Journal of Hazardous Materials 176 (2010) 85–91

Contents lists available at ScienceDirect

Journal of Hazardous Materials

journal homepage: www.elsevier.com/locate/jhazmat

Isolation and characterization of hyper phenol tolerant *Bacillus* sp. from oil refinery and exploration sites

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ARTICLE INFO

Article history:
Received 25 June 2009
Received in revised form 31 October 2009
Accepted 2 November 2009
Available online 10 November 2009

Keywords:
Bacillus cereus
Growth kinetics
Inhibition effect
Phenol degradation
Phylogenetic position

ABSTRACT

Bacillus cereus MTCC 9817 strain AKG1 and *B. cereus* MTCC 9818 strain AKG2 were isolated from petroleum refinery and oil exploration site, respectively. The 16S rDNA sequence of strain AKG1 showed the closest relation to *B. cereus* 99.63% and *Bacillus coagulans* 99.63% followed by 99.34% homology with *Bacillus thuringiensis* strain 2PR56-10. AKG2 is mostly related to *B. thuringiensis* strain CMG 861 with 99.37% homology. The similarity search between AKG1 and AKG2 gave the lowest similarity 99.19% among same genus similar sequences. At phenol concentration of 1000 mg/L, the optimum growth conditions for AKG1 were found to be 37 °C and pH 7.0 and the same were found to be 37 °C and pH 7.5 for AKG2. The growth kinetics of the strains AKG1 and AKG2 are best fitted by Yano model (maximum growth rate, $\mu_{max} = 1.024\ h^{-1}$ and inhibition constant, $K_I = 171{,}800$ mg/L) and Edward model ($\mu_{max} = 0.5969\ h^{-1}$ and $K_I = 1483$ mg/L) respectively. Growth kinetics of both the strains are also well fitted by the Haldane model with $\mu_{max} = 0.4396\ h^{-1}$ and $K_I = 637.8$ mg/L for AKG1 and $\mu_{max} = 0.9332\ h^{-1}$ and $K_I = 494.4$ mg/L for AKG2.

1. Introduction

With ever increasing demand for petroleum and associated products, the environmental pollution due to the petroleum spillage and wastes originated from the production and transportation of refinery products would remain a critical issue. Phenols and phenolic compounds, prevalent in petroleum waste, are potentially toxic causing critical health hazards. Hence, it is of utmost importance to reduce the phenol level in industrial effluents to tolerant limits prior to being released into the environment [1]. Conventional physical and chemical methods, such as granular or biological activated carbon filtration, ozonation, chlorination, H_2O_2/UV process, solvent extraction and membrane separation, used to eliminate waste organic compounds from industrial wastewater are costly and have inherent drawbacks of producing secondary hazardous products [2]. On the other hand, biodegradation that exploits the ability of microorganism (generally bacteria) to convert organic pollutants to water, carbon dioxide and biomass under aerobic or anaerobic condition appears to be the most environmentally benign method of removal of oil pollutants avoiding undesirable by-products or secondary pollutants like in chemical scrubbing or thermal waste gas treatment [3].

Recent studies on the biodegradation of mono-aromatic hydrocarbons such as benzene, toluene and phenol using mixed-substrate system revealed a number of substrate interactions during hydrocarbon degradation [4]. Meyer et al. [5] observed diauxic degradation of benzene in the presence of phenol while Reardon et al. [6] observed that benzene and toluene were better growth substrates for *Pseudomonas putida* F1 than phenol, resulting in faster growth and higher yield coefficients. Therefore, microorganisms with high biodegrading capability for phenols and phenolic compounds have been subjects of recent research [7]. In order to obtain high phenol tolerance, microorganisms have also been isolated from phenol-rich wastes [8–11]. In this regard, *Pseudomonas* is the most widely reported bacteria for the biodegradation of phenolic compounds [12,13] and the inhibitory effect of phenol became predominant at the concentration of 500 mg/L or above. On the other hand, very little is known for the degradation of phenol derivatives by *Bacillus* sp. The thermophilic, phenol-degrading *Bacillus theroleovorans* strain A2 was found to be able to degrade phenol at temperature as high as 70 °C [14]. The studies on phenol degradation by *Bacillus stearothermophilus* were mainly focused on the enzymatic pathways involved during such degradation [15,16]. Information on degradation behaviour of species like *Bacillus cereus* for phenol and its derivatives in the literature are scant.

