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Phenol Degradation using *Candida tropicalis* SSK01 Isolated from Petroleum Contaminated Soil under Optimized Medium Composition

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The microbial degradation of phenol by pure culture of *Candida tropicalis* SSK01 cells isolated from petroleum contaminated soil is studied in batch mode. Response surface methodology was used to optimize the medium composition for phenol degradation. Initial screening of variables such as initial phenol concentration, NH_4NO_3, KH_2PO_4, $CaCl_2$,K_2HPO_4, $MgSO_4$, KCl and Yeast extract was done by using Plackett–Burman experimental design .It was found that initial phenol concentration ($p=0.016$), NH_4NO_3 ($p<0.011$), $CaCl_2$ ($p<0.018$), KH_2PO_4 ($p<0.018$) and Yeast Extract ($p<0.047$) had a significant effect on phenol degradation. Then Central Composite Design was employed to determine optimal concentration of each significant variable. Based on CCD the optimum concentrations of the critical components were found to be initial phenol concentration 600 g/l, NH_4NO_3-2.5 g/l , $CaCl_2$-0.015 g/l ,KH_2PO_4-0.75 g/l and Yeast Extract -0.07 g/l for 98.5 % phenol degradation in 72 hours. The R^2, adjusted R^2 and Predicted R^2 values calculated by CCD were 0.9965, 0.944 and 0.99860 showed that experimental values are in good agreement with predicted values.

Key words: Phenol degradation, *Candida tropicalis* SSK01, Medium optimization.

Phenol is used in many industries for in the production of polycarbonate resins, paints, explosives, inks, perfumes, textiles and antibacterial agents. All industries producing or using phenol discharge this pollutant into the environment. Phenolic compounds are toxic and carcinogenic by ingestion, contact, or inhalation, and they have high stability[1-2].Therefore biodegradation of phenol at high concentrations has been an interesting topic of research for many years. A wide range of literature is available on phenol biodegradation kinetics by pure cultures of bacteria such as *Pseudomonas putida*, *Acinetobacter* sp., *Bacillus* sp.Up to date, many pure culture microorganisms has been isolated and identified to utilize phenol as the sole carbon and energy source[3-5]. Among this *Candida* species has been proved to possess high potential to degrade phenol.*Candida tropicalis* has the ability to degrade high-strength phenol by utilizing it as sole carbon and energy source[6]. Therefore, it has broad prospects in the treatment of high strength phenolic effluents. The ability of microorganisms to degrade pollutants is strongly influenced by nutritional parameters such as carbon and nitrogen sources. However, as far as we know, there is limited knowledge about nutritional requirements for phenol degradation by *Candida tropicalis*. Therefore, it is necessary to design an appropriate media composition for maximizing the removal efficiency of phenol by C*andida tropicalis*. RSM generally used to investigate a combined effect of several variables and to determine optimum conditions for a

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multivariable system [7]. The first step of statistical approach in optimization was to establish the criteria that will define the experimental factors that have a significant effect on the response variables. In this work *Candida tropicalis* capable of degrading high concentration of phenol was isolated from petroleum contaminated soil. Optimizatation of medium composition for phenol degradation was also carried out. Initial screening of medium components was done using Plackett–Burman design. Then the screened medium composition was optimized by Central Composite Design for phenol degradation using *Candida tropicalis* SSK01.

MATERIAL AND METHOD

Chemical

Analytical grade phenol was purchased from Merck, Mumbai, India. Synthetic phenol solutions were prepared for the desired concentration in distilled water before each experimental run. The solutions were always kept in a brown flask to avoid light oxidation of the phenol. All other chemicals were of analytical grade and were also obtained from Merck, Mumbai, India.

Sample collection

Petroleum contaminated soil used in this study was collected from local petrol bulk (Sholinganallur, Chennai, Tamil Nadu, India). Samples were collected in sterile polythene bags, labelled and stored in refrigerator at -4°C before analysis.

Isolation and enrichment of isolated yeast

Petroleum contaminated soil samples were homogenized, air dried and screened to remove stones. Soil was mixed thoroughly and passed through 2 mm sieve (M.B.Instruments, Delhi) to remove gravel. Zhang *et al* method was followed to isolate potential strain [8] .Serial dilution was performed and plating was done on Ramsay agar media (Phenol=500 mg/l, NH_4NO_3=2.0g/l, KH_2PO_4=0.5g/l, K_2HPO_4=1.0g/l, $MgSO_4.7H_2O$=0.5g/l, $CaCl_2.2H_2O$=0.01g/l, KCl=0.1g/l, Yeast Extract=0.06g/l) by spread plate technique. The plates were then incubated at 27°C for 5 days and examined for pure culture isolate(s) on agar plates. The isolated strain was maintained in YEPD (Yeast extract, Peptone, Dextrose) agar slants at 28 °C and sub cultured every two weeks.

