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Biodegradation of *m*-cresol in alkaline wastewater by resting photosynthetic bacteria

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ABSTRACT

The application of various micro-organisms to metabolize organic compounds is one potentially effective method in treating hazardous and toxic wastewater. The effects of doses of cells, pH, initial substrate concentrations, and the addition of metal ions (Mn^{2+} and Cu^{2+}) on *m*-cresol biodegradation by the resting cells (non-growth cells without immobilizing) of photosynthetic bacteria (PSBr) are investigated on a rotary shaker at 120 rpm at 30°C. The maximum biodegradation rate for a 200 mg/L *m*-cresol solution was 98% with a PSBr dose of 0.17 g/L, pH 7.5 at 30°C in dark conditions. The degradation capability of PSBr was drastically reduced in the presence of Mn^{2+} or Cu^{2+}, and the inhibitory effect of Cu^{2+} was greater than that of Mn^{2+} for the same concentration. Specific degradation rates were fitted with the Haldane kinetic model. The parameter values were; $r_{max} = 0.05$/h, $K_m = 254.15$ mg/L, and $K_i = 112.18$ mg/L, with a coefficient of determination (R^2) greater than 0.89. Our results suggest that PSBr shows good potential for degrading *m*-cresol in wastewater.

Keywords: Photosynthetic bacteria; *m*-Cresol; Biodegradation; Alkaline wastewater; Resting cells

1. Introduction

Environment pollution, especially with hazardous and recalcitrant toxic chemicals, is one of the major problems in developing countries like China. *m*-Cresol is a methylated derivative of phenol, which is listed as a priority pollutant by the United States Environmental Protection Agency and considered to be a toxic compound by the Agency for Toxic Substances and Disease Registry [1–3]. The presence of a methyl group in cresol molecules markedly inhibits biodegradation of cresol molecules by micro-organisms [4]. *m*-Cresol enters the environment through wastewater discharged by various industries including textiles, dying, leather processing, phenol-formaldehyde resin and pesticide manufacture, oil refinery, pharmaceuticals, coal conversion, wood-pulp, paper mills, etc. [5,6]. Wastewater containing *m*-cresol enters the receiving water bodies and causes considerable damage to the aquatic biota because of its toxic properties [7]. Hence, *m*-cresol removal from aqueous effluents prior to release into the environment is strongly desirable. Among the available and efficient treatment methods for degradation of phenolic wastewaters as *m*-cresol, biological treatment processes seem to be more promising and economical [8,9]. Biodegradation of *m*-cresol (as the sole carbon and energy source) by pure microbial cultures, including *Alcaligenes faecalis*

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[7], *Candida albicans* PDY-07 [10], and *Lysinibacillus cresolivoran*s [11], has been reported. *Pseudomonas* sp. CP_4 can mineralize all three isomers of cresol, and the degradation rates for the three isomers were in the order: *o*- > *p*- > *m*-cresol [12]. However, reports on biodegradation of *m*-cresol solutions employing mixed microbial cultures are limited. Aerobic degradation of *m*-cresol using mixed microbial communities may be advantageous for complete assimilation (CO_2 and H_2O), hence, yielding no toxic by-products [7]. Indigenous mixed microbial culture was found capable of utilizing 900 mg/L of *m*-cresol (as sole carbon source) in 136 h [1], and phenol-acclimated aerobic granules could completely remove *m*-cresol at concentrations of up to 1,500 mg/L [13]. However, no research has focused on the biodegradation of *m*-cresol by resting photosynthetic bacteria (PSBr).

PSBr, the general name of a large category of procaryotic organisms with the original photosynthesis system, which can utilize organic compounds either under anaerobic-light or aerobic-dark condition, are widely distributed in nature. PSBr have been employed by some researchers to treat high organic loads, and recalcitrants including oil, soybean, pharmaceutical, and aniline in wastewater [14–17]. However, few researchers have attempted to characterize the biodegradation of aromatic compounds by the resting cells (non-growth cells without immobilizing) of PSBr. In this study, we explored optimal conditions for *m*-cresol degradation by PSBr at various cell dosages, pH, and initial substrate concentrations in flasks on a rotary shaker at 120 rpm at 30˚C under aerobic-dark conditions. The biodegradation kinetics were calculated, and the effects on *m*-cresol degradation in the presence of metal ions (Mn^{2+} and Cu^{2+}) were investigated. Thus, we demonstrated degradation characteristics of PSBr in dark conditions, and the feasibility of PSBr treatment of *m*-cresol containing wastewater.