Phenol-degrading microorganisms has been observed to display substrate inhibition at high phenol concentrations. The maximum specific cell growth rate, substrate-affinity constant K_s and

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doi:10.1016/j.jhazmat.2009.11.002

substrate-inhibition constant K_I vary over a wide range depending on cell type and culture environments. The specific phenol-degradation rate is often modeled using the Haldane equation by coupling substrate removal rate to cell growth rate with a constant yield coefficient [17], which is valid only within a very narrow range of initial phenol concentrations. Variations in cell mass yield are expected when cells grow very slowly during substrate inhibition [17,18]. Hence, the assumption of a constant cell yield coefficient should be used with caution when the specific substrate degradation rate is modeled as it is directly related to the specific cell growth rate. Thus, isolation of bacteria with high phenol-degrading capability and investigation of their detailed growth as well as degradation kinetics in presence of phenolic substrates merit immense environmental importance.

As a part of the study on the microbial treatment of petroleum wastewater, we concentrate here on the microbial treatment of phenol, a potential pollutant in petroleum wastewater. Two bacterial strains which can grow with phenol as sole carbon and energy sources were isolated from oil refinery and oil exploration sites. The phylogenetic positions based on the 16S rDNA sequence analysis are reported. The optimum growth conditions for the isolates *B. cereus* AKG1 MTCC9817 and *B. cereus* AKG2 MTCC9818 from the refinery and exploration sites respectively were examined during the degradation of phenol in a batch system. Finally, the batch growth kinetics of the isolates in presence of phenol as the sole substrate was studied using wide range of substrate concentration (viz. 100–2000 mg/L).

2. Materials and methods

2.1. Chemicals and culture medium

A basic mineral salt medium (MSM) containing 4.0 g/L sodium nitrate ($NaNO_3$), 3.61 g/L disodium hydrogen phosphate (Na_2HPO_4), 1.75 g/L potassium dihydrogen phosphate (KH_2PO_4), 0.2 g/L magnesium sulfate ($MgSO_4{\cdot}7H_2O$), 0.05 g/L calcium chloride ($CaCl_2{\cdot}2H_2O$), 1.0 mg/L ferrous sulfate ($FeSO_4{\cdot}5H_2O$), 50 μg/L copper sulfate ($CuSO_4{\cdot}5H_2O$), 10 μg/L sodium molybdate (Na_2MoO_3), 10 μg/L manganese sulfate ($MnSO_4$) and 3 g/L yeast extract was used for the growth of the microorganisms. Solid media was prepared by the addition of 15 g/L (1.5%) agar to the medium. Prior to use, the media were sterilized in an autoclave at 121 °C for 15 min. All inorganic chemicals were of analytical grade unless specified otherwise and were obtained from E-Merck India. The growth media were prepared by adding phenol of required concentration (100–2000 mg/L) to the MSM. Initially for isolation process, the pH of the medium was maintained at 6.5 and the working volume was 100 mL in all experiments.

2.2. Isolation and culture conditions

For the isolation of phenol-degrading bacteria, wastewater samples were obtained from two different origins namely the refinery site (Indian Oil Corporation Limited, Guwahati Refinery, Guwahati, India) and the exploration site (Oil India Limited, Duliajan, India). 1 mL of each sample was added separately to 250 mL Erlenmeyer flask containing 100 mL of carbon free MSM and phenol at initial concentration of 500 mg/L. The pH of the media was adjusted to 6.5 with 1N NaOH and 1N HCl solution. The flasks were incubated at 37 °C in a rotary shaker at 120 rpm. When the cultures became turbid, 1 mL of each culture was transferred to 100 mL of fresh medium for next 48 h. The above procedure was repeated three times. At the end of the incubation process, the enriched culture was suitably diluted and plated on solid media. Each separated colony appearing on the agar plates was re-streaked on fresh agar plate (containing high concentration of phenol than previous) until pure colony was obtained. The isolated bacteria from the refinery site and the exploration site were named AKG1 and AKG2, respectively. After acclimatization for 2 months, one colony from each sample was isolated on the basis of their ability of growing at high phenol concentration (1800 and 1650 mg/L) as a sole carbon source.

2.3. Characterization and identification of strain

The isolated pure bacteria cultures were sent to Microbial Type Culture Collection (MTCC) & Gene Bank Chandigarh, India for biochemical characterization and 16S rDNA sequence determination (Table 1). The sequencing results were submitted to Gene Bank database to carry out similarity search for nucleotides by online BLAST tool (http://www.ncbi.nlm.nih.gov). The thermodynamic properties of the isolated cultures were also determined by using the online tool BIOTOOL (http://www.unc.edu/~cail/biotool/oligo/index.html). Pure cultures were stored in 50 mM KH_2PO_4:K_2HPO_4 buffer (at pH 7.0 and 7.5 respectively for strain AKG1 and AKG2) containing 30% (v/v) glycerol at −20 °C. Working cultures were maintained by subculturing every 6 weeks on phenol-agar plates.