Morphological and Molecular Characterization of isolated yeast

Microscopic (Radical Model RXLR-3T Fluorescent Microscope) examination of colonies was done. Colonies showing yeast morphology was selected. Sequence analysis was done using ITS1 and ITS4 primers which is more conserved in eukaryotes and used for molecular characterisation of organisms. The genomic DNA was isolated and the ITS region of 5.8sRNA was amplified using primer ITS1 TO 5'TCCGTAGGTGAACCTGCGG3' and primer ITS5 5' TCCTCCGCTTATTGATATGC 3' 7 and sequenced using automated sequencer. The sequences were aligned with the GenBank database using the Basic Local Alignment Search Tool (BLAST) algorithm at NCBI server. Sequences of closely related taxa were retrieved and aligned using the Clustalx. A phylogenetic tree was constructed by the Neighbour-joining method.

Plackett–Burman (PB) Design

PB design is very useful for selecting the most important variables from a list of variables such as initial phenol concentration, NH_4NO_3, KH_2PO_4, $CaCl_2$, K_2HPO_4, $MgSO_4$, KCl and Yeast Extract.PB design requires fewer runs than a comparable fractional design. Each independent variable was tested at two levels, a high (+) and a low (-) level, as shown in Table 1. Plackett–Burman design was based on first order model

$$Y_i = \beta_0 + \sum_{i=1}^{k} \beta_i X_i \quad ..(1)$$

Y_i is the predicted response, β_0 model intercept and β_1 is the linear coefficient, X_i is coded independent variable estimate or factor and k is the number of involved variables. This model is unable to describe the interaction among factors but can be used to screen for factors significantly affecting the measured response [9].In the present work eight components of the medium were evaluated in twelve experimental trials. All the trials were carried out in triplicate and percentage degradation for each trial was used as the response variable. Regression analysis determined the components that had a significant (95% level ($P < 0.05$)) effect on phenol degradation. The critical compositions of medium components were evaluated by CCD. The initial screening of eight variables was done by PB method using Minitab-15 software.

Optimization by Central Composite Method

After finding the significant parameters by PB data analysis, CCD was employed for further optimization of the medium composition. These data was used to fit the polynomial regression equation

$$Y_i = b_0 + b_1X_1 + b_2X_2 + b_3X_3 + b_4X_4 + b_5X_5 + b_{11}X_1^2 + b_{22}X_2^2 + b_{33}X_3^2 + b_{44}X_4^2 + b_{55}X_5^2 + b_{12}X_1X_2 + b_{13}X_1X_3 + b_{14}X_1X_4 + b_{15}X_1X_5 + b_{23}X_2X_3 + b_{24}X_2X_4 + b_{25}X_2X_5 + b_{34}X_3X_4 + b_{35}X_3X_5 + b_{45}X_4X_5 \quad ..(2)$$

where Y_i is the predicted response, X_1, X_2, X_3, X_4, X_5 are independent variables, b_0, is the offset term, b_1, b_2, b_3, b_4, b_5 are coefficient of linear terms, b_{11}, b_{22}, b_{33}, b_{44}, b_{55} are coefficient of squared terms and b_{12}, b_{13}, b_{14}, b_{15}, b_{23}, b_{24}, b_{25}, b_{34}, b_{35}, b_{45} are coefficient of interaction terms. The regression equation contain five linear term (X_1, X_2, X_3, X_4, X_5), five quadratic term (X_1^2, X_2^2, X_3^2, X_4^2, X_5^2), and ten cross-interactions (X_1X_2, X_1X_3, X_1X_4, X_1X_5, X_2X_3, X_2X_4, X_2X_5, X_3X_4, X_3X_5, X_4X_5) terms plus 1 block term. Subsequently Analysis of variance (ANOVA) was performed to determine whether the constructed model was significant to explain the observed data. The lack of fit of the model was tested by an F-test.

Validation of the model

The correctness of the model was tested by using the statistically optimized medium. The maximum experimental response for phenol degradation was determined to check the validity of model.

