Enhanced removal of triclosan from contaminated water by indigenous isolate *Burkholderia* sp. L303

Lan Qiu[1], Xiaoyuan Guo[1], and Hojae Shim[1,2]

[1] Department of Civil and Environmental Engineering, Faculty of Science and Technology, University of Macau, Macau SAR 999078, China.

[2] Corresponding author: hjshim@um.edu.mo

Abstract. Triclosan (TCS), one of the most widely used antimicrobial agents, is frequently detected at wastewater treatment plants and environmental matrices including soil, water, sediment, and biota samples. In this study, a TCS-degrading bacterium was isolated from local activated sludge and identified as *Burkholderia* sp. L303. Strain L303 could degrade TCS (0.5-8 mg/L) as sole carbon source. The optimal condition was 35℃ and pH 7. The *in-vitro* assay with the glucose-enriched cells showed the ability of TCS degradation in real water samples, indicating the functional enzyme expressed in the absence of TCS. The bioaugmentation of strain L303 in non-sterile wastewater showed better degradation rate than that in the control groups. The community profiles showed the potential cooperative interactions between strain L303 and indigenous bacteria, thereby enhancing the TCS degradation in the real polluted water. The finding of this study could facilitate in developing appropriate bioaugmentation strategy by using live bacteria or active enzyme and in designing beneficial community interactions within native and external species for treating TCS-laden waters.

1. Introduction

With the rapid development of industrialization and the corona virus pandemic in recent years, the demand for the antibacterial and antiviral products has further expanded. Triclosan (5-chloro-2-(2,4-dichlorophenoxy)-phenol, TCS) is a widely used antimicrobial agent and is added in many consumer products like textiles, plastics, deodorants, soaps, and toothpastes [1-3]. Accordingly, TCS can be released into the ecosystem and lead to wide distribution and pollution in various environmental matrices like ground water, wastewater, sediment, river, and sea, as well as the biotic samples like fish, birds, and human serum [4-9]. TCS has been reported to show weak androgenic activity against aquatic organisms and revealed estrogenic responses in human breast cancer cells [10, 11]. In addition, potential endocrine disruption and antibiotic cross-resistance was also reported in human bodies and in the ecological system [12]. TCS might also be transformed into chlorodioxins, dibenzofurans, and even dioxins which show higher toxicities than the parent compound [13-15].

Biodegradation of TCS has been found in the environment, which plays an important role for its natural attenuation. Until now, a total of eight bacteria have been reported to degrade TCS aerobically [13, 16-21], while only *Dyella* sp. WW1 and *Sphingomonas* sp. YL-JM2C could degrade TCS metabolically [20, 21]. To better understand the metabolic degradation mechanism for TCS in the environment and to know more about these functional TCS degraders, more works are warranted.

2. Materials and methods

2.1. Cultivation, isolation, and cell counting

Triclosan (2,4,4'-Trichloro-2'-hydroxydiphenyl ether, TCS, 97% purity) and other chemicals and solvents used were of pure analytical-grade or highest grade available. The mineral salt medium (MSM) and trace elements were used as previously reported [22, 23]. The stock solution of TCS was prepared at 10 g/L with methanol used as solvent and stored at -20°C. The activated sludge was used as the source for isolation and TCS (5 mg/L) was added as the sole carbon source. Pure culture showing the TCS degradation from the streak plate, designated L303, was selected for further study. The cell growth of strain L303 was counted with defined serial dilution on the nutrient agar plate. Experiments were conducted in replicates for cell counting.

2.2. Experimental setup

Strain L303 was cultured in MSM containing 5 mg/L TCS with methanol evaporated in advance. Different concentrations of TCS (0.5, 2, 5, 8, and 10 mg/L) and different levels of pH (5, 6, 7, 8, and 9) and temperatures (20, 25, 30, 35, and 40°C) were set to investigate their effects on TCS degradation. *In vitro* assays with the crude cell lysates of strain L303 were conducted as described previously [24]. The crude cell lysates together with TCS-laden waters were mixed intermediately for the aerobic reaction before analyses. The *in vivo* test of the bioaugmentation with strain L303 was conducted in polluted river water. The initial inoculum size was set at 1% (v/v) for the initial cell density of 2×10^5 CFUs (colony forming units)/mL. The residual TCS was analyzed, and water samples were collected for the community analysis.

