1
1	A	Ammonium Nitrate (g/l)	59.762	61.141	60.433	1.378	58.001	59.863	58.788	1.862
2	B	$MgSO_4$,$7H_2O$ (g/l)	59.586	60.623	61.127	1.036	57.791	59.079	59.782	1.288
3	C	$CaCl_2$, $2H_2O$ (g/l)	60.157	60.416	60.762	0.259	58.731	58.871	59.051	0.14
4	D	Phosphate ion (g/l)	59.662	60.742	60.932	1.079	58.098	58.691	59.864	0.593
5	E	Trace element Solution (g/l)	60.179	60.423	60.734	0.243	58.787	58.879	58.986	0.091
6	F	Inoculum size (v/v %)	59.222	61.707	60.407	2.484	57.983	60.053	58.616	2.07
7	G	Temperature(°C)	59.179	61.293	60.864	2.113	57.69	59.874	59.088	2.184
8	H	pH	60.041	61.346	59.948	1.304	58.404	59.756	58.492	1.351
9	I	Incubation time(H)	58.791	60.293	62.251	1.502	57.168	58.871	60.614	1.703
10	J	Agitation (RPM)	60.124	61.268	59.944	1.143	58.528	59.943	58.18	1.414

interaction. In this table, the "columns" represents the locations to which the interacting factors are assigned. The 100% SI indicates 90° angle between the lines (factors), while, 0% SI for parallel lines. Reserved column shows the column that should be reserved if this interaction effect has to be studied. "Levels" indicated the level of factors desirable for the optimum conditions. The interaction between two factors gives a better view for overall process analysis. In culture condition, any individual factor may interact with any or all of the other factors, creating the possibility of a large number of interactions [34]. In case of growth and biomass production, the highest interaction (SI 68.33%) was observed in between ammonium nitrate and trace element solution (at levels 2 and 1) followed by ammonium nitrate and agitation (SI 62.37% at levels 2 and 1), pH and agitation (SI 60.77% at levels 2 and 1), $CaCl_2$,$2H_2O$ and pH (SI 54.16% at levels 3 and 2), $CaCl_2$,$2H_2O$ and agitation (SI 51.77% at levels 1 and 2), $CaCl_2$,$2H_2O$ and phosphate ion (SI 51.18% at levels 1 and 2) and phosphate ion and trace element solution (SI 44.24% at levels 3 and 3) (Table 4). It was interesting to note that the trace element solution and ammonium nitrate which have low and relatively high impact factor, respectively, showed a higher SI. In case of $CaCl_2$,$2H_2O$ (lower impact factor), the combination with pH resulted in a higher SI (SI 54.16%). Ammonium nitrate and agitation are high impact factor, and a good SI (SI 62.37%) was found in combination of them. pH and agitation are high impact factor and showed good SI (SI 60.77%) in combination also. An SI of 15.41% was obtained when the high impact factor incubation time combined with low impact factor trace element solution. On the contrary, the SI between $MgSO_4$,$7H_2O$ (a high impact factor) and inoculum size (high impact factor) showed a significantly lower interaction (SI 2.13%) (Table 4). In case of phenol biodegradation, the highest interaction (SI 66.5%) was observed in between ammonium nitrate and trace element solution followed by $MgSO_4$,$7H_2O$ and $CaCl_2$,$2H_2O$ (SI 62.84%), $CaCl_2$,$2H_2O$ and phosphate ion (SI 60.07%), pH and agitation (SI 58.85%), ammonium nitrate and agitation (SI 58.29%), phosphate ion and trace element solution (SI 48.49%), $CaCl_2$,$2H_2O$ and pH (SI 48.12%) and trace element solution and pH (SI 42.53%) (Table 4). Like in case of growth and biomass production, combination of ammonium nitrate which is high impact factor and trace element solution having low impact factor resulted in highest SI (SI 66.5%). However, unlike in case of growth and biomass production, $MgSO_4$,$7H_2O$ and $CaCl_2$,$2H_2O$, in combination gave the second highest SI (SI 62.84%). Trace element solution has least impact factor but showed higher SI, besides ammonium nitrate, in combination with phosphate ion, pH and agitation where phosphate ion, pH and agitation are comparatively high impact factors. Inoculum size showed good SI in combination with $CaCl_2$,$2H_2O$, phosphate ion and ammonium nitrate. On the contrary, inoculum size, being high impact factor, but showed lowest (SI 0.49%) with $MgSO_4$,$7H_2O$ (High impact factor). It is evident from the results that the growth and biodegradation of phenol are quite independent of the individual influence but dependent on interaction of factors. The relative influences of selected factors are depicted in Fig. 3.

3.3. Analysis of variance

ANOVA was used to analyze the experimental data and to determine the variation of result due to each factor. Based on F ratio, it can be observed that

