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Evaluation of *o*-cresol degradation potential by a strain of *Pseudomonas aeruginosa* S8

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ABSTRACT

The aim of this study was to evaluate the potential of a selected *Pseudomonas aeruginosa* strain (strain S8) isolated from an Algerian soil to degrade *o*-cresol in water. This strain was used to study the biodegradation of *o*-cresol in batch aerobic experiments under variable pH values, heavy metals ions (Zn^{2+}, Cd^{2+} and Cu^{2+}) and NaCl concentrations. The results showed that *P. aeruginosa* S8 strain has a high *o*-cresol biodegradation potential; it could degrade completely 1250 mg L^{-1} *o*-cresol within 85 h. Furthermore, *o*-cresol biodegradation potential of the strain was slightly affected by alkalinity (pH 9), presence of heavy metals and salinity (NaCl). Growth kinetics and degradation activity data obtained for the concentrations ranging between 250–1250 mg L^{-1} fitted well to Andrews growth kinetic model with a coefficient of determination (R^2) higher than 0.99. Due to its high *o*-cresol biodegradation potential and its relative tolerance to alkalinity, salinity and heavy metals, the strain S8 seems to be a good candidate for elimination of *o*-cresol from industrial wastewaters.

Keywords: Biodegradation; *o*-cresol; *Pseudomonas aeruginosa* S8; Kinetics; Andrews's model

1. Introduction

Cresols are a class of organic pollutants which enter the environment mainly through industrial and human activities. Oil refineries and chemicals plants are principally responsible for discharging cresols in their aqueous effluents [1,2]. They are ubiquitous phenolic pollutants, highly toxic either by ingestion, contact or inhalation [3]. They appear in many lists of priority hazardous substances, such as those edited by the Agency for Toxic Substances and Disease Registry (ATSDR, 2007) and US Environmental Protection Agency [4].

Among these compounds, *o*-cresol is one of the most toxic and frequent because it is widely used in industry as a solvent and intermediate for the production of herbicides, dyes, resins, pharmaceuticals, etc. [5] For these reasons, the removal of *o*-cresol from industrial water effluents is strongly recommended by several organizations and institutions to preserve aqueous reserves and their ecosystems [4].

Several conventional physicochemical processes for removal of *o*-cresol from water can be used at industrial scale (chlorination, flocculation, adsorption,

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membrane processing, etc.) [6,7]. These techniques are therefore costly and release toxic end products [8,9]: inconvenients that give rise a great interest for cheap and ecofriendly alternatives for eliminating *o*-cresol from water.

Biodegradation i.e. use of the catabolic potential of micro-organisms such as bacteria or fungi to eliminate organic molecules seems to be the most promising alternative to replace conventional process of *o*-cresol and other phenolic compounds removal processes from water.

Indeed, despite the toxicity of cresols, some micro-organisms can tolerate and use these compounds as energy/carbon source, even at relatively high concentration (500 mg L^{-1} or even higher) [10–13] and doing this leads to the breakage and mineralization of cresols and ensure their removal for water without generating any by-products except carbon dioxide and water [14]. Promises of biodegradation processes had induced these last years an increasing interest for phenolic compounds metabolizing micro-organisms [8] and several interesting species, mainly represented by bacteria which were isolated from samples of clean or phenols contaminated soils and waters [15–17].

Among this species, representatives of the genus *Pseudomonas*, well known for their biodegradation potential [18], seem to be the most interesting bacterial group for removal of cresols and some strains of this genus were found able to tolerate and metabolize *o*-cresol at concentrations exceeding 1,000 mg L^{-1} [10].

Despite the potential of use of bacteria for removing cresols from water, studies on bacterial biodegradation of these compounds are rather scarce. We report in this paper the isolation of a strain of a *Pseudomonas aearuginosa* with a high *o*-cresol biodegradation potential and the effect of pH, salinity and heavy metals on its *o*-cresol degrading potential.

2. Materials and methods

2.1. Isolation and identification

Nine samples of activated sludge and soils were collected from different locations in Algiers, and diluted to 1/10th in distilled water. One millilitre of each diluted samples was spread under aseptic conditions onto agar plates containing a minimal salt medium and 500 mg L^{-1} of *o*-cresol. Colonies appeared on plates after 2–3 days of incubation at 30˚C.

