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Isolation and characterization of pure cultures for metabolizing 1,4-dioxane in oligotrophic environments

Ermias Gebrekrstos Tesfamariam [a], Dennis Ssekimpi [a], Sarajeen Saima Hoque [a], Huan Chen [b], Joshua D. Howe [c], Chao Zhou[d], Yue-xiao Shen[e] and Youneng Tang [a,*]

[a] Department of Civil and Environmental Engineering, FAMU-FSU College of Engineering, Florida State University, Tallahassee, Florida 32310, USA
[b] National High Magnetic Field Laboratory, Tallahassee, Florida 32310, USA
[c] Department of Chemical Engineering, Texas Tech University, Lubbock, Texas 79409, USA
[d] Geosyntec Consultants Inc., Costa Mesa, California 92626, USA
[e] Department of Civil, Environmental, and Construction Engineering, Texas Tech University, Lubbock, Texas 79409, USA
*Corresponding author. E-mail: ytang@eng.famu.fsu.edu

EGT, 0000-0002-5438-1385; DS, 0009-0009-7465-5839; SSH, 0000-0002-5775-2831; HC, 0000-0002-6032-6569; JDH, 0000-0002-6628-8532; YT, 0000-0001-8494-2908

ABSTRACT

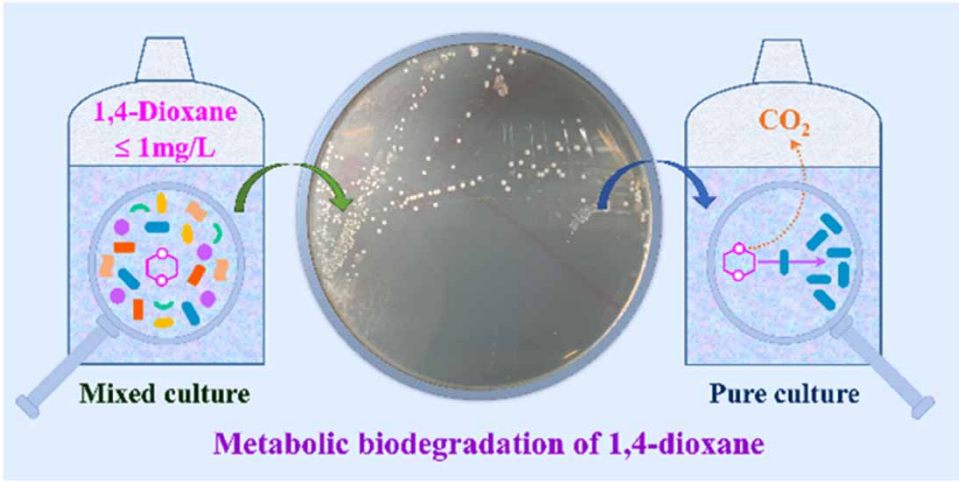
1,4-Dioxane concentration in most contaminated water is much less than 1 mg/L, which cannot sustain the growth of most reported 1,4-dioxane-metabolizing pure cultures. These pure cultures were isolated following enrichment of mixed cultures at high concentrations (20 to 1,000 mg/L). This study is based on a different strategy: 1,4-dioxane-metabolizing mixed cultures were enriched by periodically spiking 1,4-dioxane at low concentrations ($\leq$1 mg/L). Five 1,4-dioxane-metabolizing pure strains LCD6B, LCD6D, WC10G, WCD6H, and WD4H were isolated and characterized. The partial 16S rRNA gene sequencing showed that the five bacterial strains were related to *Dokdonella* sp. (98.3%), *Acinetobacter* sp. (99.0%), *Afipia* sp. (99.2%), *Nitrobacter* sp. (97.9%), and *Pseudonocardia* sp. (99.4%), respectively. *Nitrobacter* sp. WCD6H is the first reported 1,4-dioxane-metabolizing bacterium in the genus of *Nitrobacter*. The net specific growth rates of these five cultures are consistently higher than those reported in the literature at 1,4-dioxane concentrations $<$0.5 mg/L. Compared to the literature, our newly discovered strains have lower half-maximum-rate concentrations (1.8 to 8.2 mg-dioxane/L), lower maximum specific 1,4-dioxane utilization rates (0.24 to 0.47 mg-dioxane/(mg-protein · d)), higher biomass yields (0.29 to 0.38 mg-protein/mg-dioxane), and lower decay coefficients (0.01 to 0.02 d^{-1}). These are characteristics of microorganisms living in oligotrophic environments.

Key words: 1,4-Dioxane, enrichment strategy, kinetics, *Nitrobacter*, oligotrophic environment, pure culture

HIGHLIGHTS

- A low 1,4-dioxane concentration-based enrichment strategy was evaluated.
- A combination of centrifugation and dilution is the best enrichment strategy.
- Five isolated strains have higher net specific growth rates compared to the literature.
- The kinetics of the five isolated strains are suitable for oligotrophic environments.
- *Nitrobacter* sp. WCD6H is the first 1,4-dioxane-metabolizing strain in *Nitrobacter*.

GRAPHICAL ABSTRACT



1. INTRODUCTION

1,4-Dioxane ($C_4H_8O_2$), a colorless cyclic ether compound (EPA 2017), was commonly used as a stabilizer of chlorinated solvents such as 1,1,1-trichloroethane (Mohr *et al.* 2010). It is also used as a solvent in some commercial and industrial processes and consumer products, leading to widespread groundwater contamination (Sei *et al.* 2013a; Pugazhendi *et al.* 2015; Zhang *et al.* 2017). According to the third Unregulated Contaminant Monitoring Rule (UCMR3), 1,4-dioxane was detected in 21% of the U.S. public water systems and exceeded the health-based reference concentration of 0.35 μg/L (EPA 2017) at 6.9% of the systems. The detection of 1,4-dioxane was ranked second among the 28 UCMR3 contaminants, only exceeded by chlorate (Adamson *et al.* 2017). The high mobility of 1,4-dioxane in groundwater has impacted relatively large regions with trace levels of contamination, which underscores the need to explore effective treatment methods applicable to environmentally relevant, low 1,4-dioxane concentrations of ≤365 μg/L (Adamson *et al.* 2014; Chiang *et al.* 2016; Wang *et al.* 2021).

Removal of 1,4-dioxane from contaminated water is challenging due to its unique physical and chemical properties such as high water solubility, low Henry's law constant (4.80×10^{-6} atm m^3/mol), low octanol–water partition coefficient (log $K_{ow} = -0.27$), and low organic carbon partition coefficient (log $K_{oc} = 1.23$) (Zenker *et al.* 2003; EPA 2017; Zhang *et al.* 2017). Bioremediation is a promising method for treating 1,4-dioxane-contaminated water as it is potentially cost-effective and ecofriendly. Plenty of studies have demonstrated aerobic biodegradation of 1,4-dioxane under metabolic and co-metabolic conditions. 1,4-Dioxane was co-metabolized in the presence of primary substrates such as ethane, propane, isobutane, tetrahydrofuran, and toluene (Zenker *et al.* 2002; Mahendra & Alvarez-cohen 2006; Sei *et al.* 2013b; Hatzinger *et al.* 2017; Zhang *et al.* 2017; Barajas-Rodriguez & Freedman 2018; Rolston *et al.* 2019; Xiong *et al.* 2019a, 2020; Luo *et al.* 2021; Wang *et al.* 2021). In the presence of propane and isobutane as primary substrates, the minimum 1,4-dioxane concentrations required to sustain steady-state biomass (S_d^{min}) for *Rhodococcus ruber* ENV425, Propanotrophic mixed culture ENV487, *Rhodococcus rhodochrous* strain 21198 were 1.0, 0.6, and 1.3 mg/L, respectively (Barajas-Rodriguez & Freedman 2018; Rolston *et al.* 2019). Co-metabolic bioremediation has the advantage of biomass growth supported by the primary substrate while treating 1,4-dioxane at low concentrations. However, the primary substrates can make the treatment process expensive and may lead to secondary contamination.

