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Biotransformation of dioxins by assembling RW1 upper pathway gene cassettes in *Escherichia coli*

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Abstract. *Mutter TY, Zylstra GJ, Huang X. 2023. Biotransformation of dioxins by assembling RW1 upper pathway gene cassettes in* Escherichia coli. *Biodiversitas 24: 3648-3656. Rhizorhabdus wittichii* RW1 (formerly known as *Sphingomonas*) is one of the few bacterial strains known to grow and metabolize dibenzofuran and dibenzo-*p*-dioxin as a carbon source. The rare ability of strain RW1 to transform both substrates suggests the involvement of unidentified genes. Its genome sequence showed that RW1 has an extreme redundancy of ring cleavage dioxygenases and hydrolases. RW1 genes were assembled on an expression vector to provide additional experimental evidence that both substrates are metabolized in RW1 by two different sets of hydrolases. Three different combinations of the ring cleavage dioxygenase gene (*dbfB2*) with three hydrolases (*dxnB1*, *dxnB2*, and *dxnB3*) were cloned on an expression vector (pET30a) in *Escherichia coli* BL21 (DE3), and the enzymes' roles were tested against DD and DF transformation. The results of the heterologous expression in *E. coli* showed that DbfB2 can transform both intermediates 2,2',3-trihydroxybiphenyl (THD) and 2,2',3-trihydroxybiphenyl ether (THDE) from DF and DD, respectively. The two hydrolases DxnB1 and DxnB2 are involved only in transforming the DF intermediate 2-hydroxy-6-oxo-6-(2-hydroxyphenyl)-hexa-2,4-dienoate (2OH-HOPDA) into salicylate. The newly identified hydrolase DxnB3 is involved only in transforming 2-hydroxy-6-oxo-6-(2-hydroxyphenoxy)-hexa-2,4-dienoate (2OH-O-HOPDA) into catechol in the DD pathway. The study clarifies and answers the question regarding the rare ability of other organisms that can degrade dibenzofuran but can't degrade dibenzo-*p*-dioxin. The results showed that the hydrolases involved in DD degradation differ from those involved in DF degradation, as previously known that the same hydrolases are involved in the two pathways. All genes are assembled on one cassette for the first time, which has never been done previously.

Keywords: Degradation, heterologous expression, hydrolase, ring cleavage dioxygenase

INTRODUCTION

Dioxins and related compounds are a class of substances that share a common structural composition, featuring two coplanar benzene rings (Van den Berg et al. 2006). Dioxins are persistent environmental toxins having a major impact on air quality that continue to cause health problems for people (Masiol et al. 2016; Knutsen et al. 2018). Studies have shown that persistent organic dioxins like Dibenzo-*p*-Dioxin (DD), Dibenzofuran (DF), and their chlorinated congeners enter the environment primarily via atmospheric transfer through improper waste incineration and natural process such as forest fires (Li et al. 2016; Alharbi et al. 2018). Chlorinated derivatives of dibenzo-*p*-dioxin, which are thought to be the most dangerous dioxins, have received a lot of attention (Hashimoto et al. 2022). Their route into the human body from the atmosphere is mainly through ingestion, food consumption, or other liquids contaminated with dioxins (Kanan and Samara 2018).

Microbial degradation of dioxins is frequently demonstrated using non-substituted Dibenzofuran (DF) or Dibenzo-*p*-Dioxin (DD) as a classic dioxin compound substrate (Meglouli et al. 2019). *Rhizorhabdus wittichii* RW1 was isolated for its ability to grow on DF and DD as a sole source of carbon and energy and capability to metabolize several chlorinated dioxins (Wittich et al. 1992). It is among the most studied microorganism in terms of the number of congeners transformed, the elucidation of dioxin catabolic pathways, and their use as prospective candidates for contaminated soil remediation (Saibu et al. 2020). Most catabolic genes and electron transport components in dioxin degradation upper pathway have been identified in RW1 strain (Hartmann and Armengaud 2014; Chai et al. 2016). Most of the enzymes thought to be involved in the upper pathway, DF into salicylate and DD into catechol, have been purified and biochemically characterized. The first enzymatic step has been characterized as a multicomponent enzyme composed of a dioxygenase two subunits *dxnA1dxnA2*, a reductase *redA2* or *redA1* and a ferredoxin *fdx1*or *fdx3* (Faisal 2019). The second enzymatic step is catalyzed by a *meta* cleavage dioxygenase (Bünz et al. 1993; Happe et al. 1993). The best studied ring cleavage enzyme, encoded by *dbfB* (SWIT4902), has a high affinity towards 2,3-dihydroxybiphenyl (2,3-DHB) and 2,2',3-trihydroxybiphenyl (2,2',3-THB) but practically no activity against 2,2',3-

