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Biodegradation of Mixed Phenolic Compounds Under High Salt Conditions and Salinity Fluctuations by *Arthrobacter* sp. W1

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Abstract High salt concentration and salinity fluctuations in wastewater challenge the efficiency of microbial strains used for cleanup of pollutants. In this study, it was investigated that the new isolated *Arthrobacter* sp. W1 degraded mixed phenolic compounds under complex salt conditions. The results showed that *Arthrobacter* sp. W1 was able to utilize various phenolic compounds as carbon source under high salt conditions. It can degrade phenol and *p*-cresol mixture at 10% NaCl, although rates of degradation and cell growth were lower compared to 5% NaCl. The presence of trace *p*-cresol significantly inhibited phenol biodegradation. When salinity fluctuations were between 1% and 10% NaCl, strain W1 was able to degrade substrates and survived. It was also suggested that the presence of salts (i.e., NaCl, KCl, Na_2SO_4, and K_2SO_4) had almost no effects on the microbial growth and biodegradation process. Therefore, *Arthrobacter* sp. W1 would be a promising candidate for bioremediation of phenolic compounds under complex salt conditions.

Keywords Biodegradation · Phenolic compounds · Salinity · *Arthrobacter* sp.

Introduction

With the rapid development of industry and agriculture, it has brought the problem of pollution and hazardous wastewater. Phenolic compounds are the hazardous pollutants which enter the environment through wastewater discharges from a variety of industries such as petrochemical, coal gasification, ceramic, pharmaceutical, and dye manufacturing [1]. Many substituted phenols including chloro/nitro and cresols have been designated as priority pollutants by the USEPA [2]. Therefore, it is necessary to eliminate the phenolic compounds in order to preserve the environmental quality.

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Among various techniques for phenolic compounds removal, biodegradation is an environment-friendly and cost-effective technology. Microorganisms for phenol degradation were isolated as early as 1908 [3]. Since then, microbial degradation of phenol and phenol derivatives by pure cultures [4–6] and mixed cultures [7–9] have been widely studied. Most of these studies were mainly focused on the biodegradation in the laboratory using single target compound. As phenolic compounds are present as mixtures in wastewater, the study of biodegradation of mixed pollutants was increased recently. In general, the microbial degradation of compound in a mixture can be strongly influenced by other coexisting compounds. As reported previously, the degradation rate of some compounds in the mixture may be stimulated [10] or repressed [11] by the presence of other compounds. Thus, the nature of pollutant interactions is variable, and it depends on the characteristics of the compounds and the degradation capacity of the involved microorganisms.

Removal of phenolic compounds by microorganisms is also affected by some external factors such as salt ions, pH, and temperature of the wastewater. Especially, high salt concentration is the most important factor which reduced microbial activity. Biodegradation of phenol in the presence of high salt concentration, particularly sodium chloride, has been reported. Lower rate of phenol degradation was observed at NaCl concentration of about 3% [12]. More than 99.5% phenol degradation was observed at 15% NaCl using halophilic bacteria in biofilm and sequencing batch reactors [13]. Halophilic bacteria have been used to remove phenol at concentrations of up to 320 mg L^{-1} in a medium containing 10% NaCl [14]. *Halomonas* sp. have been reported to degrade 100 mg L^{-1} phenol in model industrial wastewater containing NaCl (varying between 1% and 14%, *w*/*v*) and showed optimum phenol utilization and cell growth at about 5% (*w*/*v*) NaCl [15]. Although the effects of NaCl on biodegradation of phenol have been well documented, the presence of mixed phenolic compounds in saline industrial effluents has been ignored. As industrial wastewater contains complex mixtures of phenolic compounds and salts, it was particularly desirable that strains are able to degrade mixed phenolic compounds under saline conditions. As previously reported, we found that there have been little studies on the degradation of mixed phenolic compounds under high salt conditions.

The objectives of this present study are to isolate microorganisms possessing abilities to degrade mixed phenolic compounds effectively under complex salt conditions, to investigate the interactions of phenolic compounds during the biodegradation, and to research the impact of other salts and salinity fluctuations on the microbial growth and degradation process.

