Aerobic biotransformation of the antibiotic ciprofloxacin by *Bradyrhizobium* sp. isolated from activated sludge

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Abstract

Ciprofloxacin (CIP) is an antibiotic that is widely used to treat bacterial infections and is poorly biodegraded in the wastewater treatment process. In this study, a CIP-degrading strain (GLC_01) was successfully retrieved from activated sludge by enrichment and isolation. The obtained bacterial strain shares over 99% nucleotide identity of the 16S rRNA gene with *Bradyrhizobium* spp. Results show that *Bradyrhizobium* sp. GLC_01 degraded CIP via cometabolism with another carbon substrate following a first-order kinetics degradation reaction. CIP degradation by *Bradyrhizobium* sp. GLC_01 increased when the concentration of the primary carbon source increased. The biodegradability of the primary carbon source also affected CIP degradation. The use of glucose and sodium acetate (i.e. readily biodegradable), respectively, as a primary carbon source enhanced CIP biotransformation, compared to starch (i.e. relatively slowly biodegradable). CIP degradation decreased with the increase of the initial CIP concentration. Over 70% CIP biotransformation was achieved at 0.05 mg L$^{-1}$ whereas CIP degradation decreased to 26% at 10 mg L$^{-1}$. The phylogenetic identification and experimental verification of this CIP-degrading bacterium can lead to a bioengineering approach to manage antibiotics and possibly other persistent organic contaminants during wastewater treatment.

Key words: Ciprofloxacin (CIP); *Bradyrhizobium*; biotransformation; cometabolism.
1. Introduction

The occurrence of trace organic contaminants (TrOCs) including pharmaceuticals, personal care products, steroid hormones, and industrial chemicals in sewage and sewage-impacted water bodies is of considerable human health and ecological concern. Some of these compounds, such as pharmaceuticals and personal care products, are indispensable in our modern society. Others, such as steroid hormones, are naturally and excreted continuously by mammals including human beings, and thus their release is unavoidable. There is a growing concern that the occurrence of TrOCs in the environment can affect aquatic ecology due to their biologically active properties (Clara et al., 2012; Dong et al., 2015; Luo et al., 2014; Tran et al., 2018). Another notable effect is the spread and proliferation of microbes that are persistent to antibiotics in the environment (Halling-Sørensen et al., 2000; Martínez, 2008).

Antibiotics are widely used in medicine and agriculture. However, only a small portion can be metabolised by humans and animals, and the rest is released into the environment (Nguyen et al., 2017). As an example, ciprofloxacin (CIP) is commonly used to treat bacterial infection and is frequently detected at elevated concentration in secondary effluent and hospital wastewater (ca. 10 – 200 µg L⁻¹) and pharmaceutical manufacturing wastewater (ca. 6.5 – 31 mg L⁻¹) (Larsson et al., 2007; Nguyen et al., 2017; Tran et al., 2018). Indeed, CIP concentrations in some of these wastewaters exceed the predicted no-effect concentrations for several aquatic organisms (Robinson et al., 2005). CIP has also been suspected to cause the development and transmission of antibiotic resistance genes in environmental microbiota (Martínez, 2008; Turolla et al., 2018; Zhang et al., 2013).

Biological treatment plays a crucial role in the removal of TrOCs prior to effluent discharge into the environment (Luo et al., 2014). Concerted research efforts in recent years have significantly improved our understanding of the biodegradation of TrOCs by biological (including both aerobic and anaerobic) treatment. For example, it has been established that...
biodegradation of TrOCs is governed by their physicochemical properties, especially the presence of either electron-withdrawing or donating functional groups in their molecular structure (Tadkaew et al., 2011; Wijekoon et al., 2015). TrOCs with electron-withdrawing functional groups are expected to be poorly removed (i.e. < 20%) while those with electron-donating functional groups are expected to be well removed (i.e. > 70%) by activated sludge treatment (Tadkaew et al., 2011). Based on this theory, Tadkaew et al. (2011) has developed a qualitative framework for the prediction of TrOC removal by activated sludge treatment.