Inoculum preparation

Candida tropicalis SSK01 was grown in 500 ml Erlenmeyer flasks containing 100 ml minimal media (NH_4NO_3-2.0 mg/l, $CaCl_2$-0.01 mg/l, KH_2PO_4-0.5 mg/l, K_2HPO_4-1.0 mg/l, $MgSO_4$- 0.5 mg/l, KCl-0.1 mg/l, yeast extract- 0.06 mg/l) supplemented with 500 mg/l of phenol. The cells are harvested during mid log phase of acclimatization phase. Cells were grown for 72 hours at 35°C.

Batch biodegradation experiments

All experiments for optimization of medium components for phenol degradation were carried out in 250 ml Erlenmeyer flasks containing 100 ml of sterile medium. The medium components optimized were initial Phenol Concentration, NH_4NO_3, KH_2PO_4, K_2HPO_4, $MgSO_4.7H_2O$, $CaCl_2.2H_2O$, KCl and Yeast Extract. The pH of medium was adjusted to 7 and sterilzed.The phenol is filter sterilized and then added to medium. The 2ml of inoculum was added and flask was incubated at temperature of 35°C at 150 rpm.The samples were collected after 72 hours and centrifuged at 10000 rpm for 10 minutes. The supernatant is used for phenol assay. The percentage degradation was found by equation 3.

$$\text{Percentage degradation} = \left(\frac{\text{Initial Phenol Concentration-Final Phenol Concentration}}{\text{Initial Phenol Concentration}}\right) x100 \quad ..(3)$$

Phenol assay

Phenol concentration was determined quantitatively by a uv spectrophotometer (Spectra scan UV2700, Thermo scientific), using 4-aminoantipyrine as colour reagent. These analyses were performed according to the procedures described in standard Method for the estimation of water and waste water [10].

RESULTS AND DISCUSSION

Isolation of yeast

In the present study, the 10^{-5} dilutions produced distinct colonies with yeast morphology. The *Candida* species was selected for further studies based on its distinct colony morphology and biochemical test results are given in table-2.

Molecular characterization of yeast

The internal transcribed spacer (ITS) region is located between the 18S and 28S rRNA genes and is used for molecular characterization of eukaryotic microbes which offers high sensitivity of detection due to the existence of multiple copies per genome. And is now perhaps the most widely sequenced DNA region in eukaryotes [11].The sequences obtained were matched with the GenBank database using the Basic Local Alignment Search Tool (BLAST) algorithm at NCBI and it was found to be novel and submitted by Sequin tool with an Accession number HQ622603. Phylogenetic analysis was performed to identify homologous sequences by Neighbour Joining method using online tool (www.pasteur.fr). The Phylogenetic tree as shown in Fig. 1 revealed *Candida* spices MCCF to be its closest related species.

Selection of significant variables by PB design

PB was used in the preliminary optimization process to determine the most influential factors that affect phenol degradation.

The experimental PB design resulted in 12 experiments variables were examined at low and high levels. A total of eight variables such as initial phenol concentration, NH_4NO_3, KH_2PO_4, $CaCl_2$, K_2HPO_4, $MgSO_4$, KCl and Yeast Extract were analysed. The matrix developed by the PB design and the resulting percentage degradation from each trial were presented in table-3. The regression analysis and t-value of the variables were shown in table-4. Based on the results, a first-order regression equation-4 was developed

$$\text{Percentage degradation} = 85.2 - 0.00204X_1 - 1.39X_2 + 116X_3 - 2.32X_4 + 0.158\ X_5 - 1.42X_6 - 1.42X_7 + 40.4X_8 \quad ...(4)$$

The R^2 and adjusted R^2 values were 0.975 and 0.910 respectively indicating that experimental values are in good agreement with predicted values. A 95% confidence interval was used for the statistical evaluation of the results. The variables that had a significant effect (confidence levels >95%, $P < 0.05$) on phenol degradation were initial phenol concentration ($p=0.016$), NH_4NO_3 ($p<0.011$), $CaCl_2$ ($p<0.018$), KH_2PO_4 ($p<0.018$) and Yeast Extract ($p<0.047$).The other variables K_2HPO_4, $MgSO_4$ and KCl with p values more than 0.05 did not affect the percentage of degradation.

Optimization of the screened variables by CCD

The significant variables utilized for phenol degradation were initial phenol concentration, NH_4NO_3, KH_2PO_4, $CaCl_2$ and Yeast Extract and these variable was assessed at three coded levels (1, 0, +1,), as is shown in Table 5. CCD was used to determine the optimum levels of these variables at three levels, with ten axis points and eight replicates at the center points leading to 50 experiments. The full experimental plan with regard to their values in actual form and the corresponding results of CCD experiments are provided in Table 6.

