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Isolation and characterization of phenol degrading organism, optimization using Doehlert design

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ABSTRACT

Phenol degrading organisms were effectively isolated from petroleum contaminated soil. Based on their resistivity to phenol up to 1500 mg l^{-1}, the bacterial strains were isolated as R1, R2, R3, R4, and R5. Morphological studies showed that most of these organisms as gram-positive, rod-shaped and aerobic. These five isolated organisms showed maximum similarity with *Pseudomonas stutzeri* NCG1, *Bacillus flexus* strain MS14-1, *Bacillus thuringiensis* strain 2PR56-10, *Bacillus anthracis* strain IHB B 18197, and *Bacillus thuringiensis* strain Bt 2 based on 16S rRNA sequencing. Preliminary experimental conditions such as pH, temperature, aeration, inoculum age and inoculum volume were studied for maximum phenol degrading organisms along with mixed culture. Under optimized conditions, gram-negative *P. stutzeri* showed the maximum capability to degrade phenol up to 81.8% at 500 mg l^{-1} initial concentration within 84 h. Also, the phenol degradation by *Pseudomonas stutzeri* was optimized using response surface methodology (RSM) for various parameters viz. pH, temperature, phenol concentration and yeast extract concentration through batch experiments according to Doehlert design. The optimized conditions of pH, temperature and yeast extract concentration were used to determine the kinetic parameters of phenol degradation using various models. The analysis of kinetic data suggests that the Haldane substrate inhibitory model can be used to fit experimental data. Additionally, phenol tolerant *Pseudomonas stutzeri* produced *cis, cis*-muconic acid which is a metabolic intermediate specifying the organism followed ortho pathway.

Keywords: Phenol degradation; *Pseudomonas stutzeri*; *Bacillus flexus*; Pollutants; Biochemical; Kinetics; Optimization; Doehlert; Ortho pathway

1. Introduction

Phenol is considered as priority pollutant that affects various aquatic life even at low concentration of 5–25 mg l^{-1} when released into the water bodies [1,2]. Hence it is highly essential to develop efficient techniques to remove the pollutant from the ecosystem. A variety of methods to treat phenolic effluent such as precipitation, osmosis, ion exchange, ultrafiltration, electrochemical, etc. are available with certain limitations [3]. Moreover, these treatment methods often produce toxic end products, requiring further processing steps [4]. Biodegradation is the most efficient and cheap method to reduce phenol from its permissible limits 0–0.05 mg l^{-1} as it leads to complete removal of phenols and is inexpensive too.

Various studies have been reported on biodegradation of phenol using bacteria such as *P. putida, P. auregenosa, P. fluorescence, Acinetobacter, Bacillus* [5–8]. These organisms followed different pathways to degrade phenol and utilize different time intervals to a complete removal of phenol from the waste stream [9,10]. Due to the increase in the release of various complex phenolic compounds into the environment, demand for anew organism is highly crucial to degrade phenol effectively.

Statistical techniques have been applied by several researchers to optimize model parameters [11]. The techniques include conducting experiments according to

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statistical design, assessing the mathematical model coefficients, proving the model adequacy along predicting the response. The traditional 'one-factor at a time' technique is a time-consuming method that requires to perform a number of experiments [12]. The number of experiments can be reduced by using one of the several experimental designs such as Plackett-Burman, Box-Behnken, Doehlert, and Central Composite (CC) [13]. The Plackett-Burman design is widely used for screening of significant factors, however, there is no consideration for interaction effects among variables [14]. The advantages of Doehlert design are (1) the sphericity in an experimental domain with a uniform space filling, (2) the ability to explore entire domain, and (3) the capability to reuse experiments sequentially when the boundaries have not been well chosen at first [15]. Only a few experiments in the experimental domain can be chosen more efficiently in Doehlert design [14]. The major advantage of the Doehlert design over the CC is expanding with another factor or shifting the design towards a new experimental domain [16].

The determination of phenol degradation pathways reported to date is either limited for certain initial phenol concentrations or low phenol concentration ranges. Since these pathways vary with strains and the phenol concentration ranges, there is a necessity to identify metabolic pathway under optimized conditions [17]. Also, the phenol concentrations in effluents may vary from 1–3000 mg l^{-1} which urges further investigation of the pathways in all possible initial phenol concentrations. The microorganism's metabolic pathway for the substrate also affects the composition of the waste stream due to the accumulation of variable metabolic intermediates. Therefore, it is essential to verify the reliability of phenol degradation pathway of microorganism for the possible phenol concentration range. Due to the toxic nature of phenol, the degradation rates by organisms will not be the same at different initial substrate concentrations. It is challenging to identify an appropriate substrate inhibition model that accurately predicts the degradation of phenol at different higher concentrations. Also, the design of a bioremediation system requires kinetic constants which determine the fate of toxic substrates.

In the present study, an effort to isolate bacterial strains capable of degrading phenol from the petroleum-contaminated soil has been undertaken. Morphological, biochemical and genotypic characterization of the isolated species were investigated in detail. Optimization of growth conditions such as pH, temperature, agitation, inoculum age, and inoculum volume on phenol degradation has been studied extensively. Interaction effect of each parameter on phenol degradation has been analyzed using RSM. Besides mechanism, phenol degradation using the microorganism is studied.

2. Materials and methods

2.1. Reagents and media

All chemicals and reagents were analytical grades. Minimal salt media (MSM) used as growth media contained $CaCl_2{\cdot}2H_2O$, 0.010 g; $FeSO_4{\cdot}7H_2O$, 0.010 g; $MnSO_4{\cdot}7H_2O$, 0.5 g; $MgSO_4{\cdot}7H_2O$, 0.5 g; NaCl, 0.5 g; NH_4SO_4, 0.5 g; NH_4NO_3, 1 g; K_2HPO_4, 1.5 g; KH_2PO_4, 0.5 g in one litre of deionized water. MSM media was supplemented with phenol as a sole carbon source.

2.2. Enrichment, isolation, and maintenance of strain

The vehicle workshops located at sector-2 Rourkela, Odisha have been disposing of various organic products such as grease, motor oil, engine oil, petrol, diesel, etc. into the soil nearby. The contaminated soil is expected to consist of organisms that adapted and survived at a higher concentration of organic products and has the potential to degrade phenol. In the present study, soil samples were collected from this contaminated soil and immediately stored in a previously sterilized sealed polyethylene bags and preserved at 4°C for further physiological and microbial assays.