Materials and Methods

Isolation and Identification of Microorganisms

The microorganism used in this work was isolated from the sludge samples of a sewage plant in China. The sample was inoculated in 100 mL mineral salts medium (MSM) consisting of (g L^{-1}): 2.0 $(NH_4)_2SO_4$, 2.0 Na_2HPO_4, 1.3 KH_2PO_4, 0.01 $FeCl_3$, and 50 NaCl, pH 7.0. Mixed phenolic compounds (phenol, catechol, and salicylic acid) were supplemented as the carbon and energy source. After 2 months of cultivation, the culture was subjected to spreading on agar plates containing the same medium and phenolic mixtures. Strain W1 was obtained after purification by sequential cultures. It was identified by physiological–biochemical characteristics and 16S rRNA gene sequencing [16]. The 16S rRNA gene of strain W1 was deposited in the GenBank data library under accession no. EU339930.

Biodegradation of Phenolic Compounds

When the cells' concentration reached 0.35–0.4 (OD_{660}), an aliquot of the culture was centrifuged at 8,000 rpm and 4°C for 10 min. To clean the biomass, it was resuspended in the phosphate buffer and centrifuged. The cells were inoculated into 100 mL MSM with phenolic compounds as sole carbon and energy source. The growth of strain W1 on single substrate including aniline, nitrobenzene, salicylic acid, catechol, cresol, *o*-nitrophenol, 2,4-dichlorophenol, aminophenol, and phenol was tested. In binary substrate systems (phenol and *p*-cresol), the total substrate level was fixed at 200 or 500 mg L^{-1} in which the mass fraction of one substrate was varied. Experiments were carried out at pH 7.0 and 30°C in an orbital shaker at 150 rpm. In the process of batch culture, all samples were periodically taken for biomass and substrate concentrations.

Experimental Design to Study the Effects of Salts on Phenolic Compounds Biodegradation

Effects of salts on the biodegradation of mixed of phenolic compounds were determined by adding NaCl, KCl, Na_2SO_4, and K_2SO4 separately to the growth medium to obtain approximately the same concentration (0.85 M). These studies were carried out in the medium containing 100 mg L^{-1} phenol and 100 mg L^{-1} *p*-cresol. The amounts of salts added separately into the medium were (g L^{-1}): 50 NaCl; 63.37 KCl; 121.37 Na_2SO_4, and 148.11 K_2SO_4. After 36 h, the samples were withdrawn and the concentrations of cells, phenol, and *p*-cresol were measured as described below.

To investigate the salt-tolerant ability of *Arthrobacter* sp. W1, several cycles of degradation were designed. The cells were pre-incubated at MSM (1% NaCl) containing 100 mg L^{-1} phenol and 100 mg L^{-1} *p*-cresol. The first degradation cycle was conducted at 1% NaCl. After total substrates were completely degraded, the cells were washed and resuspended in the fresh MSM with mixed substrates and were directly re-incubated at 5% NaCl. The third degradation cycle was conducted at 10% NaCl after the second one completed. These alternating salinity cycles were repeated two times.

Analytical Methods

Cell density was monitored spectrophotometrically by measuring the absorbance at a wavelength of 660 nm with an UV–visible spectrophotometer (Jasco UV-550, Japan). Concentration of phenolic compounds in all cultures were periodically measured with a high-performance liquid chromatograph (System Gold, Beckman instruments, Fullerton, CA, USA) equipped with a Hypersil ODS column (4.6×250 mm, Agilent Technologies, CA, USA) using 40% water–60% methanol as the mobile phase. The flow rate was 1.0 ml min^{-1} and UV absorbance spectrum was obtained online at 254 nm. The retention time for phenol was 4.89 min and for *p*-cresol was 8.12 min.