2.4. Analysis of cell concentration

Cell concentrations in the samples were analyzed by measuring the optical density (OD) at 600 nm using UV–vis spectrophotometer (Perkin Elmer, lamda 35, USA) with the culture medium as reference.

2.5. Effects of pH, temperature and initial phenol concentration on growth of the isolate

Optimum temperature, pH and phenol concentration for the growth of the microorganisms were determined. Each experiment was carried out in batch mode in 250 mL Erlenmeyer flask containing 100 mL mineral media along with Yeast extract (3 g/L) at 120 rpm. First, the experiments were carried out at various temperatures ranging from 31 to 39 °C, keeping initial phenol concentration at 1000 mg/L and pH 6.5 under shaking condition to find out the optimum temperature for the growth of the microbes. At this optimum temperature further experiments were also carried out at various pH values ranging from 6 to 8 with initial phenol concentration of 1000 mg/L to find out the optimum pH for the growth of the microbes. In order to find out the optimum initial phenol concentration for the growth of the isolated microbes and analyze their growth kinetics, additional experiments were carried out with various initial phenol concentrations ranging from 100 to 2000 mg/L at optimum temperature and pH. The optimum value of a parameter corresponds to the maximum specific growth rate of cell, μ (h^{-1}) as observed from the experiments. Calculation procedure of μ is discussed in the subsequent section.

2.6. Growth kinetics of isolated microorganisms

Each 250 mL shake flask containing phenol as a carbon source, 100 mL of MSM media, and 1 mL of cell suspension (OD_{600} 0.017–0.023) was incubated in a shaker (120 rpm) at 37 °C. The initial concentration of phenol in culture was maintained in the range of 100–2000 mg/L. Samples were collected at designated intervals for cell concentration measurement.

Growth kinetics is an essential and mandatory input for the design of any biological reactor where microbial degradation is carried out. Since microbial growth kinetics is highly sensitive to the microbes of concern and other physicochemical conditions of the reactor, it is extremely important to have suitable growth kinetics for a microbial process. In order to represent the growth kinetics of

Table 1
Biochemical and physiological characteristics of isolated AKG1 and AKG2 identified as *Bacillus cereus* MTCC9817 and *B. cereus* MTCC9818 (+: positive; –: negative; (+) weak growth).

	Colony morphology			Physiological tests	
	AKG-1	AKG-2		AKG-1	AKG-2
Configuration	Round	Round	Growth at temperature	>10 to 55 °C	4 to 55 °C
Margin	Entire	Entire	Growth at pH	>5 to <9	>5 to <9
Elevation	Raised	Raised	Growth on NaCl (%)	2.0% to <10.0%	2.0% to <10.0%
Surface	Flat	Flat			
Pigment	Cream	Cream		Acid production from	
Opacity	Opaque	Opaque	Salicin	(+)	(+)
Gram's reaction	+ve	+ve	Xylose	–	–
Cell shape	Rods	Rods	Mannitol	–	–
Size (μm)	2–4	2–4	Dextrose	+	+
Spore	+	+			
Motility	+	+			
Biochemical tests					
Indole test	–	–	Growth on MacConkey agar	N.G.	N.G.
Methyl red test	(+)	(+)	Esculin hydrolysis	+	+
Voges proskauer	–	–	Gelatin hydrolysis	+	+
Citrate utilization	(+)	(+)	Starch hydrolysis	+	+
H_2S production	–	–	Catalase test	+	+
Gas production	–	–	Tween 20 hydrolysis	+	+
Oxidase test	+	+	Tween 40 hydrolysis	(+)	(+)
Urea hydrolysis	+	+	Tween 80 hydrolysis (Lipase test)		
Nitrate reduction	–	–	Arginine dihydrolase	+	+

inhibitory compound in the present study, several available kinetics models were fitted to the experimental data for selecting the best model(s) (Table 2). The specific growth rate of cells in a batch system, μ (h^{-1}), is defined as

$$\mu = \frac{\mu_{max} S[1 + (S/K)]}{S + K_s + (S^2/K_I)} \quad (1)$$

where S is the substrate concentration (mg/L), μ_{max} is the maximum growth rate (h^{-1}), K_s is the substrate-affinity constant (mg/L) and K_I is the substrate-inhibition constant (mg/L). A larger K_I value indicates that the culture is less sensitive to substrate inhibition [24]. The value of μ is determined at the exponential phase of the growth curve. From the linear plot of X vs. $\ln(S/S_0)$, after a short lag phase, the value of μ for an initial phenol concentration (S_0) is obtained. Where, X is the cell concentration in either g/L (dry basis) or in absorbance unit at 600 nm (OD). Such plot indicates that the phenol was the limiting substrate in this region and the cultures were growing exponentially [25]. From the values of μ vs. S_0, the values of μ_{max}, K_s and K_I are obtained using regression analysis in MATLAB 7.0©.