Purification of strains was done by transferring several times a single colony on the same medium as described above and the pure culture obtained was preserved in nutrient agar slants at 4˚C.

The strain used in this study i.e. *Pseudomonas aeruginosa* S8 was selected amongst all other *o*-cresol degrading isolated strains for its high *o*-cresol degradation rate even when the concentration of medium in *o*-cresol reached 1,250 mg L^{-1} (data not shown).

The S8 strain was identified on the basis of its morphological, biochemical and physiological characters according to *Bergey's Manual of Determinative Bacteriology* rules [19].

2.2. Culture medium and inoculum preparation

The pH of minimal salt medium (MSM) (K_2HPO_4, 0.8 g L^{-1}; KH_2PO_4, 0.2 g L^{-1}; $CaSO_4.2H_2O$, 0.05 g L^{-1}; $MgSO_4{\cdot}7H_2O$, 0.5 g L^{-1}; $(NH_4)_2SO_4$, 1.0 g L^{-1}; $FeSO_4$, 0.01 g L^{-1}) in distilled water [20] was adjusted to 7 and sterilized at 120˚C for 20 min. Microfiltered solution of *o*-cresol was added after sterilization at desired concentration.

The pure bacterial culture was pre-cultured in 250 mL Erlenmeyer flasks containing 100 mL of minimal salt medium supplemented with 500 mg L^{-1} *o*-cresol and incubated at 30˚C on a rotary shaker at 150 rpm until it reached an initial cell concentration (OD_{600}) of 0.5 to serve as inoculum.

2.3. Experimental

The study of the effect of pH, heavy metals and salinity on the biodegradation of *o*-cresol was performed by inoculating (5% (v/v)) Erlenmeyer flasks containing 100 mL of MSM at 500 mg L^{-1} *o*-cresol concentration.

Flasks were cotton plugged and incubated for 72 h on a rotary shaker at 30˚C and 150 rpm. The pH of the medium was set to 4, 5, 6, 7, 8 and pH 9. Heavy metals effect was studied by adding separately 5, 25 and 50 100 mg L^{-1} of Zn^{2+} ($ZnCl_2$), Cd^{2+} ($CdCl_2$) and Cu^{2+} ($CuCl_2$) to the medium. The effect of salt on the biodegradation potential of the strain was assessed adding 250, 500, 750 and 1,000 mM NaCl to the culture media.

The kinetics of *o*-cresol biodegradation was monitored in polypropylene bottles used as batch reactors. The polypropylene bottles containing 500 mL of MSM supplemented with *o*-cresol at different concentrations (250, 500, 750, 1,000 and 1,250 mg L^{-1}) were inoculated by 5% (v/v) of strain S8 inocula as described previously. Culture samples were taken at appropriate times to measure cell density (OD_{600}) and residual *o*-cresol after centrifugation at 8000 g of the culture sample for 20 min.

2.4. Analytical procedures

Cell density was monitored by measuring absorbance at 600 nm using an UV/visible spectrophotometer (JENWAY 6,505). For the modelling of cell growth kinetic, the OD_{600} of the culture was converted to protein concentration ($\mu g_{proteins}\,mL^{-1}$) with the help of a calibration curve, obtained by plotting OD_{600} vs. proteins concentration determined according to Bradford method [21].

The concentration of *o*-cresol in the culture medium was determined by colorimetric method based on its condensation with 4-aminoantipyrene (*4-Amino-1,5-dimethyl-2-phenyl-4-pyrazolin-3-one*) and oxidation with potassium ferricyanide [22].

3. Results and discussion

3.1. Selected strain identification

The aerobic *o*-cresol degrading strain used in this study was isolated from a soil sample originating from a botanical garden in Algiers (Algeria). This strain, the strain S8, was identified as *P. aeruginosa* on the basis of its morphological, biochemical and physiological traits.