About 20 bacterial strains (Supporting Information Table S1) have been reported in the literature to use 1,4-dioxane as the sole carbon and energy source (Parales *et al.* 1994; Mahendra & Alvarez-Cohen 2005, 2006; Kim *et al.* 2009; Sei *et al.* 2013a; Huang *et al.* 2014; Pugazhendi *et al.* 2015; Chen *et al.* 2016; Inoue *et al.* 2016; Matsui *et al.* 2016; He *et al.* 2017b; Barajas-Rodriguez & Freedman 2018; Inoue *et al.* 2018; Yamamoto *et al.* 2018; Tusher *et al.* 2020; Ma *et al.* 2021; Simmer *et al.* 2021; Tusher *et al.* 2021; Dai *et al.* 2022; Ramos-García *et al.* 2022). The majority of them are gram-positive bacteria that belong to the genera *Pseudonocardia*, *Mycobacterium*, and *Rhodococcus*. The remaining are gram-negative bacteria that belong to the genera *Acinetobacter*, *Afipia*, *Ancylobacter*, *Dokdonella*, *Rhodanobacter*, *Variovorax*, and *Xanthobacter*. These strains were enriched and isolated under high concentrations of 1,4-dioxane (i.e., 20 to 1,000 mg/L). Only a few strains' 1,4-dioxane

metabolism kinetics have been fully characterized (Barajas-Rodriguez & Freedman 2018; Ramos-García *et al.* 2022). There is a need to characterize the 1,4-dioxane metabolism kinetics of more cultures. Isolation and identification of 1,4-dioxane-metabolizing bacteria that can thrive in dilute 1,4-dioxane environments are of particular interest.

In this study, a different enrichment strategy was evaluated: 1,4-dioxane-metabolizing mixed cultures were sub-cultured by periodically spiking 1,4-dioxane at low concentrations (i.e., $\leq$1 mg/L). Three methods – centrifugation, dilution, and combination of centrifugation and dilution – were used to enrich 1,4-dioxane-metabolizing mixed cultures from two sources. After enrichment, five 1,4-dioxane-metabolizing pure cultures were isolated and characterized in terms of phylogenetics and 1,4-dioxane metabolism kinetics and intermediates.

2. MATERIALS AND METHODS

2.1. Chemicals and medium composition

All mixed cultures enrichment, growth of pure cultures, and kinetic experiments were performed in nitrate mineral salts medium. The medium was composed of 1.18 mM $NaNO_3$, 6.1 mM K_2HPO_4, 3.9 mM KH_2PO_4, 1.28 mM Na_2SO_4, 0.08 mM $FeSO_4{\cdot}7H_2O$, 0.15 mM $MgCl_2{\cdot}6H_2O$, 0.07 mM $CaCl_2{\cdot}2H_2O$, 0.002 mM $ZnCl_2$, 0.002 mM $MnCl_2{\cdot}4H_2O$, 0.004 mM $CoCl_2{\cdot}6H_2O$, 0.001 mM $CuCl_2{\cdot}2H_2O$, 0.001 mM $NiCl_2{\cdot}6H_2O$, 0.002 mM H_3BO_3, 0.004 mM $Na_2MoO_4{\cdot}2H_2O$, 0.001 mM $Na_2WO_4{\cdot}2H_2O$, 0.001 mM $Na_2SeO_4{\cdot}5H_2O$, and 0.001 mM KI in deionized water (Xiong *et al.* 2019a). The pH of the medium was adjusted to ~7.0 using 0.1 mM H_2SO_4. The deionized water was autoclaved at 121 °C for 20 min and cooled to room temperature before the stock chemicals were added. 1,4-Dioxane was purchased from Acros Organics as American Chemical Society (ACS) grade with >99% purity. Agar powder was obtained from Thermo Fisher Scientific. All other chemicals and solvents used in this study were purchased as ACS grade.

2.2. Mixed culture enrichment

Two microbial cultures originally from a landfill (L) and activated sludge of a wastewater treatment plant (W) were separately cultured by regularly adding 1,4-dioxane for more than 300 days. Each culture was maintained in three 500 mL serum bottles containing 200 mL of nitrate mineral salts medium. Before each culture was added into the serum bottle, 20 mg of the original culture was centrifuged at 5,000 × *g* for 10 minutes and washed with the nitrate mineral salts medium twice. The objective was to remove residual carbon carried over from the seed material. 1,4-Dioxane was added as a sole electron donor at 1 mg/L whenever its concentration dropped below 0.2 mg/L. The cultures were incubated aerobically at room temperature (25 °C) on a shaker rotating at 120 revolutions per minute (Sei *et al.* 2013a, 2013b; Nam *et al.* 2016; Yamamoto *et al.* 2018). To minimize contamination, the bottles were closed with rubber stoppers and were opened inside a biosafety cabinet every two days to allow oxygen replenishment.

Each culture was enriched by three methods, corresponding to the three serum bottles mentioned above. In the first method (i.e., centrifugation), centrifugation was applied to the cultures after every five cycles of 1,4-dioxane degradation. One bottle from each culture (LC and WC) was centrifuged at 5,000 × *g* for 10 minutes. The pellet was washed with the nitrate mineral salts medium and resuspended into 200 mL fresh medium containing 1 mg/L 1,4-dioxane (Sei *et al.* 2013a; Nam *et al.* 2016; Yamamoto *et al.* 2018). In the second method (i.e., dilution), one bottle from each culture (LD and WD) was sub-cultured through 2–10 times dilution with fresh nitrate mineral salts medium containing 1,4-dioxane at 1 mg/L (Kim *et al.* 2009; Sei *et al.* 2013b; Tusher *et al.* 2020). In the third method (i.e., centrifugation and dilution), one bottle from each culture (LCD and WCD), was sub-cultured through centrifugation followed by dilution. After every 10 cycles of 1,4-dioxane degradation, half of the culture medium was centrifuged at 5,000 × *g* for 10 minutes. The pellet was washed with and then resuspended into 200 mL fresh medium containing 1 mg/L 1,4-dioxane. The three enrichment methods were selected because they create environmental conditions similar to that of 1,4-dioxane contaminated groundwater and allow the growth of the microorganisms of interest.

2.3. Isolation and phylogenetic characterization of 1,4-dioxane-metabolizing pure cultures

1,4-Dioxane-metabolizing bacteria from the six culture bottles described in Section 2.2 (i.e., LC, WC, LD, WD, LCD, and WCD) were isolated by streaking 100 μL of the enriched cultures on solid agar plates supplemented with nitrate mineral salts medium containing 100 mg/L 1,4-dioxane. A 100 mg/L 1,4-dioxane concentration was used during the isolation stage to achieve good growth of colonies. The plates were sealed with a parafilm and incubated upside down at 25 °C. A total of 54 colonies were isolated from the six microbial consortia: nine colonies for each consortium. Single colonies were inoculated into 50 mL cell

culture flasks (with vented caps) containing 10 mL of nitrate mineral salts medium with 100 mg/L 1,4-dioxane. The flasks were incubated aerobically at room temperature (25 °C) on a shaker rotating at 120 revolutions per minute. The concentrations of 1,4-dioxane in the flasks were monitored to evaluate their 1,4-dioxane degradation ability. Forty-seven of the 54 colonies showed 1,4-dioxane degradation. Among them, 30 colonies were further selected, based on their faster 1,4-dioxane degradation, for phylogenetic identification. Genomic DNA were extracted using EZNA® Bacterial DNA Kit following the manufacturer's protocol. The extracted DNA were analyzed at Florida State University, Department of Biological Science, Core Facilities. Partial sequences of 16S rRNA gene were amplified from the extracted DNA by polymerase chain reaction using the 27F and 1492R primer set. The amplicons were purified and sequenced using bi-directional Sanger sequencing. The forward and backward sequences were initially analyzed using Molecular Evolutionary Genetics Analysis version 11 (MEGA11) (Tamura *et al.* 2021). Chimera analysis was then performed using vsearch 2.21.1. Five strains that passed the chimera check were further studied for 1,4-dioxane degradation kinetics and intermediates. The aligned sequences were compared with sequences in the National Center of Biotechnology Information database using the Basic Local Alignment Search Tool. A phylogenetic tree was constructed using MEGA11 for the five 1,4-dioxane-metabolizing strains identified in this study and the bacteria reported in the literature that could metabolically degrade 1,4-dioxane. The partial 16S rRNA sequences were deposited to the GenBank database of the National Center of Biotechnology Information.

2.4. Kinetic experiments and modeling for the pure cultures

The kinetic experiments were conducted in sealed 250 mL serum bottles. The biomass from the pure cultures was centrifuged at 5,000 × *g* for 10 minutes. After removing the supernatant, the pelleted biomass was washed with the nitrate mineral salts medium, centrifuged, and resuspended into 100 mL fresh medium with 100 mg/L 1,4-dioxane. The initial biomass concentrations ranged from 10 to 20 mg/L. This range was chosen due to the 5 mg/L detection limit of the Pierce BCA Protein Assay Kit used. A 100 mg/L 1,4-dioxane concentration was used so that the 1,4-dioxane concentration range (0–100 mg/L) covered the median half-maximum-rate constant of 1,4-dioxane reported in the literature (42.5 mg/L) (Tang 2023). This helps to increase the accuracy of the parameter estimation. The concentrations of 1,4-dioxane and protein (representing biomass) were monitored daily.