One gram of soil sample was added to 9 ml sterilized water, and 0.1 mL of the diluted sample was subsequently transferred to 250 mL Erlenmeyer flask containing 100 mL of nutrient broth andincubated (120 rpm) at 30°C for 24 h in an incubator shaker. Cell growth was observed at 600 nm (OD_{600}) with time. About one mL of the incubated culture was aseptically inoculated into a 250 mL flask containing MSM medium. The content in this flask was further supplemented with phenol (100–2000 mg l^{-1}) which was used as a sole carbon source and incubated at 30°C in an incubated shaker at 120 rpm for 48 h. Selection of organisms was based on their ability to grow at higher phenol concentration and utilized as asole carbon source. The enriched culture observed with more biomass (in the flask with 1000 mg l^{-1} phenol) was further diluted serially and spread on agar plates. Single colonies with significant morphological differences were chosen and streaked on new agar plates. The developed colonies were stored at 4°C until further study.

2.3. Morphological characterization

The morphological and physiological properties of the selected strains such as motility, cell shape, cell size, colony color, colony texture, pH, temperature, oxygen dependency were systematically performed. The bacterial physiology and biochemical characteristics viz. Motility, Gram staining, starch hydrolysis were investigated, as described in Bergey's manual of determinative bacteriology [18]. Typical biochemical tests like oxidase, nitrate reduction, urease, gelatin liquefaction, catalase, indole, citrate, methylred, hydrogen sulfide were also performed [19]. Morphological properties of the selected colonies were observed by optical microscopy [Leica, DM2500]. Further, the strain with desired ability to degrade phenol was examined by FESEM (Nova Nanosem 450). For cell preparation, it was fixed in 0.1 M phosphate buffer solution (PBS) containing 2% glutaraldehyde solution at 4°C. The cells were washed with PBS followed by distilled water and finally subjected to tannic acid treatment for 5 min. For dehydration, the samples were treated with series of ethanol at increasing concentration (30%, 50%, 70%, 90% and 100% v/v). Dehydrated cells were dried and placed in a brass tube coated with platinum and subsequently observed by FESEM.

2.4. Phenol degradation

The bacterial culture was inoculated aseptically into a 100 mL MSM in 250 mL flask containing 500 mg l^{-1} phenol as a sole carbon source. It was incubated at 30°C, 120 rpm shaking speed for 84 h to study the phenol degradation potential of each isolated strain and mixed culture of R1 to R5. Samples were collected periodically to estimate the phenol concentration and biomass growth. The phenol concentrations were estimated using the 4-aminoantipyrene (4-AAP) method at wavelength 510 nm using a UV-Visible spectrophotometer, Shimadzu, 1800 [20].

2.5. Characterization by 16S rDNA sequencing and phylogenetic analysis

Genomic DNA of the selected colony was extracted, and 16S rDNA was amplified with universal primers derived from eubacteria 8F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-GGTTACCTTGTTACGACTT-3′) on a Veriti® 99 well Thermal Cycler (Model No. 9902) [21]. A single discrete PCR amplicon band of 1500 bp was observed. The amplicon was enzymatically purified and subjected to sequencing. PCR amplicon was subjected to forward and reverse RNA sequencing reaction with 704F and 907R primers using BDT v3.1 Cycle Sequencing kit and ABI 3730xl Genetic Analyzer (Applied Biosystems, California). 16S rDNA consensus sequence was generated from forward and reverse sequence data using aligner software. BLAST alignment search tool of NCBI GenBank database was used to search the 16s rDNA sequence. First fifteen sequences were selected based on maximum identity score and aligned using multiple alignment tools called Clustal W. The Distance matrix was generated using RDP database and the phylogenetic tree was constructed using MEGA5 [22]. The evolutionary history was inferred using the Neighbor-Joining method [23]. The bootstrap consensus tree inferred from 1000 replicates is taken to represent the evolutionary history of the taxa analyzed [24]. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates were collapsed. The evolutionary distances were computed using the Kimura 2-parameter method and are in the units of the number of base substitutions per site [25]. The analysis involved 16 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. Evolutionary analyses were conducted in MEGA5 [22]. The 16S rRNA nucleotide sequences of R1 to R5 were deposited in GenBank with accession numbers MG230258, MG230259, MG230260, MG230261, and MG230262 respectively.

2.6. Parameter standardization

Organisms capable of degrading maximum phenol were subjected to optimization of growth conditions such as pH, temperature, shaking speed, inoculum age, inoculum volume, etc. Keeping all other parameters constant, the influence on phenol degradation was studied for pH (6, 6.5, 7, 7.5, 8, and 8.5); temperature (25, 30, 35, 40, and 45°C); agitation speed (60, 100, 140, and 180 rpm). By using optimized pH, temperature and agitation speed the two parameters inoculum volume (2, 3, 4, 5, and 6% v/v) and inoculum age (6, 12, 18, and 24 h) were studied.

2.7. Design of experiments

The experimental design was generated using a Doehlert matrix with Xlstat 2018 trial version. The set of experiments with independent variables viz., Phenol (X_1, 100–1500 mg l^{-1}), Yeast extract (X_2, 1–3 g/l), pH (X_3, 6–8) and temperature (X_4, 28–37°C) obtained by Doehlert experimental design are shown in Table 3. The factors and corresponding levels were chosen based on parameter standardization experiments done. A feature of Doehlert design is that the number of levels of each experimental factor is not the same. The total number of points for four factors is 21 ($N = k^2 + k + 1$). A full quadratic model containing 14 coefficients including interaction terms were used to describe relationships between response and experimental factors:

$$\eta = \beta_0 + \sum_{i=1}^{4}\beta_i X_i + \sum_{i=1}^{4}\beta_{ii} X_i^2 + \sum_{i=1}^{3}\sum_{j=i+14}^{4}\beta_{ij} X_i X_j \quad (1)$$

where η is the response, β_0 is the constant coefficient, X_i (i = 1 to 4), are uncoded variables, β_i is the linear coefficient, β_{ii}(i = 1 to 4) is the quadratic coefficient, and β_{ij} (i and j = 1 to 4) is the second-order interaction coefficient. Additionally, four repetitions were conducted for a central point to assess the fit the model and precise prediction analysis. The data was analyzed by using Minitab 17 trial version including ANOVA to obtain interaction effects of variables on phenol degradation (Y, %). The statistical significance of the coefficients and the model were determined by Student's t-test and Fisher's test.