3.2. Biodegradation studies

o-cresol biodegradation (%) was calculated according to the following Eq. (1):

$$o\text{-cresol biodegradation }(\%) = \frac{S_0 - S}{S_0} \times 100 \qquad (1)$$

where S is the substrate concentration ($mg\,L^{-1}$) and S_0, the initial substrate concentration ($mg\,L^{-1}$).

3.2.1. Effect of initial pH

The growth of *P. aeruginosa* S8 and its ability to degrade *o*-cresol was monitored during 72 h in media containing 500 $mg\,L^{-1}$ *o*-cresol at pH ranging between pH 4 to pH 9.

Degradation of *o*-cresol was greatly influenced by initial pH (Fig. 1). The maximum values of biodegradation rates of 99.96 and 99.91% were observed, respectively, at pH 7 and pH 8.

However, at pH values of 4 and 5 biodegradation efficiency was affected drastically and reached only 3.6 and 2.8%, respectively, and thus because the growth of *P. aeruginosa* S8 strongly reduced in acidic conditions which is in accordance with the literature [23].

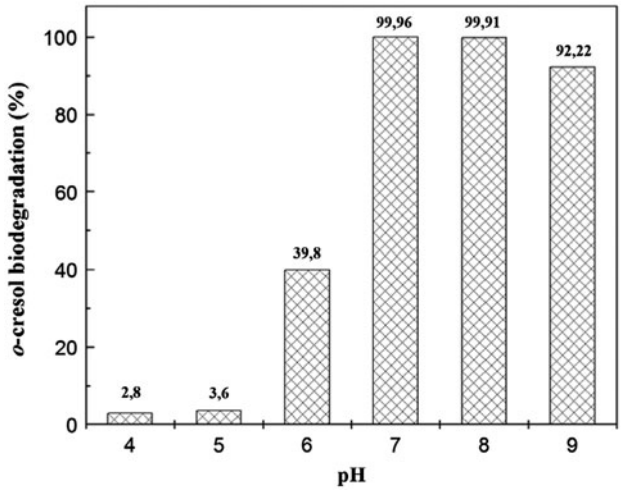

Fig. 1. Effect of pH on *o*-cresol biodegradation by *P. aeruginosa* S8.

These results indicate that growth and o-cresol biodegradation potential of *P. aeruginosa* strain S8 is much more affected by acidic conditions than alkaline conditions.

3.2.2. Effect of heavy metals

Based on the results shown in Fig. 2, the biodegradation of *o*-cresol by strain S8 was stimulated at low concentration of Zn^{2+} (5 $mg\,L^{-1}$). In this case, the biodegradation was of about 97.86% while in absence of metals this value was of 96.38%. Hassen et al. [24] demonstrated that at very low doses, zinc appeared toxic, except for some relatively zinc-tolerant bacterial strains including *P. aeruginosa*.

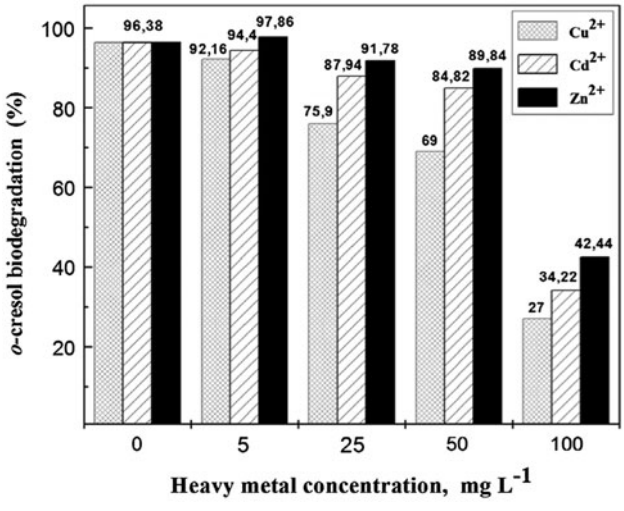

Fig. 2. Effect of heavy metals on *o*-cresol biodegradation by *P. aeruginosa* S8.