3. Results and discussions

3.1. Isolation and identification of bacteria

Both strains AKG1 and AKG2 were characterized as rod-shaped, gram positive, oxidase and catalse positive bacteria (Table 1). When examined using Scanning Electron Microscopy (SEM), after 12-h growth, cells measured 1.24 μm in width and 3.38 μm in length for strain AKG1 and 1.41 μm in width and 3.6 μm in length for strain AKG2 (Fig. 1). The complete details of the biochemical and physiological characteristics of both strains are given in Table 1. Partial 16S rDNA sequencing results obtained from MTCC, Chandigarh, India, showed that AKG1 and AKG2 had 1369 and 1444 base pairs (bp) respectively. Gene analysis by online BLAST tool indicates that both the strains were equally similar with *B. cereus*, *Bacillus thuringiensis* and *Bacillus subtilis*. But, their morphological and biochemical characteristics were more similar to *B. cereus*. On the basis of these reports, MTCC, Chandigarh, India, characterized them as *B. cereus* and was designated as strain AKG1 *B. cereus* MTCC 9817 (gb FJ841975) and AKG2 *B. cereus* MTCC 9818 (gb FJ841976). The high bootstrap support of the tree shown in Fig. 2 derived from the 16SrDNA analysis [26] demonstrated that

Table 2
Kinetic parameters obtained by different models.

Strain	Model	μ_{max} (h^{-1})	K_s (mg/L)	K_I (mg/L)	K (mg/L)	R^2	SD_{avg}
AKG1	Haldane model: $\mu = \frac{\mu_{max} S}{K_s + S + (S^2/K_I)}$ [19]	0.4396	129.4	637.8	–	0.8094	0.066718147
	Yano model: $\mu = \frac{\mu_{max} S}{K_s + S + (S^2/K_I)[1 + (S/K)]}$ [20]	1.024	66.68	1.718×10^5	5.863	0.899	0.047751826
	Aiba model: $\mu = \frac{\mu_{max} S}{K_s + S} \exp\left(\frac{-S}{K_I}\right)$ [21]	3.142×10^4	4.548×10^4	2390	–	0.3095	0.106403372
	Edward model: $\mu = \mu_{max} S\left[\exp\left(\frac{-S}{K_I}\right) - \exp\left(\frac{-S}{K_s}\right)\right]$ [22]	0.1702	208	845.1	–	0.8864	0.041252628
	Webb model: $\mu = \frac{\mu_{max} S[1 + (S/K)]}{S + K_s + (S^2/K_I)}$ [23]	4.208	602	135.5	4.259×10^5	0.8089	0.066953807
AKG2	Haldane model	0.9332	110.5	494.4	–	0.6623	0.043396717
	Yano model	0.9246	108.4	503.4	2.727×10^5	0.663	0.043353489
	Aiba model	9231	2.226×10^4	3014	–	0.2404	0.065623138
	Edward model	0.5969	69.93	1483	–	0.7076	0.04104596
	Webb model	0.412	8.542	3.054	4.136	0.1812	0.079121219

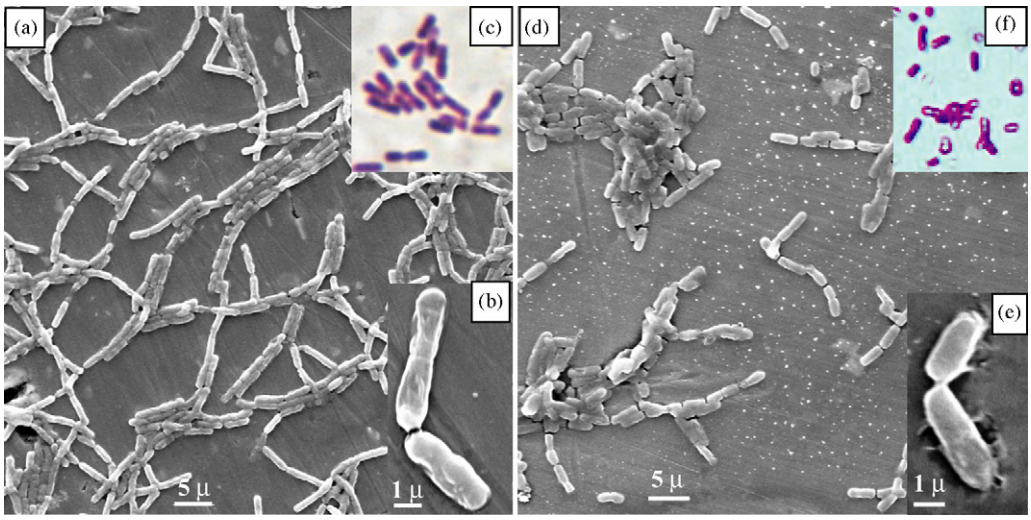

Fig. 1. (a) and (d) represents SEM images of AKG1 and AKG2 respectively with 1.01k× magnification. (b) and (e) SEM images of AKG1 and AKG2 respectively with 7.0k× magnification. Snaps (c) and (f) represent gram staining pictures of AKG1 and AKG2 respectively at 100×.