2.3. Analytical methods

TCS was analyzed using a high-performance liquid chromatography (HPLC; ThermoScientific) equipped with a diode array detector and a XBridge BEH C18 column (5 µm, 4.6 x150 mm, Waters, U.S.A.). The community genomic DNA (gDNA) extraction was performed using FastDNA Spin Kit for Soil (MP Biomedicals, Carlsbad, CA, U.S.A.) according to the manufacturer' s instructions. The U515F/U909R primer set was used to amplify the V4-V5 region of 16S rRNA genes. The Illumina Miseq sequencing (Illumina, San Diego, CA, U.S.A.) service was provided by Magigene (Shenzhen, China). The provided pair-end (2×250 nd) demultiplexed sequences were filtered, denoised, and assembled into amplicon sequence variants (ASVs) with Dada2 package (version 1.16) [25]. Taxonomy was assigned by blasting the representative non-chimeric sequence with SILVA v132 database [26]. All statistical analyses were performed with the statistical software R (version 4.0.1).

3. Results and discussion

3.1. Isolation and identification of TCS degrader

Activated sludge sample from a regional wastewater treatment plant was serially cultivated in MSM containing TCS (at the increased concentrations) as the sole carbon source. After several transfer and dilution, a relatively homogeneous collection of colonies was seen on plate. One isolate, designated as strain L303, was selected for further study. Strain L303 was gram-negative, short rod-shaped, and motile with a polar flagellum based on its morphological characteristics [27]. Its colonies were light green, circular, and convex with a diameter of about 1 mm after 48 h incubation on nutrient agar plate. The 16S rRNA sequence of strain L303 exhibited 99.73% sequence similarity to *Burkholderia cenocepacia* VC12802 (CP019670.1) and *B. cenocepacia* MSMB384WGS (CP013450.1), and above 99.5% sequence similarity to other *Burkholderia* species. Therefore, it was preliminarily identified as *Burkholderia* sp. L303.

3.2. Effect of culture condition on TCS degradation

Strain L303 could degrade TCS at a certain range of concentration. As shown in **Figure 1a**, TCS at 5 mg/L or less could easily be degraded by this strain within 5 days, while higher concentration (8 mg/L) showed a long lag phase (about 6 days) followed by quick degradation in 3 days. TCS at 10 mg/L

showed severe inhibition and toxicity to the strain and no TCS degradation was observed. On the other hand, neutral pH of 7 showed the highest degradation compared to other pH values and more than 95% of TCS were degraded within 4 days. The more acidic or alkaline condition (i.e., pH of 5 or 9) slowed the degradation and lengthened the lag phase for the initial TCS degradation (**Fig. 1b**). TCS degradation efficiency increased significantly while temperature increased from 20 to 35°C (**Fig. 1c**). The best condition for the TCS degradation was at 35°C. However, further increase 40°C suddenly caused a significant decline in TCS degradation efficiency. Compared to the reported optimal temperatures for other known TCS degradation, 30°C for *Sphingomonas* sp. YL-JM2C and 15°C for *Dyella* sp. WW1 [20, 21], strain L303 might involve novel functional enzyme accounting for the TCS degradation, which needs further experimental evidence.

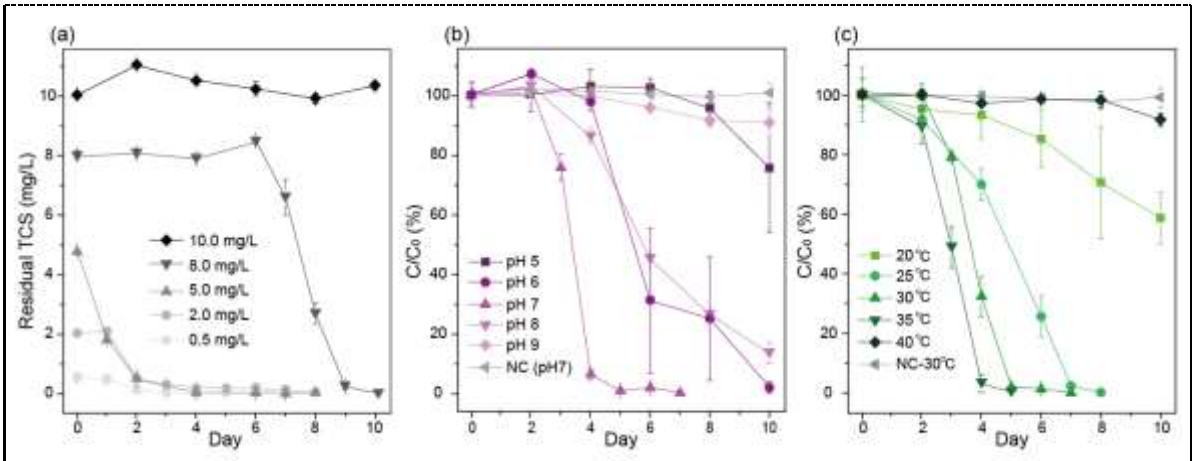

Figure 1. Degradation of TCS by strain L303 at different initial concentrations (a); pH levels (b); and incubation temperatures (c).