2.8. Phenol biodegradation and kinetics

The study of the batch degradation of phenol was carried out using statistically optimized parameters of pH 7, temperature 32.5°C, and yeast extract concentration of 2% in MS media with inoculum volume of 1 ml (OD_{600}0.02). The initial concentration of phenol was varied from 0 to 1500 mg l^{-1} with an interval of 125 mg l^{-1}. The batch experiments were conducted for 72 h under optimized conditions. The samples were withdrawn every 3 h, centrifuged (5000 g for 10 min) and filtered to determine the residual phenol concentration. The data obtained from the phenol biodegradation experiments were fitted to suitable kinetic models viz., Haldane, Yano, and Webb [26–28] . The degradation rate, q (h^{-1}) and fitted models are listed in Table 5. Where, q_{max} is the maximal degradation rate (h^{-1}), S_o the initial substrate concentration (mg l^{-1}), k_s half-saturation constant for maximal degradation (mg l^{-1}), and k_i substrate-inhibition constant, k curve parameter in Yano and Webb models. The degradation rate, q for each initial phenol concentration was determined from the slope of a semi-logarithmic plot of phenol concentration, S vs time. The kinetic parameters of available models were obtained using curve fitting of experimental values of q vs. S_o using CurveExpert Professional 2.4 trial version.

2.9. Phenol degradation pathway

On the other hand, the degradation pathway was studied with the culture media containing varying concentrations of phenol (250, 500, 750, 1000, 1250, and 1500 mg l^{-1}). For determining activities of intracellular enzymes, the samples were taken at mid of exponential phase and filtered through 0.2 µm nylon membrane filters (Whatman).

The collected cells were washed with 50 mM phosphate buffer of pH 7, and the cell suspension of phosphate buffer was subjected to sonication. Thus, the crude extract obtained was centrifuged for 30 min at 10,000 rpm and 4°C. The enzyme activities of catechol-2,3-dioxygenase and catechol-1,2-oxygenase were determined based on the presence of 2-Hydroxymuconic semialdehyde and *cis*, *cis*-muconic acid that was identified spectrometrically (Shimadzu analytical, UV-1800, India) at 375nm and 260 nm, respectively. A unit of enzyme is defined as the amount of enzyme producing 1 µmol of product formed per minute at 25°C.

3. Results and discussion

3.1. Morphological and biochemical characteristics of isolated bacteria

Soil samples collected from vehicle workshop were inoculated in the medium containing phenol for enrichment and isolation of phenol-degrading bacteria. The phenol resistant organism in soil samples was exposed to increasing concentration of phenol 100 mg l^{-1}–2000 mg l^{-1}. The bacteria were isolated separately that could utilize phenol as a sole carbon source in the enrichment study using the mineral salt medium. After four weeks of enrichment and a week of isolation, a total of 5 different colonies (R1, R2, R3, R4, and R5) were observed after 48 h on nutrient agar plates. These isolates were stored separately on agar slants at 4°C in refrigerator until further study. The growth and biochemical characteristics of the five isolates are shown in Table 1. These organisms were similar in shape (Rod), and cream in color but R3 was whitish to cream color. While R2 reported transparent color that helped to distinguish the organism easily. The growth temperature and pH for all the organisms were found to be suitable in the range of 35–40°C and 6–10 respectively. All of them were aerobic and were gram-positive except for R1 which was gram-negative.

Table 1
Morphological, biochemical characteristics and BLAST search query attributes of isolated organisms

	R1	R2	R3	R4	R5
Staining reaction	–	+	+	+	+
Cell shape	Rod	Rod	Rod	Rod	Rod
Cell size	3–4 µm	3–5 µm	2–7 µm	3–5 µm	2–6 µm
Colony color	Cream	Transparent	Whitish to cream	Cream	Cream
Colony texture	Wrinkled	Smooth, opaque	Matt or granular	Smooth	Granular
Motility	+	–	+	–	–
Oxygen dependency	Aerobic	Aerobic	Aerobic	Aerobic or anaerobic	Aerobic
Oxidase test	+	+	–	–	–
Nitrate reduction	+	–	+	+	+
Urease test	–	+	–	–	–
Starch hydrolysis	+	+	+	–	+
Gelatin liquefaction	+	+	+	+	+
Catalase test	+	+	–	–	–
Indole test	+	–	–	+	–
Citrate test	–	–	–	–	–
Methyl red test	–	–	–	–	–
Hydrogen Sulfide test	+	+	–	–	–
Secondary metabolites production	+	–	–	–	–
BLAST search with query attributes at NCBI GenBank					
Query ID (lcl|Query_ID)	96015	232447	227849	214975	200979
Query length	1266 bp	1501 bp	1516 bp	1181 bp	1331 bp
Maximum score	2250	2721	2647	2165	2429
Total score	2250	2721	2647	2165	2429
Query coverage	98%	99%	99%	100%	100%
Expect value	0.0	0.0	0.0	0.0	0.0
Maximum identity	99%	99%	98%	99%	99%
Identified strain with accession number	*Pseudomonas stutzeri* strain NCG1 (JN712254.1)	*Bacillus flexus* strain MS14-1 (KJ496372.1)	*Bacillus thuringiensis* strain 2PR56-10 (EU440975.1)	*Bacillus anthracis* strain IHB B 18197 (KM817219.1)	*Bacillus thuringiensis* strain Bt 2 (KY777762.1)

The biochemical test results for the isolates are interpreted in Table 1 using positive and negative signs. Based on the morphological and biochemical characteristics, the isolate R1 was identified as *Pseudomonas sp.* Similarly, the remaining isolates R2 to R5 were identified as *Bacillus sp.*

3.2. Identification of phenol resistant organisms and phylogenetic analysis

The 16S rDNA sequence of the isolate was used, and relative strains were identified. The five isolates along with attributes of BLAST search are shown in Table 1. The phylogenetic trees were constructed using the Megafive tool. The phylogenetic trees are shown in Fig. 1 represents the closest relatives of R1, R2, R3, R4, and R5 with accession numbers followed by names. Based on homology match of 16s rDNA, it showed the closest relation with *Pseudomonas stutzeri* strain NCG1. It has been known to degrade chlorobenzenes and also used in the production of melanin [29, 30]. Similarly, the isolate R2 showed up to 99% similarity with *Bacillus flexus* strain MS14-1. The applications of this version of the organism haven't been reported earlier, but Bacillus flexus have been known for degrading 3-Nitrobenzoate and microcystin RR [31,32]. The third isolate R3 was 98% similar to *Bacillus thuringiensis* strain 2PR56-10 which is a PAH (polycyclic aromatic hydrocarbon) degrading bacteria [33]. The remaining two isolates R3 and R4 were found to have the closest relationship with *Bacillus anthracis* strain IHB B 18197 and *Bacillus thuringiensis* strain Bt 2.

