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Original Article

Biodegradation of carbamazepine by bacterial mixed culture: An enzymatic insight

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ABSTRACT

A mixed culture of *Leifsonia shinshuensis* and *Rhodococcus zopfii* was assessed on its ability to degrade carbamazepine (CBZ). The mixed culture was found to metabolise CBZ by utilizing two enzymes, oxygen oxidoreductase and aldehyde oxidase. Correlation analysis showed that the degraded CBZ was strongly correlated ($R^2 \approx 0.9$) with bacterial culture growth. However, only the concentrations of oxygen oxidoreductase is strongly correlated ($R^2 \approx 0.9$) with both degradation of CBZ and bacterial growth, whereas the aldehyde oxidase concentration was less strongly correlated ($R^2 \approx 0.8$) with both degradation of CBZ and bacterial growth. This suggests that the mixed culture adopted two different strategies simultaneously to metabolise CBZ to support growth and reduce toxicity.

KEYWORDS: aldehyde oxidase, catechol, growth, oxygen oxidoreductase, toxicity.

INTRODUCTION

Carbamazepine and its intermediates are very toxic and carcinogenic [1, 2]. Conventional sewage treatment using chemical and physical technologies is only capable of removing less than 50% of carbamazepine, with the residual carbamazepine and its intermediates remaining in wastewater [3, 4]. With a half-life of approximately 82 days [5], carbamazepine can remain in the environment for a long time and causes negative effects on aquatic life.

Several studies reported on the drastic change in aquatic life behaviour, including inhibition of hormones in the marine organisms after being exposed to carbamazepine. Carbamazepine found in the water was reported to inhibit pupation in aquatic organisms [6], inhibit swimming activities of fish *Oryzia latipes* and induce production of the stress hormone cortisol in bluegill fish *Lepomis macrochirus* [5]. Furthermore, intermediate metabolites of carbamazepine such as acridine and hydroxyacridine can cause DNA strand breakage, inducing gene mutation and cancer malignancies [7]. Consumption of drinking water contaminated by carbamazepine causes its accumulation in the human body leading to liver dysfunction and possible foetal malformation in pregnant females [8-10]. Thus, an efficient method to remove carbamazepine needs to be developed in order to overcome this problem.

Due to the inefficiency of conventional wastewater treatment technologies [6, 11] the use of bacteria to biodegrade carbamazepine is becoming

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an interesting alternative. Biodegradation of carbamazepine using bacteria has been conducted extensively by researchers because bacteria is fast-growing and produces pharmaceuticals-degrading enzymes [6, 12]. However, the efficiency varied between species that were reported, including *Paraburkholderia xenovorans* [1], *Pseudomonas* sp. [13], *Rhodococcus rhodochrous* [14], and *Streptomyces* sp. [15]. Thus, the aim of this study was to see if a different strategy was utilised by the two different bacteria, *Leifsonia shinshuensis* and *Rhodococcus zopfii* to biodegrade carbamazepine, from bacterial growth and enzymatic point of view.

MATERIALS AND METHODS

Source of bacteria and starting inoculum

Stock culture of *Leifsonia shinshuensis* and *Rhodococcus zopfii* was obtained from Bioremediation Research Laboratory Faculty of Civil Engineering, UiTM Malaysia. The stock culture was preserved in glycerol at -80 ºC. The stock culture was revived by streaking onto nutrient agar and grown overnight at room temperature. Colonies formed on the agar were transferred to the nutrient broth for incubation at room temperature for 24 hours. The culture was resuspended in saline to give a standard concentration of approximately $X10^8$ CFU/mL. The starting inoculum of the mixed culture was prepared by mixing the standard concentration of both *L. shinshuensis* and *R. zopfii* in the ratio of 1:1 (v/v) [16].

Degradation of carbamazepine

The mixed culture was inoculated into flasks containing minimal salt solution with 10 mg/L of carbamazepine and incubated for seven days at 40 °C and shaking at 200 rpm (Infors HT Multitron Standard, Switzerland). Culture growth was determined *via* serial dilution and plating on nutrient agar and expressed as colony forming unit per millilitre (CFU/mL). Carbamazepine residue in the culture was extracted using the liquid-liquid extraction method (1:1 ratio of methanol and acetonitrile), and the extract was evaporated in a water bath (90 °C) to a final volume of 1.5 mL [17]. The sample was then injected into a Gas Chromatography-Mass Spectrophotometer (Perkin Elmer Clarus 600) to determine the residual concentration of carbamazepine.