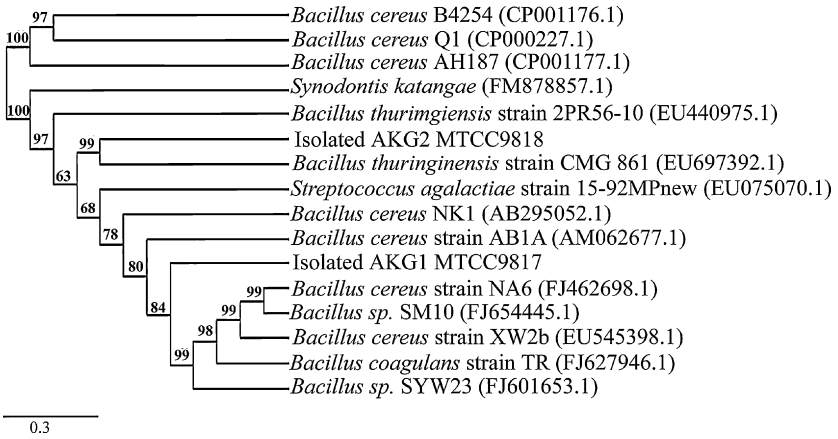

Fig. 2. The neighbor-joining Phylogenetic tree was constructed and bootstrapped (1000 iterations) using Robust Phylogenetic Analysis for the Non-Specialist [26] to represent the relationship between the phenol-degrading strain AKG1 and AKG2 and representative species of the genus *Bacillus* and related genera. Bootstrap values are noted on the branch and the scale bar (=0.3) represents nucleotide substitution per 100 nucleotide.

strains AKG1 and AKG2 are typical members of the genus *Bacillus*. The 16S rDNA sequence of strain AKG1 showed the closest relation to *B. cereus* 99.63% and *Bacillus coagulans* 99.63% followed by 99.34% homology with *B. thuringiensis* strain 2PR56-10. AKG2 was related to *B. thuringiensis* strain CMG 861 with 99.37% homology. The similarity search between AKG1 and AKG2 gave the lowest similarity 99.19% among all. Eight gaps and three mismatches were found in total similarity search among AKG1 and AKG2. The three gaps lies in between 1320 and 1369 bp of AKG1 with 1383–1431 bp of AKG2. The thermodynamic properties as obtained by the online tool, BIOTOOL indicated that strain AKG1 has 54% GC content with −2275.9 kcal/mol Gibbs free energy (ΔG), −12074.5 kcal/mol enthalpy (ΔH) and 31576.1 cal/mol-K entropy (ΔS). Strain AKG2 shows entropy $\Delta S = 33253.8$ cal/mol-K, Gibbs energy $\Delta G = 2396.8$ kcal/mol and enthalpy $\Delta H = 12715.8$ kcal/mol with lower GC content (53%) than AKG1 (54%).

3.2. Effect of incubation temperature on bacterial growth on phenol

Fig. 3 shows the growth rate profiles of isolated bacteria at initial pH 6.5 and at varying temperatures. It can be noticed from the figure that the growth rate was increasing with temperature till 37 °C for both the cases. At temperature higher than 37 °C growth rate decreased. This is attributed to the fact that at higher temperature the microbe may not be able to survive, therefore, shows lower rate of growth. For each isolate, the optimum growth temperature, 37 °C, indicates that, isolated strains are mesophilic by nature.

3.3. Effect of initial pH on bacterial growth

Fig. 4 shows the growth profile of the phenol-degrading bacteria at various pH values. It can be seen that the microbes grew effi-

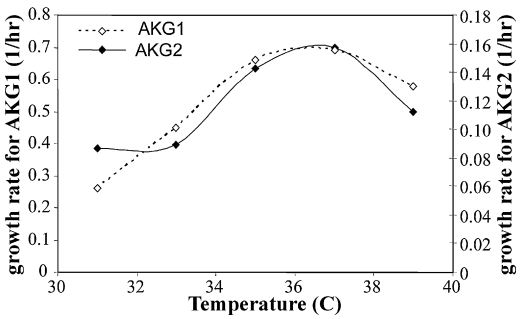

Fig. 3. Specific growth rates of AKG1 and AKG2 for various temperatures at starting pH 6.5.