3.3. Application of strain L303 in TCS-laden wastewater

Bioaugmentation of the isolate was performed using real water samples. Strain L303 was first cultivated in MSM containing glucose as the sole carbon source, without exposure to TCS during the cell growth. Then, the isolate was inoculated into deionized (DI) water and regional wastewater containing TCS (5 mg/L), and the results showed 98.14% and 94.02% TCS removed in the DI and river water respectively within 24 h, compared to the negative control (NC) without the isolate (**Fig. 2a**). The difference between the two waters might be caused by such unknown factors in wastewater as heavy metals [28] and/or co-existing unknown organic pollutants [29]. In addition, considering strain L303 was not exposed to TCS during cultivation, the functional enzyme for TCS degradation might have been pre-expressed when cultivated in the glucose added culture. Bifunctional or multifunctional enzyme for different kinds of organic compounds was also reported in other bacteria [24, 30, 31].

When strain L303 was inoculated into the river water with TCS added, TCS was gradually decreased at the degradation rate of 0.069 mg/L TCS·h (inoculated with strain L303) and 0.025 mg/L TCS·h (without inoculum), respectively (**Fig. 2b**). Interestingly, the non-sterile water without the inoculum also showed 35.33% TCS removal efficiency in three days and up to 99% removal within five days, suggesting the contribution from the indigenous microbes.

When the microbial community involved in the TCS degradation was monitored (**Fig. 3**), a total of fifteen genera including previously reported TCS-degrading genera (i.e., *Citrobacter*, *Mycobacterium*, *Rhodococcus*, and *Parashingopyxis*) were observed [13, 19, 32]. The relative abundance of *Burkholderia* in the inoculated group increased from 0.79% to 3.55% in three days, while its

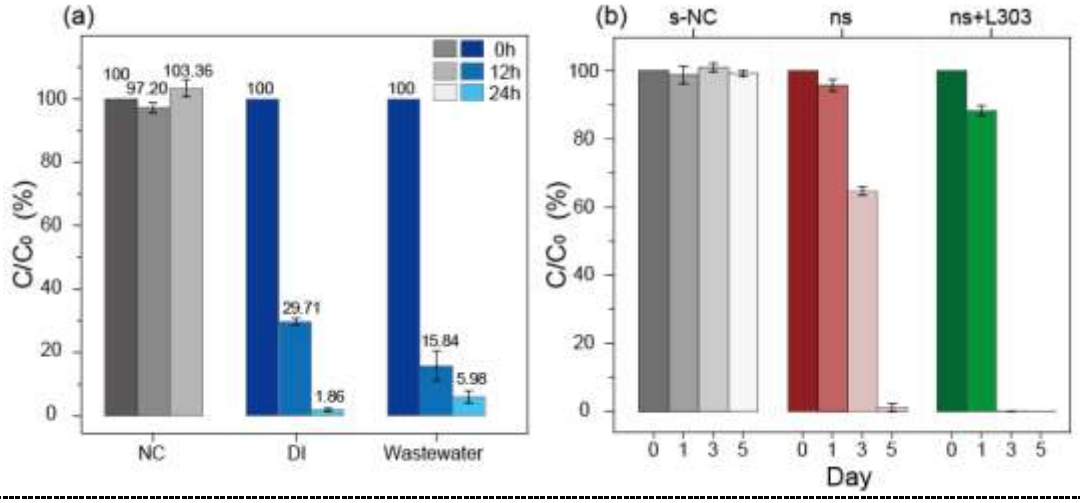

Figure 2. Bioaugmentation of strain L303 in TCS-laden water. NC, negative control; s, sterile; ns, non-sterile.

abundance unchanged in the without inoculum group, further indicating the contribution from the inoculated *Burkholderia*. Other genera (*Rheinheimera*, *Acidovorax*, and *Pseudomonas*) and the unknown genus from the family of *Conmamonadaceae* were also observed with increase in two groups, while it was difficult to identify which genera contributed to the TCS degradation in the uninoculated group. On the other hand, the relative abundance of *Flavobacterium* and *Sphingomonas* in the inoculated groups showed a significant increase compared to the uninoculated, which might account for the TCS degradation, in agreement with some previous reports [16, 18]. In comparison, *Acinetobacter* which occupied more than 50% of relative abundance showed the most decrease in both groups probably due to the TCS toxicity. The presence of TCS would show toxicity to some specific bacteria and greatly change the community composition of the water bodies. The potential interactions might exist between the inoculated strain L303 and the indigenous bacteria for more efficient TCS degradation, which needs further experimental evidences.