Regression analysis was carried out considering full quadratic model equation- (1) on the responses to evaluate the adequacy of fit is as follows.

$$\text{Percentage Degradation} = +98.71 - 2.36X1 - 9.484E - 003X_2 - 0.32X_3 + 0.27X_4 - 0.76X_5 - 0.025X_1X_2 + 0.38X_1X_3 - 0.31X_1X_4 + 0.83\ X_1X_5 + 0.41X_2X_3 + 0.26X_2X_4 + 0.000X_2X_5 + 0.43X_3X_4 + 0.26\ X_3X_5 - 0.34X_4X_5 - 10.20X_1^2 - 0.35X_2^2 - 2.55X_3^2 - 6.00X_4^2 - 0.43X_5^2 \quad ..(5)$$

Analysis of Variance

The adequacy of the model was checked using ANOVA as shown in table-7. The "*F*-value" of the model was 407.86, and the value of "Prob > F" < 0.0001, suggesting that the model was highly significant. Linear terms of X_1, X_3, X_4, X_5 and quadratic terms of X_1^2,X_3^2, X_4^2 were significant for phenol degradation. Interactive terms of X_1X_3, X_1X_4, X_1X_5, X_2X_3, X_2X_4, X_3X_4, X_3X_5 and X_4X_5 were also significant for phenol degradation with $p<0.05$. Whereas linear term X_2, interactive term X_1X_2,X_2X_5 and X_2^2, X_5^2 were not significant with $p>0.05$.The model was statistically valid with a low probability value ($P_{model} < 0.0001$). The lack-of-fit value was

Table 1. The concentrations of involved variables at different levels

No	Variables	Low (mg/l)	High (mg/l)
1	Phenol	300	900
2	NH_4NO_3	2.0	3.0
3	$CaCl_2$	0.01	0.02
4	KH_2PO_4	0.5	1
5	K_2HPO_4	1.0	2
6	$MgSO_4$	0.5	1
7	KCl	0.1	0.2
8	Yeast Extract	0.06	0.08

Table 2. Morphological and biochemical characteristics of isolates

Characters	*Candida tropicalis*
Shape	Round ,smooth
Size	0.2-0.5 mm
Colour	Cream
Texture	Dull
Margin	Round
Elevation	Round
Starch hydrolysis	Negative
Ester production	Positive
Citrate utilization	Negative
Tolerance of 1% acetic acid	Negative
Acid production from glucose	Negative
Production of ammonia from urea	Positive
Nitrate reduction	Positive
Glucose	Positive
Sucrose	Negative
Maltose	Positive

not significant (P = 0.2071), indicating that the equation was adequate. The low coefficient of variation (CV =0.8%) suggested that the model was precise and reliable.R^2, adjusted R^2 and Predicted R^2 values calculated by CCD 0.9965, 0.944 and 0.99860 respectively that showed experimental values are in good agreement with predicted values as shown in the figure-2.

Interaction effect of independent variables

Three dimensional response surface plots graphically represent regression equations and are generally used to demonstrate relationships between the response and experimental levels of each variable. These surface plots, therefore, allow for visualization of the optimum levels of each variable for the maximum production of microbial metabolites[12].From the 3D response surface plots and the corresponding contour plots, the optimal values of the independent variables could be observed, and the interaction between each independent variable pair can be easily understood. The maximum predicted value is indicated by the surface confined in the smallest ellipse in the contour diagram [13].

In a five parameter study, the 3D surface plots are drawn by taking any two variables and the other three variables constant at mid level.Figure-3.a shows the 3D surface plot for the interaction effect between initial phenol concentration (X_1) and $CaCl_2$(X_3) toward phenol degradation. The interaction between initial phenol concentration (X_1) and $CaCl_2$(X_3) was significant (P=0.0036).The maximum percentage of phenol degradation 98.73% was found at 602 mg/l and 0.015 g/l of initial phenol concentration and $CaCl_2$(X_3). The percentage degradation increased when the initial phenol concentration was increased from 300 mg/l to 600 mg/l with increase in $CaCl_2$(X_3) concentration. Then the percentage degradation decreased with increase in phenol concentration due to inhibitory effect of phenol at higher concentration [14-15].Figure -3.b shows the

Table 3. Plackett–Burman design for eight variables with coded values along with the predicted and observed results