2. Materials and methods

2.1. Chemicals and cultures

m-Cresol (CAS# 108-39-4, purity > 98.0%) used in the study was of analytical grade, and was supplied by the Sinopharm Chemical Reagent Co., Ltd (Shanghai, China). High-performance liquid chromatography (HPLC) grade methanol was obtained from Ludu Chemical Reagent Plant Co., Ltd (Shanghai, China). All other chemicals and reagents were of analytical grade and used as received. A concentrated stock solution of *m*-cresol was prepared.

Media for degradation (DM) contained K_2HPO_4 (1.55 g/L), KH_2PO_4 (0.65 g/L), $MgSO_4$ (0.2 g/L), $CaCl_2$ (0.06 g/L), and $FeSO_4$ (0.01 g/L). *m*-Cresol was added as required. Media for enrichment (EM) was formed by adding 2.8 g/L of sodium acetate to the DM.

2.2. Analytical methods

m-Cresol concentration in cultures was determined using HPLC (Jasco LC2000, Japan). The analysis was performed with a Yilite BDS C8 column (4.6 mm × 250 mm × 5 μm) with methanol/water (60/40 V/V) as the mobile phase at a flow rate of 0.7 mL/min, and measured using a UV detector at 270 nm at room temperature. *m*-Cresol concentration was estimated by comparison with the standard curve. The bacterial concentration was determined by examining OD_{510} using a UV–vis spectrophotometer (Type 759s, Shanghai Precision Scientific Instrument Co., Ltd, China), and pH in cultures were measured by meter (PHS-3C, China).

2.3. Preparation of PSBr cells

PSBr were obtained from a local chemical company (Zhejiang Dragon Chemical Group Co., China), where PSBr are used for wastewater treatment. In order to obtain a community with high *m*-cresol degradation efficiency, acclimatization was performed (Fig. 1). First, the original PSBr suspension was centrifuged at 10,000 r/min for 10 min, at room temperature. The resultant pellets were washed twice with 0.05 mol/L K_2HPO_4–0.05 mol/L KH_2PO_4 buffer (pH 7.0), centrifuged, and resuspended in the same buffer. The suspension was adjusted to the desired optical density 4.0 at 510 nm (OD_{510}), equivalent to 0.85 g dry cell weight

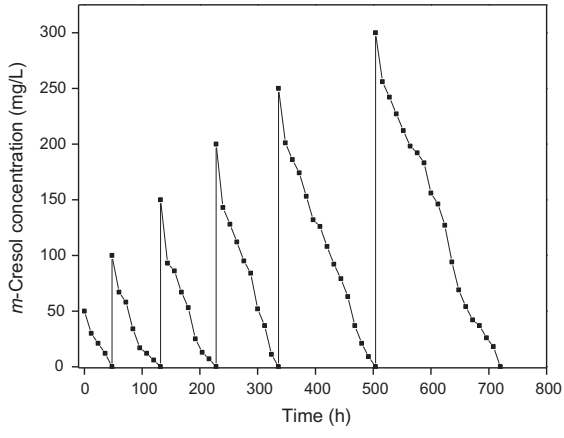

Fig. 1. *m*-Cresol degradation profile followed during the acclimatization period by the resting cells (non-growth cells without immobilizing) of PSBr.