Enzyme assay

Briefly, the culture was centrifuged (3000 x g, 15 minutes) and the cell-free supernatant was used in the detection of oxygen oxidoreductase and aldehyde oxidase. The oxygen oxidoreductase was assayed using 2,2'-azino-bis 3-ethyl benzthiazoline-6-sulphonate as substrate, and the formation of the product was detected using spectrophotometry with a wavelength set at 370 nm [15, 18]. The aldehyde oxidase was assayed using *p*-dimethyl-amino benzaldehyde and the Ehrlich Reagent (Sigma Aldrich). The formation of the product was detected using spectrophotometry with a wavelength set at 372 nm [19].

Data analysis

Statistical analyses were conducted using Statistical Package for Social Sciences 20 (SPSS 20) using one-way analysis of variance (ANOVA) with post-hoc Tukey's test, and the values were reported as mean ± standard deviation.

RESULTS AND DISCUSSION

The mixed culture degraded 100% carbamazepine within the 7-day of incubation. The mixed culture was able to biodegrade carbamazepine and used it as a carbon source to support growth, as seen in the increment of bacterial population from $x10^8$ CFU/mL to $x10^{10}$ CFU/mL (Table 1). Further supporting evidence is from the correlation analysis that shows a strong correlation ($R^2 \geq 0.9$) between the degradation of carbamazepine and the bacterial growth (Figure 1). This shows that the bacteria in the mixed culture rapidly convert the degraded carbamazepine into bacterial biomass.

The ability of the mixed culture to biodegrade carbamazepine can be attributed to the presence of oxygen oxidoreductase and aldehyde oxidase. Both enzymes were detected in the culture suggesting that these two enzymes played a vital role in the carbamazepine degradation pathway. Correlation statistical analysis shows that the concentrations of oxygen oxidoreductase are strongly correlated ($R^2 \approx 0.9$) with both degradation of carbamazepine and bacterial growth (Figure 2).

Table 1. Number of colonies and the concentration of enzymes expressed in the mixed culture of *R. zopfii* and *L. shinshuensis*.

	Colonies at day-7 ($X10^{10}$ CFU/mL)	**Enzymes' maximum concentration ($x10^{-5}$ M)**	
		Oxygen oxidoreductase	**Aldehyde oxidase**
Mixed culture	3.61 ± 4.21	1.89 ± 0.11	4.20 ± 0.21

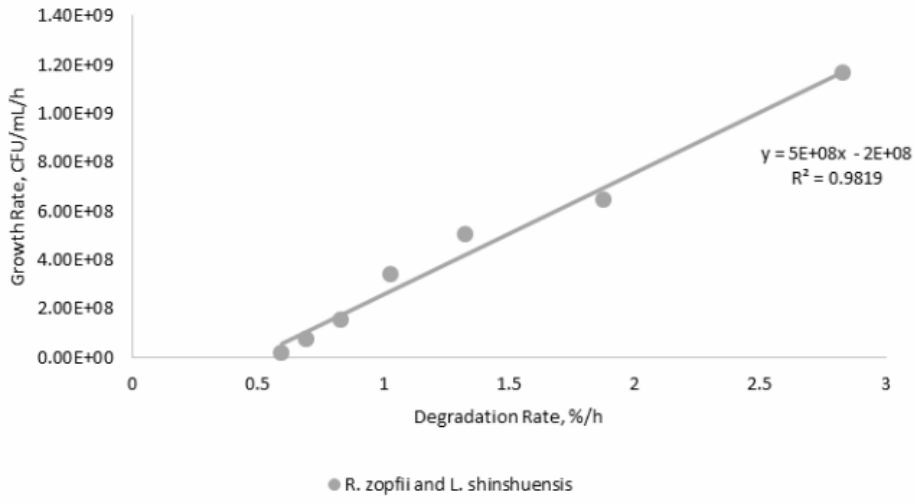

Figure 1. Correlation between growth rate and degradation rate of carbamazepine.