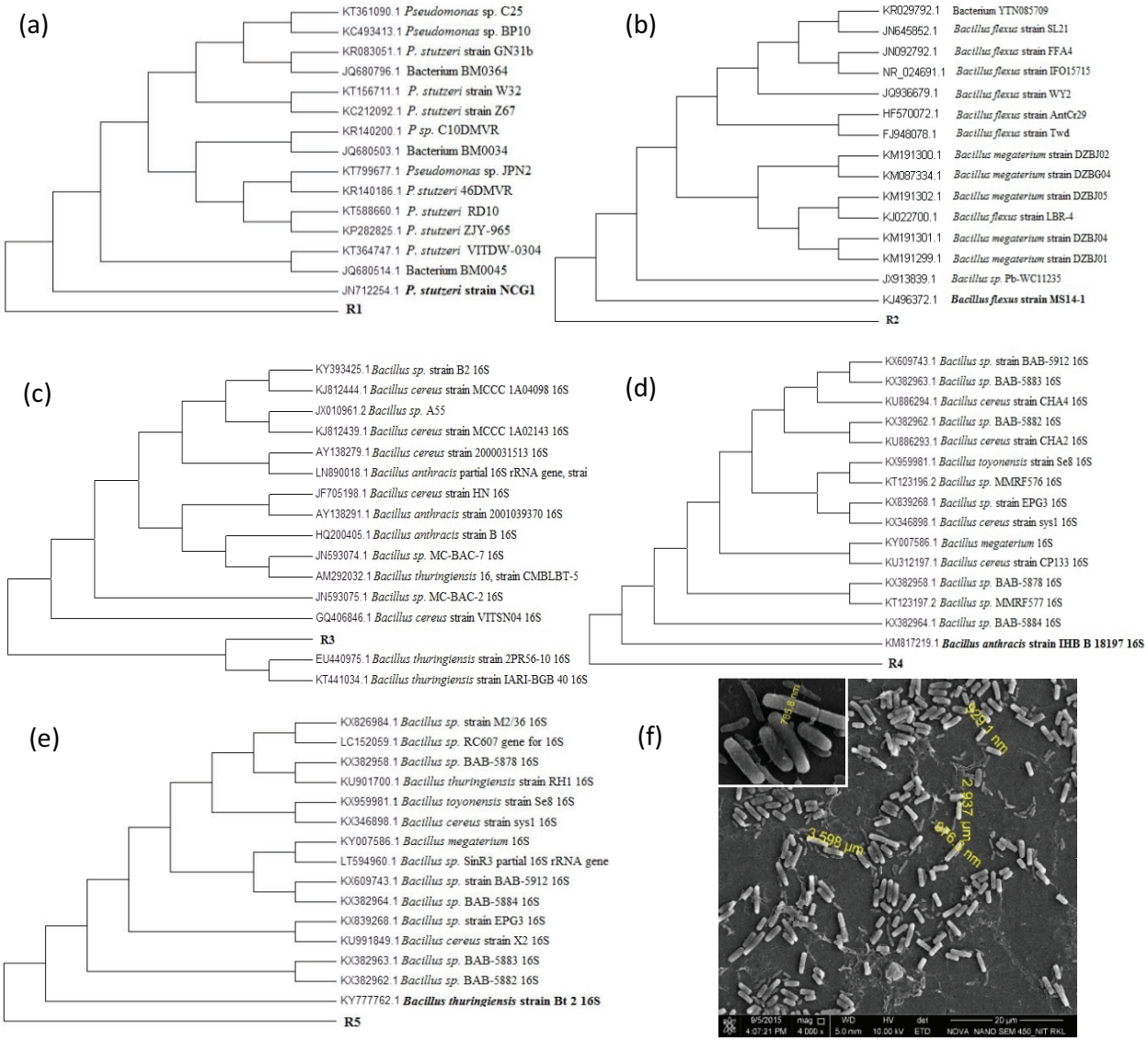

Fig. 1. Phylogenetic trees showing the similarity of isolated organisms R1, R2, R3, R4 and R5 with (a) *Pseudomonas stutzeri* NCG1, (b) *Bacillus flexus* strain MS14-1, (c) *Bacillus thuringiensis* strain 2PR56-10, (d) *Bacillus anthracis* strain IHB B 18197, and (e) *Bacillus thuringiensis* strain Bt 2 respectively. The Genbank accession numbers along with organism names are shown at the end of the boxes (f) Field emission scanning electron microscope (FESEM) images of *Pseudomonas stutzeri* NCG1 showing rod-shaped cells magnified up to 16000×.

3.3. Phenol degradation study

Pseudomonas stutzeri NCG1, *Bacillus flexus* strain MS14-1, *Bacillus thuringiensis* strain 2PR56-10, *Bacillus anthracis* strain IHB B 18197, and *Bacillus thuringiensis* strain Bt 2 representing the five isolated microorganisms were resistant to 1500 mg l^{-1} of phenol. To assess the ability of phenol degradation, these organisms were studied at an initial phenol concentration of 500 mg l^{-1} as shown in Fig. 2a. *Pseudomonas stutzeri* showed degradation up to 81.8% in 84 h, and *Bacillus flexus* strain showed degradation up to 75.8% in 84 h. The mixed culture also showed the highest degradation of up to 81.7% in 84 h which suggests the dominance of *P. stutzeri* in degradation. The parameter standardization for maximum phenol degradation by R1, R2, and mixed cultures was further investigated.

3.4. Effect of temperature on phenol degradation

Temperature is an important factor affecting the metabolism of an organism and plays a significant role in the degradation of organic pollutants [34]. The time course of maximum degradation of phenol by *P. stutzeri* (R1), *Bacillus flexus* (R2), and mixed culture were observed over a temperature range of 25°C to 45°C as shown in Fig. 2b. The degradation of phenol was found to be maximum at 30°C and 35°C temperatures for *P. stutzeri* (R1), *Bacillus flexus* (R2) and mixed culture. The phenol degradation was prolonged from 96 to 133 h at 45°C for the above cultures. The degradation percentages for the three cultures were higher than those interepreted in Fig. 2a. Polymenakou et al. have also observed the highest degradation of phenol at 30°C [35,36]. Some researchers observed that phenol degradation was ceased beyond 30°C because of cell decay [37].