Although the qualitative prediction framework proposed by Tadkaew et al. (2011) has been successfully validated by other authors (Li et al., 2015; Naghdi et al., 2018; Tran et al., 2018), it has not yet been able to account for occasionally peculiar and unusually high removal values of persistent TrOCs reported in the literature. Indeed, negligible removal efficiency (<15%) of CIP by activated sludge treatment has been widely reported (Jia et al., 2012; Li & Zhang, 2010; Lindberg et al., 2006) possibly due to the presence of fluoro which is a strong electron-withdrawing functional group in its molecular structure. On the other hand, CIP removal as high as 52.8% by a laboratory-scale membrane bioreactor has been reported by (Dorival-García et al., 2013). Recent research suggests that these occasionally and unusually high removal values of persistent TrOCs by biological treatment might be attributed to the microbial composition of the biomass (Vuono et al., 2016). In other words, there are rare microbial strains that can effectively metabolise otherwise poorly biodegradable TrOCs. The identification of these microbial strains and elucidation of their metabolic pathways can provide new insights into a bioaugmentation approach for the treatment of persistent TrOCs.

Although CIP is poorly biodegradable, a few CIP-degrading strains have been reported. A fluorobenzene-degrading bacterium *Labrys portucalensis* F11 could substitute the fluoride group in CIP with a hydroxyl group. This strain was isolated from an industrially contaminated site, however, the site characteristics were not provided (Amorim et al., 2014). Another CIP-
degrading strain *Thermus* sp. was isolated via a serial enrichment of pharmaceutical sludge with CIP concentration of 1, 5 and 20 mg L\(^{-1}\) (Pan et al., 2018). This strain was a thermophilic microbe (70 °C), making it difficult to apply in wastewater treatment which commonly operate at 20 to 30 °C (Pan et al., 2018). Freshwater microalgae *Chlamydomonas mexicana* showed 13% removal of CIP after 11 days of cultivation (Xiong et al., 2017). A mixture of anaerobic sulfate-reducing bacteria showed moderate degree of CIP biodegradation (Jia et al., 2018). Liao et al. (2016) reported that activated sludge could harbour CIP-degrading strains in the classes of *Gammaproteobacteria*, *Bacteroidia* and *Betaproteobacteria*. Identifying CIP-degrading strains from activated sludge is an important step towards the improvement of CIP removal.

Previous studies have demonstrated that long-term exposure of activated sludge microbiome to TrOCs can alter the microbial community and in some cases selectively enrich specific microbes with enhanced affinity for TrOCs biodegradation (Moreira et al., 2014; Navaratna et al., 2012; Qu & Spain, 2010; Terzic et al., 2018; Zhou et al., 2013). An early example was observed for acesulfame (ACE), a synthetic sweetener. ACE was reportedly persistent to biological degradation in German WWTPs with less than 5% removal in 2010 (Kahl et al., 2018) when it was first introduced in the market. Over time, the activated sludge microbial community seemingly evolved to biotransform ACE. Recently, more than 85% ACE removal by conventional wastewater treatment has been reported (Kahl et al., 2018). Exposure of activated sludge microbiome to TrOCs (at a level that is higher than the environmentally relevant concentration) could increase selective pressure and shorten the evolution time. The initial activated sludge was unable to degrade macrolide antibiotics at concentration of 1 – 10 mg L\(^{-1}\). After two months of exposure at 10 mg L\(^{-1}\), the removal efficiency was increased to 99% (Terzic et al., 2018). Accordingly, TrOC-degrading strains have been identified for the removal of previously reported persistent compounds (Moreira et al., 2014; Mulla et al., 2016; Pan et al., 2018; Yu et al., 2007).
This study aims to retrieve CIP-degrading strains from activated sludge and subsequently characterise the degradation of CIP by the strains. The strains are obtained using enrichment and isolation methods, and then further characterised in terms of their genotypes using 16S rRNA gene-based sequencing. Phenotypes of the strains are characterised against a number of abiotic factors (i.e. CIP concentrations, concentration and types of primary carbon sources). CIP removal mechanisms (i.e. abiotic, adsorption and biodegradation) are evaluated. By identifying and comprehensively examining CIP-degrading strains from activated sludge, this study provides new insights that can be used to enhance the removal persistent TrOCs by biological treatment.