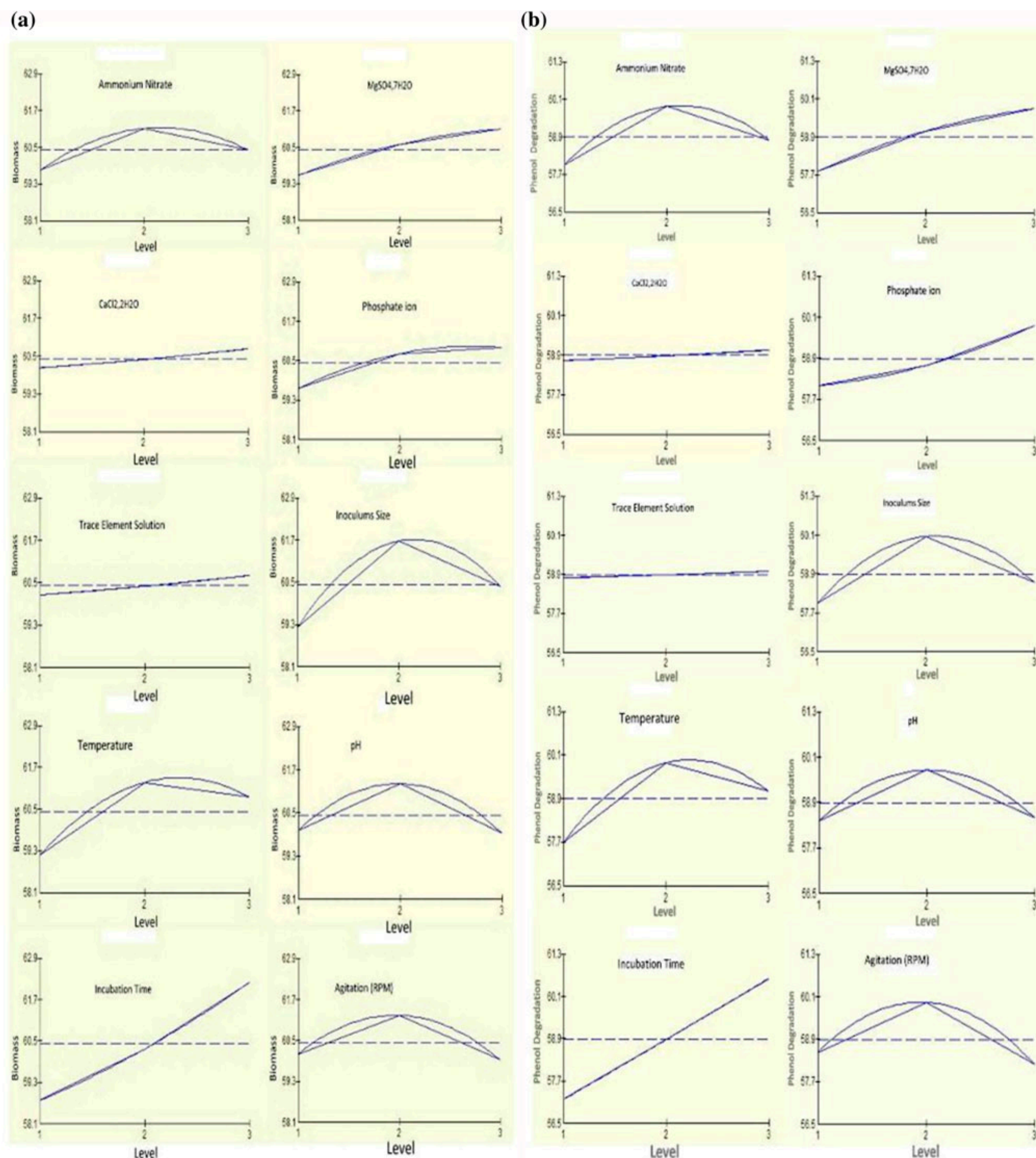

Fig. 2. Individual factors performance at different levels for biomass production (a) and phenol degradation (b).

all factors and interactions considered in the experimental design were statistically significant within 95% confidence level. ANOVA with the percentage of contribution of each factor to interaction is shown in Table 5. Result showed that incubation time contributed the maximum impact (34.132%) on growth and biomass production followed by inoculum size (17.363%), temperature (13.974%), $MgSO_4,7H_2O$ (6.748%), pH (6.677%), agitation (5.593%), ammonium nitrate (5.126%), phosphate ion (5.058%), $CaCl_2,2H_2O$ (0.74%), and trace element solution (0.579%). Likewise, incubation time (30.922%) was the most significant