3.5. Effect of pH on phenol degradation

Some organisms may tolerate varying pH and accomplish their physiological activities, but the extent of activity varies with pH. Most organisms cannot perform metabolic activities below pH 4, above pH 9 and believed to perform in the approximately neutral environment [38] . This behavior was based on the fact that the strength and ion concentration increases with pH which in turn increases permeability across cell membranes as a direct result of the electrostatic force that cannot be prevented [37–39]. The phenol degradation ability by *Pseudomonas stutzeri* (R1), *Bacillus flexus* (R2) and mixed culture were studied over a pH range of 6 to 8.5 as shown in Fig. 2c. In the present work, the maximum phenol was degraded within 84 h at neutral pH by *Pseudomonas stutzeri* (R1) whereas *Bacillus flexus* (R2) found to degrade phenol within 72 h in alkaline pH. The mixed culture showed the highest degradation within 60 h near neutral pH. Also, all the three cultures showed minor variation in the maximum phenol degradation in the studied pH range.

3.6. Effect of agitation on phenol degradation

The mass transfer influences uniform utilization of nutrient sources and depend on agitation during fermentation of aerobic organisms. Also an increase in agitation results in disruption of cells and formation of debris. Hence, the effect of shaking speed on the degradation of phenol by *Pseudomonas stutzeri* (R1), *Bacillus flexus* (R2) and mixed culture was investigated in the range of 60–180 rpm. The maximum degradation was obtained at 140 rpm for all the organisms. At this definite speed (140 rpm) *Pseudomonas stutzeri* (R1), *Bacillus flexus* (R2) and mixed culture showed maximum degradation at different time interval viz. 72 h, 84 h, and 60 h respectively. Earlier researchers also observed the complete degradation of phenol by *P. aeruginosa* MTCC 4996 at an agitation of 100–125 within 66 h (Kotresha and Vidyasagar, 2008).

3.7. Effect of inoculum concentration and age on phenol degradation

The inoculum concentrations of 2% to 6% v/v were chosen to determine their influence on phenol degradation in 500 mg l^{-1} phenol containing media using above optimized pH, temperature and agitation for respective cultures. The inoculum concentration of 6% v/v showed highest phenol degradation in short duration compared to other concentrations as shown in Fig. 2d. This may be due to the reduction of lag phase with increased concentration perhaps helped the system to attain the exponential phase rapidly. Hill and Robinson also showed the effect of inoculum concentration on the lag phase [40]. In the present study, after an optimum inoculum concentration (6%), the effect on phenol degradation was marginal. The organisms were grown in mineral media for different time periods viz. 6 h, 12 h, 18 h and 24 h and respective ages were investigated for efficient phe-

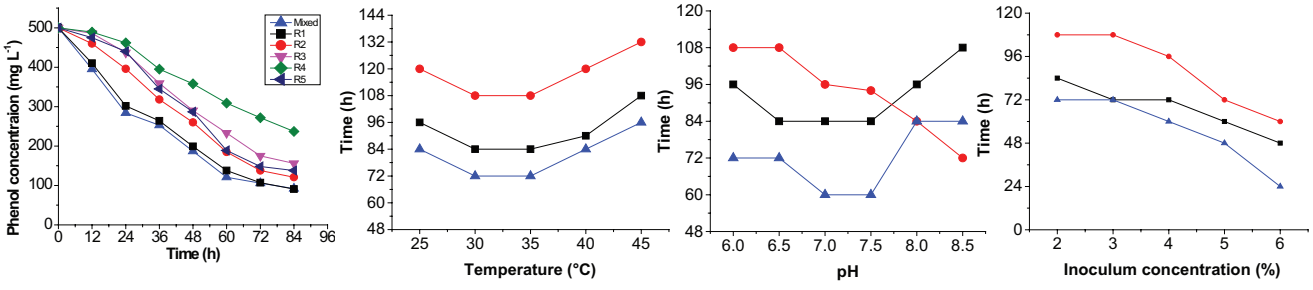

Fig. 2. (a) Phenol degradation profiles of ■R1, ●R2, ▼R3, ◆R4, ◄R5, and ▲ mixed culture (b, c, and d) Effect of temperature, pH, and inoculum concentration on the time required for maximum phenol degradation by *Pseudomonas stutzeri* (R1), *Bacillus flexus* (R2) and mixed culture.

nol degradation in 500 mg l^{-1} phenol containing media. The phenol degradation by *Pseudomonas stutzeri* (R1), *Bacillus flexus* (R2) and mixed culture were highest with an inoculum age of 12 h which is related to the exponential phase of cell growth. Similar results reported earlier for phenol degradation in exponential phase by Bandyopadhyay et al. using *Pseudomonas putida* [41]. The organisms isolated in the present investigation demonstrates significant potential to degrade phenol when compared with other organisms reported by previous researchers as shown in Table 2. In particular, *P. stutzeri* was proven to be the highest phenol degrading organism. Further the major factors affecting phenol degradation by *P. stutzeri* through statistical parameter optimization, phenol degradation kinetics and mechanism were also investigated.

3.8. Response surface methodology

Response surface methodology (RSM) is extensively used to evaluate the relationship between factors and responses based on the models derived. A four-factor multilevel Doehlert design consisting of 24 experiments for RSM was used to study the linear, square and interaction effects of variables on phenol degradation. The experimental and predicted values are shown in Table 3. The quadratic polynomial model shown in Eq. (1) is found to be suitable for the data obtained with $R^2 = 0.9961$ and adjusted $R^2 = 0.9871$.

$$\begin{aligned} Y = {} & -192.0 - 0.01866X_1 - 6.14X_2 + 56.07X_3 + 5.82X_4 \\ & + 0.000005X_1^2 - 2.06X_2^2 - 4.275X_3^2 - 0.0612X_4^2 \\ & - 0.000668X_1X_2 + 0.00333X_1X_3 - 0.000812X_1X_4 \\ & + 2.351X_2X_3 + 0.015X_2X_4 - 0.134X_3X_4 \end{aligned} \quad (2)$$

The initial phenol concentration (X_1) and yeast extract concentration (X_2) showed a negative effect on the percentage of phenol degradation (Y). It suggests an increase in initial phenol concentration produced a toxic effect on the cells and reduced their ability to degrade phenol. Also, the increase in the initial concentration of yeast extract influenced organisms to minimize the phenol intake. The pH of the medium (X_3) had a greater effect on phenol degradation followed by temperature (X_4). The percentage phenol degradation increased with increase in pH from acidic to neutral and also from room temperature to higher temperature. But, a further increase in pH and temperature showed a negative effect on phenol degradation as suggested by quadratic terms in the model. Therefore, a near neutral pH and moderate temperature could enhance the phenol degradation. Additionally, the increased concentration of yeast extract continued to have a negative effect on phenol degradation as suggested by its squared term in the model.