2. Materials and methods

2.1 Chemicals

Analytical grade (> 98% purity) of ciprofloxacin hydrochloride monohydrate was purchased from Sigma-Aldrich (Singapore). A stock solution containing 1 g L⁻¹ was prepared in Milli-Q water for all subsequent experiments. A growth medium containing glucose (1.8 g L⁻¹), urea (35 mg L⁻¹), KH₂PO₄ (17.5 mg L⁻¹), MgSO₄ (17.5 mg L⁻¹), and FeSO₄ (10 mg L⁻¹) was prepared following a procedure previously described by Oh et al. (2013). R2A agar was purchased from (DB Diagnostics, Singapore).

2.2 Enrichment protocol

Three identical laboratory scale reactors (0.9 L active volume each) were seeded with activated sludge from an aeration tank of wastewater treatment plant in Singapore. The reactors were aerated to achieve a dissolved oxygen content of 3 mg L⁻¹ and maintained at 22.5 ± 0.5 °C. The reactors were fed every 3.5 days by withdrawing 0.3 L mixed liquor and replacing with a freshly-prepared growth medium, resulting in 10.5 days of retention time. Soluble chemical oxygen demand (sCOD) removal and volatile suspended solids (VSS) were monitored every...
3.5 days and the system achieved a steady period with sCOD removal >90% and VSS 0.98 ± 0.2 g L\(^{-1}\) after 30 days.

The reactors were then fed with growth medium containing 5 mg L\(^{-1}\) CIP for 4 months to encourage the proliferation of CIP degrading bacterial strains. The sludge was then obtained from these reactors, mixed together into an inoculum source, and incubated on agar plate (R2A agar) supplementing with 5 mg L\(^{-1}\) CIP. The derived colonies were subsequently transferred to another agar plate four times by repeated streaking culture until a single colony was confirmed. This enrichment and isolation procedure resulted in two separated colonies (designated as strain GLC_01 and GLC_02). They were then evaluated for CIP removal. Strain GLC_01 demonstrated CIP removal capacity and was selected for future experiments. A single colony of strain GLC_01 was suspended into 50 mL growth medium containing CIP and incubated on a rotary shaker at 25 °C and 150 rpm. This culture was kept as the inoculum source for genotype and phenotype characterised experiments.

2.3 Evaluation of CIP removal routes

The potential removal routes of CIP including biotransformation (experiment I), adsorption (experiment II), and utilization of CIP as sole carbon source (experiment III) by the strain GLC_01 were elucidated by batch experiments. In experiment I, the growth medium was inoculated with active GLC_01 cells at the initial OD\(_{620}\) nm of 0.1 that was equivalent to 10\(^5\) colony forming unit (CFU/mL). In experiment II, inactive (heat-killed) GLC_01 cells were added to get an OD\(_{620}\) nm of 1.6 ± 0.01. This value was pre-determined to maintain the same level of cell biomass in experiment I and II, and thus adsorption of CIP would be comparable in these two experiments. In experiment III, the growth medium was inoculated with same amount of active GLC_01 cells as in experiment I but did not have any glucose - which was the primary carbon source. All experiments were prepared with 50 mL growth medium into 250 mL-sterile flasks. Experiment IV was prepared without GLC_01 cells to determine the
removal of CIP by abiotic factors (i.e. photolysis, hydrolysis and volatilization). CIP was added in all experiments at concentration of 4.89 ± 0.01 mg L\(^{-1}\) \((n=12)\). All experimental flasks were covered and incubated on a rotary shaker at 25 °C and 150 rpm. Samples were collected at interval time of 1 day for 8 consecutive-days. All laboratory apparatuses were autoclaved at 121°C and 15 min to avoid any contamination. Residues of CIP in each experiment were measured using a HPLC method (see Section 2.5.2). COD removal was measured in experiment I (active cells). Cell growth rate in experiment I, II and III was measured using methods as described in Section 2.5.1.