Results and Discussion

Identification and Characterization of Strain W1

Strain W1 capable of growth on the mixed phenolic compounds was isolated under high salt conditions (5% NaCl). It was a Gram-positive, non-motile, rod-shaped aerobic

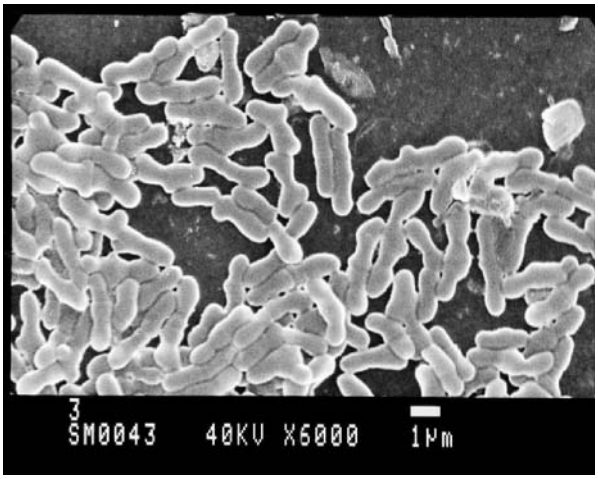

Fig. 1 Micromorphological structure of *Arthrobacter* sp. W1

bacterium. Colonies were smooth, convex, opaque, and wet which was slight yellow and circular with diameter 1–2 mm within 2–3 days. It was catalase-positive, oxidase-positive, and nitrogenase-negative. Figure 1 shows the scanning electron micrograph picture of strain *Arthrobacter* sp. W1.

In addition, a partial 16S rRNA sequence was obtained and sequence alignment revealed that strain W1 showed the highest similarity (99%) to *Arthrobacter nicotianae* (X80739). According to the physiological–biochemical characteristics and 16S rRNA gene sequence, the strain was identified as *Arthrobacter* sp. W1. It has been reported that *Arthrobacter* sp. can metabolize phenol up to 22 mM concentration [17], and *Arthrobacter chlorophenolicus* A6 [18] could degrade 4-chlorophenol at low temperature. However, there was no report on the utilization of *Arthrobacter* sp. in the treatment of mixed phenolic compounds under saline conditions. Figure 2 shows the phylogenetic relationship between different members of the genus *Arthrobacter* and isolated W1.

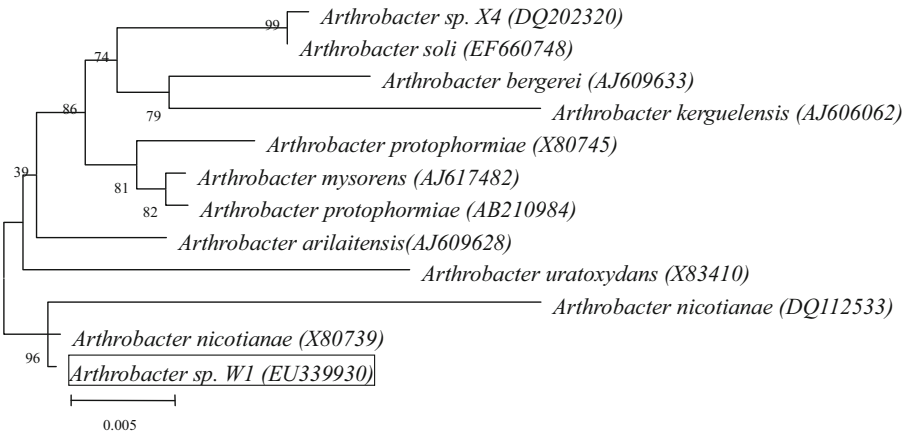

Fig. 2 Phylogenetic tree based on 16S rRNA sequences of strain W1 and related *Arthrobactrer* species. *Numbers at the nodes* indicate the percentages of bootstrap samplings

From Table 1, it was obvious that *Arthrobacter* sp. W1 was able to use various phenolic compounds as sole carbon and energy source. Strain W1 was able to utilize salicylic acid, catechol, *o*-cresol, *p*-cresol, and phenol at different concentrations. The growth of strain W1 was inhibited by the *o*-aminophenol and *p*-aminophenol at the concentration higher than 200 mg L^{-1}. It was also shown that strain W1 could not use the aniline, nitrobenzene, *o*-nitrophenol, and so on. It could be explained that the nitro group is electron-attracting group, which could cause decrease of benzene ring electron density and hamper the oxidase attacking. Therefore, the aromatic compounds with nitro group were more difficult to biodegrade under aerobic conditions. Although several researchers demonstrated that the *Arthrobacter* sp. was able to degrade variety of phenolic compounds [19], there are no reports on the degradation in saline solutions. In our study, it was investigated that strain W1 could tolerate high salt concentrations. Therefore, we tried to prove the ability of strain W1 for phenolic compounds removal under saline conditions.

