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# Unraveling the benzylsuccinate synthase catalytic properties: modeling vs the experimental approach

## AUTHORS

Gabriela OLEKSY / JERZY HABER INSTITUTE OF CATALYSIS AND SURFACE CHE, NIEZAPOMINAJEK ST. 8, CRACOW
Anna SEKUŁA / JERZY HABER INSTITUTE OF CATALYSIS AND SURFACE CHE, NIEZAPOMINAJEK ST. 8, CRACOW
Kai KRÄMER / FACULTY OF BIOLOGY, UNIVERSITY OF MARBURG, GERMANY, KARL-VON-FRISCH-STRASSE 1, MARBURG
Ivana ALEKSIĆ / JERZY HABER INSTITUTE OF CATALYSIS AND SURFACE CHE, NIEZAPOMINAJEK ST. 8, CRACOW
Maciej SZALENIEC / JERZY HABER INSTITUTE OF CATALYSIS AND SURFACE CHE, NIEZAPOMINAJEK ST. 8, CRACOW
Johann HEIDER / FACULTY OF BIOLOGY, UNIVERSITY OF MARBURG, GERMANY, KARL-VON-FRISCH-STRASSE 1, MARBURG
Antonio PIERIK / FACULTY OF CHEMISTRY, TECHNICAL UNIVERSITY OF KAISERSLAUTERN, GERMANY, ERWIN-SCHRÖDINGER-STRASSE 54, KAISERSLAUTERN

## PURPOSE OF THE ABSTRACT

Benzylsuccinate synthase (BSS) is a model Fumarate Adding Enzyme (FAE), which belongs to the glycyl radical enzyme (GRE) family. The presence of a glycyl radical in the active centres of FAE makes those enzymes extremely sensitive to oxygen exposure. The role of FAEs in the environment is the initiation of anaerobic degradation pathways of aliphatic and aromatic hydrocarbons. BSS is produced by various anaerobic microorganisms, such as Thauera aromatica K172 and catalyzes the stereospecific addition of toluene to fumarate yielding (R)-benzylsuccinate. Due to the severe susceptibility of BSS to inactivation, the best strategy to study its catalytic mechanism is a combination of experimental and in silico techniques.
The presented research focuses on the investigation of the catalytic properties of BSS produced natively by toluene-grown T. aromatica K172 and a recombinant BSS produced in the related species Aromatoleum evansii, which does not degrade toluene. The goal of this experiment was to verify the probable mechanism for the formation of an intermediate radical product with the inhibitors benzyl alcohol/benzaldehyde and fumarate.
We followed changes in the glycyl-radical EPR signal in the presence of two inhibitors i.e., benzyl alcohol (B-OL) and benzaldehyde (B-ADH). B-OL and B-ADH inhibition kinetics were determined with T. aromatica K172 cell extracts under a strictly anaerobic environment at a pH of 7.8, at constant substrate concentrations (5 mM fumarate and 2 mM toluene) and inhibitors added to 5-100 µM. Benzylsuccinate production was quantified by the LC-DAD method, while product identification was conducted by LC-MS/MS.

Interpretation of the observed EPR signals was supported by the simulation of spectra using the EasySpin program. The experimental spectra obtained with unlabeled and isotopically labelled (2H and 13C) B-OL and fumarate were analyzed. Isotropy and anisotropy of the hyperfine coupling constants (A) were tested, as well as a varying range of A, g value and linewidth parameters.

Inhibition tests showed that adding 5 µM B-OL results in a 70% decrease of BSS activity and 100 µM led to almost complete inhibition. The B-ADH was a weaker BSS inhibitor caucusing a 50% and 95% decrease in activity, respectively, at 5 and 100 µM. The modelling result suggests the formation of a resonance-stabilized dehydrated

allylic radical intermediate.

The substrate preference of BSS for cresols and xylenes was investigated with cell extracts of recombinant WT and Y193S and Y193F mutants of BSS from A. evansii. The MS/MS analysis confirmed the activity of Y193F mutants with cresols and xylenes. A substrate preference shift from m-xylene to o-xylene was observed for the Y193F mutant.

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## FIGURES

### FIGURE 1

### FIGURE 2


## KEYWORDS

anerobic enzymes | biodegradation | EPR


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