A Monod-based kinetic model was used to describe the rates of change in the concentrations of 1,4-dioxane (Equation (1)) and biomass (Equation (2)). Descriptions of the model input and model output parameters are presented in Table 1.

Table 1 | Description of model parameters

Parameter	Description	Unit
(a) Model input parameters		
N	Total number of experimental measurements	–
$S^e_{d(t)}$	1,4-Dioxane concentration in the experiment at time t	mg-dioxane/L
t	Time	d
T	Time at the end of each experiment	d
$X^e_{(t)}$	Biomass concentration in the experiment at time t	mg-protein/L
(b) Model output parameters		
b	Biomass decay coefficient	1/d
K_d	Half-maximum-rate concentration of 1,4-dioxane	mg-dioxane/L
$MSRR$	Mean sum of squared relative residuals	–
q_d	Maximum specific 1,4-dioxane utilization rate	mg-dioxane/(mg-protein·d)
S_d or $S^m_{d(t)}$	1,4-Dioxane concentration at time t of the model	mg-dioxane/L
S^{min}_d	Minimum 1,4-dioxane concentration	mg-dioxane/L
X or $X^m_{(t)}$	Biomass concentration at time t of the model	mg-protein/L
Y_d	Biomass yield of 1,4-dioxane	mg-protein/(mg-dioxane)
μ	Net specific growth rate of active biomass	1/d

Equations (1) and (2) were solved numerically by a numerical code written in FORTRAN. Initial conditions included the concentrations of 1,4-dioxane and biomass that were experimentally determined at day 0 (i.e., $S^e_{d(0)}$ and $X^e_{(0)}$). The unknown kinetic parameters q_d, K_d, Y_d, and b were determined by fitting the experimentally measured concentrations (i.e., $S^e_{d(t)}$ and $X^e_{(t)}$) to the model simulated concentrations (i.e., $S^m_{d(t)}$ and $X^m_{(t)}$) using a model-independent parameter estimator (Doherty 2019). The goodness of fit between the experiment and the model simulation was determined using the mean sum of squared relative residuals (Equation (3)). This approach differs from previous work reported by Barajas-Rodriguez & Freedman (2018) in that the four kinetic parameters were estimated simultaneously. The estimated parameters were then evaluated by comparing a new set of experimental data with their corresponding simulated results. The conditions of the experiments used for the model evaluation were the same as the kinetics estimation, except that the initial 1,4-dioxane concentration was 10 mg/L in the evaluation experiments.

$$\frac{dS_d}{dt} = -X\, q_d \left[\frac{S_d}{K_d + S_d}\right] \tag{1}$$

$$\frac{dX}{dt} = Y_d\, X\, q_d \left[\frac{S_d}{K_d + S_d}\right] - b\, X \tag{2}$$

$$MSRR = \frac{1}{N}\left[\sum_{t=0}^{T}\left(\frac{S^m_{d(t)} - S^e_{d(t)}}{S^e_{d(t)}}\right)^2 + \sum_{t=0}^{T}\left(\frac{X^m_{(t)} - X^e_{(t)}}{X^e_{(t)}}\right)^2\right] \tag{3}$$

To evaluate the growth potential of the isolated cultures in oligotrophic environments (i.e., < 1 mg/L in this study) and compare it to that of the literature-reported cultures, the net specific growth rates of the cultures and the minimum 1,4-dioxane concentration required to sustain steady-state biomass were calculated by Equations (4) and (5) (Rittmann & McCarty 2020).

$$\mu = Y_d\, q_d \left[\frac{S_d}{K_d + S_d}\right] - b \tag{4}$$

$$S_d^{min} = K_d\, \frac{b}{Y_d\, q_d - b} \tag{5}$$

2.5. Identification and quantification of 1,4-dioxane intermediates for the pure cultures

The experiments for identification and quantification of 1,4-dioxane intermediates were performed in triplicate for each of the five pure cultures. The biomass was centrifuged at 5,000 × *g* for 10 minutes. After removing the supernatant, the pelleted biomass was washed with the nitrate mineral salts medium, centrifuged, and resuspended into 200 mL fresh medium with 100 mg/L 1,4-dioxane. The bottles were incubated aerobically at room temperature (25 °C) on a shaker rotating at 120 revolutions per minute. Liquid samples were filtered using 0.20 μm Acrodisc® syringe filters and stored in a refrigerator at 4 °C before they were analyzed for 1,4-dioxane and its degradation intermediates following the analytical methods in Section 2.6.

2.6. Analytical methods

The concentrations of 1,4-dioxane in all experiments were measured using solid phase micro-extraction followed by gas chromatography/mass spectrometry, which is described in our previous publication (Xiong *et al.* 2019a). The quantification limit was 0.5 μg-1,4-dioxane/L. 1,4-Dioxane intermediates were analyzed by adapting the methods in our previous publication (Xiong *et al.* 2019b). First, chemical oxygen demand (COD) concentrations were measured by Hach DR 3900 spectrophotometer using low range (3 to 150 mg-COD/L) and ultra-low range (1 to 60 mg-COD/L) COD kits. The COD concentration was compared to the 1,4-dioxane concentration (after being converted to mg-COD/L) for each sample to estimate whether intermediates were contributing to COD. A paired t-test at a *p*-value of 0.05 was used to evaluate the statistical significance of the difference between the concentrations of 1,4-dioxane and total COD. Second, the presence of organic acids, including formic, glycolic, glyoxylic, methoxyacetic, and oxalic acids, as potential 1,4-dioxane degradation intermediates were checked using a DIONEX ion chromatography system. The detection limits of the organic acids were approximately 0.1 mg/L. All aqueous samples used for COD and ion chromatography measurements were filtered by

0.20 μm Acrodisc® syringe filters. Third, the presence of potential 1,4-dioxane degradation intermediates including the organic acids, ethylene glycol, and 1,4-dioxan-2-ol, were checked using gas chromatography/mass spectrometry operated in the scan mode.

To evaluate the biomass concentration change in all the experiments, protein concentrations were measured by a Nanodrop spectrophotometer using Pierce BCA Protein Assay Kit with Albumin Standard Ampules. The detection limit for protein measurement was 5 mg-protein/L. The concentrations of the extracted DNA were measured using the same spectrophotometer.

3. RESULTS AND DISCUSSION

3.1. The five isolated 1,4-dioxane-metabolizing strains

Six microbial consortia capable of utilizing 1,4-dioxane as a sole carbon and energy source were enriched. They corresponded to two sources (L and W) and three enrichment methods (C, D, and CD) for each source. Figure S1 shows the pictures of the six mixed cultures during the enrichment: the three cultures based on the L source were green, while the three cultures based on the W source were brown. The 1,4-dioxane degradation profiles of different generations of the consortia are presented in Figure S2. The six mixed cultures were able to degrade 1,4-dioxane from 1 mg/L to below 0.01 mg/L. Figure S3 compares the 1,4-dioxane utilization rates of the cultures. At the beginning of the enrichment, the 1,4-dioxane utilization rates of the six cultures slightly varied between 2.1×10^{-3} and 2.9×10^{-3} mg-dioxane/(mg-protein · d). During the 300 days of enrichment, the two CD-based cultures (LCD and WCD) had the highest utilization rates, approximately five times higher than their initial rates. On the other hand, the utilization rates of the two C-based (LC and WC) and D-based (LD and WD) cultures increased by two and four times, respectively, compared to their initial rates. The lower utilization rates of the cultures enriched by centrifugation (C-based) could be due to the retainment of the inert biomass from one generation to the next. Therefore, dilution or combination of centrifugation and dilution should be considered during culture enrichments to enhance the growth of active biomass and minimize retainment of inert biomass.