trihydroxybiphenyl ether (2,2',3-THBE) (Happe et al. 1993). Recently, a research showed that the second enzymatic step in DF degradation is driven by *dbfB* and that this enzyme has no role in DD degradation. Gene knockout of the newly identified *meta* cleavage enzyme SWIT3046 (dbfB2) abolished the growth of RW1 on DD when used as a substrate (Mutter and Zylstra 2021a). Another study found that DbfB2 was superior in THBE transformation compared to other extradiol dioxygenases in RW1 (Hassan et al. 2022).

The third enzymatic step of dioxin degradation in RW1 involves two hydrolases named DxnB1 (SWIT4895) and DxnB2 (SWIT3055) in the DF pathway and a newly identified hydrolase named DxnB3 (SWIT0910) in the DD pathway (Mutter and Zylstra 2021b). The genes *dxnB1* and *dxnB2* are involved only in transformation of 2-hydroxy-6-oxo-6-(2-hydroxyphenyl)-hexa-2,4-dienoate (2OH-HOPDA) into salicylic acid while only *dxnB3* is involved in transformation of 2-hydroxy-6-oxo-6-(2-hydroxyphenoxy)-hexa-2,4-dienoate (2OH-O-HOPDA) into catechol.

Microbial bioremediation of pollutants have attracted significant global interest due to its eco-friendliness, social acceptance, and lower health risks. Furthermore, with recent advances in biotechnology and microbiology, genetically engineered bacteria with a high ability to remove environmental toxins are frequently used, resulting in powerful bioremediation (Liu et al. 2019; Mishra et al. 2021). Engineering RW1 genes on a transmissible plasmid is a promising technique that can be used in an efficient strain like *Escherichia coli* to improve dioxins bioremediation, especially since no studies have tried to assemble all these genes together on one plasmid.

The present work aims to assemble different combinations of the *dbfB2* gene for the *meta* cleavage enzyme with the *dxnB1*, *dxnB2*, *dxnB3* genes for the three hydrolases along with the genes for the initial angular dioxygenase on an expression vector (pET30a) in *E. coli* BL21 (DE3) to provide additional experimental evidence of the involvement of the above-mentioned enzymes in DD and DF metabolism. Dioxins upper pathway genes have never been assembled on one plasmid and all other works speculate the functions of those genes. This work indicates the enzymes' exact function and role in dioxins metabolism.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions

Rhizorhabdus wittichii RW1 is the source of the genes that were PCR amplified. Clones were routinely transformed into *E. coli* DH5α and then transformed into *E. coli* BL21 (DE3) for expression from the T7 promoter. All plasmid transformations into competent *E. coli* were performed by the calcium chloride-glycerol transformation procedure (Green and Sambrook 2012). The pGEM-T easy vector series (Promega, Madison, Wisconsin) of cloning vectors were used to construct subclones and pET30a was used to construct subclones used for protein overexpression. Plasmid DNA was purified using NucleoSpin Plasmid Kit, Machery-Nagel following the manufacturers' instructions. DNA fragments were purified from PCR amplification and gels using the GeneClean III Kit, MP Biomedical following the manufacturers' instructions. DF and DD were used as crystals or dissolved in acetone in a sterile flask to a final concentration of 3 mM and the flasks were left in a fume hood for 6 hours to allow for complete evaporation of acetone. LB agar and LB broth were used as an enrichment media. *Escherichia coli* strains were aerobically cultured in LB medium at 37°C. Ampicillin and kanamycin were added to the medium when needed at 100 and 50 µg/mL, respectively.