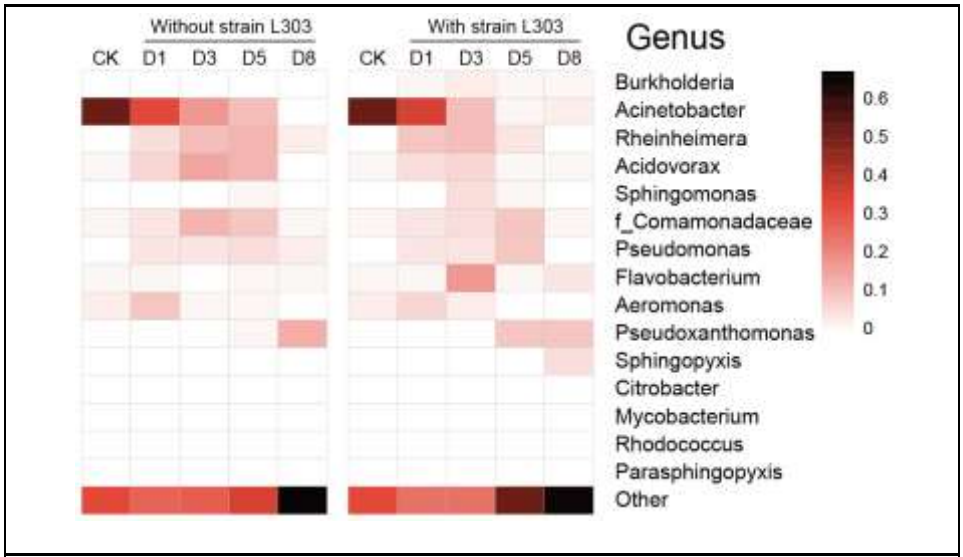

Figure 3. Microbial community profiles in the two experimental groups inoculated with strain L303 and without the inoculum. CK, the raw river water before use.

4. Conclusion

A novel TCS degrading bacterium designated as *Burkholderia* sp. L303 was isolated. This isolate could metabolically degrade TCS up to 8 mg/L and efficiently degraded 5 mg/L TCS within three days. The optimal condition for the TCS degradation was at 35℃ and pH 7. The TCS degrading functional enzyme might have been pre-expressed in the glucose-enriched culture and the strain L303 showed TCS degrading ability, suggesting the potential of developing enzyme for remediation of high TCS concentrations. TCS pollution in the water bodies would pose toxicity to some species and greatly change the microbial community, and bioaugmentation with the strain L303 could positively cooperate with the indigenous bacteria to enhance the remediation of the TCS-laden waters.

Acknowledgement

This work was supported by the grant from the Science and Technology Development Fund of Macao (FDCT0034/2021/AFJ).

References

[1] H. Singer, S. Müller, C. Tixier, L. Pillonel, Environ. Sci. Technol. **36**, 4998-5004 (2002)

[2] D. Sabaliunas, S.F. Webb, A. Hauk, M. Jacob, W.S. Eckhoff, Water Res. **37**(13), 3145-3154 (2003)

[3] J.V. Rodricks, J.A. Swenberg, J.F. Borzelleca, R.R. Maronpot, A.M. Shipp, Crit. Rev. Toxicol. **40**, 422-484 (2010)

[4] I.B. Lolas, X. Chen, K. Bester, J.L. Nielsen, Microbiology **158**, 2796-2804 (2012)

[5] Q. Sun, M. Lv, A. Hu, X. Yang, C.P. Yu, J. Hazard Mater. **277**, 69-75 (2014)

[6] M. Lv, Q. Sun, A. Hu, L. Hou, J. Li, X. Cai, C.P. Yu, J. Hazard Mater. **280**, 696-705 (2014)

[7] B.R. Ramaswamy, G. Shanmugam, G. Velu, B. Rengarajan, D.G.J. Larsson, J. Hazard. Mater. **186**, 1586-1593 (2011)