Biodegradation of Phenol and *p*-Cresol Mixture Under Different Salt Conditions

The ability of *Arthrobacter* sp. W1 to degrade the mixture of phenol (200 mg L^{-1}) and *p*-cresol (100 mg L^{-1}) at different salt concentration (0%, 5%, and 10% NaCl) was investigated. As shown in Fig. 3a, the mixture was completely degraded by strain W1 within 32 h without salt addition. When the culture was incubated at 5% NaCl, phenol and *p*-cresol mixture was degraded within 36 h (Fig. 3b). It was indicated that there were almost no effects on the microbial growth and biodegradation process with salinity less than 5%. At the same time, it was observed that complete degradation of the same mixtures at 10% NaCl was within 60 h and the lag phase increased to 20 h (Fig. 3c). It was also exhibited that the cell growth rate and final concentration at 5% NaCl were higher than that at 10% NaCl. Although more than 5% NaCl would inhibit the strain growth rate and degradation efficiency, the strain still maintained activity under high salt conditions (i.e., 10%). Thus, it was concluded that *Arthrobacter* sp. W1 could degrade mixed phenolic compounds under high salt conditions. Furthermore, many studies showed that phenol and *p*-cresol were metabolized by the β-ketoadipate pathway through ortho-fission of catechol [20, 21]. The activities of the first two enzymes involved in the degradation of phenolic compounds were

Table 1 Effects of different carbon sources on the growth of *Arthrobacter* sp. W1.

Substrates	Concentration (mg L^{-1})				
	50	100	150	200	300
Phenol	+	+	+	+	+
Aniline	−	−	−	−	−
Nitrobenzene	−	−	−	−	−
Salicylic acid	+	+	+	+	+
Catechol	+	+	+	+	+
o-Cresol	+	+	+	+	+
p-Cresol	+	+	+	+	+
o-Nitrophenol	−	−	−	−	−
2,4-Dichlorophenol	−	−	−	−	−
o-Aminophenol	+	+	+	−	−
p-Aminophenol	+	+	+	−	−

