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Kinetics and metabolic versatility of highly tolerant phenol degrading *Alcaligenes* strain TW1

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ABSTRACT

A bacterium that could completely metabolize phenol in batch culture supplied with up to 1200 mg phenol l^{-1} at room temperature (25 °C) was isolated from the activated sludge of the industrial wastewater treatment plant of a Coke company (Cairo, Egypt). Morphological and physiological characterization showed strain TW1 was a motile, strictly aerobic, gram negative and short-rod occurring singly or in clusters. Partial 16S rRNA gene sequence analysis revealed strain TW1 belonged to the *beta* group of *Proteobacteria*, showing 100% identity to *Alcaligenes* SCTI. Strain TW1 aerobically grew on a number of monocyclic aromatic compounds (hydroquinone, catechol and o-cresol) as well as polycyclic aromatic compounds (pyrene, phenanthrene and naphthalene). The growth of *Alcaligenes* TW1 on phenol as sole carbon and energy source (25 °C) was well described by the Haldane kinetics model with a maximal specific growth rate of 0.58 h^{-1}, a half-saturation constant of 10 mg l^{-1}, and a substrate inhibition constant of 152–550 mg l^{-1}. The biomass yield coefficient ranged from 0.55 to 0.64 mg dry cell mass/mg phenol. Due to its high tolerance to phenol and high metabolic versatility, *Alcaligenes* sp. TW1 is considered an excellent candidate for the biotreatment of high strength phenol-laden industrial wastewaters.

1. Introduction

Phenolic compounds such as phenol, chlorophenols, and nitrophenol are massively used in the chemical and pharmaceutical industries and are therefore often found in many industrial wastewaters [1,2]. Many of these compounds are highly toxic and have been listed by the USA Environmental Protection Agency (EPA) as priority pollutants [3]. It is therefore crucial to efficiently remove them from contaminated streams in order to prevent their entry in the environment.

Suitable methods for the treatment of phenol-laden wastewater include chlorination, ozonation, adsorption, solvent extraction, membrane process, coagulation, flocculation and biological degradation [4,5]. Physicochemical methods are generally efficient but costly and might generate secondary pollutants such as substituted phenols or dioxins [6,7]. Biological methods are therefore generally preferred for being considered as more economical and environmentally friendly [8]. Unfortunately, direct batch treatment (which allows a good control of the effluent quality) is virtually impossible if the influent is too toxic whereas continuous treatment instability under conditions of fluctuating influent composition can lead to poor efficiency [9]. In such cases, the use of acclimatized microorganisms especially adapted to metabolize the contaminants at high concentration can provide an attractive solution to improve process performance [10–13].

With this perspective, the present work reports the isolation and characterization of a highly resistant phenol degrading bacterium from the activated sludge process of the wastewater treatment plant of a Coke factory located near Cairo, Egypt. The metabolic versatility of the isolate was also screened on various aromatic compounds. This isolate can be useful for the biological treatment of high strength phenol-laden industrial wastewater and the *in situ* remediation of phenol contaminated soils.

2. Materials and methods

All chemicals were reagent grade; phenol was obtained from Sigma–Aldrich (Steinheim, Germany). Unless otherwise specified, all experiments were conducted in triplicate at room temperature (23 ± 2 °C) under sterile conditions.

2.1. Culture medium

A Mineral Salt Medium (MSM) with composition as described in [6] was used for screening, isolation and maintenance of strain

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TW1. The pH was adjusted to approx. 7 with 2N NaOH. When needed, the MSM was enriched with 500 mg phenol l^{-1} and/or solidified with 2% (w/v) agar.

2.2. Enrichment, isolation and maintenance of strains

An activated sludge sample was collected from the aeration tank of the wastewater treatment plant of a Coke factory (Cairo, Egypt). Then, 15 ml of the sludge sample was aseptically transferred into a conical flask filled with 100 ml of MSM medium containing 500 mg phenol l^{-1} as a sole organic carbon source and incubated on a rotary shaker at 150 rpm. The optical density at 600 nm (OD_{600}) of the culture broth was periodically monitored and once microbial growth was established ($OD_{600} \approx 0.9–1.0$), 8 ml of culture was transferred to a new shaking flask containing 100 ml of a fresh MSM amended with 500 mg phenol l^{-1}. This procedure was repeated 3 times before 1 ml of the final culture was aseptically spread onto plates containing MSM with 500 mg phenol l^{-1} and solidified with 2% agar. Colonies, visible after 2–3 d of incubation (25 °C), were then picked and successively subcultured in MSM supplemented with increasing concentration of phenol (successively 200, 400, 600, 800, 1000, and 1200 mg l^{-1}). Finally, 1 ml of the final subculture broth was mixed with 9 ml of saline (sterile 0.9% NaCl) and 1 ml of the dilute suspension was aseptically streaked on phenol agar plates for isolation. A pure culture of strain TW1 was observed after 2–3 d of incubation. Purity was confirmed by microscopic examination of the colonies. Strain TW1 was preserved in enriched solid medium at 4 °C or in aqueous MSM containing 20% sterilized glycerol at −80 °C.