### 2.4 Influence of abiotic factors on CIP removal

To evaluate the variance in influence of initial COD concentrations, a range of COD concentration (i.e. 150, 500, 1000 and 2000 mg L\(^{-1}\)) was used. Glucose was used as the primary carbon source with different amounts that were equivalent to the desired COD levels. All experimental flasks were covered and incubated on a rotary shaker at 25°C and 150 rpm. Samples were collected at interval time of 1 day for 8 consecutive-days. Other experimental conditions were maintained as in experiment I (see Section 2.3).

Influence of primary carbon sources on the performance of strain GLC_01 was elucidated in this study. Glucose, sodium acetate and starch were selected to have diverse chemical structures and biodegradable levels. Each carbon source was prepared to achieve 2000 mg L\(^{-1}\) initial COD. Other experimental conditions were maintained as in experiment I (see Section 2.3).

Impact of initial CIP concentrations on the performance of strain GLC_01 was investigated. The concentration of CIP varied from 0.05 to 10 mg L\(^{-1}\). This range was selected to represent environmentally relevant concentrations and occasionally high concentrations (e.g. in pharmaceutical manufacturing wastewater) (Halling-Sørensen et al., 2000; Jia et al., 2012; Larsson et al., 2007). Glucose was used as the primary carbon source in this experiment at a
concentration of 2000 mg L$^{-1}$ (as expressed by COD). Other experimental conditions were maintained as in experiment I (see Section 2.3).

2.5 Analytical methods

2.5.1 COD and microbial growth

COD concentration was measured by using digestion vials (Hach, Singapore) and Hach DR3900 spectrophotometer following the manufacturer’s instruction. Two analytical ranges (20 – 1500 mg L$^{-1}$) and (0 – 150 mg L$^{-1}$ COD) were used for initial and after treatment samples, respectively. VSS in the reactors was measured following the standard method 2540A. Cell growth was quantified by optical density (OD) at 620 nm and was measured using a Shimadzu DR 6000 spectrophotometer.

2.5.2 CIP concentration

CIP concentration was measured by a Shimadzu HPLC system equipped with Shim-Pack GIST Phenyl, 5 µm, 4.6 x 250 mm column (Shimadzu Asia Pacific Pte Ltd). The detection wavelength and sample injection volume were 280 nm and 100 µL, respectively. The mobile phase comprised of 60% acetonitrile and 40% Milli-Q water buffered with 25 mM NaH$_2$PO$_4$ at pH 2.5. The mobile phase was delivered in an isocratic elution mode, at 1.8 mL/min through the column for 3.5 min. The limit of quantification for CIP using these conditions was approximately 10 µg L$^{-1}$. CIP removal was calculated using the following equation: Removal ($\%) = [(C_0 - C_t) \times 100] \div C_0$, where $C_0$ and $C_t$ denote the concentration of CIP at day 0 and $t$, respectively. Statistical testing for differential CIP removal by different experiments was conducted using the Student’s t-test in Excel.

A LC-MS/MS system (Agilent 6400 Series Triple Quadrupole LC/MS-MS) was used to detect any by-products from the treatment. Electrospray ionisation (ESI) source is applied technique.

To improve MS outcomes, several preliminary experiments were conducted to optimize the
LC-MS parameters. The target components were separated on a C18 column (particle size 1.5 µm, ID 2.1 µm, L 10 cm). Two eluents, A (acetonitrile + 0.1% (v/v) formic acid) and B (water + 0.1% (v/v) formic acid) were delivered at 0.2 mL min⁻¹ through the column for 11 min in the following time-dependent gradient proportions: [Time (min), % of B] = [0, 90], [1, 90], [6, 10], [7, 10], [7.1, 90], [10, 90]. The column temperature was maintained at 35°C. The mass spectrometric data were collected from 100 to m/z 1000 in positive and negative ion mode. The cone voltage for each sample was optimised in both positive and negative ion mode. Additional detector parameters were held constant for all samples: interface temperature 350 °C; nebulizing gas flow 1.5 L min⁻¹; dry gas flow 3 L min⁻¹; DL temperature 250 °C and heating block 200 °C.