The five isolated bacterial strains were *Dokdonella* sp. LCD6B, *Acinetobacter* sp. LCD6D, *Afipia* sp. WC10G, *Nitrobacter* sp. WCD6H, and *Pseudonocardia* sp. WD4H, respectively. They represent successful isolation from both mixed cultures and the three enrichment methods. The five identified strains were related to genera *Dokdonella* sp. (98.3%), *Acinetobacter* sp. (99.0%), *Afipia* sp. (99.2%), *Nitrobacter* sp. (97.9%), and *Pseudonocardia* sp. (99.4%) respectively. The partial 16S rRNA gene sequences of the five pure strains are deposited at the NCBI GenBank database under accession numbers OP362562, OP362563, OP362564, OP362565, and OP362566, respectively. The phylogenetic tree shown in Figure 1 presents the evolutionary relationships among the five 1,4-dioxane-metabolizing bacteria isolated in this study and bacteria reported in the literature that can metabolically degrade 1,4-dioxane. The 24 literature-reported 1,4-dioxane-metabolizing pure cultures are summarized in Table S1. The upper 14 in the tree are gram-negative bacterial strains that belong to the phylum Proteobacteria while the lower groups are gram-positive bacteria from the Actinobacteria phylum. This study isolated *Nitrobacter* sp. WCD6H as the first 1,4-dioxane-metabolizing bacterium that belongs to the *Nitrobacter* genus.

1,4-Dioxane biodegradation is initiated by enzymes encoding soluble di-iron monooxygenase (SDIMO) genes. SDIMO genes also have the ability to catalyze the initial oxidation of a wide variety of hydrocarbons (Li *et al.* 2014; Inoue *et al.* 2016; Tusher *et al.* 2021). To date, three SDIMO gene clusters (Group-2, Group-5, and Group-6) have been confirmed to be involved in the metabolic biodegradation of 1,4-dioxane (Table S1). Li *et al.* (2014) found a positive correlation between the biodegradation of 1,4-dioxane and the abundance of Group-5 SDIMO genes, tetrahydrofuran/dioxane monooxygenase (*thmA*/*dxmA*). Genes *thmA*/*dxmA* also were identified in the 1,4-dioxane-metabolizing bacteria that belong to the genera *Dokdonella*, *Afipia*, and *Pseudonocardia* (Inoue *et al.* 2016; Tusher *et al.* 2021) (Table 2). Similarly, tetrahydrofuran monooxygenase gene cluster (*thmADBC*) was expressed by *Pseudonocardia dioxanivorans* CB1190 during growth on 1,4-dioxane (Grostern *et al.* 2012; Sales *et al.* 2013). However, the molecular mechanisms of 1,4-dioxane biodegradation are only partially understood. The genes associated with the metabolism of 1,4-dioxane by *Acinetobacter*, *Nitrobacter*, and several 1,4-dioxane degrading pure cultures in the literature are unknown (Table S1). Future studies that incorporate genomic and proteomic analyses will help to further elucidate the metabolic pathways and enzymatic mechanisms underlying 1,4-dioxane degradation.

3.2. Kinetic parameters estimation and evaluation

The 1,4-dioxane biodegradation kinetics of the five strains were described well by the Monod-based equations with low MSRR values ranging from 0.01 to 0.11. The estimated kinetic parameters for the five strains are presented in Table 3. Figure 2

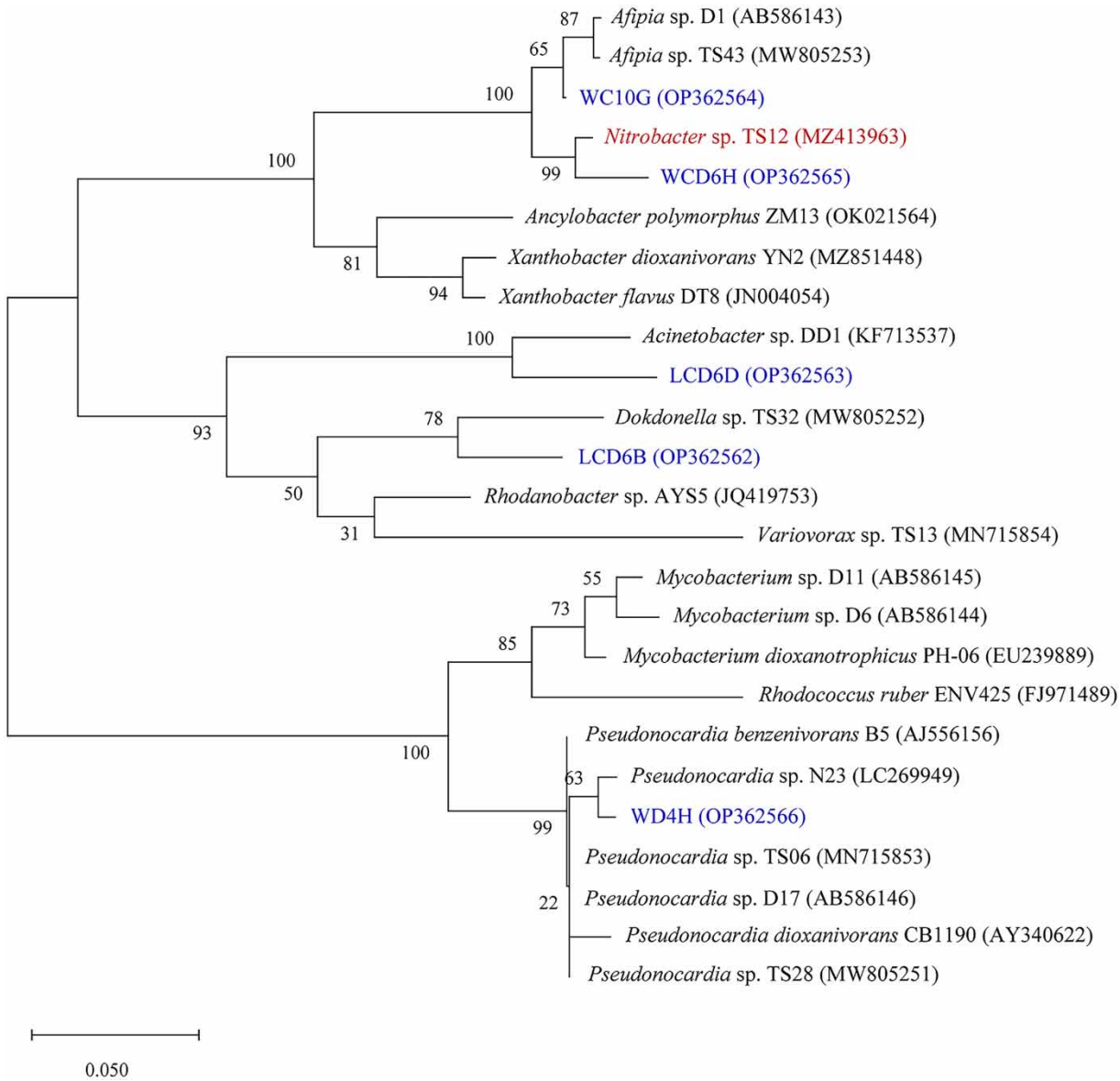


Figure 1 | Phylogenetic tree based on partial 16S rRNA gene sequences showing the evolutionary relationship among five 1,4-dioxane-metabolizing bacteria isolated in this study (LCD6B, LCD6D, WC10G, WCD6H, and WD4H) and bacteria reported in the literature that can metabolically degrade 1,4-dioxane. Note: *Nitrobacter* sp. TS12, which cannot degrade 1,4-dioxane (Tusher *et al.* 2022), is added to the tree to show the evolutionary relationship of WCD6H.

further compares the experimental and modeling results for 1,4-dioxane and biomass concentrations in the kinetic experiments of the five strains. The parameterized model captured all major trends for the concentrations of 1,4-dioxane and biomass. In general, the variability of the kinetic parameters is quite small, especially Y_d and b. The variability for q_d and K_d are slightly greater, with approximate 2- and 4.5-fold difference between the largest and smallest values, respectively. The reason for the narrow range of kinetic parameters may be associated with the enrichment strategy. As the mixed cultures were enriched with ≤ 1 mg/L 1,4-dioxane for more than 300 days, it may have created an environment to enrich strains with similar kinetic parameters. The kinetic parameters presented in Table 3 will be discussed in this section by comparing them with corresponding values from the literature.