2.3. Morphological and physiological characterization

Morphological characterization and motility test were done using light microscopy (Nikon Optiphot-2 microscope, Japan). Gram stain reaction was done using Difco Gram stain set according to the standard protocol [14]. Further morphological examination was done using Scanning Electron Microscopy (SEM) (JSM-5600 LV scanning electron microscope JEOL, USA Inc. at 3000–13,000× magnification) according to [15].

Biochemical characterization (Table 1) was done using API 20 NE kit system (bioMérieux, France) according to the manufacture's instructions. The presence of oxidase was determined using a test strip (Microbiology Bactident Oxidase, Merck, Germany). Catalase activity was evaluated by transferring a loop of bacterial cells onto a microscope slide and adding a drop of 3% hydrogen peroxide solution [16].

2.4. Partial 16S rRNA sequence analysis

Gnomic DNA was obtained and purified according to [17]. Two universal primers 28f 5′-AGAGTTTGATCCTGGCTCAG-3′ (positions 8-28 in *E. Coli* numbering) and 1512R 5′-ACGGCTACCTTGTTACGACT-3′ (positions 1512-1493 in *E. Coli* numbering) were used to amplify 16S rRNA [18]. PCR products were purified using a QIA quick PCR purification kit (Qiagen) and resuspended in 40 μl of sterile pure water. DNA sequencing on both strands was performed by the dideoxy chain termination method with an ABI prism 3100 DNA Analyzer, using the ABI Prism BigDye Terminator Cycle Sequencing Ready Reactions kit (PE Biosystems) according to the manufacturer's protocol. The GenBank database (NCBI, USA) was used to search for 16S rRNA sequence similarities.

2.5. Metabolic versatility

Under sterile conditions, various organic compounds (Table 2) were supplied as sole carbon source at 5–50 mg l^{-1} in 250 ml flasks containing 100 ml MSM. Each flask was then inoculated with 5 ml cells grown on phenol to the late exponential phase, and incubated on a rotary shaker at 150 rpm. All cultivations were performed in duplicate. A significant increase in the optical density at 600 nm was considered as positive growth. Cells grown on phenol supplied at a similar final Theoretical Oxygen Demand concentration (ThOD) were used as positive controls and flasks that were inoculated, but not supplied with any organic carbon source, served as negative controls. The purity of cultures showing signs of growth was checked microscopically.

Table 1
Physiological characteristics of *Alcaligenes* sp. TW1 (+, positive reaction; −, negative reaction; (+), weakly positive reaction).

Characteristics		Present study	[21]	[22]
Morphology		Rods	Rods	Rods
Gram reaction		G−	G−	G−
Motility		+	+	+
Nitrate reduction (denitrification)		+	+	+
Indol reduction		−	−	ng
Glucose fermentation		−	+	−
Enzyme production	Arginine dihydrolase	(+)	ng	−
	Urease	+	−	+
	β-Glucosidase	+	−	−
	Protease (gelatine liquifaction)	+	+	−
	β-Galactosidase	(+)	ng	+
	Cytochrom oxidase	+	+	+
	Catalase	+	+	+
Sugars assimilation	Glucose	(+)	+	−
	Arabinose	−	−	−
	Mannose	−	+	+
	Mannitol	+	−	ng
	N-Acetyl-glucosamine	−	ng	ng
	Maltose	−	−	−
	Potassium gluconate	+	ng	+
Acids assimilation	Capric acid	+	ng	ng
	Adipic	+	ng	+
	Malic	−	ng	+
	Phenyl acetic acid	−	ng	ng
	Trisodium citrate	+	+	+

2.6. Phenol biodegradation and cell growth kinetics determination

The Haldane kinetic model (Eq. (1)) is frequently used to describe growth rate on inhibitory substrates such as phenol in

Table 2
Substrate screening test of *Alcaligenes* sp. TW1 (+, growth; −, no growth).