Nevertheless, at high concentration of Zn^{2+}, the *o*-cresol biodegradation varies inversely with the concentration of metal. When Zn^{2+} concentration increased to 100 mg L^{-1}, *o*-cresol biodegradation rate reached 42.44%. Similarly for Cd^{2+} and Cu^{2+}, the biodegradation of *o*-cresol by strain S8 decreased when metal concentration in the medium increased. In these conditions, the biodegradation potential of strain S8 was lowered and reached only 27 and 34.22% in presence of 100 mg L^{-1} of cadmium and copper respectively.

The decrease in *o*-cresol biodegradation at high concentrations of metals (above 5 mg L^{-1}) is probably due to adsorption of these metals to the bacterial cells and the modification of the *o*-cresol enzymes degrading active sites [25]. The other possible explanation is that the metals ions may inhibit pollutants biodegradation through their interaction enzymes involved in general metabolism [26].

In this study, the results showed that the copper ion Cu^{2+} was the most toxic for the growth of strain S8 and consequently led to a considerable decrease in *o*-cresol biodegradation potential of the strain.

Several previous reports have shown that copper was more toxic than other heavy metals [24,27,28].

3.2.3. Effect of salinity

Industrial wastewater may contain various salts that can inhibit the activity of micro-organisms involved in the biodegradation of phenolic compounds. Biodegradation of phenols under saline conditions have been reported by few authors [15,29,30]. However, no studies on bacterial biodegradation of cresols have been performed under salinity.

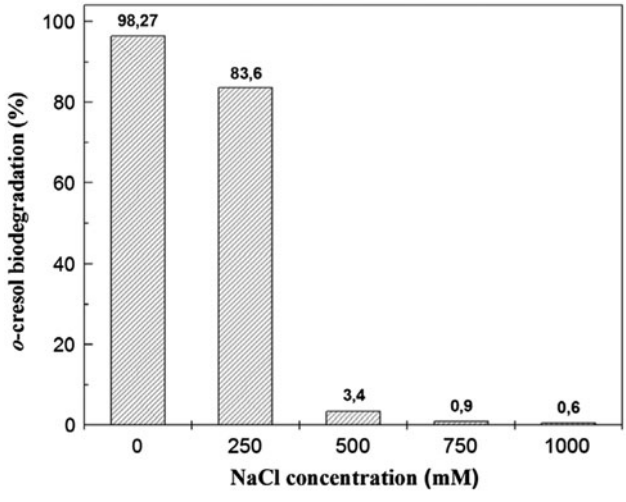

Fig. 3. Effect of salinity on *o*-cresol biodegradation by *P. aeruginosa* S8.

Fig. 3 shows the effect of varying NaCl concentrations on *o*-cresol biodegradation at 500 mg L^{-1}. It can be seen that at NaCl concentration of 250 mM, the biodegradation was of 83.6% indicating that *o*-cresol biodegradation is affected by high concentration of salt. The NaCl concentration of 500, 750 and 1,000 mM decreased considerably the *o*-cresol biodegradation. It may be attributed to the variation of enzyme activity under different saline conditions. Low sodium concentration is necessary for the growth of many micro-organisms, but at high concentration it inhibits the microbial growth, conditions where physiology of bacteria is strongly impaired by the decrease of water activity and the plasmolysis phenomenon [31].

From these results, it can be deduced that *P. aeruginosa* S8 strain is able to grow on *o*-cresol in presence of NaCl at 250 mM corresponding at about 15 g L^{-1}. The salt tolerance of this species (up to 3% NaCl) has been reported by several authors as observed in this study, even if culture conditions slightly differ [30,32].

3.2.4. Effect of initial concentration on o-cresol biodegradation and on the growth of the culture

Five batch cultivation experiments were performed using *o*-cresol as single limiting substrate for culture of *P. aeruginosa* S8. Different initial *o*-cresol concentrations were in the range of 250 to 1,250 mg L^{-1}.

The growth profile of *P. aeruginosa* S8 growth and biodegradation of *o*-cresol is shown in Fig. 4. It could be seen that at different initial *o*-cresol concentrations ranging from [250–1250 mg L^{-1}] there was a complete conversion after 30, 45, 51, 75, 85 h, respectively.