The net specific growth rates of the five cultures isolated in this study and two pure cultures from the literature are shown in Figure 3. *Pseudonocardia dioxanivorans* CB1190 (Barajas-Rodriguez & Freedman 2018) and *Pseudonocardia dioxanivorans* BERK-1 (Ramos-García *et al.* 2022) are the only pure cultures with a full set of kinetics in the literature and are used

Table 2 | Summary of 1,4-dioxane-metabolizing pure cultures and their corresponding 1,4-dioxane degrading genes

Pure cultures in this study	Pure cultures in the literature	1,4-Dioxane degrading genes	Notes	References
Dokdonella sp. LCD6B	*Dokdonella* sp. TS32	*thmA*/*dxmA*	Tetrahydrofuran/dioxane monooxygenase	Tusher *et al.* (2021)
Acinetobacter sp. LCD6D	*Acinetobacter baumannii* DD1	–	No study on 1,4-dioxane degrading genes	Huang *et al.* (2014)
Afipia sp. WC10G	*Afipia* sp. TS43	*thmA*/*dxmA*	Tetrahydrofuran/dioxane monooxygenase	Tusher *et al.* (2021)
Nitrobacter sp. WCD6H	–	–	No study on 1,4-dioxane degrading genes	–
Pseudonocardia sp. WD4H	*Pseudonocardia dioxanivorans* CB1190	*thmADBC*	Tetrahydrofuran monooxygenase	Grostern *et al.* (2012)
	Pseudonocardia dioxanivorans JCM 13855	*thmA*/*dxmA*	Tetrahydrofuran/dioxane monooxygenase	Inoue *et al.* (2016)
	Pseudonocardia sp. D17	*thmA*/*dxmA*	Tetrahydrofuran/dioxane monooxygenase	Inoue *et al.* (2016)
	Pseudonocardia sp. TS28	*thmA*/*dxmA*	Tetrahydrofuran/dioxane monooxygenase	Tusher *et al.* (2021)

Table 3 | Summary of kinetic parameters and minimum 1,4-dioxane concentrations required for the 1,4-dioxane-metabolizing strains isolated in this study

Pure culture	$q_d\left(\frac{\text{mg dioxane}}{\text{mg protein}\cdot\text{d}}\right)$	$K_d\left(\frac{\text{mg dioxane}}{\text{L}}\right)$	$Y_d\left(\frac{\text{mg protein}}{\text{mg dioxane}}\right)$	$b\left(\frac{1}{\text{d}}\right)$	$S_d^{min}\left(\frac{\text{mg dioxane}}{\text{L}}\right)$	MSRR
Dokdonella sp. LCD6B	0.24	7.7	0.34	0.01	1.5	0.04
Acinetobacter sp. LCD6D	0.27	5.6	0.38	0.02	1.3	0.02
Afipia sp. WC10G	0.47	8.2	0.36	0.02	1.0	0.11
Nitrobacter sp. WCD6H	0.24	1.8	0.30	0.01	0.4	0.08
Pseudonocardia sp. WD4H	0.26	5.3	0.29	0.01	0.8	0.01

for comparison. *P. dioxanivorans* CB1190 has about 2- to 8-fold higher net specific growth rates at 1,4-dioxane concentrations ≥ 0.5 mg/L, while all the five strains in this study have about four times higher net specific growth rates at concentrations < 0.5 mg/L. This supports our hypothesis that enrichment with 1,4-dioxane at lower concentration helps to isolate oligotrophs. The minimum 1,4-dioxane concentration required to sustain steady-state biomass in a continuous-flow, suspended-growth reactor (S_d^{min}) ranged from 0.4 to 1.5 mg/L (Table 3). This is at the lower end of the range (0.4–20 mg/L) reported in the literature (Tang 2023). Based on the S_d^{min}, two of the five cultures in Table 3 are able to grow at 1,4-dioxane < 1 mg/L. In an attached-growth reactor (i.e., biofilm reactor) that enhances biomass attachment and growth, the S_d^{min} values should be much lower than 0.4 mg/L (Tang 2023). Simmer *et al.* (2021) reported low S_d^{min} value of 0.49 μg/L for *Rhodococcus ruber* strain 219. However, the decay coefficient was assumed to be the same as that of *P. dioxanivorans* CB1190 (Simmer *et al.* 2021).

Figure 4 compares the 1,4-dioxane biodegradation kinetic parameters determined in this study to the same parameters reported in the literature for all 1,4-dioxane metabolizing mixed cultures (Zenker *et al.* 2002; Mahendra & Alvarez-cohen 2006; Nam *et al.* 2016; Barajas-Rodriguez & Freedman 2018; Lee *et al.* 2020; Xiong *et al.* 2022) and pure cultures (Mahendra & Alvarez-cohen 2006; Sei *et al.* 2013a; Chen *et al.* 2016; He *et al.* 2017a; Barajas-Rodriguez & Freedman 2018; Inoue *et al.* 2018; Yamamoto *et al.* 2018; Ma *et al.* 2021; Simmer *et al.* 2021). Figure 5 further compares the kinetic parameters of *Afipia* and *Pseudonocardia* isolated in this study and the literature. Only two genera are compared in Figure 5 because no kinetics data is available in the literature for biodegradation of 1,4-dioxane by *Dokdonella*, *Acinetobacter*, and *Nitrobacter*. Figures 4 and 5 show the same comparison results: compared to the 1,4-dioxane-metabolizing cultures in the literature, the five pure cultures have lower half-maximum rate constants, higher biomass yields, lower maximum specific 1,4-dioxane utilization

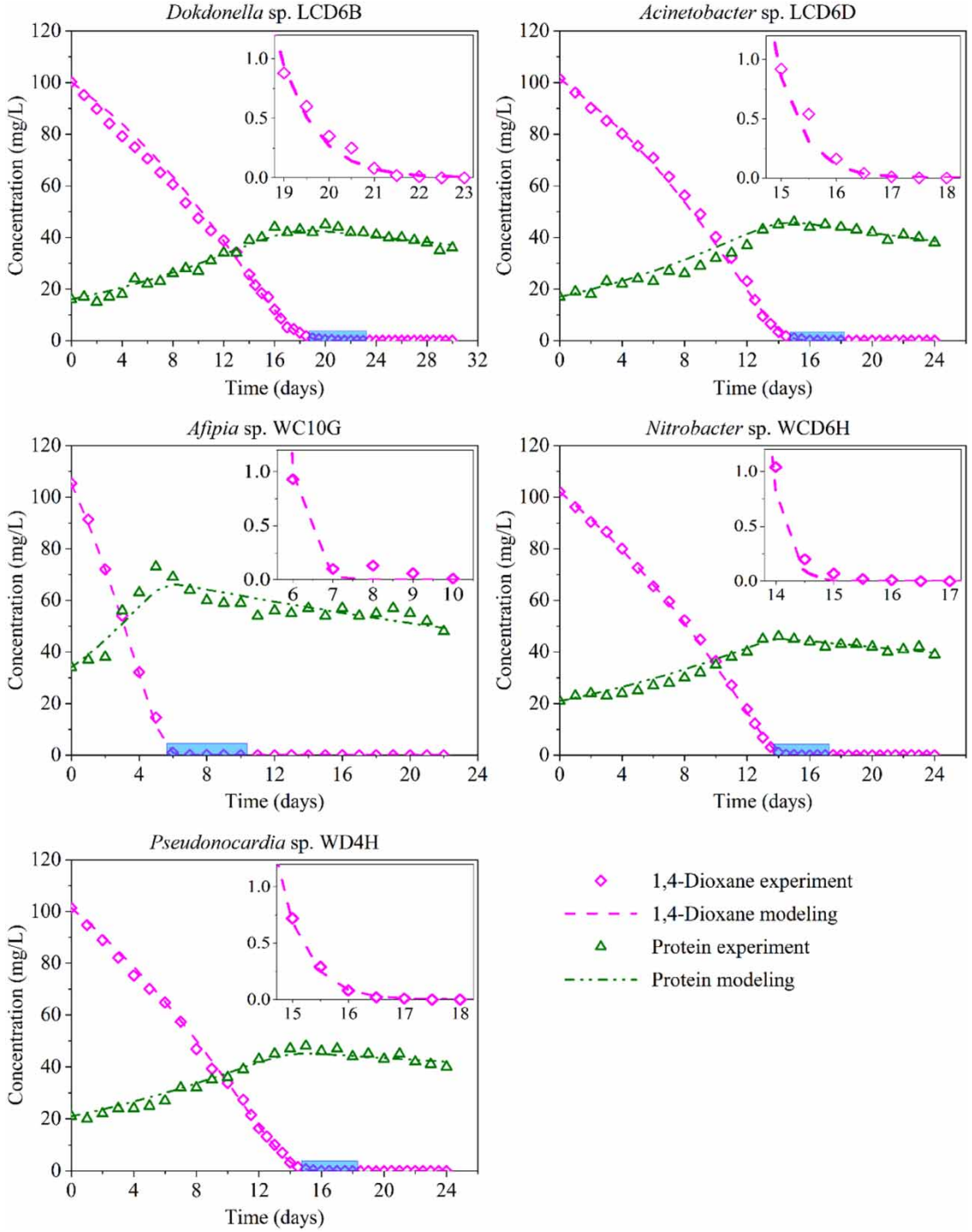


Figure 2 | Comparison of experimental data and model-simulated results for 1,4-dioxane and biomass concentrations in the kinetic experiments used for parameter estimation. The inset graphs show fittings for 1,4-dioxane concentrations below 1 mg/L.

rates, and lower decay coefficients. These are characteristics of microorganisms living in oligotrophic environments (Koch 2001; Xiong *et al.* 2022; Tang 2023).