3.9. Analysis of variance (ANOVA)

The results of the experiments were analyzed using F-test and ANOVA (analysis of variance) to assess the fit of the model. The main advantage of this analysis approach is that the model suggests the interaction effects besides main effects [47]. The estimated effects listed in Table 4 are constituted by four linear, four quadratics, six interaction effects. Also, student's *t*-test and P-values were also determined to understand the statistical significance of the model terms. The smaller the P-value ($p < 0.05$) and larger the *t*-value of a term, the more significant is the term in the model [48]. The results of the ANOVA revealed that all the main factors, quadratic effects, and X_1X_3, X_1X_4, X_2X_3 interactions were statistically significant at 95% confidence level with $p \leq 0.05$. However, the interactions of X_1X_2, X_2X_4, X_3X_4 have $p > 0.05$ elucidates their statistical insignificance which was a consequence of low values of corresponding coefficients in the model. The quality of fit is explained by R-squared statistic also known as the coefficient of determination which was found to be 0.9957. The value of adjusted R-squared is also in good agreement with the predicted R-squared to support the significance of the model. The predicted R-squared also suggests that the model can precisely predict new observations. The standard deviation (S) between a data point and the fitted value was found to be less. The P-value for Lack-of-fit is 0.143 indicates the model is adequate for all experimental conditions performed.

3.10. Interaction effects

The effect of independent variables on the phenol degradation was evaluated using RSM. The interactions of the factors were interpreted in 3D surface plots and 2D contour plots to understand their effect on the response. Generally,

Table 2
Illustrates the comparison of phenol degradation potential of different organisms with the present study

Microorganisms	Initial phenol conc. (g l^{-1})	Conditions	Maximum percentage phenol degradation (time, h)	References
Pseudomonasstutzeri strain NCG1 (R1)	0.5	Optimum	81.8 (84)	Present study
Bacillus flexus	0.5	Optimum	75.8 (84)	Present study
Mixed consortium (R1 to R5)	0.5	Optimum	81.7 (84)	Present study
Chlorella pyrenoidosa	0.8	Ambient	97 (96)	[42]
Halophilic bacteria	0.28	Hypersaline	100 (120)	[43]
Mixed consortium	0.5	Anaerobic	99.5 (42)	[44]
Pseudomonas putida	2.1	Aerobic	100 (72)	[45]
Pseudomonas sp.	2.66	Aerobic	98.28 (32)	[46]

Table 3
Experimental and predicted values in phenol degradation experiment design along with coded variables

Run	Initial phenol concentration (X_1), mg l^{-1}	Yeast extract concentration (X_2), g l^{-1}	pH (X_3)	Temperature (X_4), °C	Percentage degradation of phenol (Y), experimental	Percentage degradation of phenol (Y), predicted
1	1500	2.00	7.00	32.50	73.73	74.317
2	100	2.00	7.00	32.50	96.69	96.103
3	1150	2.86	7.00	32.50	77.90	78.066
4	450	1.13	7.00	32.50	85.59	85.424
5	1150	1.13	7.00	32.50	75.38	74.936
6	450	2.86	7.00	32.50	88.92	89.364
7	1150	2.28	7.81	32.50	76.90	76.420
8	450	1.71	6.18	32.50	86.92	87.400
9	1150	1.71	6.18	32.50	75.15	74.737
10	450	2.28	7.81	32.50	85.13	85.543
11	800	2.57	6.18	32.50	80.32	80.086
12	800	1.42	7.81	32.50	76.23	76.464
13	1150	2.28	7.20	36.05	77.85	77.577
14	450	1.71	6.79	28.94	85.32	85.593
15	1150	1.71	6.79	28.94	76.11	76.380
16	450	2.28	7.20	36.05	90.42	90.150
17	800	2.57	6.79	28.94	81.58	81.204
18	800	1.42	7.20	36.05	80.17	80.546
19	800	2.00	7.61	28.94	79.52	79.353
20	800	2.00	6.38	36.05	82.15	82.317
21	800	2.00	7.00	32.50	83.00	82.900
22	800	2.00	7.00	32.50	82.62	82.900
23	800	2.00	7.00	32.50	82.70	82.900
24	800	2.00	7.00	32.50	83.36	82.900

Table 4
Analysis of variance (ANOVA) along with coefficients in the phenol degradation model obtained by multiple linear regression

Source	DF	Adj SS	Adj MS	F-Value	P-Value	T-Value
Model	14	692.098	49.436	147.24	0	
X_1: Initial phenol concentration	1	593.287	593.287	1767.04	0	–42.04
X_2: Yeast extract concentration	1	20.827	20.827	62.03	0	7.88
X_3: pH	1	3.002	3.002	8.94	0.015	–2.99
X_4: temperature	1	8.12	8.12	24.19	0.001	4.92
$(X_1)^2$	1	6.992	6.992	20.83	0.001	4.56
$(X_2)^2$	1	5.658	5.658	16.85	0.003	–4.11
$(X_3)^2$	1	27.413	27.413	81.65	0	–9.04
$(X_4)^2$	1	2.559	2.559	7.62	0.022	–2.76
X_1*X_2	1	0.164	0.164	0.49	0.502	–0.7
X_1* X_3	1	3.266	3.266	9.73	0.012	3.12
X_1* X_4	1	3.632	3.632	10.82	0.009	–3.29
X_2* X_3	1	3.317	3.317	9.88	0.012	3.14
X_2* X_4	1	0.003	0.003	0.01	0.932	0.09
X_3* X_4	1	0.203	0.203	0.6	0.457	–0.78
Lack-of-Fit	6	2.683	0.447	3.96	0.143	
Pure Error	3	0.338	0.113			

3D surfaces or 2D contours for response are plotted by taking two interacting factors while keeping the remaining constant in multi-parameter model studies. The changes in values of response can be examined with changes in independent parameters using surface plots. The variations in the response area covered can be visualized with a change in levels of independent variables using contour plots. The interactions that are statistically significant with $p < 0.05$ are shown in Fig. 3.