2.5.3 DNA sequencing

A single colony of the GLC_01 strain was obtained from the agar plate and cultured in 50 mL growth medium supplemented with CIP on rotary shaker for 3 days, 25 °C and 150 rpm. The culture was collected into sterile centrifuge tube and centrifuged at 6000 rpm for 5 min. Bacterial pellet was collected and subjected to DNA extraction (MoBio PowerSoil® DNA isolation kit -MOBIO, Carlsbad, CA, USA) following the manufacture’s instruction. Then polymerase chain reaction (PCR) was conducted using 1 µL of 10 pM/mL each of universal primer (27F, 5′ AGAGTTTGATCMTGGCTCAG 3′; and 1492R, 5′ TACGGYTACCTTGTTACGACTT 3′) (Lane, 1991), a pre-mixed solution (dNTP, buffer, Taq polymerase and dye), 1 µL of extracted DNA and 18 µL of Milli-Q water into 50 µL strip cap tubes. A thermocycler (Eppendorf Mastercycler) was programmed time-dependent gradient proportions: pre-heating [95°C, 5 min], denaturation [95°C, 30 second], annealing [55°C, 30 second], extension [72°C, 1.5 min], repeat 30 cycles, additional extension [72°C, 7 min], and hold [4°C, ∞ min]. PCR products were purified by an Ultraclean PCR clean up DNA
purification kit (Mo Bio Laboratories, USA). Finally, PCR products were confirmed by DNA electrophoresis. The gene sequence was conducted by 1BASE Asia (Singapore).

The 16S rRNA sequence was submitted to the NCBI BLAST database (National Center for Biotechnology Information http://www.ncbi.nlm.nih.gov/BLAST/ and run with 16S ribosomal RNA (Bacteria and Archaea) to identify close relatives with strain GLC_01. The 16S rRNA gene sequences of 11 species (99% nucleotide identity) and one outlier were used to construct the phylogenetic tree in MEGA 7.0 with a maximum likelihood method (Kumar et al., 2016). The 16S rRNA gene sequence of strain GLC_01 was deposited in GenBank under the accession number of MH297488.

3. Results and discussion

3.1 Identification of a CIP-degrading bacteria

Two bacterial strains were retrieved by enrichment and isolation (Section 2.2) from activated sludge continuously exposing to 5 mg L\(^{-1}\) of CIP in the feed. However, only strain GLC_01 showed the ability to degrade CIP in a growth medium. The DNA sequencing of the 16S rRNA gene (1326 bp) showed that strain GLC_01 shares over 99% nucleotide identity with the genus *Bradyrhizobium* (Fig. 1). This strain was classified as *Bradyrhizobium* sp. strain GLC_01.

Species of the genus *Bradyrhizobium* have been isolated from soil, contaminated site, drinking water filtration system (e.g. sand filter, granular activated carbon column) (Hayashi et al., 2016; Oh et al., 2018; Sudtachat et al., 2009).