As an example of these four parameters, the half-maximum-rate concentration is discussed in detail here. The half-maximum-rate concentrations obtained in this study ranged from 1.8 to 8.2 mg-dioxane/L. The 1,4-dioxane-metabolizing pure

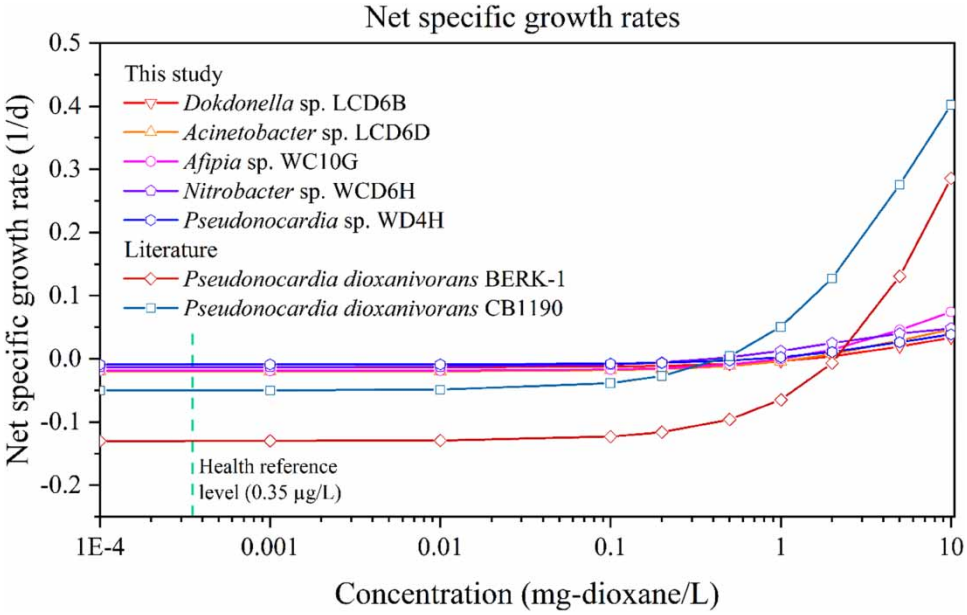


Figure 3 | Net specific growth rates for 1,4-dioxane-metabolizing pure cultures isolated in this study and reported in the literature. *Note: P. dioxanivorans* CB1190 (Barajas-Rodriguez & Freedman 2018) and *P. dioxanivorans* BERK-1 (Ramos-García *et al.* 2022) are the only pure cultures with a full set of kinetic parameters reported in the literature.

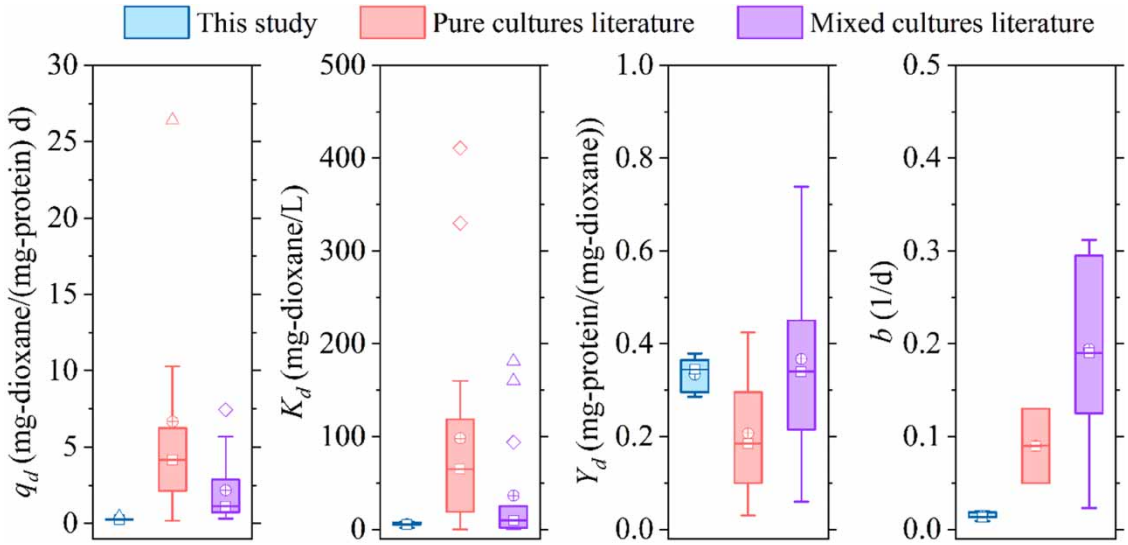


Figure 4 | Comparison of kinetic parameters of the 1,4-dioxane-metabolizing pure cultures isolated in this study and 1,4-dioxane-metabolizing pure and mixed cultures in the literature.

cultures reported in the literature have high half-maximum-rate concentrations ranging from 6.3 to 411 mg-dioxane/L (Mahendra & Alvarez-cohen 2006; Sei *et al.* 2013a; Chen *et al.* 2016; He *et al.* 2017a; Barajas-Rodriguez & Freedman 2018; Inoue *et al.* 2018; Yamamoto *et al.* 2018; Ma *et al.* 2021). *Nitrobacter* sp. WCD6H showed the lowest half-maximum-rate concentration of 1.8 mg-dioxane/L among the five strains isolated in this study. The half-maximum-rate concentration of *Afipia* sp. WC10G was 8.2 mg-dioxane/L, about 3-fold lower than that of *Afipia* sp. D1, 26 mg-dioxane/L (Sei *et al.* 2013a). Similarly, *Pseudonocardia* sp. WD4H had a half-maximum-rate concentration of 5.3 mg-dioxane/L. This was about 1.2 to 30-fold lower than those of *P. dioxanivorans* BERK-1 (14.7 mg-dioxane/L) (Ramos-García *et al.* 2022),

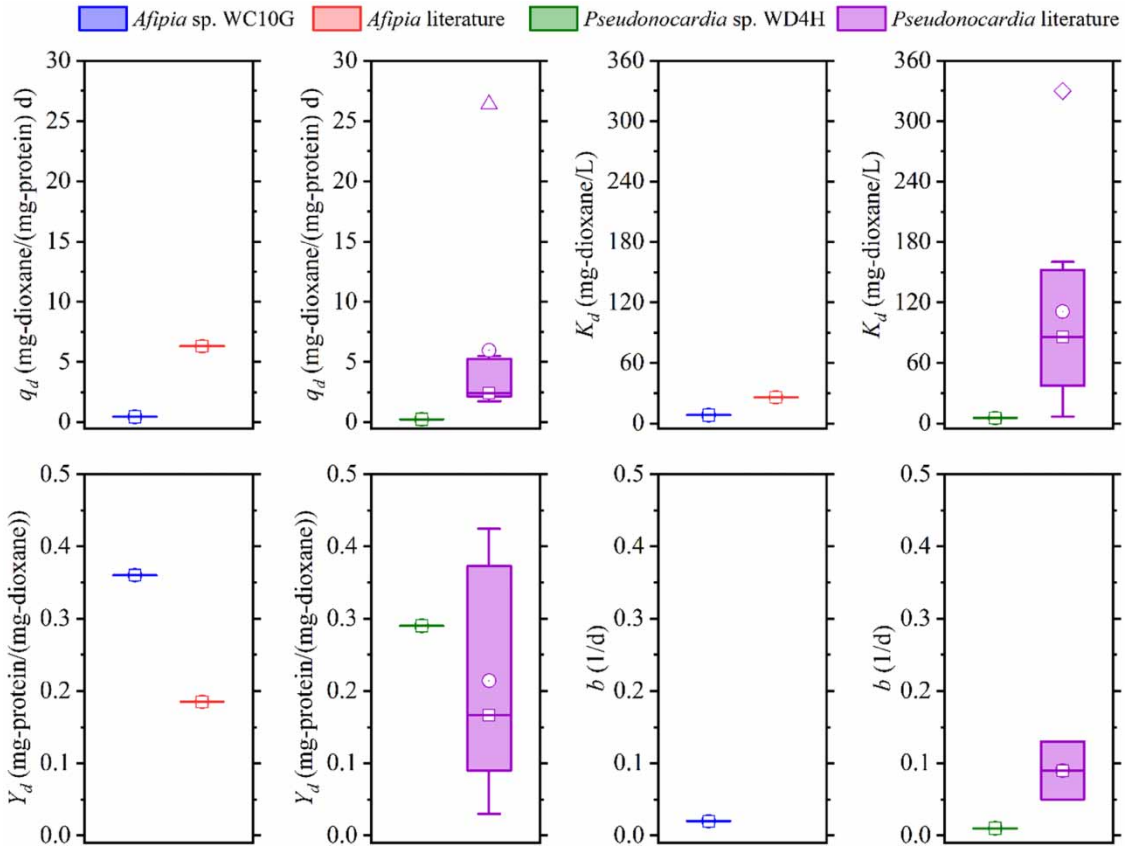


Figure 5 | Comparison of kinetic parameters for *Afipia* and *Pseudonocardia* isolated in this study and reported in the literature. *Note: Afipia* in the literature has no reported biomass decay value.