These results show the high potential of this strain to degrade *o*-cresol, even at relatively high concentrations. Similar observation was made by Kotresha and Vidyasagar [33]. They reported that *P. aeruginosa* MTCC 4,996 strain was able to degrade phenol up to 1,300 mg L^{-1} within 156 h. In *Pseudomonas*, many of induced enzymes are non-specific and its metabolic pathways contain a high degree of convergence [34].

For the same initial amount of inocula (OD_{600} = 0.5), the *o*-cresol biodegradation and the time required for *o*-cresol degradation varied as function of initial *o*-cresol concentration in the medium.

Bacterial growth results indicate that lag time increased with increasing *o*-cresol concentration which probably is due to the reason that micro-organisms take some time for adaptation to the new environment. In fact, in the presence of *o*-cresol concentration of 250, 500 and 750 mg L^{-1}, the lag time of the strain was below 8 h. However, at high substrate concentrations 1,000 and 1,250 mg L^{-1}, the lag time reached more than

30 h. Ahamad and Kunhi [10] have also reported an increase of lag time of *Pseudomonas* sp. strain CP4 in presence of 1,100 and 1,400 mg L^{-1} *o*-cresol.

The increase in lag time depends on the inoculum size and on substrate inhibition as reported by Bajaj et al. [35] and Geng et al. [36].

Moreover, it was found that after complete *o*-cresol degradation at initial *o*-cresol concentration of 1,000 and 1,250 mg L^{-1} (Fig. 4(d) and (e)), the biomass of the S8 cultures increased. Sàez and Rittmann [37] and Hao et al. [38] have reported the same behaviour for *Pseudomonas*. sp. strains. This increase may be explained by the fact that phenol is first converted into intermediate metabolites until fully degraded by the bacteria [39,40].

3.3. Growth kinetics

Based on the growth curves of *P. aeruginosa* S8, the specific growth rate was obtained for each initial