Table 4
Estimated interaction of the SIs for different factors

SN	Biomass production					Phenol degradation				
	Factors	Columns	SI (%)	RC	Levels	Factors	Columns	SI (%)	RC	Levels
1	Ammonium Nitrate X Trace element solution	1 X 5	68.33	4	[2,1]	Ammonium Nitrate X Trace element solution	1 X 5	66.5	4	[2,1]
2	Ammonium Nitrate X Agitation	1 X 10	62.37	11	[2,1]	$MgSO_4$, $7H_2O$ X $CaCl_2$, $2H_2O$	2 X 3	62.84	1	[3,1]
3	pH X Agitation	8 X 10	60.77	2	[2,1]	$CaCl_2$,$2H_2O$ X Phosphate ion	3 X 4	60.07	7	[1,2]
4	$CaCl_2$, $2H_2O$ X pH	3 X 8	54.16	11	[3,2]	pH X Agitation	8 X 10	58.85	2	[2,1]
5	$CaCl_2$,$2H_2O$ X Agitation	3 X 10	42.06	9	[1,2]	Ammonium Nitrate X Agitation	1 X 10	58.29	11	[2,1]
6	$CaCl_2$,$2H_2O$ X Phosphate ion	3 X 4	51.18	7	[1,2]	Phosphate ion X Trace Element Solution	4 X 5	48.49	1	[3,3]
7	Phosphate ion X Trace Element Solution	4 X 5	44.24	1	[3,3]	$CaCl_2$, $2H_2O$ X pH	3 X 8	48.12	11	[1,2]
8	$MgSO_4$, $7H_2O$ X $CaCl_2$, $2H_2O$	2 X 3	43.87	1	[3,1]	Trace Element Solution X pH	5 X 8	42.53	13	[3,2]
9	Ammonium Nitrate X $CaCl_2$, $2H_2O$	1 X 3	43.34	2	[2,1]	Trace Element Solution X Agitation	5 X 10	39.54	15	[3,2]
10	Trace Element Solution X Agitation	5 X 10	40.63	15	[3,2]	$CaCl_2$, $2H_2O$ X Temperature	3 X 7	38.88	4	[1,2]
11	$CaCl_2$, $2H_2O$ X Temperature	3 X 7	39.8	4	[3,3]	$CaCl_2$, $2H_2O$ X Inoculum Size	3 X 6	36.32	5	[1,2]
12	$MgSO_4$, $7H_2O$ X Phosphate ion	2 X 4	37.52	6	[3,2]	$MgSO_4$, $7H_2O$ X Agitation	2 X 10	35.99	8	[3,2]
13	$MgSO_4$, $7H_2O$ X Trace Element Solution	2 X 5	36.91	7	[3,3]	$MgSO_4$, $7H_2O$ X Phosphate ion	2 X 4	35.41	6	[3,2]
14	Phosphate ion X pH	4 X 8	36.4	12	[3,2]	Ammonium Nitrate X Incubation Time	1 X 9	34.66	8	[2,3]
15	Inoculum Size X pH	6 X 8	36.02	14	[3,2]	Phosphate ion X pH	4 X 8	34.36	12	[3,2]
16	Trace Element Solution X pH	5 X 8	34.58	13	[3,2]	$CaCl_2$,$2H_2O$ X Agitation	3 X 10	33.25	9	[1,2]
17	Temperature X Agitation	7 X 10	33.59	13	[3,2]	Temperature X Agitation	7 X 10	32.32	13	[3,2]
18	$MgSO_4$, $7H_2O$ X Agitation	2 X 10	32.8	8	[3,2]	Phosphate ion X Inoculum Size	4 X 6	30.89	2	[3,3]
19	Ammonium Nitrate X Temperature	1 X 7	32.45	6	[2,3]	Ammonium Nitrate X Inoculum Size	1 X 6	30.61	7	[2,2]
20	$CaCl_2$, $2H_2O$ X Inoculum Size	3 X 6	31.83	5	[1,2]	Trace Element Solution X Inoculum Size	5 X 6	30.58	3	[1,2]
21	Ammonium Nitrate X Inoculum Size	1 X 6	30.11	7	[2,2]	Ammonium Nitrate X $CaCl_2$, $2H_2O$	1 X 3	30.35	2	[2,1]
22	Ammonium Nitrate X Incubation Time	1 X 9	29.95	8	[2,3]	$MgSO_4$, $7H_2O$ X Temperature	2 X 7	29.98	5	[3,3]
23	Trace Element Solution X Inoculum Size	5 X 6	29.78	3	[1,2]	Inoculum Size X pH	6 X 8	29.14	14	[3,2]
24	Phosphate ion X Incubation Time	4 X 9	28.79	13	[2,3]	$MgSO_4$, $7H_2O$ X Trace Element Solution	2 X 5	28.02	7	[3,3]
25	Phosphate ion X Inoculum Size	4 X 6	28.32	2	[3,3]	Inoculum Size X Temperature	6 X 7	27.73	1	[2,3]
26	Ammonium Nitrate X $MgSO_4$, $7H_2O$	1 X 2	28.273	3	[2,3]	Inoculum Size X Incubation Time	6 X 9	26.44	15	[2,3]
27	$MgSO_4$, $7H_2O$ X Incubation Time	2 X 9	26.96	11	[2,3]	Ammonium Nitrate X $MgSO_4$, $7H_2O$	1 X 2	26.08	3	[2,3]
28	Inoculum Size X Temperature	6 X 7	26.28	1	[2,3]	Phosphate ion X Incubation Time	4 X 9	25.83	13	[2,3]
29	$MgSO_4$, $7H_2O$ X Temperature	2 X 7	21.69	5	[3,3]	Ammonium Nitrate X Temperature	1 X 7	25.21	6	[2,3]
30	Inoculum Size X Incubation Time	6 X 9	18.9	15	[2,3]	$MgSO_4$, $7H_2O$ X Incubation Time	2 X 9	18.29	11	[2,3]
31	Ammonium Nitrate X Phosphate ion	1 X 4	17.44	5	[2,2]	Trace Element Solution X Temperature	5 X 7	17.3	2	[1,3]
32	Trace Element Solution X Temperature	5 X 7	17.44	2	[1,3]	Temperature X Incubation Time	7 X 9	16.86	14	[2,3]
33	Temperature X Incubation Time	7 X 9	15.96	14	[2,3]	Ammonium Nitrate X Phosphate ion	1 X 4	11.75	5	[2,2]

(*Continued*)

Table 4 (*continued*)