Runs	X_1	X_2	X_3	X_4	X_5	X_6	X_7	X_8	Predicted	Observed
1	+	+	-	+	+	-	+	-	80	79.82
2	+	-	-	-	+	+	+	-	81.5	81.67
3	+	-	+	+	-	+	-	-	82	81.65
4	+	+	+	-	+	+	-	-	82.4	82.38
5	-	+	+	-	+	-	-	-	83.5	83.51
6	+	-	+	-	-	-	+	+	84	84.18
7	-	-	-	+	+	+	-	+	82.5	82.68
8	-	-	+	+	+	-	+	+	84.6	84.41
9	-	+	+	+	-	+	+	-	81	81.34
10	-	+	-	-	-	+	+	+	82.5	82.15
11	-	-	-	-	-	-	-	-	83.6	83.58
12	+	+	-	+	-	-	-	+	80.45	80.62

Table 4. Regression analysis of the Plackett–Burman design

No	Variables	Estimate	t-value	p-value
X_1	Phenol	-0.00204	-4.95	0.016
X_2	NH_4NO_3	-1.3917	-5.62	0.011
X_3	$CaCl_2$	115.83	4.68	0.018
X_4	KH_2PO_4	-2.3167	4.68	0.018
X_5	K_2HPO_4	0.1583	0.64	0.568
X_6	$MgSO_4$	-1.4167	-2.86	0.065
X_7	KCl	-1.417	-0.57	0.608
X_8	Yeast Extract	40.42	3.26	0.047

Table 5. Coded levels for independent factors used in the Central Composite Design

Factor	Symbol	Coded Levels		
		-1	0	+1
Initial phenol concentration (mg/l)	X_1	300	600	900
NH_4NO_3 (g/l)	X_2	2	2.5	3.0
$CaCl_2$(g/l)	X_3	0.01	0.015	0.02
KH_2PO_4(g/l)	X_4	0.25	0.50	0.75
Yeast Extract(g/l)	X_5	0.10	0.15	0.20

Table 6. Experimental designs used in RSM studies by using four independent variables showing observed and predicted values of Phenol degradation

Runs	Initial Phenol Con.. mg/l	NH_4NO_3 g/l	$CaCl_2$ g/l	KH_2PO_4 g/l	Yeast extractg/l	Experimental % degradation	Predicted % degradation	Residue
1	600	2.5	0.022	0.75	0.07	93.6	93.15	0.45
2	600	2.5	0.008	0.75	0.07	94.5	94.07	0.43
3	600	2.5	0.015	0.75	0.07	97.6	98.71	-1.11
4	900	3	0.01	1	0.08	76	75.57	0.43
5	600	2.5	0.015	0.75	0.08	98	96.79	1.21
6	900	2	0.02	0.5	0.08	76.5	77.04	-0.54
7	300	2	0.01	1	0.08	79.6	80.33	-0.73
8	300	2	0.01	0.5	0.06	84.4	84.26	0.14
9	600	2.5	0.015	0.75	0.07	98.5	98.71	-0.21
10	600	2.5	0.015	0.4	0.07	86.6	86.33	0.27
11	300	3	0.02	1	0.08	80.6	80.85	-0.25
12	900	3	0.02	0.5	0.06	76.5	75.92	0.58
13	900	3	0.02	1	0.06	77.5	77.91	-0.41
14	900	3	0.02	1	0.08	77.9	77.89	0.012
15	300	3	0.01	1	0.08	79.5	80.06	-0.56
16	300	2	0.02	1	0.08	79	79.47	-0.47
17	600	3.21	0.015	0.75	0.07	98.4	97.99	0.41
18	300	3	0.02	0.5	0.08	79.6	79	0.6
19	600	2.5	0.015	0.75	0.07	98.5	98.71	-0.21
20	900	2	0.02	1	0.06	76.5	76.63	-0.13
21	900	2	0.01	0.5	0.08	77.8	78.1	-0.3
22	600	2.5	0.015	0.75	0.07	98.7	98.71	-7.6210^{-03}
23	300	2	0.01	1	0.06	85	84.7	0.3
24	300	3	0.02	0.5	0.06	80.3	80.98	-0.68
25	300	3	0.02	1	0.06	84.5	84.2	0.3
26	300	2	0.02	1	0.06	83.3	82.82	0.48
27	300	3	0.01	0.5	0.06	83.2	82.94	0.26
28	900	3	0.02	0.5	0.08	76.7	77.27	-0.57
29	900	2	0.01	1	0.08	76.5	75.94	0.56
30	175.8	2.5	0.015	0.75	0.07	82.5	81.65	0.85
31	900	3	0.01	0.5	0.06	76.5	76.36	0.14
32	900	2	0.01	1	0.06	76.5	76.99	-0.49
33	300	2	0.02	0.5	0.06	79.6	80.65	-1.05
34	300	3	0.01	1	0.06	84.5	84.44	0.065
35	1024.2	2.5	0.015	0.75	0.07	75	74.97	0.032
36	300	3	0.01	0.5	0.08	79.5	79.94	-0.44
37	900	2	0.02	0.5	0.06	76.7	75.69	1.01
38	900	3	0.01	1	0.06	76.5	76.62	-0.12
39	300	2	0.01	0.5	0.08	81.5	81.26	0.24
40	600	2.5	0.015	0.75	0.07	98.5	98.71	-0.21
41	600	2.5	0.015	0.75	0.07	98.9	98.71	0.19
42	900	3	0.01	0.5	0.08	76.5	76.68	-0.18
43	600	2.5	0.015	0.75	0.07	97.5	98.71	-1.21
44	900	2	0.02	1	0.08	76.5	76.61	-0.11
45	600	1.79	0.015	0.75	0.07	98.5	98.02	0.48
46	600	2.5	0.015	1.1	0.07	87.7	87.08	0.62
47	900	2	0.01	0.5	0.06	77.6	77.78	-0.18
48	300	2	0.02	0.5	0.08	79	78.67	0.33
49	600	2.5	0.015	0.75	0.06	98.6	98.93	-0.33
50	600	2.5	0.015	0.75	0.07	98.8	98.71	0.092