The surface plot and contour plot for the effect of the interaction between initial phenol concentration (X_1) and pH (X_3) on percentage phenol degradation is illustrated in Figs. 3a, 3d. The hold values of yeast extract concentration and temperature were 2%w/v and 32.5°C. A maximum percentage degradation >95% was observed below pH of 7.2 and initial phenol concentration <200 mg l^{-1}. As the initial phenol concentration increases from 1150 to 1500 mg l^{-1}, the area under < 75 % phenol degradation narrows from pH 6.2 to 7.2 and expands from pH 7.2 to 7.8. Conversely, the percentage phenol degradation increases from acidic or basic end to neutral pH. A similar trend was observed with phenol concentration ranging from 200 to 1150 mg l^{-1}. The effect of the interactions between initial phenol concentration (X_1) and temperature (X_4) on percentage phenol degradation is shown in Figs. 3b, 3e. The values of yeast extract concentration and pH were kept constant at 2%w/v and 7. A maximum percentage degradation > 95 % was observed above the temperature of 31.3°C and initial phenol concentration < 200 mg l^{-1}. As the initial phenol concentration increases from 1150 to 1500, the area under 75% phenol degradation narrows from temperature 28 to 32.5°C and expands from temperature 32.5 to 36°C. Conversely, the percentage phenol degradation increases from lower or higher end of temperature to optimum at 32.5°C. The effect of the interactions between yeast extract concentration (X_2) and pH (X_3) on percentage phenol degradation is shown in Figs. 3c, 3f. The values of initial phenol concentration and temperature were kept constant at 800 mg l^{-1} and 32.5°C. The elliptical nature of the plot indicates the mutual effect of both the independent variables on phenol degradation. The plot area is covering lower phenol degradation <75% was observed from pH 7.6 to 7.8 and yeast extract concentration of 1.2 to 1.4 %w/v. The higher phenol degradation >85% was observed from pH 6.5 to 7.5 and yeast extract concentration above 1.8 %w/v.

3.11. Validation of optimum conditions obtained from phenol degradation model

The response optimizer in Minitab was used by assigning target value of 80% for phenol degradation with initial phenol concentration of 1000 mg l^{-1}. The optimized conditions obtained were yeast extract concentration: 2%w/v, pH: 6.9753 and temperature: 32.5453 with the desirability of 1. The experiment was performed using predicted conditions of independent variables suggested by response optimizer. The percentage phenol degradation was found to be 78.65 which is in good agreement with the target assigned.

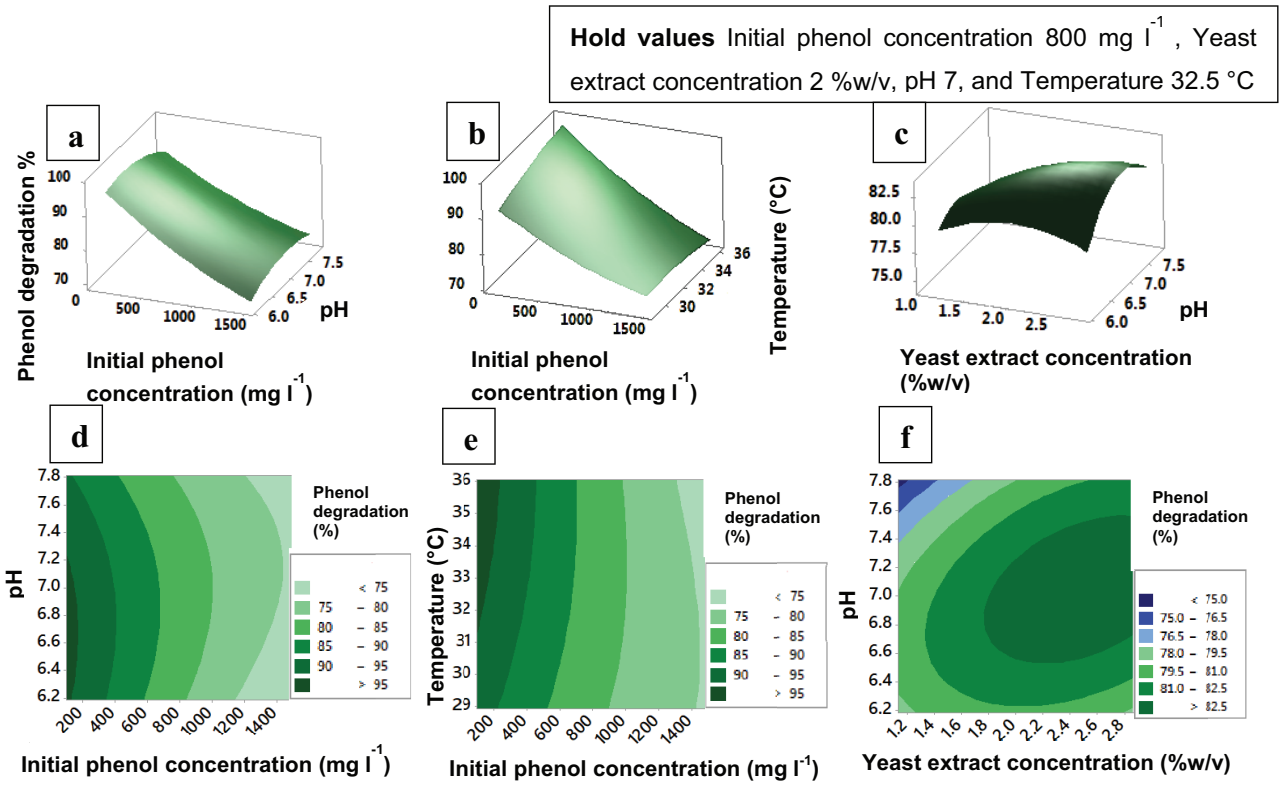

Fig. 3. The (a, b and c) surface plots and (d, e, and f) contour plots showing the interaction effects of X_1 (Initial phenol concentration, mg l^{-1}): X_3(pH), X_1 (Initial phenol concentration, mg l^{-1}): X_4 (temperature, °C), and X_2 (yeast extract concentration, %w/v): X_3 (pH) on phenol degradation.