(DCW)/L). The relationship between OD_{510} and DCW is shown in Fig. 2. Second, 0.17 g (DCW)/L of the suspension was incubated in 200 mL of DM supplemented with 50 mg/L of *m*-cresol (as the sole carbon source) in Erlenmeyer flasks, and cultivated in a light incubator at 30°C and 4,800 Lux. The initial pH in the flasks was adjusted to 7.0 with NaOH or HCl (5 mol/L). When the residual *m*-cresol concentration in the cultures was 0 mg/L, the bacterial suspension was treated in the same way described above, and 0.17 g(DCW)/L of the bacterial suspension was transferred to 200 mL fresh DM with 100 mg/L of *m*-cresol and cultivated in a light incubator at 30°C and 4,800 Lux. Further acclimatization of PSBr was carried out by gradual (50 mg/L) increments of *m*-cresol after each reincubation. When the PSBr were acclimatized to 250 mg/L of *m*-cresol, the suspension was treated in the same way described above, and utilized for degradation experiments.

2.4. m-Cresol biodegradation by PSBr

For all experiments, PSBr were inoculated into DM supplemented with various *m*-cresol concentrations in flasks, and cultivated at 30°C under aerobic-dark conditions. The total liquid volume was 200 mL. The effect of PSBr doses (*m*/*v*), namely 0.043, 0.085, 0.13, 0.17, and 0.21 g/L in flasks, on *m*-cresol degradation at pH 7.0 was investigated. The influence of initial pH value (6.0, 6.5, 7.0, 7.5, 8.0, 8.5, and 9.0) on *m*-cresol degradation, was studied, for PSBr dosage 0.17 g/L. Initial substrate concentrations (50, 100, 150, 200, and 250 mg/L) with PSBr dosage 0.17 g/L, and pH 7.5, were investigated. In addition, the influence of metal ions (Cu^{2+} and Mn^{2+}) on *m*-cresol degradation was investigated at metal ion concentrations of 0, 5, 10, 15, and 20 mg/L, PSBr dosage of 0.17 g/L, and pH 7.5.

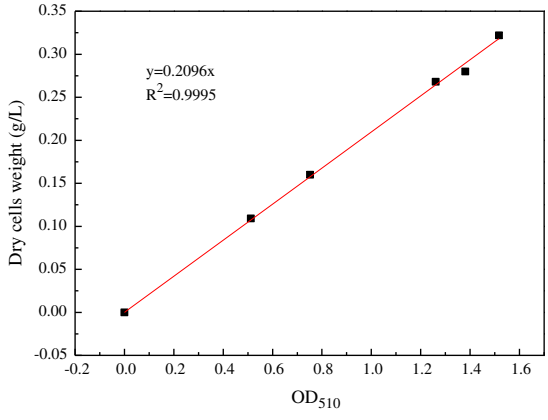

Fig. 2. Relationship between OD_{510} and DCW.

All tests were performed in duplicate under non-sterile conditions.

To enumerate residual *m*-cresol concentration, aliquots of test solution were taken every 12 h and treated by centrifugation at 10,000 r/min for 10 min. The resulting supernatants were membrane filtered at 0.45 μm, and a 20 μL aliquot immediately analyzed by HPLC. The average of the duplicate experimental results was used in calculations and data analyses.

2.5. Kinetics experiments

Batch experiments were conducted to investigate degradation kinetics in open Erlenmeyer flasks in darkness. Flasks were amended with various initial substrate concentrations from 50 to 250 mg/L, PSBr dose 0.17 g/L, pH 7.5, and incubated at 30°C. The Haldane kinetics model was selected due to its mathematical simplicity and wide acceptance for representing the kinetics of inhibitory substrates. Haldane's inhibitory kinetics equation associated with a specific degradation rate is as follows [18]:

$$r_i = \frac{r_{\max}}{1 + \frac{K_m}{c} + \frac{c}{K_i}} \quad (1)$$

where c is the initial substrate concentration (mg/L), r_i is the specific degradation rate (h^{-1}), K_m is the Michaelis–Menten constant (mg/L), K_i is the inhibition constant (mg/L), and $r_{\max}$ is the maximum specific degradation rate (h^{-1}).