interaction effect between initial phenol concentration (X_1) and KH_2PO_4 (X_4)which was significant (p=0.0164).The maximum percentage of 98.60% degradation was obtained at 604.12 mgl^{-1} and 0.73 g/l of initial phenol concentration (X_1) and KH_2PO_4 concentration respectively. The effects of initial phenol concentration (X_1) and yeast extract (X_5) on phenol degradation are shown in Figure-3.c Phenol degradation increases as yeast extract increases from 0.050 to 0.075 g/l, but further increase in yeast extract content showed a declining trend for phenol degradation. This was likely because high concentrations of yeast extract could impede the activity of enzymes needed degrade phenol [16]. A similar observation was reported in earlier literature[17].The maximum of 98.65%

Table 7. Analysis of variance (ANOVA) for the response surface model developed for Phenol degradation by CCD

Source	Sum of Squares	df	Mean Square	F-Value	p-value Prob> F	
Model	3772.39	20	188.62	407.86	< 0.0001	significant
X_1-Initial Phenol Concentration	200.72	1	200.72	434.03	< 0.0001	
X_2-NH_4NO_3	3.24E-03	1	3.24E-03	7.00E-03	0.9339	
X_3-$CaCl_2$	3.78	1	3.78	8.18	0.0078	
X_4-KH_2PO_4	2.54	1	2.54	5.48	0.0263	
X_5-Yeast Extract	20.63	1	20.63	44.6	< 0.0001	
X_1 X_2	0.02	1	0.02	0.043	0.8367	
X_1 X_3	4.65	1	4.65	10.06	0.0036	
X_1 X_4	3	1	3	6.49	0.0164	
X_1 X_5	22.11	1	22.11	47.81	< 0.0001	
X_2 X_3	5.44	1	5.44	11.77	0.0018	
X_2 X_4	2.2	1	2.2	4.77	0.0372	
X_2 X_5	0	1	0	0	1	
X_3 X_4	5.95	1	5.95	12.87	0.0012	
X_3 X_5	2.1	1	2.1	4.54	0.0416	
X_4X_5	3.78	1	3.78	8.18	0.0078	
X_1^2	989.03	1	989.03	2138.61	< 0.0001	
X_2^2	1.17	1	1.17	2.53	0.1229	
X_3^2	61.83	1	61.83	133.7	< 0.0001	
X_4^2	342.25	1	342.25	740.06	< 0.0001	
X_5^2	1.72	1	1.72	3.72	0.0636	
Residual	13.41	29	0.46			
Lack of Fit	11.44	22	0.52	1.84	0.2071	not significant
Pure Error	1.98	7	0.28			
Cor Total	3785.8	49				