P. dioxanivorans CB1190 (6.3 to 160 mg-dioxane/L) (Mahendra & Alvarez-cohen 2006; He *et al.* 2017a; Barajas-Rodriguez & Freedman 2018), *Pseudonocardia* sp. D17 (60 mg-dioxane/L) (Sei *et al.* 2013a), and *Pseudonocardia* sp. N23 (80 mg-dioxane/L) (Yamamoto *et al.* 2018).

The kinetic parameters presented in Table 3 were evaluated by comparing results from a second set of independent experiments with their corresponding model-simulated results. Figure 6 compares the experimentally measured data and the model simulated results for the five isolated strains. The parameterized model captured the major trends for the concentrations of 1,4-dioxane and biomass. The MSRR values obtained during model evaluation were 0.22, 0.27, 0.26, 0.22, and 0.13, respectively. These results were 2- to 13-fold larger than those obtained during parameter estimation. The slower degradation rates in the evaluation experiments than the model predictions could be due to the effect of lag phase in the biomass growth.

3.3. 1,4-dioxane intermediates

Figure 7 compares the 1,4-dioxane concentrations (converted from mg-1,4-dioxane/L to mg-COD/L by a conversion factor of 1.82) and the total COD of the corresponding water samples for the five isolated strains. When 1,4-dioxane was metabolized from 180 to 20 mg-COD/L, the difference between the concentrations of 1,4-dioxane and total COD was not statistically significant at a confidence level of 95%, suggesting negligible production of 1,4-dioxane intermediates. When the 1,4-dioxane was < 20 mg-COD/L (i.e., 11 mg-1,4-dioxane/L), the total COD concentration was higher than the 1,4-dioxane concentrations, suggesting that some chemicals other than 1,4-dioxane were present. The base COD value of the nitrate mineral salts medium without 1,4-dioxane was 1.33 ± 0.58 mg-COD/L. The other chemicals could be 1,4-dioxane metabolism intermediates or soluble microbial products due to endogenous respiration of the bacteria (Rittmann & McCarty 2020). Further analyses of the liquid samples by the gas chromatography/mass spectrometry and ion chromatograph showed no presence of any 1,4-dioxane intermediates discussed in Section 2.6 above the detection limits. Therefore, we inferred that soluble

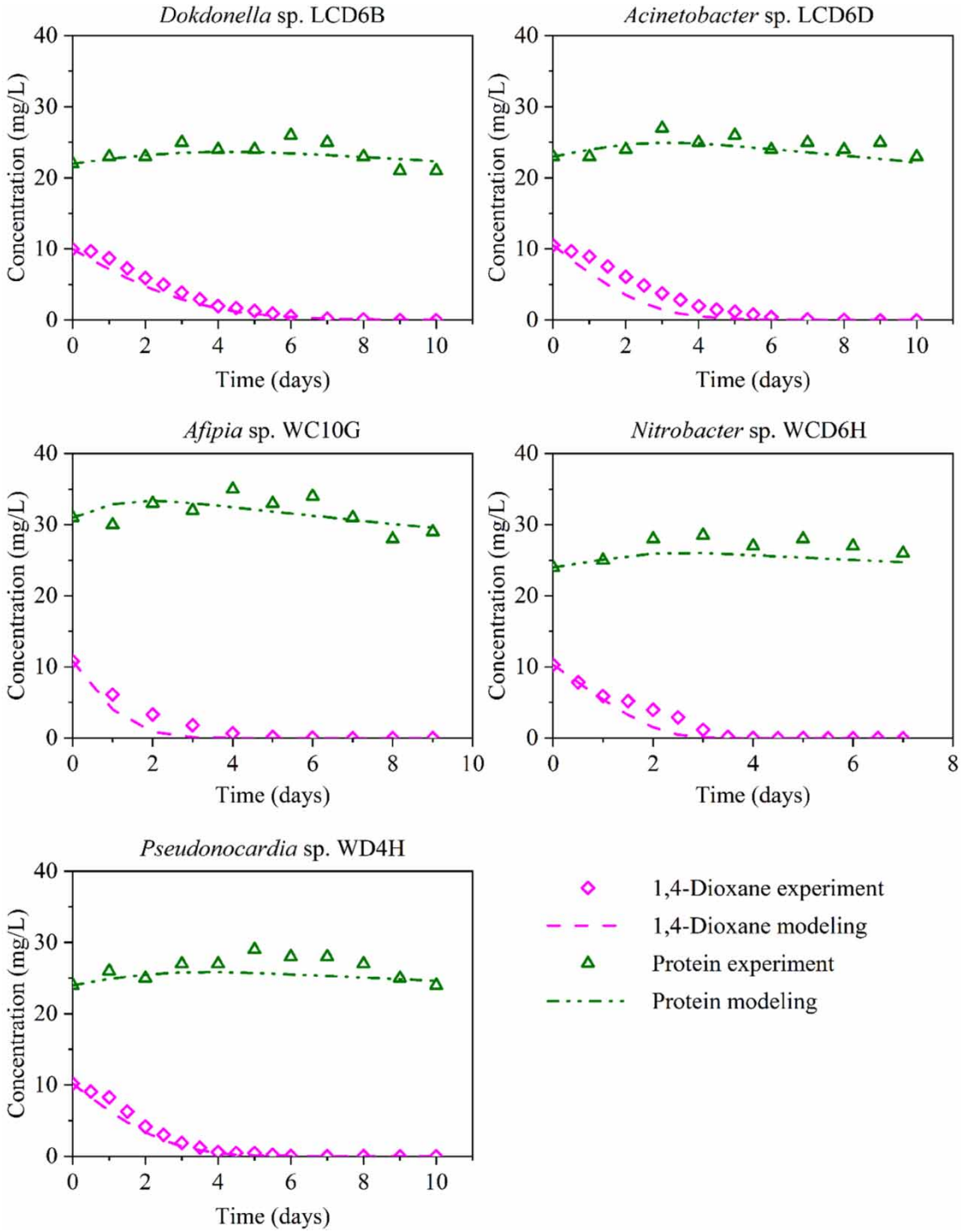


Figure 6 | Comparison of experimental data and modeling results for 1,4-dioxane and biomass concentrations during model evaluation.

microbial products generated from endogenous respiration of the active biomass might have contributed to the total COD during the decay phase of the experiment.