SN	Biomass production					Phenol degradation				
	Factors	Columns	SI (%)	RC	Levels	Factors	Columns	SI (%)	RC	Levels
34	Trace Element Solution X Incubation Time	5 X 9	15.41	12	[2,3]	Temperature X pH	7 X 8	11.12	15	[2,2]
35	Phosphate ion X Agitation	4 X 10	15.05	14	[3,2]	Incubation Time X Agitation	9 X 10	11.05	3	[3,1]
36	$CaCl_2$, $2H_2O$ X Trace Element Solution	3 X 5	12.98	6	[2,2]	Ammonium Nitrate X pH	1 X 8	7.7	9	[2,2]
37	Temperature X pH	7 X 8	12.88	15	[3,2]	Phosphate ion X Temperature	4 X 7	5.35	3	[2,2]
38	$MgSO_4$, $7H_2O$ X pH	2 X 8	12.23	10	[3,2]	$CaCl_2$, $2H_2O$ X Trace Element Solution	3 X 5	4.59	6	[2,2]
39	Inoculum Size X Agitation	6 X 10	8.91	12	[2,2]	pH X Incubation Time	8 X 9	4.03	1	[2,3]
40	Phosphate ion X Temperature	4 X 7	4.86	3	[2,2]	Trace Element Solution X Incubation Time	5 X 9	3.88	12	[2,3]
41	Incubation Time X Agitation	9 X 10	4.55	3	[3,1]	Inoculum Size X Agitation	6 X 10	2.84	12	[2,2]
42	pH X Incubation Time	8 X 9	4.44	1	[2,3]	$CaCl_2$, $2H_2O$ X Incubation Time	3 X 9	2.28	10	[2,3]
43	Ammonium Nitrate X pH	1 X 8	4.13	9	[2,2]	Phosphate ion X Agitation	4 X 10	1.18	14	[3,2]
44	$MgSO_4$, $7H_2O$ X Inoculum Size	2 X 6	2.13	4	[2,2]	$MgSO_4$, $7H_2O$ X pH	2 X 8	1.04	10	[3,2]
45	$CaCl_2$, $2H_2O$ X Incubation Time	3 X 9	1.67	10	[2,3]	$MgSO_4$, $7H_2O$ X Inoculum Size	2 X 6	0.49	4	[2,2]

contributory factor for phenol biodegradation. The next significant factors for the degradation in order of importance were temperature (12.646%), inoculum size (11.603%), $MgSO_4,7H_2O$ (10.507%), ammonium nitrate (8.97%), agitation (8.956%), phosphate ion (8.291%), pH (5.813%), $CaCl_2,2H_2O$ (0.1%), and trace element solution (0%).

The factors (above 95% of confidence level) were pooled until the degree of freedom for the error term was approximately half the total degree of freedom of experiment. A pooled ANOVA values revealed that $CaCl_2,2H_2O$ and trace element solution were not significant factors for growth and phenol degradation. Individually, each significant factor influenced the production of biomass and phenol degradation at certain level. However, in combination, the level of significant factors was different for maximum growth and biodegradation, which may be due to the interactive effect of different factors. The contribution of selected factors to the growth and phenol degradation at optimum performance is shown in Fig. 4.

3.4. Optimum process parameters

The optimum conditions and their performances in terms of contribution for achieving higher growth of the organism and phenol degradation by it are shown in Table 6. Result showed that incubation time was the most important contributory factor for the degradation of phenol. Taguchi DOE design suggested that the higher levels of growth and consequent phenol degradation can be achieved with ammonium nitrate (0.5 g/l), $MgSO_4,2H_2O$ (0.3 g/l), phosphate ion (0.6 g/l), inoculum size (2%), temperature (30° Celsius), pH (6), agitation (150 RPM), and incubation time (56 h). The expected growth and phenol degradation at optimum condition in S/N ratio were found to be 67.944 and 67.555, respectively, (total contribution from all the factors being found 7.499 and 8.671 with grand average performance of 60.445 and 58.884, respectively). The estimated biomass produced and phenol degradation from the S/N ratio was 2,386.43 mg/l and 2,495.74 mg/l with MSD of 0.0 and 0.0, respectively, [by Equation (3)].

3.5. Validation experiments

Fig. 5(a) and (b) showed the frequency distribution of current condition along with improved condition. It is obvious from the figure that the biomass could be increased from 1,051.96 to 2,495.744 mg/l and the amount of phenol consumed could be increased from 879.42 up to 2,386.43 mg/l based on Taguchi DOE methodology. The overall 137.24% enhancement in the