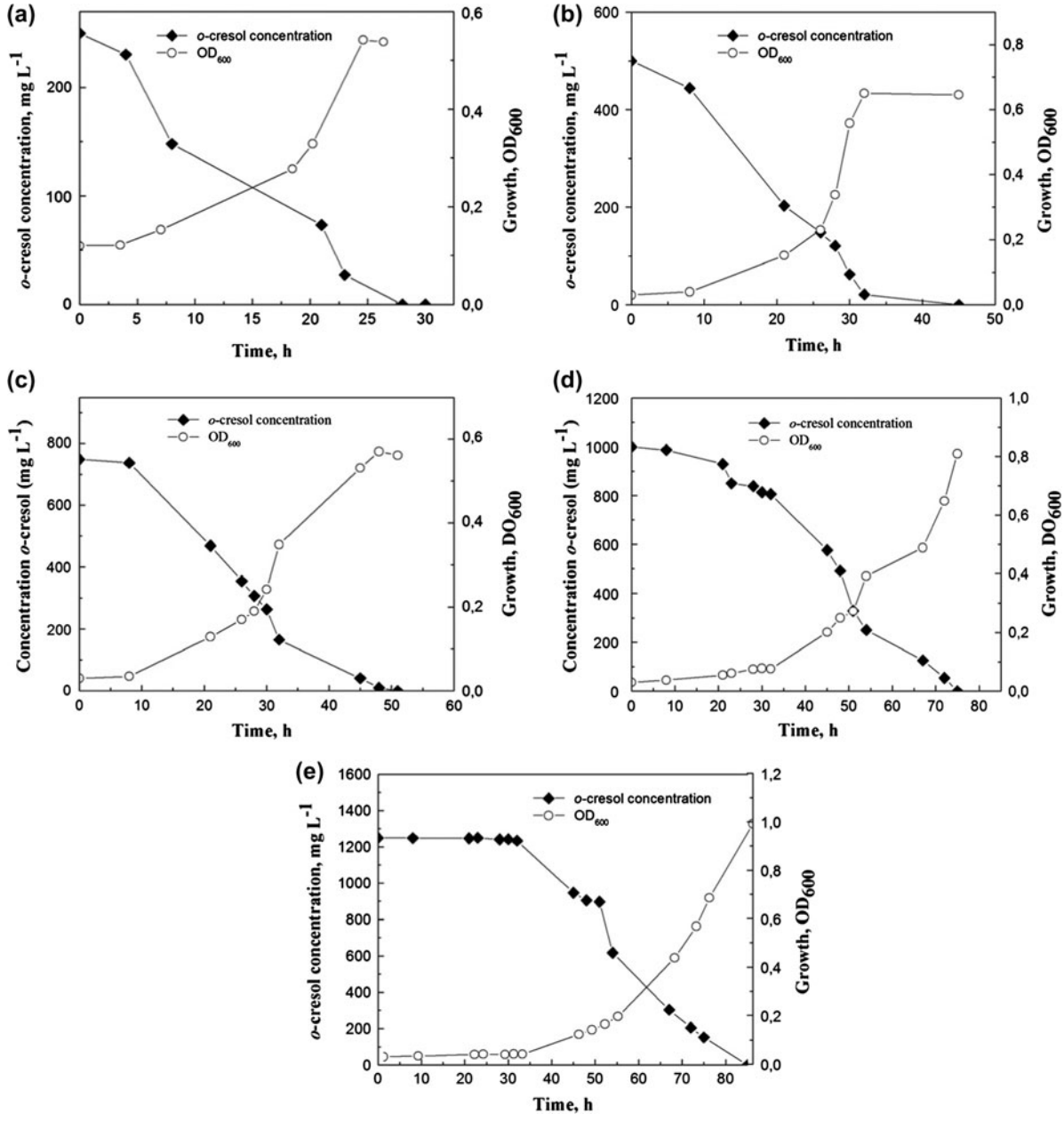

Fig. 4. Effect of *o*-cresol concentrations on *P. aeruginosa* S8 growth and *o*-cresol biodegradation: (a) 250, (b) 500, (c) 750, (d) 1,000 and (e) 1,250 mg L^{-1}.

concentration using the following relation given in Eq. (2):

$$\mu = \frac{1}{X}\frac{dX}{dt} \tag{2}$$

where μ is the specific growth rate (h^{-1}), X is the biomass concentration ($\mu g_{proteins}\, mL^{-1}$) at time t (h). After integration, Eq. (2) can be represented by Eq. (3):

$$\ln X = \ln X_0 + \mu t \tag{3}$$

where X_0 is the initial biomass concentration ($\mu g_{proteins}\, mL^{-1}$) at $t = 0$.

The specific growth rate (μ) was determined by taking in consideration only exponential growth phase. It was calculated for each initial concentration of *o*-cresol from the slope of linear logarithmic plots of biomass concentration ($\ln X$) vs. time (t). The experimental values of μ are plotted against initial concentration of *o*-cresol in Fig. 5.

From this figure, the specific growth rate decreased with increasing substrate concentration. The value of maximum specific growth rate is obtained at initial *o*-cresol concentration of about 250 mg L^{-1} and it is found to be equal to 0.117 h^{-1}. Above this concentration, the specific growth rate decreased. This type of behaviour is a result of substrate inhibition which occurred because of excess substrate concentration [41,42].

At high *o*-cresol concentrations some cells could be damaged leading to decreased metabolic activity. Moreover, the dead part of cells present in the culture seems to contribute further to substrate inhibition of growth [43].

3.3.1. Andrews's model

Substrate utilization can be quantitatively described on the basis of growth models. The relationship between the specific growth rate (μ) of microorganisms and the substrate concentration (S) is a valuable tool in biodegradation processes.

In this present study, Andrews's model [44] equivalent to the Haldane model [45] for enzymatic kinetics has been adopted for its mathematical simplicity and it is a good representation of experimental data. It is widely used to describe bacterial growth in presence of substrate inhibition [46–49].

The Andrews inhibitory growth kinetics equation is as follows Eq. (4):

$$\mu = \mu_{max}\frac{S}{K_S + S + \left(\frac{S^2}{K_I}\right)} \tag{4}$$

where μ_{max} is the maximum specific growth rate (h^{-1}), K_S is substrate affinity constant (mg L^{-1}) and K_I is the substrate inhibition constant (mg L^{-1}).

The model parameters were evaluated using nonlinear regression method using *MATLAB 7.8*. This software utilizes the Levenberg–Marquard algorithm for minimizing the sum square of residuals.