Vector construction of the gene encoding the meta-cleavage enzyme

Unless otherwise specified, RW1 chromosomal DNA was used as a template for PCR reactions used in plasmid constructions. Total genomic DNA was extracted using Ultra Clean Microbial Kit, Qiagen. Phusion High-Fidelity kit from New England Biolabs was used to amplify the genes for overexpression studies. A 0.98 kb of SWIT3046 (*dbfB2*) was amplified using the primers dbfB2_F and dbfB2_R_containing restriction sites *BamHI* and *NotI*, respectively (Table 1). The resulting fragment was TA cloned into the pGEM-T Easy vector to form pGfB2, transformed into DH5α and sequenced. pGfB2 plasmid was isolated and digested with *BamHI* and *NotI*, and ligated into similarly digested pET30a to make pETfB2. The final construct was transformed into BL21 (DE3). A 3.46 kb *XbaI* and *BglII* fragment of a clone containing the initial angular dioxygenase (with genes *dxnA1A2*, *fdx3*, and *redA2*) (Faisal 2019) was ligated into pETfB2 after digestion with *XbaI* and *BamHI* (*BamHI* compatible end with *BglII*) to make pETDfB2. The final construct was transformed into BL21 (DE3). All constructs were confirmed by PCR and plasmid digestion.

Vector construction of the genes encoding the meta-cleavage enzyme dbfB2 plus a hydrolase

The PCR primers for all genes were designed using DNASTAR Lasergene 8.1, DNASTAR Inc, USA (Table 1). All genes were amplified using RW1 genomic DNA and a Phusion HF DNA polymerase kit. Three constructs were assembled using PCR overlap extension that consists of *dbfB2* plus a hydrolase as following: a 0.93 kb of *dbfB2* was amplified using the primers dbfB2_F and dbfB2xB1_R. A 0.89 kb of *dxnB1* was amplified using the primers dxnB1fB2_F and dxnB1_R. The two fragments were PCR purified and mixed in one PCR at 1:1 ratio without adding any primer using the following conditions: 10 cycles of denaturation for 30 s at 98°C, annealing for 30 s at 72°C, and extension for 2 min at 72°C. Then, the primers dbfB2_F and dxnB1_R were added to the reaction mixture and returned to the PCR machine for another 25-cycle using the same conditions. The resulting 1.8 kb fragment was PCR purified and TA cloned into pGEM-T vector to yield pGfB2xB1, transformed into DH5α and sequenced. A 0.93 kb of *dbfB2* was amplified using the primers dbfB2_F and dbfB2xB2_R. A 0.89 kb of *dxnB2* was amplified using the primers dxnB2fB2_F and dxnB2_R. The two fragments were ligated by PCR overlap extension as mentioned above by using the primers

dbfB2_F and dxnB2_R. The resulting 1.8 kb fragment was PCR purified and TA cloned into pGEM-T vector to yield pGfB2xB2, transformed into DH5α and sequenced. A 0.93 kb of *dbfB2* was amplified using the primers dbfB2_F and dbfB2xB3_R. A 0.81 kb of *dxnB3* was amplified using the primers dxnB3fB2_F and dxnB3_R. The two fragments were ligated by PCR overlap extension as mentioned above by using the primers dbfB2_F and dxnB3_R. The resulting 1.7 kb fragment was PCR purified and TA cloned into pGEM vector to yield pGfB2xB3, transformed into DH5α and sequenced.