Members of the genus *Bradyrhizobium* are aerobic microbes and are diverse in biochemical functions such as nitrification, sulphur oxidation and aromatic degradation (Hayashi et al., 2016; Oh et al., 2018; Sudtachat et al., 2009). The *Bradyrhizobium* sp. strain GLC_01 could not grow under anaerobic conditions, and thus it is an aerobic microbe. A number of genus *Bradyrhizobium* has demonstrated the ability to biotransform aromatic compounds. For
example, *Bradyrhizobium japonicum* has multiple gene copies for aromatic degradation in its genome. This strain could degrade vanillate for energy and carbon source (Sudtachat et al., 2009). *Bradyrhizobium* sp. strain JS329 has been successfully isolated from soil supplement with 5-Nitroananthranilic acid, and it expressed a number of enzymes such as dioxygenase, deaminase that cleaved the benzene ring of 5-Nitroananthranilic acid (Qu & Spain, 2010). The soybean root *Bradyrhizobium elkanii* USDA94 encodes tfdAα and cadABC gene cluster, which have been reported as degrading genes for herbicides like 2,4-dichlorophenoxyacetic acid (2,4-D)- and 2,4,5-trichlorophenoxyacetic acid (Hayashi et al., 2016). It is noted that prior to our study, no CIP-degrading strain has been isolated from activated sludge. The CIP degrading strain obtained in this study can be a supplementary bacteria source for strategic bioaugmentation of activated sludge treatment.

3.2 CIP biotransformation by the *Bradyrhizobium* sp. strain GLC_01

The time-course removals of CIP over 8 days in four different batch experimental settings that represent removal by biotransformation (I), adsorption (II), utilization of CIP as sole carbon source (III) and abiotic (IV) were presented in Fig. 2a. The observed CIP removal in each experiment were 46.7 ± 1.9% (I), 5.2 ± 1.0 (II), 3.0 ± 1.5 (III) and 2.5 ± 1.0 (IV). The results suggest that the removal pathway of CIP was mainly biotransformation (38 ± 2%). Only a small fraction of CIP was removed by adsorption and abiotic means. The removal of CIP via adsorption and abiotic means depends on its physicochemical properties (e.g. volatility and hydrophobicity). The Henry’s constant of CIP is $5.09 \times 10^{-19}$ atm m$^3$/mol (Table S1), indicating that CIP has low volatility. The log octanol-water partition coefficient (log $K_{ow}$) of CIP is 0.28 suggesting that it is hydrophilic and adsorption to activated sludge is insignificant. The results further showed that the biotransformation of CIP occurred via cometabolism. Cometabolism is the transformation of a non-growth substrate in the presence of a growth
substrate or another transformable compound. The term 'non-growth substrate' describes compounds that are unable to support cell growth as sole carbon source (Tobajas et al., 2012).

Cometabolic transformation of CIP is evident upon comparing experiment I (fed with medium + CIP) and III (fed with CIP only). There was no microbial growth in experiment III, whereas intensive growth occurred in experiment I (OD increases from 0.1 to 1.6). CIP removal at the end of incubation period in experiment III was significantly less ($P < 0.05$ by Student’s t-test) than that of experiment I ($3.0 \pm 1.5\%$ vs. $46.7 \pm 1.9\%$) (Fig. 2a). CIP removal in experiment I increased sharply after 3 days incubation (from $8.3 \pm 0.23\%$, day 1 to $37.4 \pm 2.3\%$, day 3), which coincided with high COD removal and cell growth rate (Fig 2b). Then, CIP removal was stable at $45 \pm 2\%$ until the end of incubation period (Fig 2a) after 100% COD was consumed (experiment I). The degradation of CIP followed a first-order kinetics reaction with the reaction rate constant $k$ of 2.53 (1/h) (Fig S1). Taken together, these observations led us to conclude that biological CIP removal occurred via cometabolism rather than direct metabolism by the *Bradyrhizobium* sp. strain GLC_01. Our results suggest that the isolated strain can be a new auxiliary bacterial source for the removal of CIP-containing wastewater.

The biotransformation of CIP via cometabolism by activated sludge strain is a notable finding from this study. Previous studies have reported that adsorption and biotransformation were the main removal mechanisms of TrOCs in activated sludge (Luo et al., 2014; Tran et al., 2018). However, biotransformation is preferable to adsorption in activated sludge, because adsorption is not detoxification or mineralization. The adsorbed TrOCs would require further treatment or monitoring in downstream of activated sludge or biosolids (Semblante et al., 2015). The currently study provides ample evidence that an activated sludge strain can perform biotransformation of CIP through cometabolism rather than direct metabolism. Biotransformation of TrOCs in activated sludge likely occurs via cometabolism due to their low concentration to serve as main growth substrate. Moreover, TrOC concentration varies in
wastewater. If a compound is not present for an extended period, the specific compound degrader via direct metabolism could be washed out or outcompeted by other species and only the cometabolic organisms can survive (Fischer & Majewsky, 2014). The biotransformation of TrOCs via cometabolism would have important implications for process optimization. For example, prior exposure to CIP is not required due to the cometabolism, but the presence of other substrates is crucial. Thus, altering environmental factors (e.g. primary carbon source concentration and types) can influence the CIP removal. The following section will provide the evaluations on the abiotic factors influencing CIP biotransformation.