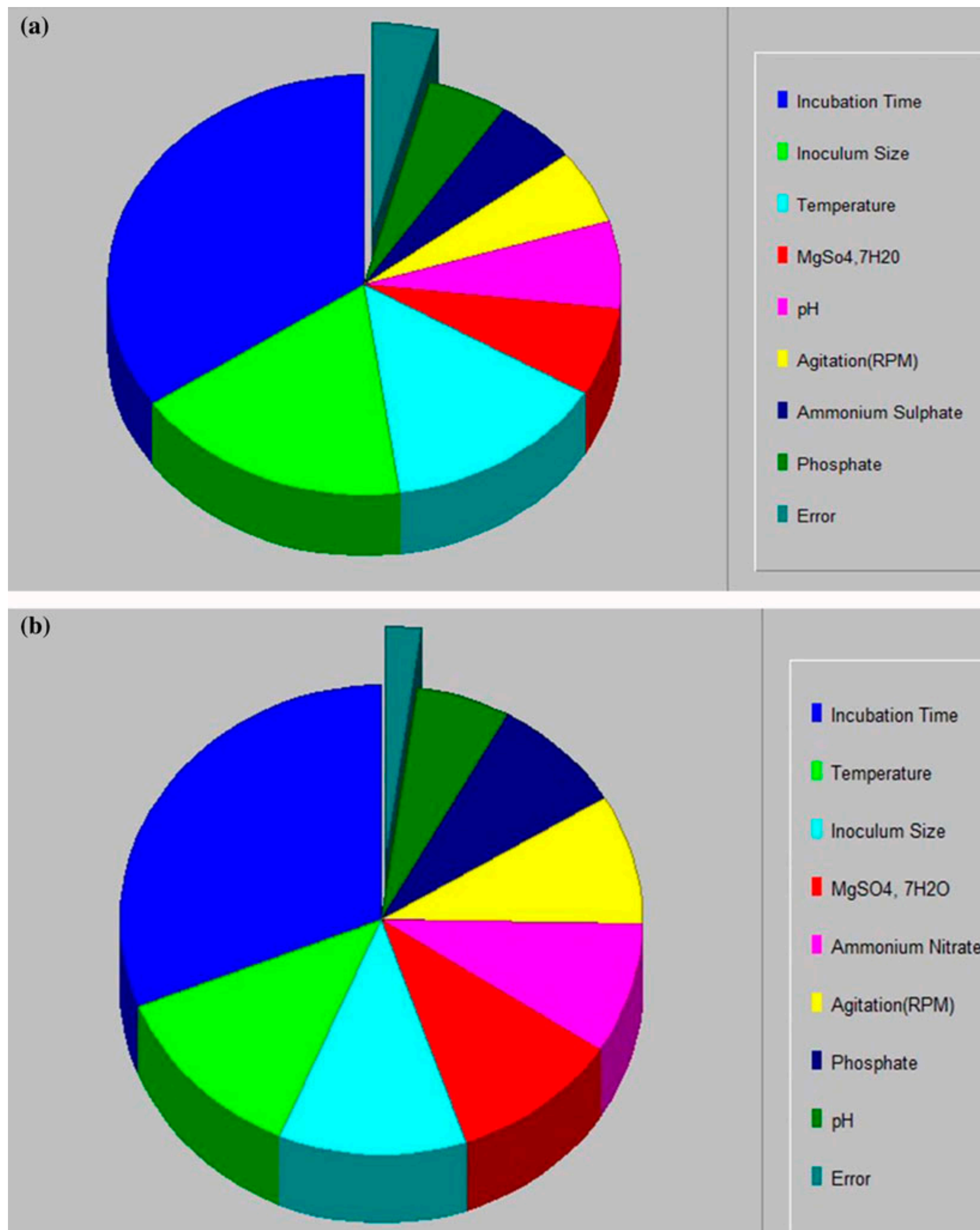

Fig. 3. Relative influence of factors and interaction for biomass production (a) and phenol degradation (b).

growth could be achieved. The overall 171.49% enhancement in the phenol degradation could be attained. Further to validate the proposed experimental methodology, degradation experiments were performed by employing the obtained optimized culture conditions. The experimental data showed that enhanced biomass yield (132.4%) and phenol degradation (165.64%) were found with the optimized culture conditions.

4. Discussion

Microbial degradation of phenol can be swayed by many typical physicochemical parameters like medium composition, temperature, pH, and aeration, since degradation of phenol reported so far has been carried out mostly by aerobic microorganisms [10–12,14,16]. Optimum amounts of nitrogen, Mg^{2+}, phosphate ion, and optimum temperature, pH, and aeration are thus necessary for enhanced phenol biodegradation.

Table 5
ANOVA

Factor	Biomass production						Phenol degradation					
	DOF	Sum of squares	Variance	F-ratio	Pure sum	Percent	DOF	Sum of squares	Variance	F-ratio	Pure sum	Percent
Ammonium Nitrate (g/l)	2	8.552	4.276	17.639	8.068	5.126	2	15.712	7.856	52.737	15.414	8.97
$MgSO_4$,$2H_2O$ (g/l)	2	11.105	5.552	22.905	10.62	6.748	2	18.353	9.176	61.602	18.055	10.507
$CaCl_2$,$2H_2O$ (g/l)	2	1.65	0.825	3.404	1.165	0.74	2	0.47	0.235	1.579	0.172	0.1
Phosphate ion (g/l)	2	8.446	4.223	17.42	7.961	5.058	2	14.545	7.272	48.821	14.247	8.291
Trace Element Solution (g/l)	2	1.396	0.698	2.88	0.911	0.579	2	0.184	0.092	0.617	0	0
Inoculum Size (v/v %)	2	27.81	13.905	57.357	27.325	17.363	2	20.236	10.118	67.923	19.938	11.603
Temperature (°C)	2	22.476	11.238	46.356	21.991	13.974	2	22.028	11.014	73.938	21.73	12.646
pH	2	10.993	5.496	22.672	10.508	6.677	2	10.288	5.144	34.532	9.99	5.813
Incubation time (h)	2	54.2	27.1	111.785	53.715	34.132	2	53.434	26.717	179.35	53.136	30.922
Agitation (RPM)	2	9.287	4.643	19.154	8.802	5.593	2	15.688	7.844	52.659	15.39	8.956
Other/error	6	1.453	0.242			4.01	6	0.893	0.148			2.192
Total	26	157.374				100%	26	171.835				100%

The parameters taken under investigation were chosen based on previous reports and the parameters used mostly in the degradation of phenol. It is obvious from Table 2 that growth of *C. tropicalis* on phenol serving as sole source of carbon and energy and ensuing degradation of it were found to be very much reliant on the culture condition, the variation observed was according to the experiments conducted based on the Taguchi DOE method. The average effects of the selected factors, along with the interaction at the assigned levels, on the growth of *C. tropicalis* and phenol degradation are shown in Table 3. It can be seen from Table 3 that, among the factor taken under investigation, temperature boasted the stronger influence on the degradation of phenol when compared to the other factors, whereas inoculum size showed a stronger influence on the growth of *C. tropicalis* when compared to other factors. Therefore, it can be concluded that both temperature and inoculum size are important factor for phenol biodegradation, since the amount of phenol consumed is directly proportional to growth of the organism owing to the fact that only phenol had been used as sole carbon source. It is also to be noted that the real industrial effluent will contain not only phenol, but a mixture of carbon source; as a result microorganisms will prefer the carbon source first which will be easier for them to assimilate and then the phenol (Diauxie) [35]. This diauxie property may have positive role on phenol degradation, since phenol is toxic to cells its degradation may be augmented if a specific amount of inoculum is attained. However, from the Table 6 and Fig. 2, it is evident that maximum growth and phenol degradation were noticed at the level 2 of inoculum size and above that it is repressive. This may be attributed to the fact that, at level 1 the concentration of inoculum is less such that the adverse effects of phenol's toxicity cannot be alleviated by the number of organism present, and at level 3, the effect is also negative because of the limitation in other medium components and key nutrients like nitrogen and phosphorus [36]. As per the data in Table 4, the SI for inoculum size interaction with $CaCl_2$, $2H_2O$ was fairly high (SI 36.32%), which was then followed by interaction with phosphate ion, ammonium nitrate and trace element solution. This reveals that inoculum size plays an important role in growth and biodegradation of phenol based on the main effect of the selected parameters represented in Table 3. Microbial growth and phenol biodegradation were also controlled by temperature. Maximum growth and phenol degradation were found at the level 2. The phenol degradation was found to decrease at any other level of these factors. It may be due to the effect of temperature on