Substrate	Tested concentration (mg l^{-1})	Growth
Phenol	50–1500	+
4-Nitrophenol (PNP)	10–50	−
2-Nitrophenol	10–50	−
4-Chlorophenol	10–50	−
2,4-Dichlorophenol (DCP)	10–50	−
2,4,6-Trichlorophenol (TCP)	5–50	−
Pentachlorophenol (PCP)	5–50	−
O-Cresol	10–50	+
P-cresol	10–50	−
Pyrene	5–20	+
Naphthalene	5–50	+
Phenanthrene	5–50	+
Catechol	10–50	+
Hydroquinone	10–50	+
Methanol	10–50	−
Ethanol	10–50	−
Benzyl alcohol	10–50	−
Resorcinol	10–50	−
Thiocyanate (CNS^-)	5–50	−
Cyanide (CN^-)	5–50	−

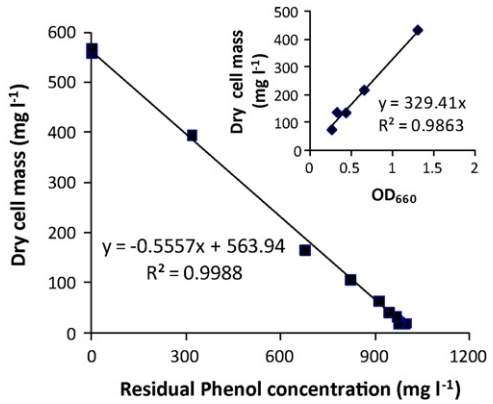

Fig. 1. Determination of growth yield for initial phenol concentration of 1000 mg l^{-1} and calibration curve between the dry cell mass (mg l^{-1}) and the optical density (600 nm) of *Alcaligenes* sp. TW1.

pure or mixed cultures [12,19].

$$\mu = \mu_m \frac{S}{K_s + S + S^2/K_i} \quad (1)$$

where μ, is the specific growth rate (h^{-1}), μ_m, is the maximum specific growth rate (h^{-1}), S, is the substrate concentration (mg l^{-1}), K_s, is the half-saturation constant of growth kinetics (mg l^{-1}) and K_i, is the inhibition constant (mg l^{-1}).

One loop of stock culture maintained on agar slants was transferred aseptically into 250 ml baffled flasks with 100 ml of sterilized MSM enriched with 500 mg phenol l^{-1}. Inocula of 5 ml were withdrawn from the late exponential growth phase and aseptically transferred into 250 ml baffled flasks containing 95 ml of sterilized MSM (pH 7.0) amended with phenol to final concentrations of 200–1500 mg l^{-1} (added from a 20 g l^{-1} stock solution using sterile 0.2 μm syringe filters). All flasks were then incubated on a rotary shaker at 150 rpm. Samples were withdrawn periodically to record cell density and phenol concentration.

2.7. Analysis

To measure phenol concentration, samples were centrifuged for 10 min at 11,300 × *g* (Biofuge 13, Heraeus) and portions of the supernatants were analyzed using a Waters 2690 HPLC system (USA) equipped with an autosampler and a diode-array detector 996 from the same manufacturer. Elution was performed using a Supelcosil LC-8 column with a mobile phase composed of methanol/water/acetic acid (60:39:1, v/v). UV detection was performed at 280 nm and external standards were used to enable quantitative determination. The limit of detection was 1 mg l^{-1}.

Cell growth was monitored by measuring the optical density at 600 nm using a UV–vis spectrophotometer (Ultrospec® 1000, Pharmacia Biotech, Uppsala, Sweden). OD (600) values were then converted into dry cell mass (mg l^{-1}) using a linear coefficient of 329.4 (Fig. 1).

3. Results

Incubation of activated sludge samples in MSM enriched with 500 mg phenol l^{-1} was followed by a significant increase in OD_{600} after 7–10 d. Following enrichment at 500 mg l^{-1}, cultivation of solid media and selection in liquid culture at increasing concentrations of phenol resulted in the isolation of strain TW1. Colonies of TW1 on phenol MSM agar plates were rough, non-pigmented, opaque, and fluorescent, with sizes ranging from 1 to 3 mm in diameter after 2–3 d incubation at 25 °C.