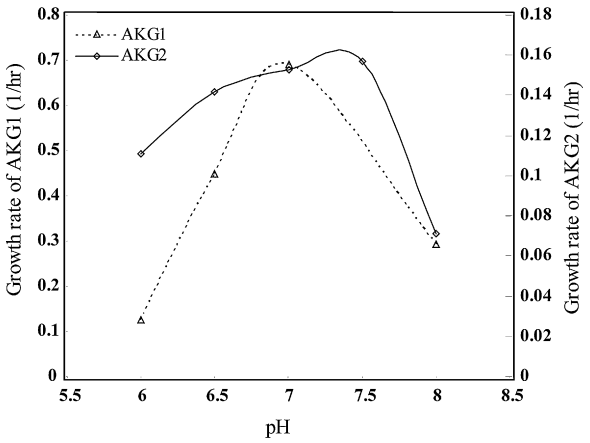

Fig. 4. Specific growth rates of AKG1 and AKG2 for various pH and at optimum temperature.

ciently at almost neutral medium. Enzymes produced by microbes are affected by the change in pH because it is an important factor in the stability of the enzymes and pH change affects the solubility of enzymatic compounds. Extremely high or low pH values generally results in complete loss of activity for most of the enzymes. Thus, for each enzyme there is also a region of optimal pH. Therefore, the optimum pH is the most favorable pH value where the enzyme is most active and the microbes are stable. Irrespective of temperature, strains AKG1 and AKG2 showed maximum growth rate at pH 7.0 and 7.5 respectively using phenol as a sole carbon source.

3.4. Effect of initial substrate (phenol) concentration on bacterial growth

For AKG1, a maximum growth rate was observed at 600 mg/L, while, for AKG2, it is observed at 1000 mg/L of initial phenol concentration (Fig. 5). Phenol has been shown to have a significant inhibitory effect on growth of microorganism at higher concentration (>1000 mg/L). No significant growth was observed for initial phenol concentration of 2000 mg/L and beyond. This suggests that the microbe is inefficient for phenol concentration beyond 2000 mg/L. Yeast extract was added in the growth medium as inducer to increase the biomass in short period of time. A typical biomass concentration profile (as OD_{600}) of AKG1 *B. cereus* MTCC 9817 and AKG2 *B. cereus* MTCC 9818 at 1000 mg/L is shown in Fig. 6. The figure does not show idealized curve, but that curve obtained when the experimental points are joined up, thus avoiding an arbitrary interpretation of the results. After the highest growth peak, it is observed that absorbance at 600 nm is decreasing. It is due to the cell death because of cell lyses. Similar phenomenon is already

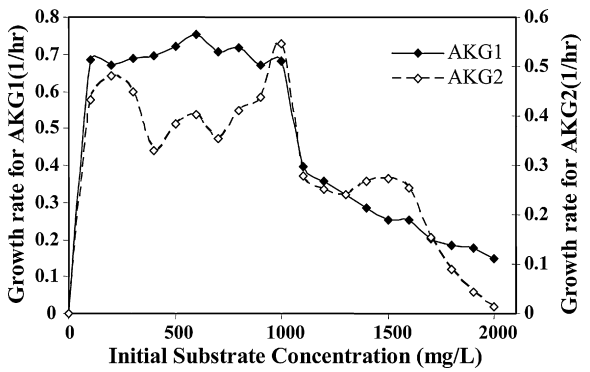

Fig. 5. Growth rate curves for AKG1 and AKG2 for various initial substrate (phenol) concentrations at optimum temperature and pH.

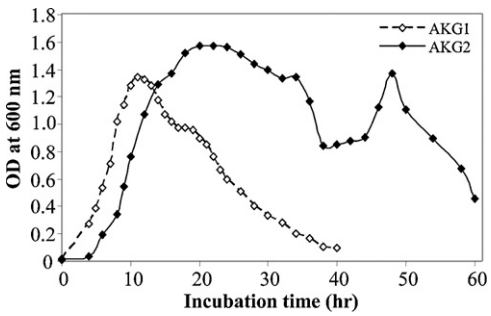

Fig. 6. Typical growth curves of AKG1 and AKG2 with 1000 mg/L phenol as a carbon source at optimum temperature and pH.

reported in the literatures [15,27,28]. For strain AKG2, a prominent late growth phase was observed, but, it is too early to comment whether phenol or any other intermediate product was responsible for this growth.