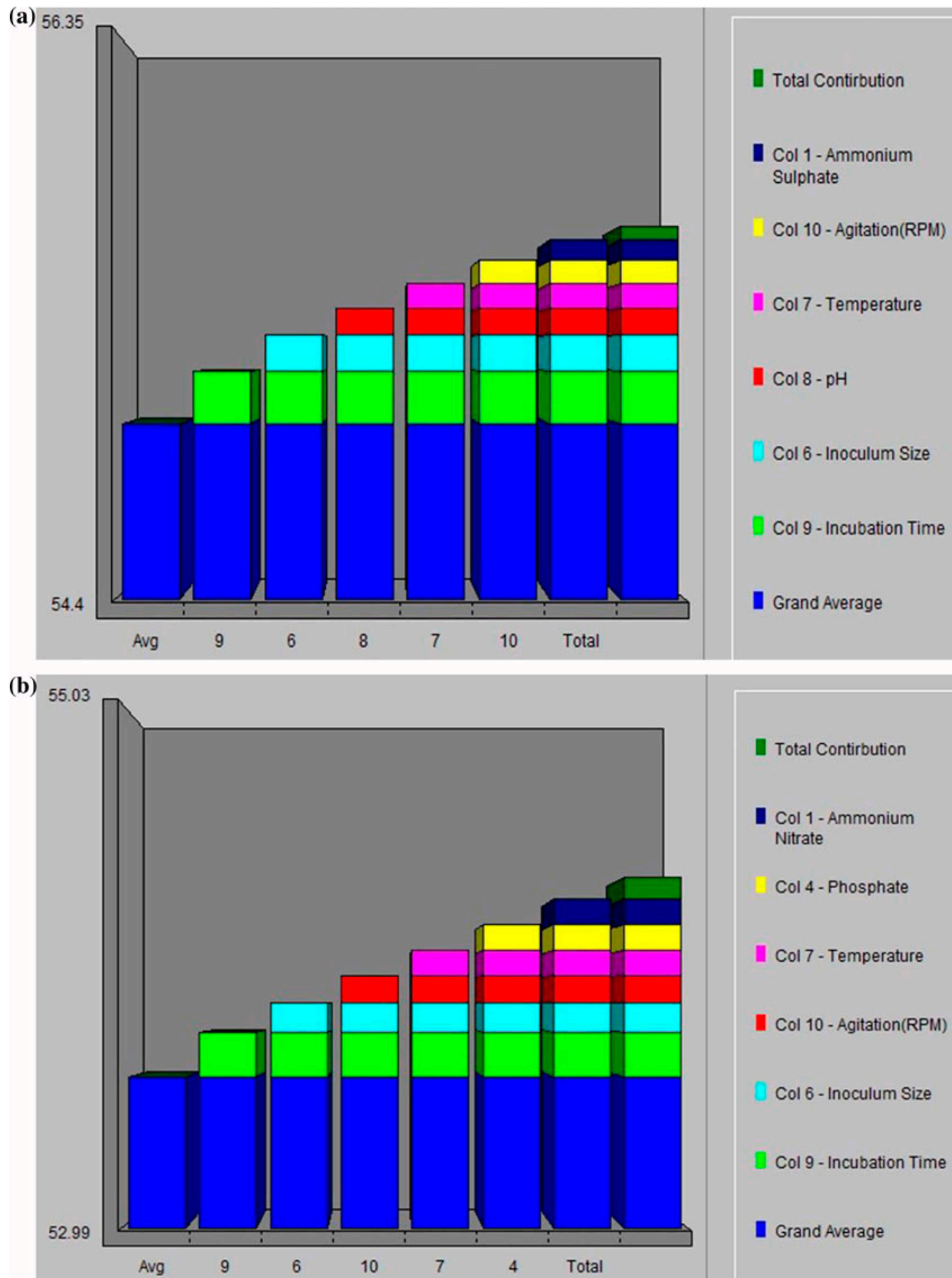

Fig. 4. Optimum performance with contribution of the major factor for biomass production (a) and phenol degradation (b).

growth of organisms since each organism has it specific optimum temperature. The contribution of temperature is also high (Table 6) as revealed from the experiment, and should be considered as an important contributing factor for phenol biodegradation.