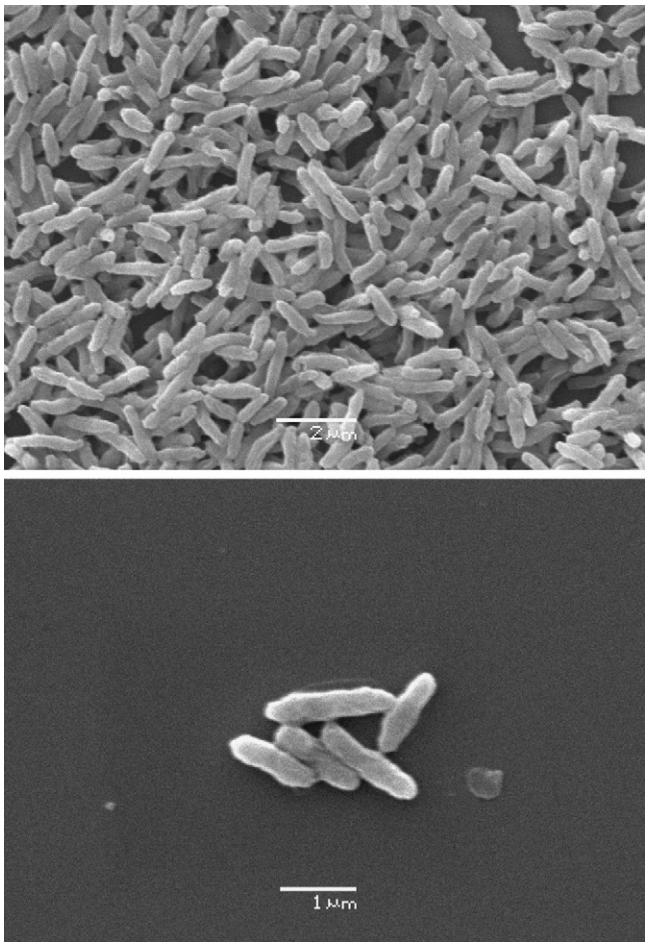

Fig. 2. Scanning electron microscope (SEM) pictures of *Alcaligenes* sp. TW1.

Light microscopy showed strain TW1 was motile, gram negative short rods. Scanning electron microscopy revealed strain TW1 was around 1 μm in length, had no visible flagella, and was arranged singly or in clusters (Fig. 2). The investigated morphological and phenotypical characteristics of strain TW1 (Table 1) were similar to those of *beta Proteobacteria* and *Alcaligenes* [20–22]. The partial 16S rRNA sequencing of strain TW1 (749 bases; Genbank accession number DQ120520) showed 100% identity to the sequence of *Alcaligenes* SCT1 [23], which confirmed the physiological and biochemical characterization (Table 1).

Strain TW1 could grow aerobically on a number of aromatic compounds such as hydroquinone, catechol, o-cresol but was not able to grow on substituted aromatics (chlorophenols, 4-nitrophenol) and a number of other compounds (Table 2).

Strain TW1 was able to grow and utilize phenol as a sole carbon source under pH of 6.5–8.5 and at temperature of 20–37 °C. Optimum growth was recorded at room temperature (23 ± 2 °C), pH 7, 150 rpm agitation, and phenol concentration of 500–700 mg l^{-1}. The cell growth kinetics and the dependence of the specific growth rate (μ) on phenol concentration were well described by the Haldane model, especially during the exponential phase (Fig. 3). The increase in initial phenol concentration was accompanied by an increase in the lag-phase (Fig. 3), a decrease in K_i (Fig. 4), and a slight decrease $Y_{x/s}$ (0.65–0.55), especially at initial phenol concentrations above 800 mg l^{-1}. Strain TW1 kinetic parameters are listed in Table 3.