3. Results and discussion

3.1. m-Cresol degradation by PSBr

A unique feature of PSBr is that they have two energy metabolic pathways and can either survive under anaerobic-light conditions or aerobic-dark conditions, and pollutant removal is higher under aerobic-dark conditions than anaerobic-light conditions. In dark conditions, carbon sources can be degraded to CO_2 by an oxidation mechanism [19]. *m*-Cresol degradation by PSBr was investigated at pH 7.0 in the dark with the cell dose 0.17 g/L, as shown in Fig. 3. In the process of *m*-cresol degradation, the value of OD_{510} was almost invariant, but 88.4% of *m*-cresol in flasks was degraded after 72 h of incubation. Equivalent and autoclaved PSBr cultured in the same conditions served as control, and the loss of *m*-cresol in the control was just 7.7%, a small contribution to the overall loss of *m*-cresol in the experimental flasks. *m*-Cresol degradation may be caused by some enzyme

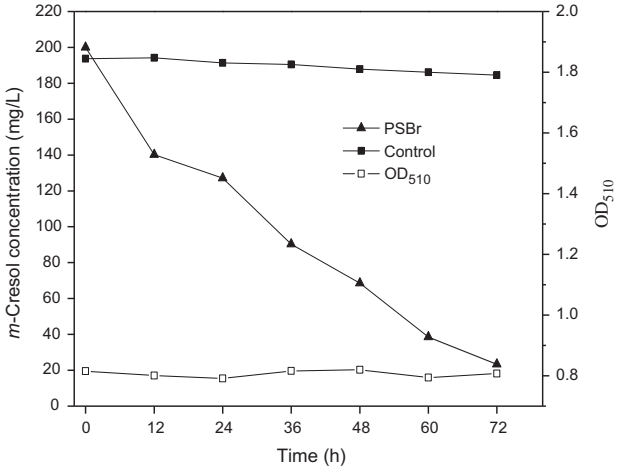

Fig. 3. *m*-Cresol degradation with PSBr dose 0.17 g/L at pH 7.0 in dark conditions.

produced by PSBr. Lee et al. reported that cresols were oxidized by dioxygenase [13]. Enzyme catalytic reaction may be the case for the degradation of 2, 4, 6-trichlorophenol by PSBr [18].

3.2. *Effect of PSBr dose on* m-*cresol degradation*

m-Cresol was degraded over the range of PSBr doses tested in the experiments (Fig. 4), and the dose significantly affected the degradation rate. After incubation for 72 h, the degradation rate of *m*-cresol with the cell dose of 0.043 g/L was just 41.2%. As the cell dose increased, the degradation rate improved, reaching a peak when the cell dose was 0.17 g/L. However, when the cell dose increased further, up to 0.21 g/L, the degradation efficiency decreased slightly. In general, larger cell doses mean higher concentrations of enzymes for *m*-cresol degradation. The optimal dosage of PSBr to degrade 2, 4, 6-trichlorophenol was 30% (V/V) (from a range of 10–50% tested in batch experiments) [18], which suggested that this phenomena probably stemmed from mass transfer resistance and aggravating circumstances. The optimal inoculation dosage (from a range of 2–20%) for *m*-cresol biodegradation by *L. cresolivorans* was 10% (V/V) [11]. Yao et al. postulated that surplus micro-organisms competed for the limited availability of nutrition which led to a declining biodegradation rate [18].

3.3. *Effect of initial pH on* m-*cresol degradation*

Initial media pH is an important factor that affects degradation of organic compounds by PSBr [20]. The highest degradation rate was achieved at pH 7.5 under the experimental conditions (Fig. 5). *m*-Cresol degradation rates exceeded 84% in the pH range from 7.0 to 8.5, indicating that a weak alkaline environment was good for degradation of *m*-cresol. Whereas, Yao et al. found that the highest biodegradation rate of *m*-cresol by *L. cresolivorans* was achieved between pH 6.8–7.3 with pH greater than 9.0 or lesser than 6.0, the degradation rate decreased [18]. Jin et al. suggested that the optimal pH for aniline degradation by *Dietzia natronolimnaea* JQ-AN was 8.0 [17]. It is likely that enzyme activity would be inhibited at high or low pH, because of alterations to the enzymes' structure.