Biotransformation of CIP by the *Bradyrhizobium* sp. strain GLC_01 was further confirmed by the detection of by-products in the aqueous phase of experiment I (i.e. active cells). The first by-product was proposed as 7-amino-1-cyclopropyl-6-fluoro-4-oxo-1,2,3,4-tetrahydroquinoline-3-carboxylic acid with m/z = 263.2 (Fig S2). This was formed due to the loss of piperazine ring. The results suggest that the N-C bond of the piperazine ring can be biologically break-down by bacteria in this study. In a previous study, N-C bond cleavage by brown-rot fungus has also been reported (Wetzstein et al., 1999). The second by-product with the m/z of 348.1 was proposed as (1-cyclopropyl-6-fluoro-8-hydroxy-4-oxo-7-(piperazinyl-1y1)-1,2,3,4-tetrahydroquinoline-3-carboxylic acid). This compound was formed by hydroxylation process. The by-products were identified based on mass spectra and the fragmentation patterns and in comparison with the previous studies (Jia et al., 2018; Paul et al., 2010; Wetzstein et al., 1999). This study appears to be the first report the biotransformation of CIP by a microbe originating from activated sludge.
3.3 Abiotic factors optimizing CIP biotransformation

3.3.1 Concentration and type of the primary carbon source

Cometabolic transformation of CIP can be influenced by the availability of cometabolites, i.e. the primary carbon source. In this study, the results revealed that COD concentration positively affected CIP biotransformation (Fig. 3a). CIP biotransformation decreased as the concentration of the carbon source (i.e. glucose) decreased. Decreasing the carbon source concentration also led to a decrease in cell growth as indicated by OD_{620 nm} measurement (Fig. 3b). High COD concentration supported strain GLC_01 growth and consequently influenced the overall CIP biotransformation.

Previous studies suggested that initial COD affected the removal of TrOCs via co-metabolic transformation (Pan et al., 2018; Tobajas et al., 2012). For instance, a thermophilic bacteria strain *Thermus sp.* degraded 52% of 5 mg L\(^{-1}\) CIP under the addition of 0.5 g L\(^{-1}\) sodium acetate and 2 days incubation (Pan et al., 2018). Increasing sodium acetate concentration to 3 g L\(^{-1}\) inhibited cell growth and CIP degradation. The inhibition of cell growth was possibly caused by high levels of sodium acetate (i.e. more carbon source) that increased the C/N ratio in the culture medium. The C/N ratio in the current study was 57:1 (for the COD of 2000 mg L\(^{-1}\)). Although this value was higher than that of a normal biological process (a mixed culture) (25-30:1), no inhibition was observed. Since the organic carbon concentration is the key operating parameter for CIP removal efficiency, strategy to increase the organic carbon concentration should also consider the C/N ratio in future study.

[FIGURE 3]

The type of primary carbon source has also been reported to affect the removal efficiency of various compounds (Xiong et al., 2017; Zhou et al., 2013). In this study, we examined the effects of different types of readily biodegradable carbon sources sodium acetate, glucose and
starch with a diverse range of chemical structures and biodegradable levels. The results showed that type of primary carbon source could affect cell growth and CIP removal. With sodium acetate and glucose in the culture medium, the strain grew at faster rate compared to starch ($P < 0.05$ by Student’s t-test). An exponential phase occurred within 3 days of incubation. The $\text{OD}_{620\text{nm}}$ measurement was much lower in starch medium ($0.14 \pm 0.01$) compared to sodium acetate ($1.47 \pm 0.03$) and glucose ($1.62 \pm 0.01$) (Fig. 4a). Conversely, above 90% of COD was consumed in sodium acetate and glucose medium, whereas only 20% of COD was used in starch ($P < 0.05$ by Student’s t-test). The removal efficiency of CIP was $46.7 \pm 1.9$, $32.5 \pm 2.8$ and $12.5 \pm 3.5\%$ (Fig. 4b) in glucose, sodium acetate and starch medium, respectively.