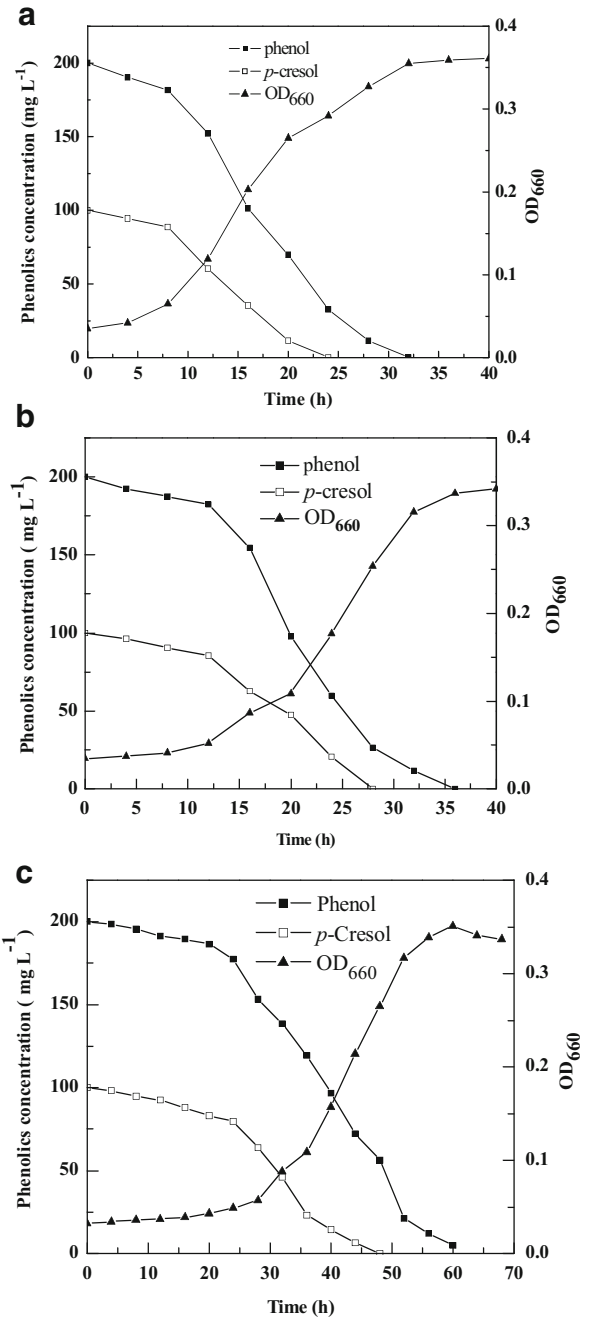

Fig. 3 Biodegradation of phenol and *p*-cresol mixture and cell growth for *Arthrobacter* sp. W1 incubated at 0% NaCl (**a**), 5% NaCl (**b**), and 10% NaCl (**c**)

investigated in our study. The results showed that the phenol hydroxylase and catechol 1,2-dioxygenase were produced during phenol and *p*-cresol mixtures degradation process (data not shown). Thus, it was concluded that phenol and *p*-cresol in the mixture were metabolized by the ortho-cleavage pathway.

Biodegradation of Phenol and *p*-Cresol Mixture with Different Concentrations

Time course of the growth and degradation at the initial phenolics concentration of 200 mg L^{-1} are shown in Fig. 4. When phenol or *p*-cresol was the sole carbon source, it was found that the microorganism could grow better with phenol than with *p*-cresol. When phenol and *p*-cresol coexisted, the phenol degradation rate was decreased. The inhibition of *p*-cresol on phenol degradation was enhanced with the increase of initial *p*-cresol concentration. In the absence of *p*-cresol, 200 mg L^{-1} phenol was completely removed within 36 h. When the mass fraction of *p*-cresol was increased from 0.25 to 1.0, the time required for complete degradation of the mixture was prolonged from 40 to 52 h (Fig. 4a). It was also clear that the growth rates decreased with the increased *p*-cresol concentration (Fig. 4b). It was postulated that increasing the concentration of *p*-cresol would enhanced the substrate inhibition on cell growth. This is consistent with the results found by Paraskevi and Polymenakou [22] in the co-metabolic transformation of phenol and *o*-cresol. It has been demonstrated that the addition of *o*-cresol strongly inhibited phenol transformation in respect to the strong competitive inhibition between the substrates.

When the total substrate (S_T) concentration was at 500 mg L^{-1}, the lag period was increased from 20 to 40 h, with the mass fraction of *p*-cresol increased from 0 to 1.0. The

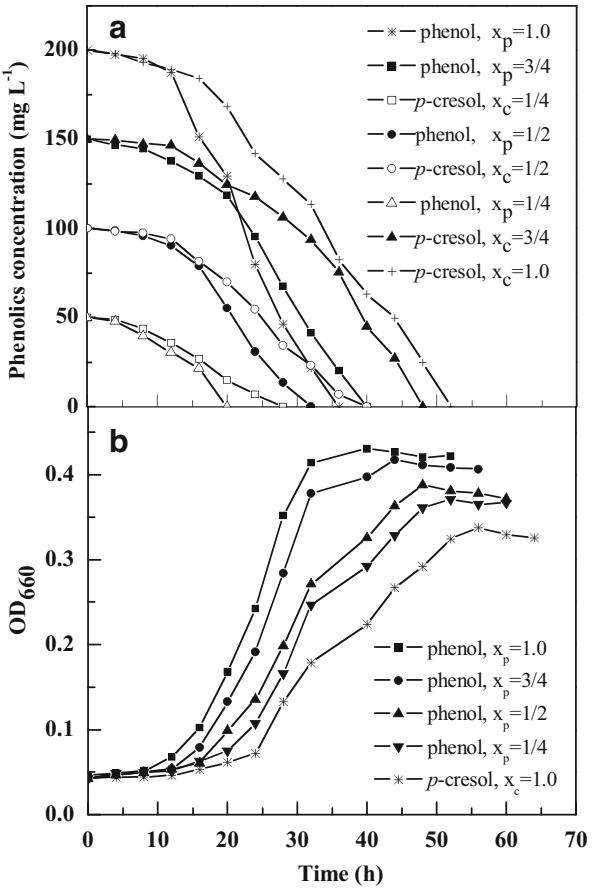

Fig. 4 Typical profiles of cell growth and biodegradation of phenol and *p*-cresol mixture (S_T=200 mg L^{-1}, 1.0 OD_{660}= 0.98 g L^{-1})