The three constructs (pGfB2xB1, pGfB2xB2, and pGfB2xB3) were digested with *BamHI* and *HindIII* restriction enzymes and at the same time the angular dioxygenase construct (pETD) was digested with *XbaI* and *BglII* (*BglII* is compatible end with *BamHI*) and cloned by three-way ligation into pET30a after digestion with *XbaI* and *HindIII* to form the final constructs pETDfB2xB1, pETDfB2xB2, and pETDfB2xB3. The final constructs were transformed into *E. coli* Bl21 (DE3) to yield Bl21_pETDfB2xB1, Bl21_pETDfB2xB2, and Bl21_pETDfB2xB3 (Figure 1).

Table 1. List of primers used in this study

Primer's name	Sequence	Tm	Descriptions
dbfB2_F	GGATCCACGATCCTGATCATGAGGTGTTCCG	72°C	Contains *BamHI* and *NotI* restriction sites to form *dbfB2*
dbfB2_R	GCGGCCGCGCAGGCTTCCGACCCGGTG		gene
dbfB2xB1_R	GACTTATTCCGTCAATGCGCGTGCGCGTGT	72°C	Contains overlap sequence with the beginning of *dxnB1*
dxnB1fB2_F	CGCATTGACGGAATAAGTCGCCCGCGAAGTGAG		Contains overlap sequence with the end of *dbfB2*
dxnB1_R	AAGCTTGCGGCATGCTAGAATTTCCGAGCG		Contains *HindIII* restriction site
dbfB2xB2_R	GCAATGCTCAATGCGCGTGCGCGTGT	72°C	Contains overlap sequence with the beginning of *dxnB2*
dxnB2fB2_F	CGCATTGAGCATTGCCGGTCGGTGAATA		Contains overlap sequence with the end of *dbfB2*
dxnB2_R	AAGCTTCGGCCATCGATCAATCCAGCTTTCCG		Contains *HindIII* restriction site
dbfB2xB3_R	AGCGATCCTCAATGCGCGTGCGCGTGT	72°C	Contains overlap sequence with the beginning of *dxnB3*
dxnB3fB2_F	CGCGCATTGAGGATCGCTGGCGAGGGGAGGATTT		Contains overlap sequence with the end of *dbfB2*
dxnB3_R	AAGCTTCGAATGTTCGTCGCTCATGCTGCCCC		Contains HindIII restriction site