3.5. Growth kinetics studies for B. cereus AKG1 and AKG2

Fig. 7 shows the prediction of the experimental specific growth rate by various kinetic models (Table 2) for various initial phenol concentrations. All the models used in this study have been generally used to describe substrate-inhibition model. From the figure, it was observed that, between the five different models, Yano model for the strain AKG1 and Edward model for the strain AKG2 fit the data well with respect to their kinetic parameters. Table 2 repre-

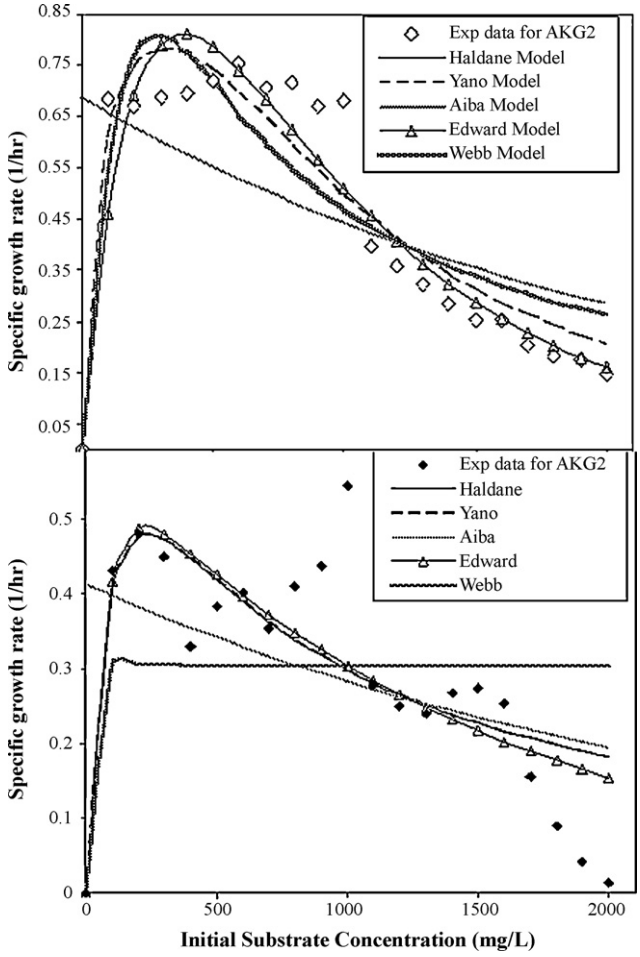

Fig. 7. Model prediction of experimental growth rates of AKG1 and AKG2 in phenol.

Table 3
Comparison of the growth kinetics parameter of microbial culture.

Sl. no.	Bacterial strain	System	Concentration range	Haldane's model			Temp/pH	Ref.
				μ_{max} (h^{-1})	K_s (mg/L)	K_I (mg/L)		
1	*Pseudomonas putida* CCRC14365	Batch	0–4.25 mM	0.245	0.129	12.6	30/7.0	[7]
2	Mixed culture I	Batch	0–900 mg/L	0.260	25.4	173.0	28 ± 0.5/6.6	[17]
3	Mixed culture II (filamentous organism)	Batch	0–1000 mg/L	0.223	5.86	934.5	28 ± 0.5/6.6	[17]
4	*P. putida* DSM548	Batch	NA	0.57	2.4	106	30/6.0	[25]
5	*P. putida* (ATCC 17514)	Continuous	0–500 mg/L	0.567	2.39	106.0	30/6.0	[25]
6	*T. cutaneum*	Continuous	0–900 mg/L	0.464	1.66	380.0	30/4.5	[25]
7	*P. putida* CCRC14365	Batch	0–600 mg/L	0.33	13.9	NA	30/6.8	[29]
8	*P. putida* DSM548	Batch	0–100 mg/L	0.44	6.2	54.1	26/6.3–6.8	[30]
9	*P. putida* DSM50222	Batch		0.53	<1.0	470	30/6.2–6.7	[31]
10	*P. putida* (ATCC 17484)	Batch/continuous	0–700 mg/L	0.534	0.015	470.0	30/(6.2–6.7)	[31]
11	*Alcaligenes faecalis*	Batch	10–1400 mg/L	0.15	2.22	245.37	30/7.2	[32]
12	Mixed culture	Batch	0–800 mg/L	0.3085	44.92	525.00	27 ± 1/7.0	[33]
13	*P. putida* (F1ATCC 700007)	Batch	750–1750 mg/L	0.051	18.0	430	30/7.0	[34]
14	*P. putida* (MTCC1194)	Batch	0–1000 mg/L	0.305	36.33	129.79	29.9 ± 0.5/7.1	[34]
15	*P. putida*	Batch	5–150 mg/L	NA	NA	NA	30/7.0	[35]
16	AKG1 *Bacillus cereus* MTCC 9817 (gb FJ841975)	Batch	0–2000 mg/L	0.4396	129.4	637.8	37/7.0	Present work
17	AKG2 *B. cereus* MTCC 9818 (gb FJ841976)	Batch	0–2000 mg/L	0.9332	110.5	494.4	37/7.5	Present work

Table 4
Calculated coefficient of determination (R^2) values for initial guess changing for each model using non-linear regression method.