Fig. 6 shows the fit of Andrews's model with the experimental results, the curve obtained had a high correlation coefficient (R^2) of 0.995 and root-mean-square error (RMSE) of 0.003929 between the substrate inhibition model predicted and experimental specific growth rate of the culture in different *o*-cresol

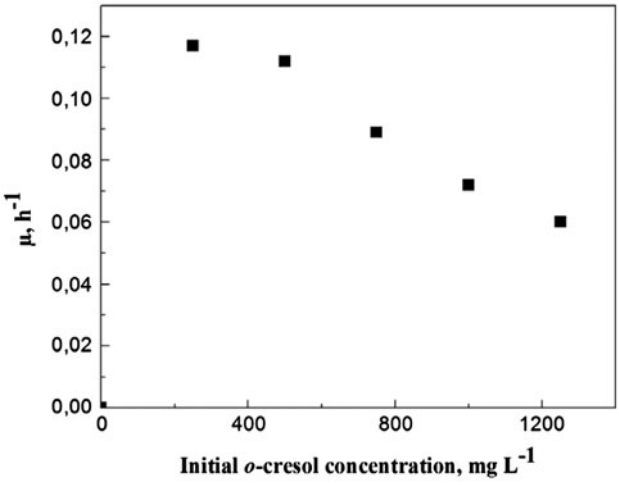

Fig. 5. Effect of *o*-cresol concentration on specific growth rate of *P. aeruginosa* S8.

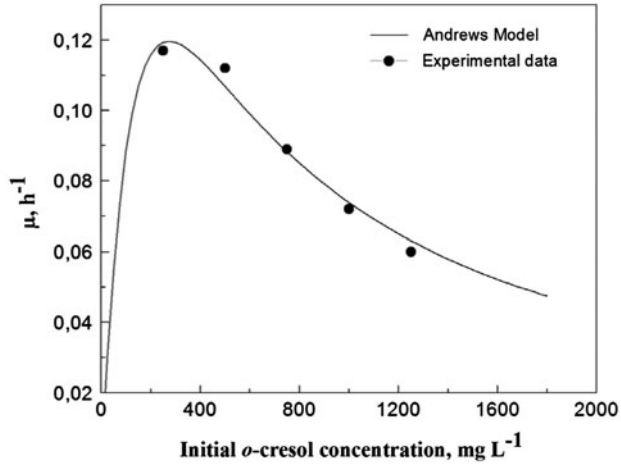

Fig. 6. Plots of the experimental data (points) and their fitting to Andrews's kinetic model (curve).

Table 1
Summary of Andrews (Haldane) kinetic parameters for the biodegradation of cresols

Authors	Substrate	Bacterial strain	Concentration range ($mg\,L^{-1}$)	Andrews's (Haldane) model			Culture conditions
				μ_{max} (h^{-1})	K_S ($mg\,L^{-1}$)	K_I ($mg\,L^{-1}$)	
Kar et al. [50]	*o*-cresol	*Arthrobacter* sp. *MTCC 1,553*	50–1,200	0.84	84.0	1,050	pH = 7, *T* = 28–30°C
Jiang et al. [51]	*m*-cresol	*Candida tropicalis*	0–320	2.78	866	4.42	pH = 6, *T* = 30°C
Ho et al. [13]	*o*-cresol	Mixed culture	50–400	0.11	15.3	192	pH = 7, *T* = 30°C
Saravanan et al. [52]	*m*-cresol	Mixed culture (predominantly *Pseudomonas sp*)	100–400	0.19	65.1	244	pH = 7, *T* = 27°C
Kar et al. [50]	*p*-cresol	*Arthrobacter* sp. MTCC 1,553	50–1,200	0.92	48	800	pH = 7, *T* = 28–30°C
Maeda et al. [5]	*o*-cresol	Mixed culture	30–600 (medium-A)	0.37	92.4	125	pH = 7, *T* = 25°C
Maeda et al. [5]	*o*-cresol	Mixed culture	30–600 (medium-B)	0.11	2.8	325	pH = 7, *T* = 25°C
Sing et al. [11]	*p*-cresol	*Gliomastix indicus* MTCC 3,869	10–700	0.8	42.37	43.28	pH = 6, *T* = 28°C
Ho et al. [13]	*p*-cresol	Mixed culture	50–1,000	0.56	12.8	211	pH = 7, *T* = 30°C
Saravanan et al. [41]	*m*-cresol	Mixed culture	0–900	0.68	79.1	204	pH = 7, *T* = 27°C
Ho et al. [13]	*m*-cresol	Mixed culture	50–500	0.14	13.1	116	pH = 7, *T* = 30°C
This work	***o*-cresol**	***Pseudomonas aeruginosa* S8**	**250–1,250**	**0.335**	**246**	**304.2**	**pH = 7, *T* = 30°C**