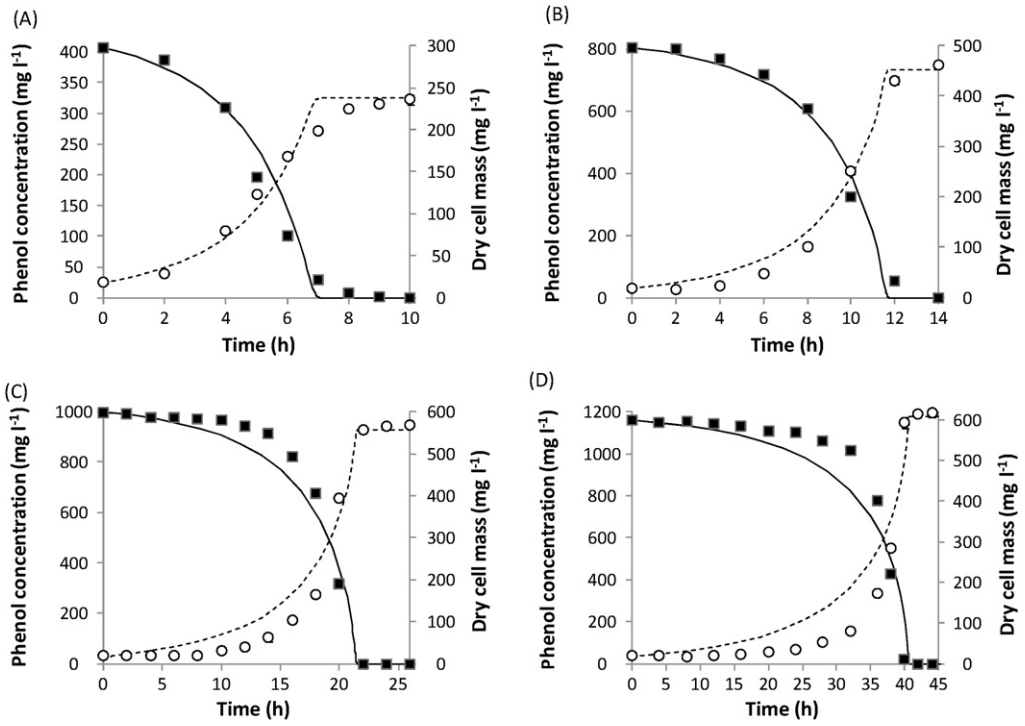

Fig. 3. Simulated (lines) and observed (symbols) changes in the concentration of phenol (■) and dry cell mass (○) in cultivation baffled 250 supplied with phenol at 400 mg l^{-1} (A), 800 mg l^{-1} (B), 1000 mg l^{-1} (C) or 1200 mg l^{-1} (D) in MSM, and inoculated with 5% (v/v) of *Alcaligenes* sp. TW1 suspension. Data shown represents average on triplicates ± standard deviations.

4. Discussion

Alcaligenes species are well known for their ability to degrade aromatic compounds and phenol in particular [24–26]. Interestingly, strain TW1 could aerobically grow on Polycylcic Aromatic Hydrocarbons (PAHs) pyrene, phenanthrene, and naphthalene. Although the ability of *Alcaligenes* to tolerate or mineralize PAHs has been reported [22,25], strain TW1 represents, to the best of our knowledge, the first *Alcaligenes* isolate capable to metabolize both phenol and polycyclic aromatics.

Strain TW1 showed high tolerance and degradation capacity to phenol as it was able to completely mineralize up to 1200 mg phenol l^{-1}. Microbial growth was inhibited at 1300–1500 mg l^{-1} and no growth was observed at concentrations higher than 1500 mg l^{-1}.

Table 3
Comparison of growth kinetics parameters and properties of selected (maximum growth with phenol as sole source of organic carbon and energy ≥1000 mg l^{-1}) phenol degrading pure strains or cultures (all data obtained from batch cultivations).

Microorganism	Origin	Temp (°C)	Max. phenol (mg l^{-1})	μ_m (h^{-1})	K_s (mg l^{-1})	K_i (mg l^{-1})	$Y_{X/S}$ (g g^{-1})	Reference
Mixed culture	Soil and activated sludge organisms	28	1000	0.223	5.86	9345	–	[30]
Acinetobacter sp.	Industrial activated sludge	25	1000	0.28	1167	58.5	0.4–0.6	[15]
Rhizobium sp.	*Astragalus c.* modules	28	1000	0.054	63.3	735	–	[31]
Rodococcus sp.	Petroleum contaminated soil	10	1175	–	–	–	–	[32]
Mixed bacterial culture	Coal wastewater treatment plant	–	1200	–	–	–	–	[33]
Alcaligenes	Coke factory activated sludge	25	1200	0.58	10	152–550	0.55–0.65	Present study
Mixed culture	Wastewater phenol plant	ng	1300	0.251	11	348	ng	[10]
Acinetobacter sp.	Industrial sewage effluent	–	1410	–	–	–	–	[34]
Triscosporon sp.	Petroleum contaminated soil	10	1410	–	–	–	–	[32]
Comamonas testosteroni wild type	Industrial site soil	32	1410	0.52	6.2	996	–	[35]
Alcaligenes faecalis mutant sp.	Activated sludge domestic gasworks	–	1600	0.22	1.55	340	–	[36]
Aureobasidium pullulans	Stainless steel production effluent	30	1692	–	–	–	–	[37]
Bacillus brevis	Phenol-formaldehyde resin factory effluent and sludge	34	1750	0.026–0.078	2.2–29.3	868–2434	0.29–0.57	[38]
Comamonas testosteroni mutant	Methyl sulfonate mutagenesis	32	1880	0.36	22.6	1908	–	[35]
Alcaligenes faecalis mutant sp.	He–Ne laser irradiation	–	2000	0.15	2.22	245	–	[36]
Candida tropicalis	Activated sludge domestic gasworks	30	2000	0.48	11.7	208	–	[39]
Candida albicans	Petrochemical plant soil	30	2256	–	160	3760	–	[40]
Pseudomonas cepacia	Phenol-formaldehyde resin factory effluent and sludge	30	2500	–	–	–	–	[38]