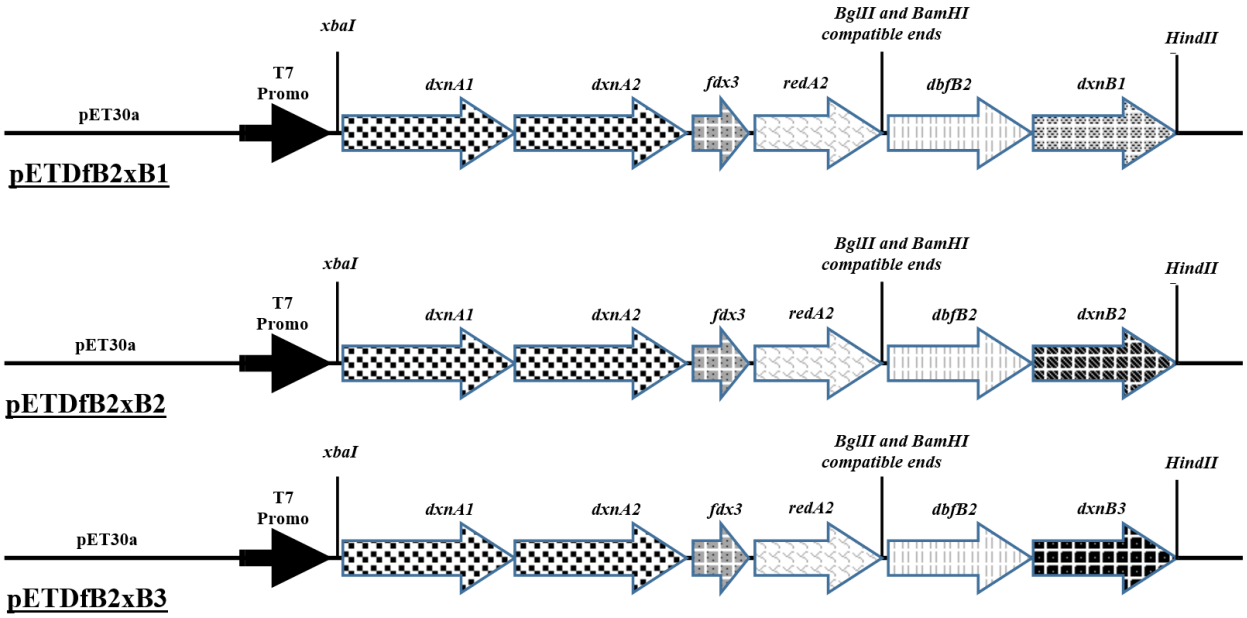

Figure 1. Gene map of the three expression constructs. pETDfB2xB1 contains the angular dioxygenase, the ring cleavage dioxygenase (*dbfB2*), and the hydrolase *dxnB1*. pETDfB2xB2 contains the angular dioxygenase, the ring cleavage dioxygenase (*dbfB2*), and the hydrolase *dxnB2*. pETDfB2xB3 contains the angular dioxygenase, the ring cleavage dioxygenase (*dbfB2*), and the hydrolase *dxnB3*

Resting cell assays

Escherichia coli strains Bl21_pETDfB2, Bl21_ pETDfB2xB1, Bl21_ pETDfB2xB2, and Bl21_ pETDfB2xB3 were cultivated overnight in 50 mL LB broth containing 50 µg/mL kanamycin (km) in a rotary shaker at 180 rpm at 37°C. 2 mL of the preculture was inoculated into 100 mL LB broth containing km. When the optical density at 600 nm reached 0.5, Isopropyl-b-D-Thiogalactopyranoside (IPTG) was added to a final concentration of 1 mM and the cells were incubated for another 1h. The cells were harvested by centrifugation, washed twice with 50 mM sodium phosphate buffer (pH 7.2) and resuspended in the same buffer supplemented with 20 mM glucose and 3 mM DF or DD. After overnight incubation, the enzyme products were assayed by observing the presence or disappearance of a bright yellow color of the supernatants since this type of intermediate is unstable and very difficult to detect (Kohler et al. 1993). The cells were removed by centrifugation and the supernatants were collected for analysis by High-Performance Liquid Chromatography (HPLC).

When needed, metabolites were extracted with ethyl acetate after acidification to pH 2.0 with HCl (concentrated). The ethyl acetate layer was dried with anhydrous sodium sulfate and then evaporated to dryness, dissolved in acetonitrile and kept at -20 for further analysis.

Monitoring of intermediates (ring cleavage products) formation

The ring cleavage products were monitored by spectral scanning of resting cell supernatants with a Beckman DU-70 spectrophotometer. Accumulation of the intermediates was followed by High Performance Liquid Chromatography (HPLC). A gradient of 0 to 100 methanol in water under acidic (1% acetic acid) conditions with a reverse-phase 5 mm C18 column (length 250 mm) was used to separate the different compounds.

RESULTS AND DISCUSSION

This study work was performed to identify and verify the enzymes involvement in the DD and DF pathways through heterologous expression in *E. coli*. Three cassettes were constructed in an expression vector under T7 promoter control that contain the angular dioxygenase system (*dxnA1A2*, *fdx3* and *redA2*), a ring cleavage dioxygenase (*dbfB2*), and one of three hydrolases mentioned in the Methods (*dxnB1*, *dxnB2*, *dxnB3*). All clones are in the T7 expression vector pET30a and all cloned fragments were verified by sequencing. RW1 angular dioxygenase was cloned in *E. coli* and showed that the enzyme has the ability to perform angular deoxygenation towards DF, DD, Xanthene, and Xanthone (Faisal 2019). Also, the angular dioxygenase used to engineer *Sphingobium yanoikuyae* B1DR to transform xanthene to 2- hydroxyphenylacetate (Faisal and Al-Shiti 2023).

