Unraveling the catalytic properties of benzylsuccinate synthase from a novel toluene-degrading strain *Aromatoleum sp.*

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The petroleum industry is one of the main causes of the excessive release of hydrocarbons into the environment. Aromatic hydrocarbons contribute to the deterioration of soil quality and biodiversity depletion. Moreover, as these compounds enter the food chain and contaminate potable water, aromatic hydrocarbons are hazardous to human and animal health, increasing the risk of carcinogenesis, miscarriage and kidney and liver damage [1]. The areas affected by oil spills are often anoxic or anaerobic. Microorganisms capable of survival under such extreme conditions have developed a unique enzymatic machinery, allowing them to incorporate recalcitrant saturated and aromatic compounds into the central metabolic pathways even without oxygen [2]. One of the biodegradation strategies is fumarate addition to the methyl group of toluene and its analogs, which is catalysed by benzylsuccinate synthase (BSS).

Benzylsuccinate synthase is a representative of the glycyl-radical enzymes family. The reaction mechanism is started by a radical transfer from Gly to Cys residue, yielding thiyl radical, which enables the activation of the methyl group of toluene. Intermediate benzyl radical addition to the double bond of fumarate results in the enantioselective formation of a product-based radical, which is quenched by hydrogen abstraction from Cys, thus forming (*R*)-benzylsuccinate and regenerating the protein-bound radical (Fig. 1) [3]. Due to the high oxygen sensitivity of BSS, reports on successful activity tests are scarce.

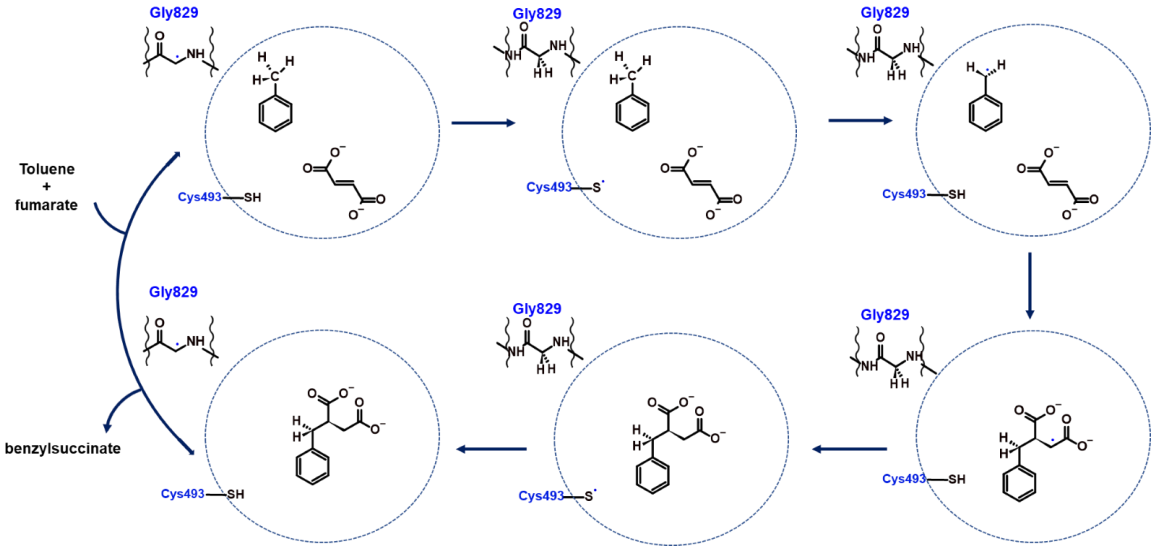

Figure 1. Proposed benzylsuccinate synthase mechanism (Heider et al., 2016, modified)

Throughout our research, a novel toluene-degrading strain of *Aromatoleum sp.* was discovered. Whole genome sequencing confirmed the presence of *bss* operon. Novel BSS isozyme shares the highest resemblance to the BSS isozyme from *Aromatoleum petrolei* ToN1. We have examined the catalytic properties of novel BSS, including the kinetic isotopic effect in a direct and competitive assay and H/D exchange on benzylsuccinate in the presence of 40% D_2O. Additionally, we have tested BSS activity with some of the toluene analogues, including cresols and xylenes isomers. All the activity tests were conducted under a strictly anaerobic atmosphere, using the cell-free extract, acquired from toluene-grown *Aromatoleum sp.* Samples collected from the reactors were subjected to LC-DAD and LC-MS/MS analysis, allowing the identification and quantification of benzylsuccinate and its derivatives.

According to our measurements, BSS isozyme produced by a novel strain *Aromatoleum sp.* exhibits a strong KIE, equal to 2.1 ±0.1 and 3.7 ±0.3 for a direct and competitive assay, respectively. These values are in agreement with the results obtained for BSS produced by *T. aromatica* T1 [4]. H/D exchange in the reaction product was proven by LC-MS/MS measurements in single ion monitoring mode, which provides experimental support for H/D exchange at the catalytic Cys493 residue [5]. At last, we have confirmed that BSS from *Aromatoleum sp.* can convert all three xylene isomers, suggesting that the strain may exhibit wider substrate preference which is an important factor for the removal of aromatic hydrocarbons from the contaminated sites.

References

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