concentrations. Thus, Andrews's model fitted well the experimental data. The maximum specific growth rate value of $0.119\,h^{-1}$ was slightly higher than that obtained by experimental. This value corresponds to a substrate concentration of $275\,mg\,L^{-1}$.

The Andrews parameters for strain grown on *o*-cresol at 30°C were obtained as follows: $\mu_{max} = 0.335\,h^{-1}$, $K_S = 246\,mg\,L^{-1}$ and $K_I = 304.2\,mg\,L^{-1}$. A comparison of these kinetics parameters with those reported in other reports on cresols isomers degradation by various micro-organisms either by pure or mixed culture is shown in Table 1; the maximum specific growth rate μ_{max} lies in the range of 0.11–2.78, which implies that growth rate of *P. aeruginosa* S8 on *o*-cresol is comparable with the growth rate of other micro-organisms used for removal of cresols in effluents.

The K_S value obtained in this work was found higher than those reported in literature on *o*-cresol degradation, with pure and mixed culture, indicating that the *P. aeruginosa* S8 has a moderate affinity with the substrate.

The degree of resistance of the micro-organism to toxic effect of *o*-cresol is indicated by the value of the kinetic parameter K_I. A high value of K_I reveals that biomass is highly resistant to inhibition by the substrate. In other works as listed in Table 1, K_I value vary in the range of 4.42 to 105. While, in our case it was estimated to be $304.2\,mg\,L^{-1}$ indicating that the inhibitory effect on *Pseudomonas* is observed at high concentration of *o*-cresol.

4. Conclusion

The biodegradation of *o*-cresol in water was investigated using a strain isolated from soil and evaluated for its tolerance and ability to degrade *o*-cresol at high concentration. This strain was identified by morphological and biochemical analyses as *P. aeruginosa* strain.

The influence of environmental parameters on *o*-cresol biodegradation studies indicates that:

(1) The optimal pH for the growth and degradation of *o*-cresol is pH 7 and *P. aeruginosa* S8 strain tolerated well the alkaline pH.
(2) This strain was resistant to heavy metals (Cu^{2+}, Cd^{2+} and Zn^{2+}) concentration of $5\,mg\,L^{-1}$ with *o*-cresol biodegradation higher than 90%.

However, in the presence of Zn^{2+} at concentration of $5\,mg\,L^{-1}$, *o*-cresol biodegradation was stimulated. High concentration of metals decreased *o*-cresol biodegradation due to the inhibitory effect for bacterial growth.

(3) The presence of salt (NaCl) in the medium influenced negatively the *o*-cresol biodegradation, a considerable decrease in *o*-cresol biodegradation potential of the *P. aeruginosa* S8 strain was recorded at NaCl concentration above 250 mM.

Kinetics of *o*-cresol biodegradation was examined in aerated batch reactors at varying initial *o*-cresol concentration ranging from 250 to $1{,}250\,mg\,L^{-1}$. *P. aeruginosa* S8 strain can degrade *o*-cresol completely at concentration of $1{,}250\,mg\,L^{-1}$ within 85 h. The Andrews kinetic model fitted well the experimental data with a correlation factor of 0.99.

We can conclude that *P. aeruginosa* S8 *strain* has a high potential to degrade *o*-cresol, and it represents to be an elite strain to develop biotechnological applications to treat industrial wastewaters containing high *o*-cresol concentrations.

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