Aerobic degradation of phenol requires oxygen to be delivered at an optimum level; therefore, agitation is a deciding factor of degradation of phenol. The highest microbial growth and phenol degradation was found with the increase in agitation up to level 2. This

Table 6
Optimum culture condition and their contribution for biomass production and phenol degradation production

Coded factor	Factor	Values	Level	Contribution for biomass production from S/N ratio	Contribution for phenol degradation from S/N ratio
A	Ammonium Nitrate (g/l)	0.5	2	0.695	0.978
B	$MgSO_4$,$2H_2O$ (g/l)	0.3	3	0.681	0.897
D	Phosphate ion (g/l)	0.6	3	0.486	0.979
F	Inoculum Size (v/v %)	2	2	1.261	1.168
G	Temperature (°C)	30	2	0.847	0.99
H	pH	6	2	0.9	0.871
I	Incubation time (h)	56	3	1.806	1.729
J	Agitation (RPM)	150	2	0.823	1.059
	Total contribution from all factors			7.499	8.671
	Current grand average performance			60.445	58.884
	Expected result at optimum condition			67.944	67.555

Table 7
Comparison of phenol biodegradation capacity between *C. tropicalis* PHB5 (this study) and other microorganisms

Microorganisms	Maximum concentration of phenol degradation reported
Acaligenes sp.	1,200 mg/l [10]
Bacillus cereus	2,000 mg/l [11]
Acinetobacter sp., *Sphingomonas* sp.	800 mg/l [12]
Pseudomonas sp.	800 mg/l [13]
Pseudomonas putida	Lower than 800 mg/l [41]
Pseudomonas resinovorans	Lower than 600 mg/l [14]
Brevibacillus sp.	Lower than 200 mg/l [14]
Corynebacterium sp.	2,000 mg/l [15]
Paecilomyces variotii JH6	1,800 mg/l [16]
C. tropicalis PHB5	2,386 mg/l [present study]

was demonstrated by fact that agitation raises the oxygen mass transfer [37]. However, the effect of agitation further decreased at level 3, which may be attributed to the fact that shear stress can afflict the cells of *C. tropicalis* since besides binary fission, it also grows by budding. Results also show that higher growth and phenol biodegradation was achieved with subsequent increase of incubation time up to level 3.

The culture pH condition is one of the important parameters in growth of microorganisms and is also expected to affect phenol biodegradation by *C. tropicalis*. The obtained result shows that the phenol degradation was higher at pH 6.0, that is, at level 2. The pH is one of the operational parameters that influence the metabolic activity of the organism, and can affect the biodegradation of phenol and it is evident from the Table 6 that the contribution of pH based on S/N ratio is fairly high. Table 6 and Fig. 2 show that higher growth and subsequent phenol consumption were found with increase of ammonium nitrate concentration up to level 2 and further increase of its concentration (level 3) decreased both growth and phenol degradation. Nitrogen source is an essential requirement for growth and production of enzyme some of which are ultimately involved in the phenol utilization and it is also an important cellular component mostly as part of the protein, DNA, and ATP [38]. From Table 6, it can be seen that contribution of ammonium nitrate for phenol degradation is second highest based on S/N ration as well as from Table 4, we see the SI for ammonium nitrate interacting with trace element solution and agitation were highest for growth and biomass production (SI 68.33 and 68.37%, respectively). For phenol degradation the interaction of ammonium nitrate with trace element solution is also highest (SI 66.5%) followed by interaction with agitation (SI 58.29%). This reveals that ammonium nitrate plays an important role in growth of *C. tropicalis* and biodegradation of phenol. However, the decrease in phenol degradation at the concentration beyond level 2 can be attributed to the verity that higher concentration of nitrogen source significantly affects the pH of the medium during growth and also influences the microbial metabolism [39].

Phosphate is necessary for the synthesis of cellular building block viz., nucleic acids, phospholipids, etc. as well as a part of ATP. Hence, phosphate along with nitrogen is considered as the limiting nutrient for microbial growth. Dependence of growth of *C. tropicalis*