3.4. *Effect of initial substrate concentrations on* m-*cresol degradation*

The degradation time by PSBr was dependent upon the initial *m*-cresol concentration (Fig. 6). *m*-Cresol is a kind of toxic substance to PSBr, and

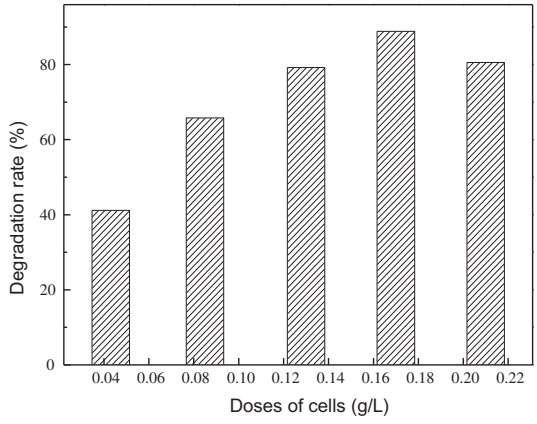

Fig. 4. The effect of PSBr dose on *m*-cresol degradation at pH 7.0.

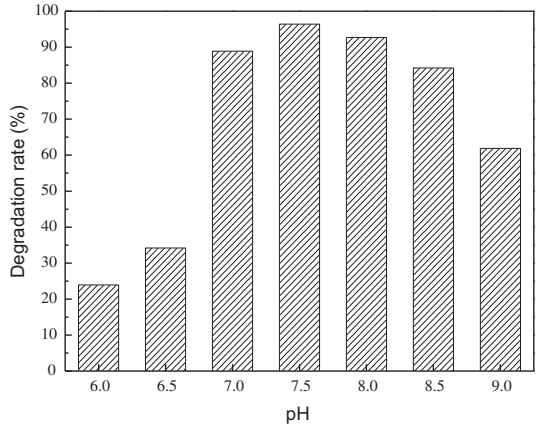

Fig. 5. The effect of initial pH on *m*-cresol degradation by PSBr.

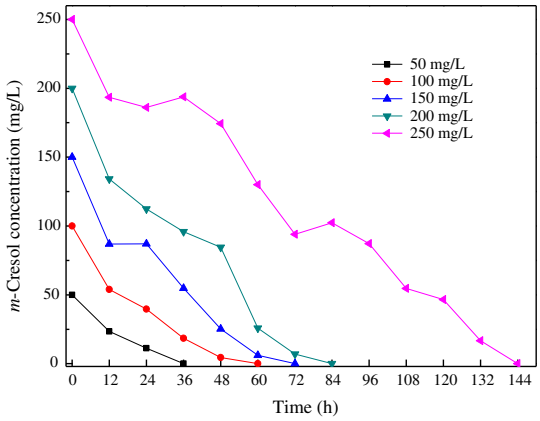

Fig. 6. Effect of initial *m*-cresol concentration on degradation.

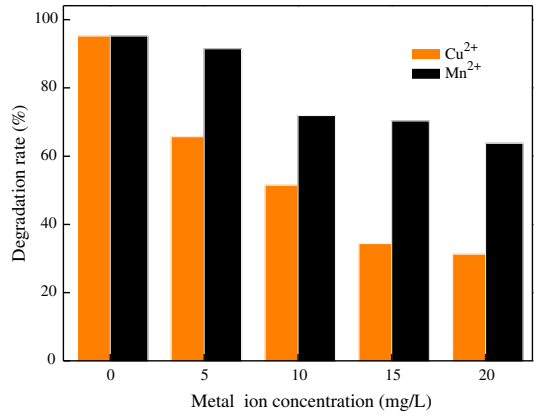

Fig. 7. Effect of heavy metals (Mn^{2+} and Cu^{2+}) on *m*-cresol degradation.

higher initial substrate concentrations mean greater inhibitory effects on degradation process. Saravanan et al. found that the complete degradation time for 100 mg/L *m*-cresol by a mixed culture was 14 h, but it took 136 h for 900 mg/L *m*-cresol degradation [1]. Concentrations of 50, 100, 150, 200, and 250 mg/L were completely degraded by PSBr within 36, 60, 72, 84, and 144 h, respectively. The maximum degradation rate of *m*-cresol was achieved at an initial concentration of 200 mg/L, and the results clearly illustrate that PSBr is effective at biodegrading *m*-cresol at concentrations less than 250 mg/L.