3.12. Phenol degradation kinetics and modeling

The phenol degradation rate, q is the slope of the straight line obtained from the plot of the negative logarithm of S/S_0 vs. time. A typical plot for initial phenol concentration of 1500 mg l^{-1} is shown in Fig. 4a. The straight line in the plot indicates first order degradation rate after three hours of dead time. Additionally, the q values obtained at different initial phenol concentrations are shown in Fig. 4b. The maximum degradation rate was observed with the initial phenol concentration of 500 mg l^{-1} and the degradation rate gradually decreased with further increase in initial phenol concentration. This is due to the inhibitory effect of the substrate. The highest degradation rate of 0.039 h^{-1} was observed at 500 mg l^{-1} when compared with the lowest value of 0.014 h^{-1} at 1350, and 1500 mg l^{-1}. The degradation rate at 500 mg l^{-1} is therefore 2.78 times that of 1500 mg l^{-1}. In other words, the degradation is fast when initial phenol is 500 mg l^{-1} in contrast to that of initial phenol concentration of 125 mg l^{-1}. This may be due to the abundance of the substrate with the lesser effect of inhibition and growth rate of the microorganisms. However, the degradation of phenol is linear with incubation time.

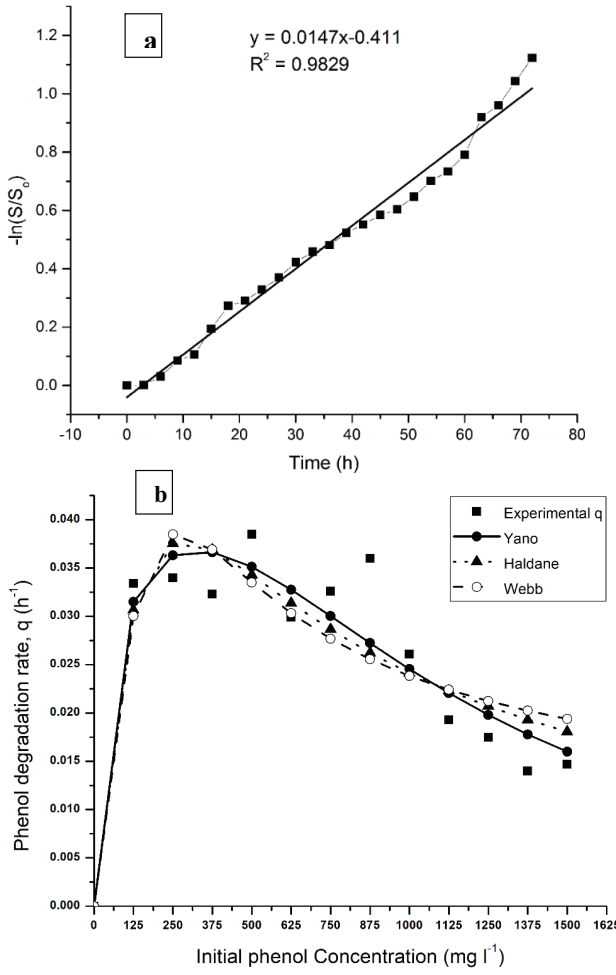

Fig. 4. (a) Typical phenol degradation plot at initial phenol concentration of 1500 mg l^{-1} (b) Phenol degradation models (Yano, Haldane, Webb) with experimental values.

Some empirical kinetic models have been used to describe the relationship between substrate degradation rate and initial phenol concentration. The experimental data obtained were fitted to the models by nonlinear regression analysis are shown in Table 5. Additionally, Fig. 4b, also shows three different predicted kinetic models along with experimental data obtained in the current study. The experimental data was fairly predicted by the Haldane model with reasonable values of kinetic parameters. But, the coefficient of determination R^2 obtained for Yano is relatively higher than that of the Haldane model as shown in Table 5. The standard error of the models was found to be less than 0.004 in all the three cases which were significant.

3.13. Mechanism of phenol degradation

The phenol degradation pathway was determined by investigating both the ortho- and meta-cleavage pathways. The presence of *cis, cis* -muconic acid confirmed by absorbance at 260 nm suggests that the *P. stutzeri* followed the ortho-cleavage pathway. Conversely, the absence of a peak at 375 nm indicates that the phenol degradation is not by the meta-cleavage pathway [10,17]. Also, the spectrometric detection carried out for different initial phenol concentrations (250, 500, 750, 1000, 1250, and 1500 mg l^{-1}) confirmed ortho-cleavage pathway irrespective of its initial phenol concentration. In ortho pathway, the secreted enzyme phenol hydroxygenase converts phenol to catechol and the catechol ring is cleaved by 1,2-dioxygenase to produce cis-cis muconic acid. Thus the cell-free extract exhibited both the enzyme activity of phenol hydroxygenase and catechol 1,2-dioxygenase. The activity of catechol 1,2-dioxygenase increased from initial phenol concentration of 250 mg l^{-1} to 750 mg l^{-1} from 0.41 U mg^{-1} to 0.68 U mg^{-1}. Further, increase in initial phenol concentration from 750 mg l^{-1} to 1500 mg l^{-1} the decline of enzyme activity observed from 0.68 U mg^{-1} to 0.09 U mg^{-1}. It could indicate the evidence of stable catechol 1,2-dioxygenase at lower phenol concentrations and a less stable or active catechol 1,2-dioxygenase at higher concentration.

4. Conclusion

Among the five phenol resistant microorganisms isolated from the soil, *P. stutzeri* showed the maximum capability to degrade phenol up to 81.8% at 500 mg l^{-1} initial concentration within 84 h. The factors affecting the phenol degradation by *P. stutzeri* were optimized using RSM with Doehlert design. All the variables showed a significant influence on the degradation of phenol. A polynomial equation was fitted and also its predicted values were verified by carrying out actual experiments. The significant interactions among experimental variables were also noticed from surface and contour plots. The kinetic parameters were derived from the Haldane substrate inhibition model which was found to be fairly suitable. Also, *P. stutzeri* followed ortho-pathway in degrading phenol at various initial phenol concentrations.

Table 5
Phenol degradation kinetic models of *P. stutzeri*

Kinetic models (*P. stutzeri*)	q_{max}(h^{-1})	k_s(mg l^{-1})	k_i(mg l^{-1})	k (mg l^{-1})	R^2	SE
Haldane $q=\frac{q_{\max}s_o}{k_s+s_o+\frac{s_o^2}{k_i}}$	0.103	248.421	329.728		0.847	0.005
Webb $q=\frac{(q_{\max}s_o)\left(1+\frac{s_o}{k}\right)}{\left(k_s+s_o+\frac{s_o^2}{k_i}\right)}$	2.539	8730.930	6.308	1727.656	0.804	0.006
Yano $q=\frac{q_{\max}s_o}{\left(k_s+s_o+\frac{s_o^2}{k_i}\right)\left(1+\frac{s_o}{k}\right)}$	504.144	652272.314	1.855	61.631	0.894	0.004

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Conflict of interests

The authors declare no conflict of interest.

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