Initial value	R^2									
	Haldane model		Yano model		Aiba model		Edward model		Webb model	
	AKG1	*AKG2*	*AKG1*	*AKG2*	*AKG1*	*AKG2*	*AKG1*	*AKG2*	*AKG1*	*AKG2*
Case 1: $\mu < 1$, $K_s < 1$, $K_I < 1$	0.8094	0.6623	−2.419	−2.514	−3.458	−3.561	−3.458	−3.561	0.1732	0.1812
Case 2: $\mu < 1$, $K_s > 1$, $K_I > 1$	0.8094	0.6623	0.899	0.663	0.3095	0.24	0.8864	0.6876	0.8089	0.3114
Case 3: $\mu = 1$, $K_s > 1$, $K_I > 1$	0.8094	0.6623	0.899	0.7354	0.3095	0.2401	0.8864	0.7076	0.8089	0.458
Case 4: $\mu > 1$, $K_s < 1$, $K_I < 1$	0.8094	0.6623	0.8989	−2.132	0.3089	0.2397	0.8864	0.7076	0.348	0.2599

sents their kinetic parameters along with their R^2 (coefficient of determination) values. Table 3 showed a comparison of the present experimental biokinetic parameters when calculated using Haldane model with those previously reported in literature, although, Haldane model is not our best fitted model. The biokinetic parameters thus estimated were found to be higher than the literature data.

The simulated growth profiles of isolated strains for various initial phenol concentrations were obtained using the estimated biokinetic parameters as input. These biokinetic parameters are highly sensitive to their initial guess values, which in turn may change the accuracy of prediction of experimental growth profiles. Therefore, in this study we have carried out sensitivity analysis for parametric estimation. Here, four different sets (Table 4) of biokinetic parameters (μ, K_s and K_I) values were used as initial guesses. Table 4 presents the R^2 obtained by various initial changes considered. The table clearly indicates that, Haldane model has the most repeatability for both the cases, but, Yano model and Edward model provided the best fit for entire data sets for each of them respectively. The latter two models also repeatedly identified the similar biokinetic parameters with negligible deviation except the case I ($\mu < 1$, $K_s < 1$, $K_I < 1$) (Table 4).

The best fitted Yano and Edward models predict that the isolated strains AKG1 and AKG2 respectively can grow on higher concentration, as high as 171,800 and 1483 mg/L initial phenol concentrations (Table 2). The values of inhibition constants (K_I) using Haldane model, the most widely used model, for both the strains in the present case are higher than the reported literature data (Table 3) for pure cultures. This reflects that the isolated strains can tolerate higher phenol concentration than the reported values (Table 3). This high phenol tolerance characteristic of the isolated strains in the present case could be due to their genetic modifications because of long time exposure in phenol contaminated wastes. Further investigations on the characteristic properties of these strains may provide real insight. Our best fitted models (Yano and Edward models for strains AKG1 and AKG2 respectively) predicted higher growth rate ($1.024\,h^{-1}$) for strain AKG1 and little lower growth rate ($0.5969\,h^{-1}$) for strain AKG2, than Haldane model with higher inhibition constants for both cases (Table 4). Webb model and Aiba model fitted very poorly to all data sets for both the strains.

4. Conclusion

Two phenol-degrading bacteria were isolated from a phenol-enriched mixed culture from two different sites. Both strains were identified as *B. cereus* MTCC 9817 strain AKG1 and *B. cereus* MTCC 9818 strain AKG2 using a method based on the 16S rDNA gene sequencing. Both the strains have a great potential to grow at very high concentration of phenol. The optimum growth temperatures for *B. cereus* AKG1 and AKG2 was 37 °C, utilizing phenol as the substrate. Though both strains belong to same genus, results show that, they have a characteristic growth difference. Strain AKG1 can easily grow on 1000 mg/L phenol without any inhibition effect but can not sustain in phenol media for more than 40 h, whereas, AKG2 has a slow growth rate with inhibition effect on 1000 mg/L phenol concentration and long time sustainability. As all these properties have a direct or indirect relation on isolates characteristics, thus, this difference may lead them to act differently in the same environment. The above experimental parameters are invariably required for the design and simulation of batch and continuous bioreactors for treatment of phenolic wastewaters. This potential of the strains towards high phenol utilization for industrial effluent treatment and decontamination of natural polluted areas are being studied and will be reported in future correspondence.

Acknowledgment

This work was supported by the Council of Scientific and Industrial Research (CSIR) (24(0302)/09/EMR-II, dated: 04-03-2009), New Delhi, Government of India, India.

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