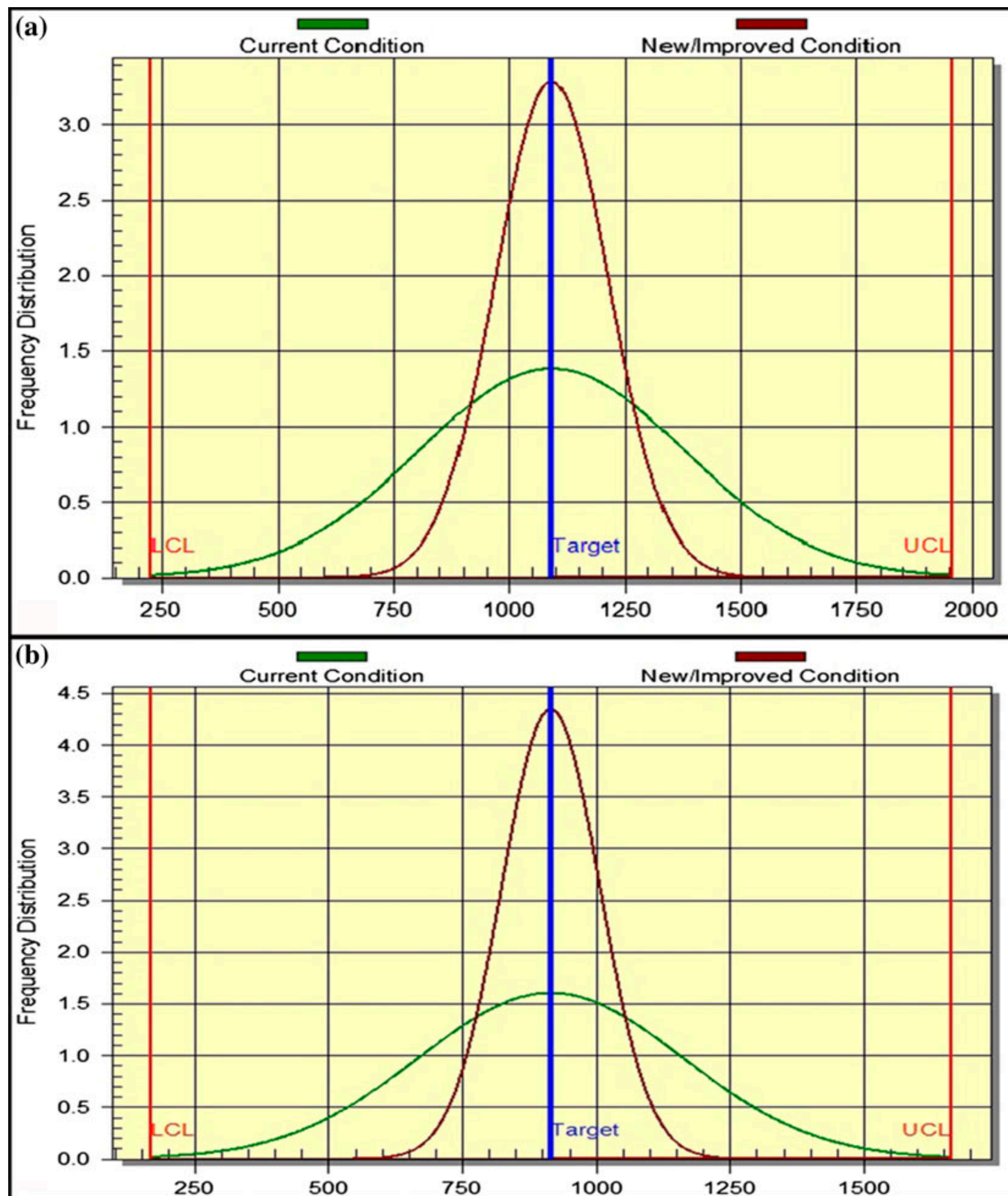

Fig. 5. Performance distribution of current and improved condition for biomass production (a) and phenol biodegradation (b).

on phosphate ion and subsequent phenol degradation by this organism can be seen from the data in Table 3 and Fig. 2. Phosphate has a reasonably high contribution toward phenol degradation as is evident from the S/N ratio (Table 6). High percentages of phenol removal and growth have been observed in the phosphate concentration of level 3. The interaction of phosphate ion with $CaCl_2,2H_2O$ bestowed a fairly high SI value (51.18%) for biomass production followed by its interaction with trace element solution, $MgSO_4,7H_2O$ and pH (44.24, 37.52, and 36.4% respectively). On the other hand, the SI value for interaction of phosphate ion with $CaCl_2,2H_2O$ showed the third highest value for phenol degradation. A possible explanation of the above observation is that phosphate ions, besides being an important nutrient, can aid in the growth of the organisms owing to the buffering action which consequently results in stabilization of the pH of the medium (pH homeostasis) which indirectly favors phenol degradation.

Incubation time showed the maximum contribution for biomass production and phenol degradation as evidenced from data based on S/N ratio. This can be explained by the fact that phenol is not a suitable carbon source to assimilate as it increases the porosity in the cell membrane and organisms may require time to appease themselves to utilize this compound. Table 6 shows the optimum incubation time (56 h, i.e. level 3) is required for highest degradation of phenol, this can be explained by the fact that growth decreases when the initial phenol concentration is increased, and it may be due to an increased maintenance coefficient and to cell lysis [40]. Mg^{2+} ion also plays significant role to some extent in biomass production and phenol degradation. It is supported by the data represented in the Table 3 (the L2–L1 value is fairly high for Mg^{2+} ion) and Table 6. As mg^{2+} plays a key role as cofactor of many enzymes, its importance is quite obvious in growth of microorganisms. Since biological cell membranes and cell walls are polyanionic surfaces, Mg^{2+} serves to shield their anionic groups which may indirectly help membrane stabilization as well which will ultimately counteract the membrane destabilization effect of phenol. Trace element solution and Ca^{2+} has role in the growth of microorganisms but their effect on biodegradation of phenol is significant at the assigned level taken under investigation (Table 3).

5. Conclusion

The optimization of the medium is one of the key factors to maximize the percentage phenol degradation. Traditional methods of optimization involve changing one independent variable while fixing the others at a certain level. This single-dimensional search is laborious, time consuming and incapable of reaching a true optimum due to interactions among variables. The Taguchi approach of OA DOE represents a simple methodology that selects the best conditions producing consistent performance.

In this work, we have successfully applied the Taguchi DOE methodology to optimize physicochemical parameters for enhancement of growth of *C. tropicalis* on phenol containing inorganic media and subsequent degradation of phenol was experimentally verified. Although, phenol degradation by different microorganisms in the presence of glucose, yeast extract, urea, etc. in the medium has been reported, this may increase the process cost and it may not be economically feasible to carry out the phenolic wastewater treatment in large scale employing those carbon sources. In this article, we have optimized the composition of inorganic mineral salt medium containing phenol as sole carbon source and other physical parameters that govern biodegradation of phenol by *C. tropicalis*. The true functional relationship between the dependent variable (phosphate, nitrogen sources, temperature, pH, etc.) and maximum percentage of phenol biodegradation have been studied and we have found that maximum growth of *C. tropicalis* (132.4%) and resultant higher percentage of phenol degradation (165.64%) can be achieved with the optimized conditions when compared to unoptimized conditions in the same unit volume. The treatment of phenol in industrial and domestic wastewater effluents is very important due to its persistence and toxic effect. The optimum culture medium obtained in these experiments gives a basis for large-scale degradation phenol present in industrial effluents in batch or fed-batch cultivation in bioreactor.

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