Adj R^2-0.9944; R^2 -0.9967;Pred R^2-0.99

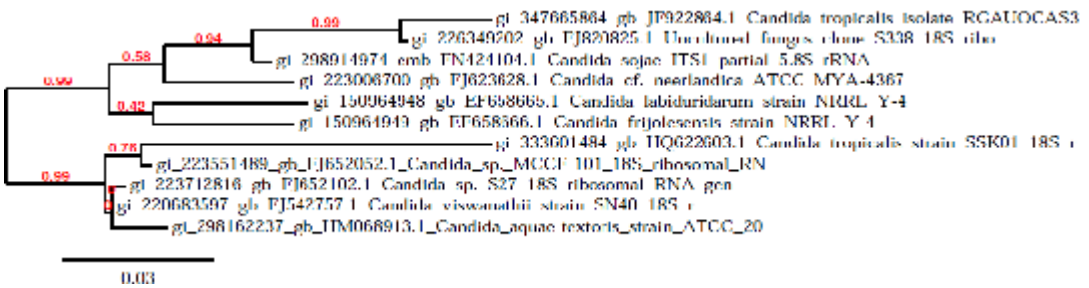

Fig. 1. Phylogenetic tree of *Candida tropicalis* SSK01 by Neighbour joining method.

percentage degradation was observed when initial phenol concentration (X_1) and yeast extract (X_5) were 604.21 mg/l and 0.075 g/l respectively. The interactions effect of NH_4NO_3 (X_2) and $CaCl_2$ (X_3) on percentage degradation was significant ($P< 0.0018$).As the concentration of NH_4NO_3 (X_2) increases from 2 to 2.5 g/l and $CaCl_2$ (X_3) from 0.015 g/l to 0.025 g/l the percentage degradation increases.Figure 3.d. Surface plot showed that maximum 98.74% degradation was observed when NH_4NO_3 (X_2) and $CaCl_2$ (X_3) concentration was 2.49 g/land 0.015 g/l respectively. Figure 3.e shows surface plot the interactions effect of NH_4NO_3 (X_2) and KH_2PO_4 (X_4) on percentage degradation. As the concentration of NH_4NO_3 (X_2) increased from 2 to 2.5 g/l and KH_2PO_4 (X_4) 0.50 g/l to 0.75 g/l the percentage degradation increases. Maximum of 98.52 % degradation was observed when NH_4NO_3 (X_2) and KH_2PO_4 (X_4) concentration was 2.54 mg/l and 0.76 g/l respectively. Phosphate serves the construction material of cellular components such as cyclic AMP, nucleic acids, phospholipids, nucleotides and coenzymes [18]. Further increase in KH_2PO_4 which serves as buffering agent and loss of buffering capacity leads to increase the pH of the medium which might be growth inhibiting [19]. The interaction effect of $CaCl_2$ (X_3) and KH_2PO_4 (X_4) was also significant ($p< 0.0012$). Figure 3.f shows interactions effect of $CaCl_2$ (X_3) and KH_2PO_4 (X_4) on percentage degradation. The maximum 98.60 % of phenol degradation was found at 0.015 g/l and 0.75 g/l of $CaCl_2$ (X_3) and KH_2PO_4 (X_4).

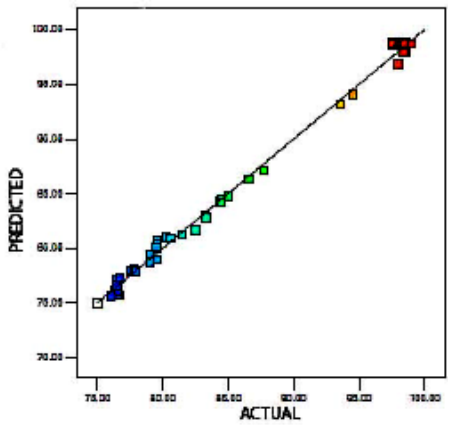

Fig 2. Graphical comparison between actual and predicted percentage of phenol degradation