3.5. Effect of heavy metals on m-cresol degradation

Toxic organic pollutants and heavy metals often coexist in wastewaters. Heavy metals usually will inhibit the metabolism of micro-organisms, and the impact of heavy metals on degradation rates is dependent on their type and concentration [21,22]. The effects of Mn^{2+} and Cu^{2+} on *m*-cresol degradation at different concentrations (0, 5, 10, 15, and 20 mg/L) in DM supplemented with 200 mg/L of *m*-cresol were investigated under the optimal conditions reported above. Cultures without Mn^{2+} and Cu^{2+} served as controls. After 72 h of incubation, the degradation rates of *m*-cresol were inhibited in the presence of Mn^{2+} or Cu^{2+} (Fig. 7), and the inhibition increased with increasing [Mn^{2+}] or [Cu^{2+}]. When [Mn^{2+}] and [Cu^{2+}] increased from 0 to 20 mg/L, the degradation rates decreased from 96.4 and 93.8% to 63.8 and 31.3%, respectively. Strong inhibitory effects on *m*-cresol degradation were observed when [Mn^{2+}] reached 10 mg/L or [Cu^{2+}] reached 5 mg/L, i.e. for a given metal concentration, PSBr were more sensitive to Cu^{2+} than to Mn^{2+}. For example, when [Mn^{2+}] and [Cu^{2+}] were both 10 mg/L, the degradation rates were 71.9 and 51.5%, respectively. Hence, the toxic inhibitory effect of Cu^{2+} was substantially greater than that of Mn^{2+} (at the tested concentrations), which was consistent with the results found by Lin et al. [23]. Heavy metals can pass through cell membranes to interact with intracellular enzymes; thus, potentially interfering with essential bacterial cellular metabolism [24].

3.6. Degradation kinetics

The degradation kinetics of *m*-cresol by PSBr was determined using Haldane's kinetic model, which is a modified version of the Monod kinetic model that takes the inhibition factor into account. Specific *m*-cresol degradation rates were plotted against initial concentrations (Fig. 8). The specific degradation rate increased with the increase of *m*-cresol concentration

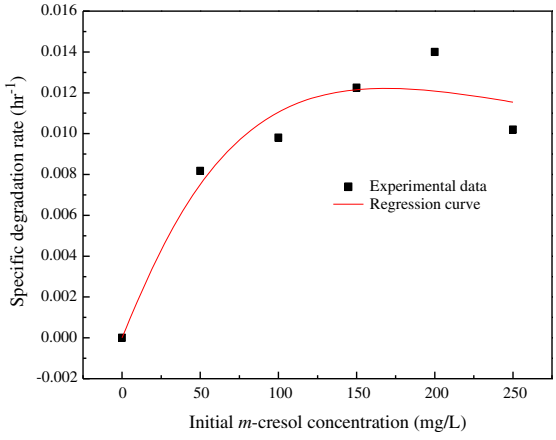

Fig. 8. Specific *m*-cresol degradation rate plotted against initial concentration.