[8] L. Carranza-Diaz, M. Schultze-Nobre, J. Moeder, P. Nivala, H. Kuschk, H. Koeser, Ecol. Eng. **71**, 234-245 (2014)

[9] A.B. Dann, A. Hontela, J. Appl. Toxicol. **31**, 285-311 (2011)

[10] R.H. Gee, A. Charles, N. Taylor, P.D. Darbre, J. Appl. Toxicol. **28**, 78-91 (2008)

[11] C.M. Foran, E.R. Bennett, W.H. Benson, Mar. Environ. Res. **50**, 153-156 (2000)

[12] S.P. Yazdankhah, A.A. Scheie, E.A. Høiby, B.T. Lunestad, E. Heir, T.Ø. Fotland, Microb. Drug. Resist. **12**, 83-90 (2006)

[13] D.G. Lee, F. Zhao, Y.H. Rezenom, D.H. Russell, K.H. Chu, Water Res. **46**(13), 4226-4234 (2012)

[14] C. Liyanapatirana, S.R. Gwaltney, K. Xia, Environ. Sci. Technol. **44**, 668-674 (2010)

[15] T. Methatham, M.C. Lu, C. Ratanatamskul, Desalin. Water Treat. **52**, 920-928 (2013)

[16] A.G. Hay, P.M. Dees, G.S. Sayler, FEMS Microbiol Ecol. **36**(2-3),105-112 (2001)

[17] H. Roh, N. Subramanya, F. Zhao, C.P. Yu, J. Sandt, K.H. Chu, Chemosphere **77**(8), 1084-1089 (2009)

[18] Y.M. Kim, K. Murugesan, S. Schmidt, V. Bokare, J.R. Jeon, E.J. Kim, Y.S. Chang, Bioresour. Technol. **102**(3), 2206-2212 (2011)

[19] D.G. Lee, K.H. Chu, Chemosphere **93**(9), 1904-1911 (2013)

[20] S.I. Mulla, A. Hu, Y. Wang, Q. Sun, S.L. Huang, H. Wang, C.P. Yu, Chemosphere **144**, 292-296 (2016)

[21] S. Wang, Y. Yin, J. Wang, Appl. Microbiol. Biotechnol. **102**(4), 1997-2006 (2018)

[22] P. Fortnagel, H. Harms, R.M. Wittich, S. Krohn, H. Meyer, V. Sinnwell, H. Wilkes, W. Francke, Appl. Environ. Microbiol. **56**, 1148-1156 (1990)

[23] Y.M. Kim, I.H. Nam, K. Murugesan, S. Schmidt, D.E. Crowley, Y.S. Chang, Appl. Microbiol. Biotechnol. **77**(1), 187-194 (2007)

[24] L. Yu, Q. Lu, L. Qiu, G. Xu, Y. Zeng, X. Luo, S. Wang, B. Mai, Appl. Environ. Microbiol. **84**, e01300-18 (2018)

[25] B.J. Callahan, P.J. McMurdie, M.J. Rosen, A.W. Han, A.J.A. Johnson, S.P. Holmes, Nat. Methods **13**, 581-583 (2016)
[26] Q. Wang, G.M. Garrity, J.M. Tiedje, J.R. Cole, Appl. Environ. Microbiol. **73**, 5261-5267 (2007)
[27] E. Yabuuchi, Y. Kosako, H. Oyaizu, I. Yano, H. Hotta, Y. Hashimoto, T. Ezaki, M. Arakawa, Microbiol. Immunol. **36**(12), 1251-75 (1992)
[28] L. Qiu, K.S. Lok, Q. Lu, H. Zhong, X. Guo, H. Shim, Environ. Technol. **4**, 1-12 (2022)
[29] G. Xu, X. Zhao, S. Zhao, C. Chen, M. J. Rogers, R. Ramaswamy, J. He, Environ. Sci. Technol. **55**, 4205-4226 (2021)
[30] L. Qiu, W. Fang, H. He, Z. Liang, Y. Zhan, Q. Lu, D. Liang, Z. He, B. Mai, S. Wang, Environ. Sci. Technol. **54**, 8791-8800 (2020)
[31] R. Shen, S. Zhang, Z. Liang, B.Mai, S. Wang, Water Res. **220**, 118725 (2022)
[32] R. Kumari, S. Ghosh Sachan, A. Sachan, Int. J. Environ. Sci. Technol. **19**(5), 3565-3580 (2021)