Several previous studies identified biodegradation intermediates of 1,4-dioxane. The major intermediates identified during 1,4-dioxane biodegradation by monooxygenase-expressing bacteria were ethylene glycol, glycolate, 2-Hydroxyethoxyacetic acid, and oxalate (Mahendra *et al.* 2007). 1,4-Dioxane degradation by *Mycobacterium* sp. PH-06 also showed 1,4-dioxane-2-ol

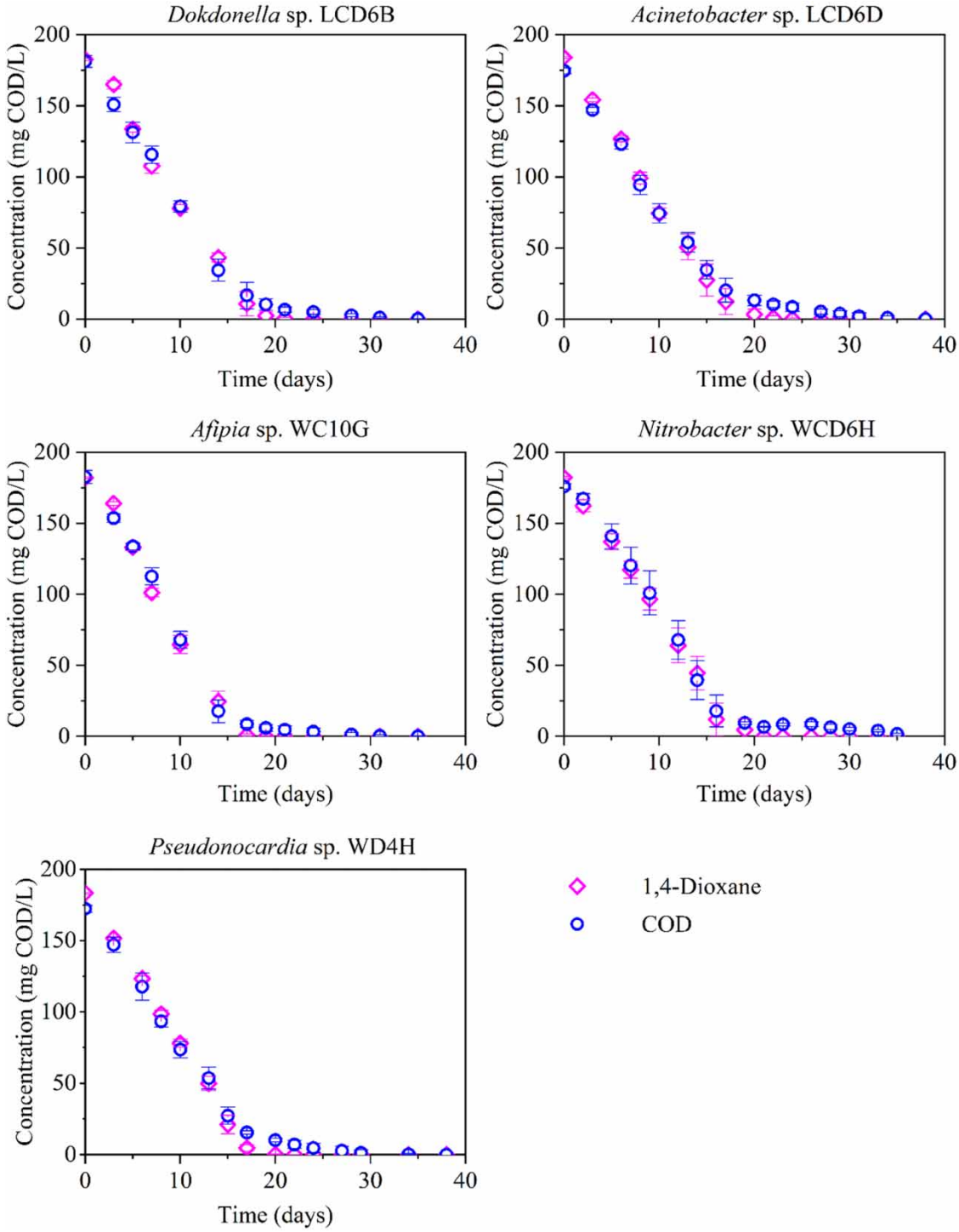


Figure 7 | Comparison of 1,4-dioxane and total COD concentrations for the 1,4-dioxane-metabolizing pure cultures isolated in this study. The 1,4-dioxane concentrations were converted from mg-1,4-dioxane/L to mg-COD/L by a conversion factor of 1.82. Aqueous samples used for COD measurements were filtered by 0.20 μm Acrodisc® syringe filters.

and ethylene glycol as the major degradation intermediates (Kim *et al.* 2009). On the other hand, 1,4-dioxene was the only 1,4-dioxane intermediate by *Acinetobacter baumannii* DD1 (Huang *et al.* 2014). Chen *et al.* (2016) found three 1,4-dioxane intermediates: 1,4-dioxene, ethylene glycol, and oxalic acid by *Xanthobacter flavus* DT8. The differences in the identification of

1,4-dioxane intermediates indicate that the degradation pathway of 1,4-dioxane may vary depending on microbial composition.

3.4. Practical implications of the study

The feasibility and practical applications of 1,4-dioxane bioremediation require profound research on the kinetics, operating parameters, and sustainability of the treatment system. This research will contribute to the current needs to identify microorganisms for treating 1,4-dioxane. The kinetic constants obtained in this study could be used in the design and assessment of large scale 1,4-dioxane remediation technologies. The minimum 1,4-dioxane concentrations required to sustain steady-state biomass (S_d^{min}) for the isolated strains are at the lower end of the range reported in the literature. However, the S_d^{min} values are still higher than most 1,4-dioxane contaminated sites. The S_d^{min} of these strains are derived based on continuous-flow stirred-tank reactor containing suspended biomass. Biofilm reactors inoculated with these microorganisms are expected to have a smaller S_d^{min} than those of suspended-growth reactors since the biofilm provides protection to the microorganisms, leading to a lower effective biomass decay rate. Incorporating adsorption and biodegradation using materials that can adsorb 1,4-dioxane, provide biomass attachment, and concentrate substrates and nutrients could also enhance the bioremediation of 1,4-dioxane.

4. CONCLUSIONS

1,4-Dioxane-metabolizing pure cultures reported in the literature were isolated following enrichment with 1,4-dioxane at high concentrations (20–1,000 mg/L). This study enriched 1,4-dioxane-metabolizing mixed cultures through periodically spiking 1,4-dioxane at low concentrations ($\leq$1 mg/L) and then isolated five bacterial strains that could metabolize 1,4-dioxane. This strategy worked well for two mixed cultures: the first one from a landfill leachate and the second one from a municipal wastewater treatment plant. It also worked well for three enrichment methods: dilution, centrifugation, and their combination, but dilution and combination of centrifugation and dilution resulted in the highest 1,4-dioxane utilization rate for the mixed cultures. The five isolated bacterial strains were *Dokdonella* sp. LCD6B, *Acinetobacter* sp. LCD6D, *Afipia* sp. WC10G, *Nitrobacter* sp. WCD6H, and *Pseudonocardia* sp. WD4H. To the best of our knowledge, WCD6H is the first known strain in the genus *Nitrobacter* that can metabolize 1,4-dioxane. Only two 1,4-dioxane-metabolizing pure cultures in the literature have a full set of kinetic parameters for calculating the net specific growth rate. Compared to these pure cultures, the five strains isolated in our study have higher net specific growth rates when the 1,4-dioxane concentrations are $<$0.5 mg/L. Further comparison of the kinetic parameters between the five strains in this study and the pure and mixed cultures reported in the literature shows that the five strains in this study have lower half-maximum-rate concentrations (1.8 to 8.2 mg-dioxane/L), higher biomass yields (0.29 to 0.38 mg-protein/mg-dioxane), lower decay coefficients (0.01 to 0.02 d^{-1}), and lower maximum specific 1,4-dioxane utilization rates (0.24 to 0.47 mg-dioxane/(mg-protein $\cdot$ d)). The minimum 1,4-dioxane concentration required to sustain steady-state biomass ranged from 0.4 to 1.5 mg/L. The five strains were also similar in that they did not produce detectable 1,4-dioxane intermediates.

In order to further elucidate the metabolic pathways and enzymatic mechanisms underlying 1,4-dioxane degradation by the isolated strains, future studies incorporating genomic and proteomic analyses are warranted. These analyses will provide a more comprehensive understanding of the metabolic capabilities of the organisms and contribute to the broader knowledge of biodegradation processes. Additionally, exploring the genomic and proteomic profiles of these strains will facilitate the identification of specific genes or enzymes critical to 1,4-dioxane degradation, ultimately aiding in the development of targeted bioremediation strategies.

AUTHOR CONTRIBUTIONS

Ermias Gebrekrstos Tesfamariam: methodology, investigation, formal analysis, software, visualization, writing – original draft, review and editing. Dennis Ssekimpi: investigation, formal analysis, writing – review and editing. Sarajeen Saima Hoque: investigation, formal analysis, writing – review and editing. Huan Chen: methodology, resources, writing – review and editing. Joshua D. Howe: methodology, writing – review and editing. Chao Zhou: methodology, writing – review and editing. Yue-xiao Shen: methodology, writing – review and editing. Youneng Tang: conceptualization, methodology, validation, supervision, funding acquisition, project administration, writing – review and editing.

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DATA AVAILABILITY STATEMENT

All relevant data are included in the paper or its Supplementary Information.

CONFLICT OF INTEREST

The authors declare there is no conflict.

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