Table 1
m-Cresol biodegradation by various micro-organisms

Bacterial strain	System	Concentration (mg/L)	Oxygen condition	Temperature (℃)	pH[a]	Efficiency	References
Desulfotomaculum sp. strain Groll	Batch	32.4	Anaerobic	32	–	100%/35 d	[3]
Pseudomonas-like strain S100	Batch	54	Anaerobic	28	7.4	100%/20 min	[25]
Alcaligenes faecalis	Batch	100	Aerobic	30	7.2	100%/32 h	[4]
Candida tropicalis	Batch	280	–	30	–	52 h	[2]
Candida albicans PDY-07	Batch	300	Anaerobic	35	7.0	100%/100 h	[10]
Aerobic activated sludge[b]	Batch	500	Aerobic	30	7.0	100%/380 h	[26]
Lysinibacillus cresolivorans	Batch	529.1	Aerobic	35	7.0	100%/44 h	[11]
Mixed microbial culture	Batch	900	Aerobic	27	7.0	100%/136 h	[1]
Pseudomonas sp. CP_4	Batch	1,100	Aerobic	30	–	100%/94 h	[12]
Phenol-acclimated granules[c]	Batch	1,500	Aerobic	30	7.0	100%/48 h	[13]
PSBr	Batch	200	Aerobic	30	7.5	98%/72 h	This study

[a]pH: the optimal pH used or tested in the study.
[b]Aerobic activated sludge: predominantly *Corynebacterium* sp., *Pseudomonas* sp., and *Alcaligenes* sp.
[c]Phenol-acclimated granules: predominantly genera *Bacillus*, *Acinetobacter*, *Corynebacterium*, and *Nocardioides*.

below 200 mg/L. When the initial concentration of *m*-cresol was 200 mg/L, the maximum specific degradation rate was approximately 0.014/h. As the initial substrate concentration increased up to 250 mg/L, the specific degradation rate decreased, which might be the result of intense substrate inhibition. The values of the parameters: $r_{max} = 0.05/h$, $K_m = 254.15$ mg/L, $K_i = 112.18$ mg/L, were derived using a non-linear regression analysis with coefficient of determination (R^2) value greater than 0.89 based on the experimental data. The low value of K_i indicated that the inhibition effect for *m*-cresol degradation could be observed in a low concentration range.

3.7. Comparison with other reported m-cresol degrading micro-organisms

The degradation of *m*-cresol by PSBr was compared with several reported *m*-cresol degrading micro-organisms (Table 1). Phenol-acclimated granules showed the highest *m*-cresol degrading activity and tolerance among the various reported strains; totally degrading 1500 mg/L of *m*-cresol in 48 h [13]. The range of degradation rates and initial concentrations in the other reported experiments vary widely.

Many of the micro-organisms that degrade *m*-cresol prefer neutral environments rather than mildly acid or alkaline conditions. Yao et al. found that *m*-cresol could be degraded by *L. cresolivorans* under aerobic conditions, the degradation rate was substantially decreased for pH greater than 9.0 or less than 5.0, and the optimal pH value was 7.0 [11]. In this study, PSBr could degrade 200 mg/L of *m*-cresol over a relatively large pH range (7.0–8.5) with degradation rate greater than 84%. The optimal pH of 7.5 achieved the degradation rate higher than 98%. The pH range of raw coking wastewater is 7.9–8.7, and the pH of paper mill wastewater is around 8.6, thus, it is necessary to adjust the initial pH of these alkaline wastewaters for most *m*-cresol degrading strains except PSBr. High degradation efficiency of *m*-cresol by PSBr over a large alkaline pH range means that PSBr has good adaptive capacity for the likely influents encountered.

4. Conclusions

The optimal conditions for *m*-cresol degradation in darkness by PSBr were cell dose 0.17 g/L and pH 7.5 in the absence of Mn^{2+} or Cu^{2+}, which achieved 98% degradation of 200 mg/L of *m*-cresol after 72 h of incubation. The data presented in this paper suggest that PSBr are suited to a weak alkaline environment, which is conducive to treatment of a range of *m*-cresol containing wastewaters, whereas other microbes would require pH adjustment and abundant aeration. The

experimental data fitted the Haldane kinetics model well. The presence of Mn^{2+} or Cu^{2+} had an adverse impact on *m*-cresol degradation by PSBr, and Cu^{2+} had stronger inhibitory effect than Mn^{2+} for a given concentration. This study demonstrates the potential of PSBr in treating wastewaters containing highly recalcitrant compounds such as *m*-cresol.

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