time for complete degradation of the phenolic mixtures was increased from 60 to 88 h, with the increase of the mass fraction of *p*-cresol (Fig. 5a). During the same periods, the maximum cell density (OD_{660}) was decreased from 0.531 to 0.342 (Fig. 5b). These results indicated that the presence of a small amount of *p*-cresol retarded cell growth and phenol and *p*-cresol mixture degradation. It was postulated that the presence of *p*-cresol might be inhibited and inactivated phenol metabolic enzymes. It is consistent with the reports of Ely et al. [23, 24] that during the co-metabolic process, the effects of a toxic substrate on bacterial activity are usually associated with the enzyme inhibition and inactivation. The inactivation phenomena of enzyme become obvious with the increase of *p*-cresol concentration. As a result, it takes a long time for the enzyme to recover activities at high initial *p*-cresol concentration.

Effects of Different Salts on Biodegradation of Phenol–Cresol Mixture

As the industrial wastewater contains many salts at high concentrations, therefore, it is essential to study biodegradation of mixed phenolic compounds in the presence of different salts. As shown in Table 2, *Arthrobacter* sp. W1 was able to degrade phenolic compounds in the presence of all selected salts. Mixture of 100 mg L^{-1} phenol and 100 mg L^{-1} *p*-cresol

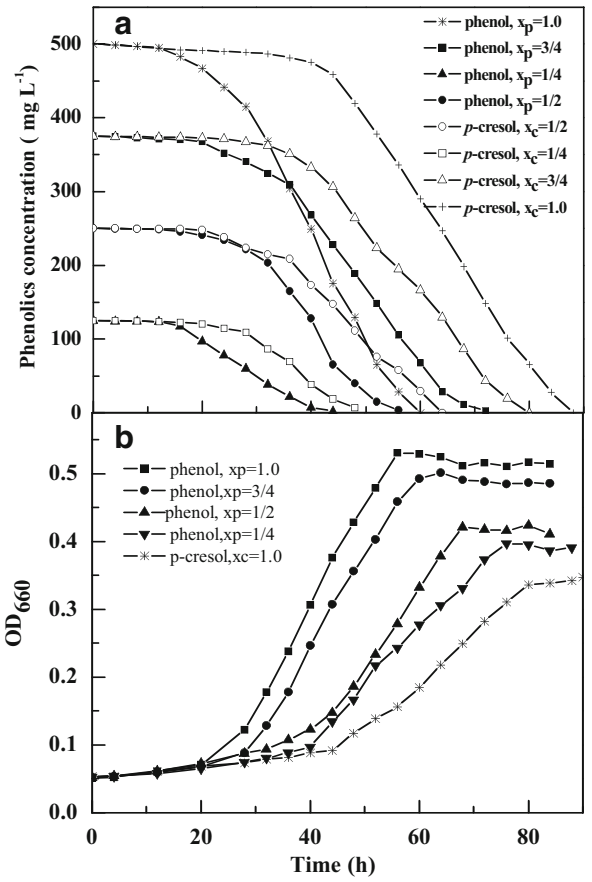

Fig. 5 Typical profiles of cell growth and biodegradation of phenol and *p*-cresol mixture (S_T=500 mg L^{-1}, 1.0 OD_{660}= 0.98 g L^{-1})

Table 2 Effects of salts on the growth and biodegradation of mixtures of phenol and *p*-cresol.