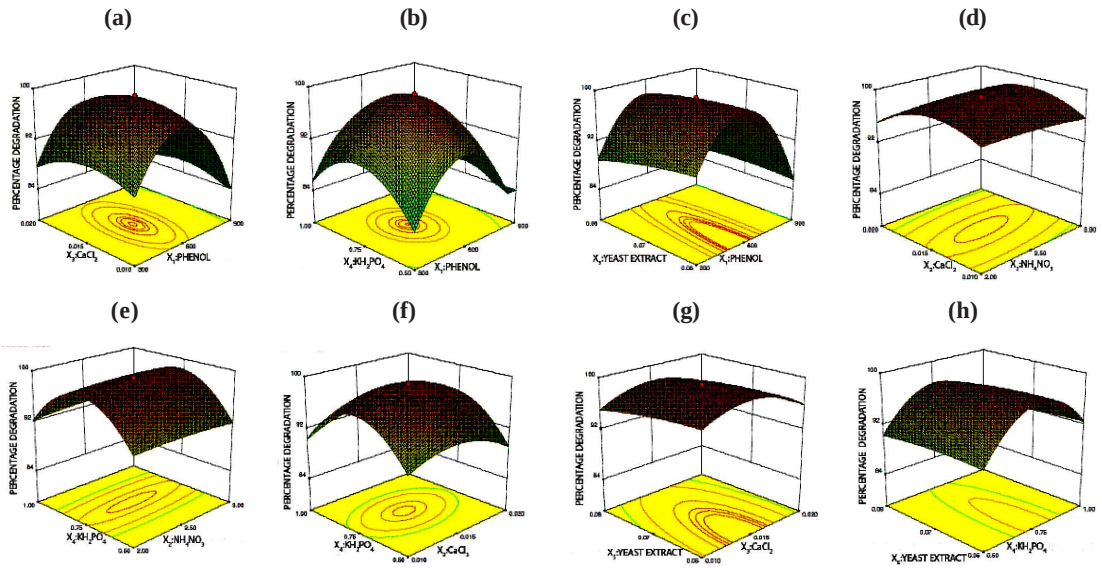

Fig .3. Surface plot showing the interactions effect of **(a)** Phenol concentration (X_1) and $CaCl_2$ (X_3) on percentage degradation **(b)**Phenol concentration (X_1) and KH_2PO_4 (X_4) on percentage degradation **(c)** Phenol concentration (X_1) and Yeast Extract (X_5) on percentage degradation **(d)** NH_4NO_3 (X_2) and $CaCl_2$ (X_3) on percentage degradation **(e)** NH_4NO_3 (X_2) and KH_2PO_4 (X_4) on percentage degradation **(f)** $CaCl_2$ (X_3) and KH_2PO_4 (X_4) on percentage degradation **(g)** $CaCl_2$ (X_3) and Yeast extract (X_5) on percentage degradation **(h)** KH_2PO_4 (X_4) and yeast extract (X_5) on percentage degradation

The interaction effect of $CaCl_2$ (X_3) and yeast extract (X_5) was also significant ($p< 0.0416$). Figure 3.g shows interactions effect of $CaCl_2$ (X_3) and yeast extract (X_5) on percentage degradation. The maximum 98.69 % of phenol degradation was found at 0.015 g/l and 0.07 g/l of $CaCl_2$ (X_3) and yeast extract (X_5).The interaction effect of KH_2PO_4 (X_4) and yeast extract (X_5) was also found to be significant (p=.0078).Maximum of 98.73% of phenol degradation was obtained when the KH_2PO_4 (X_4) and yeast extract (X_5) concentration were 0.74 g/l and 0.07 g/l respectively as shown in figure-3.h.

Validation of the model

In order to determine the correctness of the model, verification experiments were performed using the statistically optimized medium. The maximum experimental response for phenol degradation 98.5% was observed at initial phenol concentration 600 mg/l, NH_4NO_3-2.5 g/l, $CaCl_2$-0.015 g/l , KH_2PO_4-0.75 g/l, and Yeast Extract -0.07 g/l. The predicted percentage degradation was found to be 98.73% indicating a strong agreement between the observed and predicted value calculated from the model that confirms the validity and precision of the model.

CONCLUSION

A new strain *Candida tropicalis*SSK01 was isolated from petroleum contaminated soil was able to degrade high initial concentration of phenol. The PB was applied to assess the effect of medium composition like initial phenol concentration, NH_4NO_3, KH_2PO_4, $CaCl_2$, K_2HPO_4, $MgSO_4$, KCl and Yeast Extract on phenol degradation. The initial screening done using P B experimental design showed that initial phenol concentration, NH_4NO_3, KH_2PO_4, $CaCl_2$ and Yeast Extract were found to affect phenol degradation. Based on CCD the optimum concentrations of the critical components was found to be initial phenol concentration 600 mg/l, NH_4NO_3-2.5 g/l, $CaCl_2$-0.015 g/l ,KH_2PO_4-0.75 gl^{-1}, and Yeast Extract -0.07 g/l for 98.5 % phenol degradation in 72 hours. The R^2, adjusted R^2 and Predicted R^2 values calculated by CCD were 0.9967, 0.944 and 0.990 respectively indicating the experimental values are in good agreement with predicted values. The combined optimization method described here is new and effective method for screening medium components as well as determining their optimum level concentration for percentage of phenol degradation. Optimization of the culture medium reduces the cost of medium components and improved the feasibility of commercial degradation of phenol in industrial scale.

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