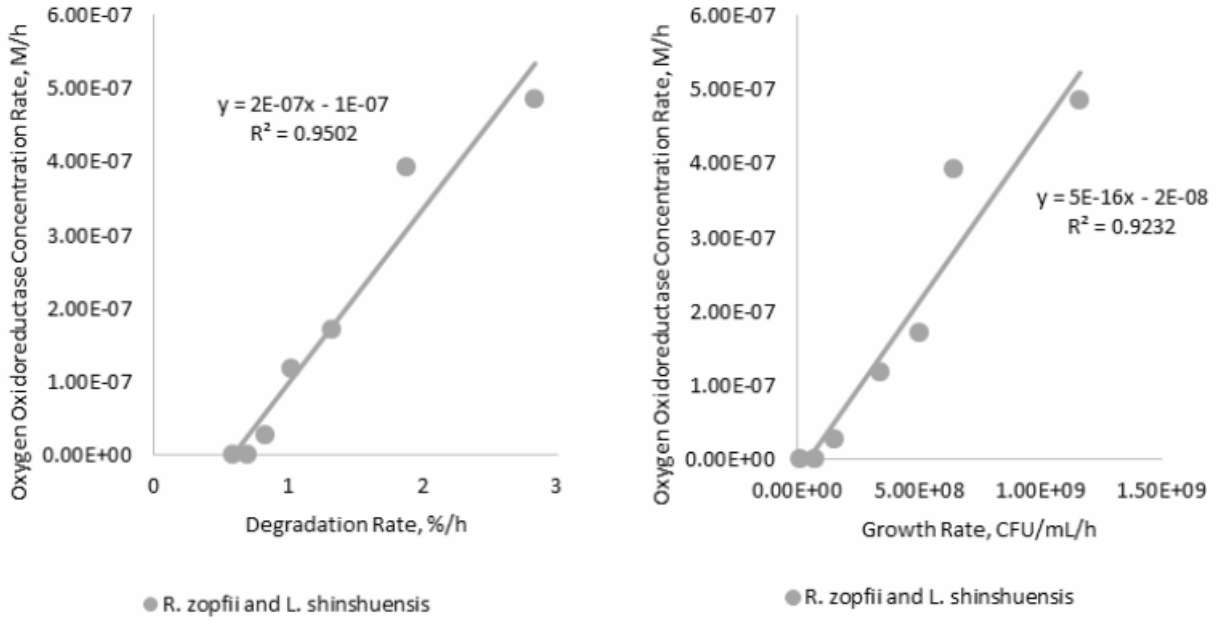

Figure 2. Correlation between oxygen oxidoreductase concentration, growth rate and degradation rate of carbamazepine.