Salt (g L^{-1})	Cell concentration (OD_{660})	Removal efficiency of phenol (%)	Removal efficiency of *p*-cresol (%)
NaCl (50)	0.314	100	93.5
KCl (63.37)	0.277	77.5	63.8
Na_2SO_4 (121.37)	0.309	90.1	84.4
K_2SO_4 (148.11)	0.264	76.1	59.2

was completely degraded within 36 h in the presence of NaCl. Nearly 90% of phenol and 84.4% of *p*-cresol was removed simultaneously by strain W1 in the presence of Na_2SO_4. Similar observations were recorded with added KCl and K_2SO_4. It was shown that while growing in the presence of NaCl and Na_2SO_4, strain W1 had higher efficiency and growth rate, as compared to the rates in the presence of KCl and K_2SO_4. From the results above, it indicated that *Arthrobacter* sp. W1 can be very efficiently used for treating phenolic wastewater containing high concentrations of cations/anions such as Na^+, K^+, Cl^-, and SO_4^{2-}.

Biodegradation of Phenol and *p*-Cresol Mixture Subjected to Salinity Fluctuations

As for bioremediation, the candidate strain should possess not only the high removal efficiency for the target compounds but also the strong abilities of adapting some conditions such as shock loading, temperature impact and salinity fluctuations, and so on. Here, it was shown that *Arthrobacter* sp. W1 was able to degrade phenol and *p*-cresol mixture during salinity fluctuations between 1% and 10% NaCl (Fig. 6). It was clear that there was no obvious difference of degradation rate with the NaCl concentration changing from 1% to 5%. However, there was obvious lag phase for degradation when the cells transferred from 5% to 10% NaCl solutions, and it was concluded that degradation rates were faster at 1% or 5% NaCl than that at 10% NaCl. Salinity fluctuation had little negative impact on the ability of the cells to degrade substrates. Moreover, *Arthrobacter* sp. W1 was able to grow and remain active and continued to degrade phenolic compounds efficiently for batch experiments. It was concluded that strain W1 has an inherent flexibility to adapt to salinity

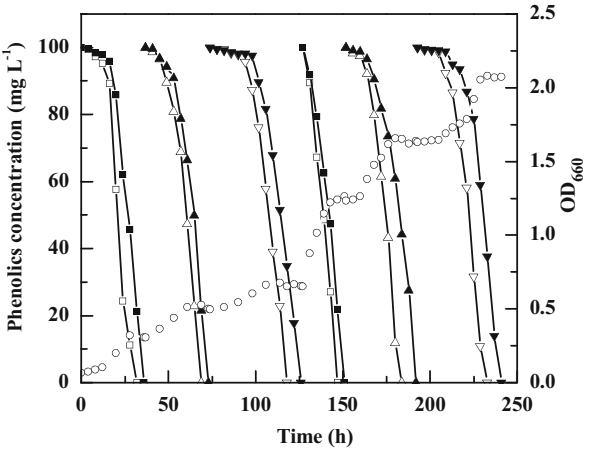

Fig. 6 Degradation of phenol (*open symbols*) and *p*-cresol (*filled symbols*) mixture and cell concentration (*circle*) at different salt concentrations of 1% NaCl (*squares*), 5% (*triangles*), and 10% NaCl (*inverted triangles*)

fluctuations. Also, the cell density was increased throughout the operational process. Therefore, *Arthrobacter* sp. W1 is a promising candidate for bioremediation of phenolic wastewater under complex salt conditions.

Conclusions

In this study, *Arthrobacter* sp. W1 was systematically characterized for biodegradation of phenolic compounds under complex salt conditions. It was shown that *Arthrobacter* sp. W1 possessed the high ability to degrade mixtures of phenol and *p*-cresol under high salt conditions and salinity fluctuations. The mixture of 200 mg L^{-1} phenol and 100 mg L^{-1} *p*-cresol was degraded by strain W1 within 60 h at 10% NaCl. The presence of small amount of *p*-cresol retarded phenol degradation. *Arthrobacter* sp. W1 is efficient in degrading mixture of phenol and *p*-cresol during salinity fluctuations between 1% and 10% NaCl. The presence of other salts had almost no effect on the microbial growth and biodegradation. The results also indicate that the strain can be very fit for treating phenolic wastewater containing high concentrations of cations/anions such as Na^+, K^+, Cl^-, and SO_4^{2-}. In a word, this paper could be helpful for the design of batch and/or continuous bioreactors for the treatment of mixed phenolic wastewater under complex salt conditions.