Temp = temperature, μ_m, maximum specific rate constant, K_s, half-saturation constant, K_i, inhibition constant, and $Y_{X/S}$, yield coefficient. The Haldane model (Eq. (1)) was used to calculate all kinetic coefficients.

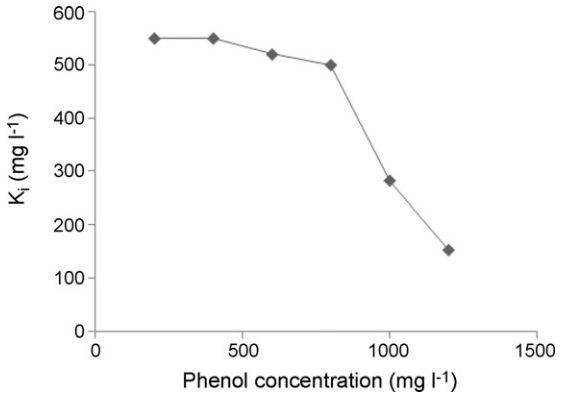

Fig. 4. Relation between the inhibition constant (K_i) of *Alcaligenes* sp. TW1 and phenol initial concentration.

Various strains that can tolerate and mineralize phenol at initial concentrations above 1000 mg l^{-1} have been isolated, most of them during the last 5 years (Table 3). The capacity to tolerate and mineralize phenol at high concentration appears to be found in many microbial species (including bacteria and yeasts) isolated from different environments. Such resistance is a prerequisite to ensure efficient and stable phenol removal in continuous and especially batch treatment. However the abilities to rapidly bring down phenol concentration at satisfactory levels and thrive in the presence of other contaminants are also crucial for efficient treatment of real industrial wastewaters. In this regard, *Alcaligenes* sp. TW1 exhibited an interesting combination of high growth rate, high substrate tolerance and high substrate affinity similar to the best tolerant microorganisms yet isolated (Table 3). The additional ability of *Alcaligenes* sp. TW1 to degrade several monocyclic and polycyclic aromatic compounds further confirms this strain represents a promising candidate for the biological treatment of contaminated effluents. The inhibition constant (K_i) reflects the magnitude of substrate inhibitory effect and its relation to the initial concentration. K_i was nearly constant or slightly decreased at low initial phenol concentrations (550–500 mg l^{-1}) whereas it decreased significantly at concentrations 800 mg l^{-1} (Fig. 4). Similar trends were reported by Okaygun et al. [27] when studying the effect of phenol on activated sludge and Hao et al. [19] when studying the kinetics of phenol and 4-chlorophenol utilization by *Acinetobacter* species. This observation, together with the decrease in $Y_{x/s}$ and increase in lag-phase observed at high phenol concentration suggest strain TW1 experienced greater stress and expressed new mechanisms of toxicity resistance (ranging from membrane composition modification to efflux pumps and pollutant detoxification [28]).

5. Conclusions

Strain TW1 was isolated from industrial wastewater treatment plant of Coke company (Cairo, Egypt). Morphological, physiological and molecular (partial 16S rRNA) identifications located strain TW1 in the *beta* group of *Proteobacteria* as *Alcaligenes*. Strain TW1 was able to tolerate and grow on various monocyclic aromatic compounds and PAHs. In particular, it was able to tolerate and utilize up to 1200 mg phenol l^{-1} and its growth kinetics using phenol as a sole carbon source was well described by the Haldane model.

Alcaligenes sp. has been successfully used for the treatment of real industrial wastewater [29] and strain TW1 was specifically used to treat artificial wastewater containing phenol and nitrophenol [41]. The present study confirms the potential of Strain TW1 as an efficient and versatile microorganism for the bioaugmentation of highly contaminated effluents.

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