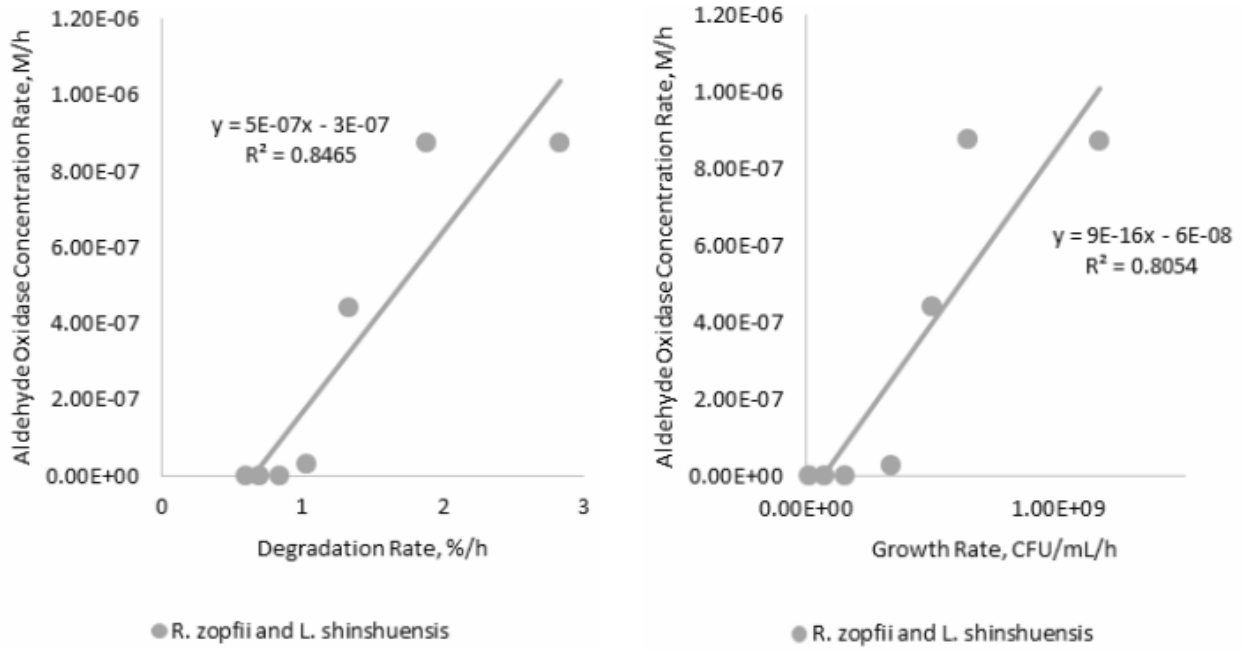

Figure 3. Correlation between aldehyde oxidase concentration, growth rate and degradation rate of carbamazepine.

However, the enzyme aldehyde oxidase concentration was less strongly correlated ($R^2 \approx 0.8$) with both degradation of carbamazepine CBZ and bacterial growth (Figure 3).

These results suggested two possible scenarios. The first is the mixed culture when exposed to carbamazepine expresses oxygen oxidoreductase to biodegrade carbamazepine and rapidly uses the metabolized carbon and converts it into bacterial biomass. The mixed culture also adopted a second strategy, whereby aldehyde oxidase was expressed to degrade carbamazepine in order to reduce the toxicity posed by carbamazepine. Aldehyde oxidase has been reported to metabolise catechol (carbamazepine central metabolite) into acetaldehyde or pyruvate, *via* meta cleavage [20]. Despite both substrates can enter the glycolytic and tricarboxylic acid cycle to produce energy that contributes to cell replication and growth [21], it is possible that the mixed culture favours other metabolites to support growth. A study on the growth of similar species *Rodococcus equi* demonstrated that this isolate prefers to metabolise organic acids such as acetate, lactate, succinate, malate, and fumarate instead of pyruvate [22], the latter being the product of aldehyde oxidase as pointed out earlier. Therefore, the mixed culture in this study containing *Rodoccocus zopfii* might also prefer to utilize acetyl-CoA or succinate, which can be generated *via* ortho cleavage of catechol, instead of meta cleavage. Only further investigation on the catechol dioxygenases activity can confirm this hypothesis. Although at present this observation does not allow the distinction whether both ortho- and meta-pathways were simultaneously utilized to biodegrade carbamazepine, it is known that the carbon from the secondary substrate may not enter all central pathways, particularly energy production, during their metabolism [23]. But the results from this study pointed to the interesting scenario that the mixed culture adopted two distinct enzymes, with two distinct aims, which are to degrade carbamazepine and convert it into bacterial biomass as well as break down carbamazepine to reduce the toxicity rapidly.

CONCLUSION

This study shows that a mixed culture containing *Leifsonia shinshuensis* and *Rhodococcus zopfii* can biodegrade carbamazepine completely and efficiently. The strong correlation of oxygen

oxidoreductase concentration and carbamazepine degradation and bacterial growth suggests that this enzyme was expressed as a strategy to obtain carbon from carbamazepine to increase bacterial biomass. On the other hand, aldehyde oxidase concentration shows a less strong correlation with carbamazepine degradation and bacterial growth, suggesting this enzyme was expressed mainly to reduce carbamazepine toxicity. Thus, the mixed culture adopted two different strategies simultaneously to remove carbamazepine more efficiently.

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CONFLICT OF INTEREST STATEMENT

There is no conflict of interest.

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