Biodegradability of carbon source and physiology of microorganism strains were two main reasons cited for different removal efficiencies by isolates or pure cultures (Fischer & Majewsky, 2014; Zhou et al., 2013). It is likely that the type of primary carbon source influenced the enzymatic system involved in the co-metabolic reaction. Further studies into the metatranscriptomic sequencing of strain GLC_01 under different primary carbon types may reveal the reason for the observation in this study. At this stage, the results provide information for the selection of primary carbon types.

3.3.2 Initial concentration of CIP

Initial CIP concentrations (0.05 to 10 mg L$^{-1}$) had no impact on the cell growth. For example, $\text{OD}_{620\text{nm}}$ values of the culture under CIP concentration of 0.05 and 10 mg L$^{-1}$ were similar (1.58 vs 1.52) ($P > 0.05$ by Student’s t-test). Consistently, over 94% of COD was consumed from all the cultures after 3 days of incubations. The results indicated that *Bradyrhizobium* sp. strain GLC_01 could tolerate high levels of CIP. The physiology of the bacterial cell (e.g. cell
membrane properties, presence of efflux pump) and the phenotype (e.g. degradation capacity) determine antibiotic resistance of microbes (Oh et al., 2013).

CIP concentration influenced the biotransformation capacity of strain GLC_01 (Fig. 5). At the highest CIP concentration (10 mg L$^{-1}$), the removal efficiency of CIP was 26.4 ± 4.3%. The removal efficiency increased further to 70.4 ± 7.4% when CIP concentration was reduced to 0.05 mg L$^{-1}$ (P < 0.05 by Student’s t-test). The removal efficiency of CIP by Bradyrhizobium sp. strain GLC_01 in this study is higher than previous reported values by other organisms (Pan et al., 2018; Xiong et al., 2017). Freshwater microalgae Chlamydomonas mexicana degraded only 13% of 2 mg L$^{-1}$ CIP after 11 days of incubation (Xiong et al., 2017). A thermophilic bacteria strain Thermus thermophilus, removed 55% of CIP (Pan et al., 2018). The higher degree of CIP removal by Bradyrhizobium sp. makes it a potential source of bacteria for bioremediation application.

The removal of CIP under the influence of its concentration followed the first-order reaction kinetics. The obtained first-order rate constants are summarized in Table S2. The results showed that the biotransformation rate constants (k) also decreased from 5.03 to 1.05 (h$^{-1}$) with increased CIP concentration from 0.05 to 10 mg L$^{-1}$. Overall, the results illustrated that initial CIP concentration negatively correlated with biotransformation rate by the Bradyrhizobium sp. strain GLC_01.

[FIGURE 5]

4. Conclusion

CIP-degrading Bradyrhizobium sp. GLC_01 was isolated from activated sludge. Our quantitative analyses revealed that biotransformation was the major removal pathway of CIP by Bradyrhizobium sp. GLC_01, and biotransformation occurred via cometabolism with the presence of another primary carbon source, rather than direct metabolism. Concentration and
biodegradability of the primary carbon substrate affected the extent and rate of CIP biotransformation. Higher concentration of the primary carbon substrate led to the higher removal of CIP. The biotransformation of CIP was influenced by the initial CIP concentration. The results of this study provided new insights into devising biological means (e.g. bioaugmentation) of treating CIP-containing wastewater.

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Conflicts of interest
The authors declare that they have no competing interests.

List of Figures:

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