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References

1. Arutchelvan, V., Kanakasabai, V., Elangovan, R., Nagarajan, S., & Muralikrishnan, V. (2006). *Journal of Hazardous Materials*, *B129*, 216–222. doi:10.1016/j.jhazmat.2005.08.040.
2. Keith, L. H., & Telliard, W. A. (1979). *Environmental Science & Technology*, *13*, 416–423. doi:10.1021/es60152a601.
3. Evans, W. C. (1947). *The Biochemical Journal*, *41*(3), 373–382.
4. Hill, G. A., & Robinson, C. W. (1975). *Biotechnology and Bioengineering*, *17*, 1599–1615. doi:10.1002/bit.260171105.
5. Westimeier, F., & Rehm, H. J. (1985). *Applied Microbiology and Biotechnology*, *22*, 301–305. doi:10.1007/BF00582412.
6. Jiang, Y., Wen, J., Bai, J., Jia, X. Q., & Hu, Z. D. (2007). *Journal of Hazardous Materials*, *147*, 672–676. doi:10.1016/j.jhazmat.2007.05.031.
7. Pawlowsky, U., & Howell, J. A. (1973). *Biotechnology and Bioengineering*, *25*, 889–896. doi:10.1002/bit.260150506.
8. Ehrhardt, H. M., & Rehm, H. J. (1985). *Applied Microbiology and Biotechnology*, *21*, 32–36. doi:10.1007/BF00252358.
9. Annadurai, G., Juang, R. S., & Lee, D. J. (2002). *Waste Management (New York, N.Y.)*, *22*, 703–710. doi:10.1016/S0956-053X(02)00050-8.
10. Abuhamed, T., Bayraktar, E., Mehmetoglu, T., & Mehmetoglu, U. (2004). *Process Biochemistry*, *39*, 983–988. doi:10.1016/S0032-9592(03)00210-3.
11. Lovanh, N., & Alvarez, P. J. J. (2004). *Biotechnology and Bioengineering*, *86*, 801–808. doi:10.1002/bit.20090.
12. Kargi, F., & Uygur, A. (1996). *Environmental Technology Journal*, *17*, 325–330. doi:10.1080/09593331708616391.
13. Woolard, C. R., & Irvine, R. L. (1995). *Water Research*, *29*, 1159–1168. doi:10.1016/0043-1354(94)00239-4.
14. Peyton, B. M., Wilson, T., & Yonge, D. R. (2002). *Water Research*, *36*, 4811–4820. doi:10.1016/S0043-1354(02)00200-2.

15. Hinteregger, C., & Streichsbier, F. (1997). *Biotechnology Letters*, *19*, 1099–1102. doi:10.1023/A:1018488410102.
16. Neilan, B. A. (1995). *Applied and Environmental Microbiology*, *61*, 2286–2291.
17. Karigar, C., Mahesh, A., Nagenahalli, M., & Yun, D. J. (2006). *Biodegradation*, *17*, 47–55. doi:10.1007/s10532-005-3048-y.
18. Backman, A., & Jansson, J. K. (2004). *Microbial Ecology*, *48*(2), 246–253. doi:10.1007/s00248-003-2026-3.
19. Unell, M., Nordin, K., Jernberg, C., Stenstrom, J., & Jansson, J. K. (2008). *Biodegradation*, *19*, 495–505. doi:10.1007/s10532-007-9154-2.
20. Kolomytseva, M. P., Baskunov, B. P., & Golovleva, L. A. (2007). *Biotechnology Journal*, *2*(7), 886–893. doi:10.1002/biot.200700013.
21. Santos, V. L., Heilbuth, N. M., Braga, D. T., & Linardi, V. R. (2003). *Journal of Basic Microbiology*, *43*(3), 238–248. doi:10.1002/jobm.200390027.
22. Polymenakou, P. N., & Stephanou, E. G (2005). *Biodegradation*, *16*, 403–413. doi:10.1007/s10532-004-3333-1.
23. Ely, R. L., Williamson, K. J., Guenther, R. B., Hyman, M. R., & Arp, D. J. (1995). *Biotechnology and Bioengineering*, *46*, 218–231. doi:10.1002/bit.260460305.
24. Ely, R. L., Hyman, M. R., Arp, D. J., Guenther, R. B., & Willianson, K. J. (1995). *Biotechnology and Bioengineering*, *46*, 232–245. doi:10.1002/bit.260460306.