Expression of the ring cleavage dioxygenase (dbfB2) in *E. coli* and verification of intermediates

Bl21_pETDfB2 was constructed and transformed into BL21 (DE3) and induced with IPTG as described in the Methods. Culture supernatants were characterized by observing the bright yellow color of the supernatants, the yellow color formation is a measurement of making the ring cleavage products (Kohler et al. 1993), and HPLC analysis through noticing disappearance of the THB and THBE peaks from DF and DD, respectively. Since the meta cleavage products are very difficult to detect by HPLC, the supernatants were compared with the authentic compounds 2,2',3-THB (retention time of 31 min) and 2,2',3-THBE (retention time of 30.2 min) from DF and DD, respectively. HPLC results showed that cells harboring the construct pETDfB2 can attack both intermediates (Figure 2 and Figure 3). Both supernatants showed bright intense yellow color indicates making the ring cleavage products. Both intermediates 2,2',3-THB and 2,2',3-THBE peaks from DF and DD; respectively, disappeared in the HPLC analysis comparing with the authentic compounds. This proved that DbfB2 is able to transform 2,2',3-THB and 2,2',3-THBE into 2OH-HOPDA (Bünz et al. 1993) and 2OH-O-HOPDA (Wittich et al. 1992); respectively, indicating the involvement of the previously mentioned ring cleavage dioxygenase on both substrates. The results are in agreement with other findings regarding *dbfB2* which indicates that *dbfB2* can substitute for *dbfB1* to enable RW1 growth on DF and also that gene knockout of *dbfB2* abolished RW1 growth on DD as a source of carbon (Mutter and Zylstra 2021a).

Expression of the ring cleavage dioxygenase (DbfB2) plus a hydrolase

The three cassettes pETDfB2xB1, pETDfB2xB2, and pETDfB2xB3 were tested for their ability to transform DF and DD into salicylate and catechol, respectively. The HPLC analysis indicated that the two constructs pETDfB2xB1 and pETDfB2xB2 transformed DF into salicylate (retention time of 29 min) (Figure 4 and Figure 5). While no salicylate was detected in the supernatant of cells containing the construct pETDfB2xB3 (Figure 6). Additionally, the yellow color disappeared from the supernatants of the cells harboring *dxnB1* and *dxnB2* while the yellow color was still present in the supernatants of cells harboring *dxnB3* (Figure 7). Which indicates that only the two hydrolases *dxnB1* and *dxnB2* are involved in DF metabolism. This confirms the previous work identifying two hydrolases involved in vivo in metabolism of DF (and not DD) by RW1(Mutter and Zylstra 2021b).

When DD was used as a substrate for the resting cells assay, the yellow color didn't disappear from the supernatants of the cells harboring *dxnB1* (Bl21_pETDfB2xB1), *dxnB2* (Bl21_pETDfB2xB2), and *dxnB3* (Bl21_pETDfB2xB3). Also, no catechol peaks showed up when the cell's supernatant was analyzed by HPLC. This means that either the three hydrolases are not involved in the DD upper pathway or that any formed catechol could be cleaved by the *meta* ring cleavage dioxygenase, *dbfB2*. So, the ability of the ring cleavage dioxygenase was tested to cleave catechol by using catechol as a substrate for cells harboring only *dbfB2* (Bl21_pETfB2). The results showed that DbfB2 was superior in cleaving catechol and forming the ring cleavage

product within a few seconds. The product was confirmed by spectrophotometry to be identical to the ring cleavage product of catechol (2-hydroxymuconic semialdehyde) with absorbance at 375 nm (Omori et al. 1986; Asimakoula et al. 2023). This result is in agreement with the finding by Hassan et al. (2022) that showed that the enzyme edo4 (DbfB2) transformed catechol with a reasonable activity. The supernatants of the cells harboring the three constructs were analyzed spectrophotometry and the results showed that only the supernatants from the cells harboring *dxnB3* showed an absorbance at 375 nm (Figure 8). Which indicates that catechol was formed only in the supernatant of the cells containing *dxnB3* and that catechol was cleaved by DbfB2 into 2-hydroxymuconic semialdehyde, the yellow-colored product.

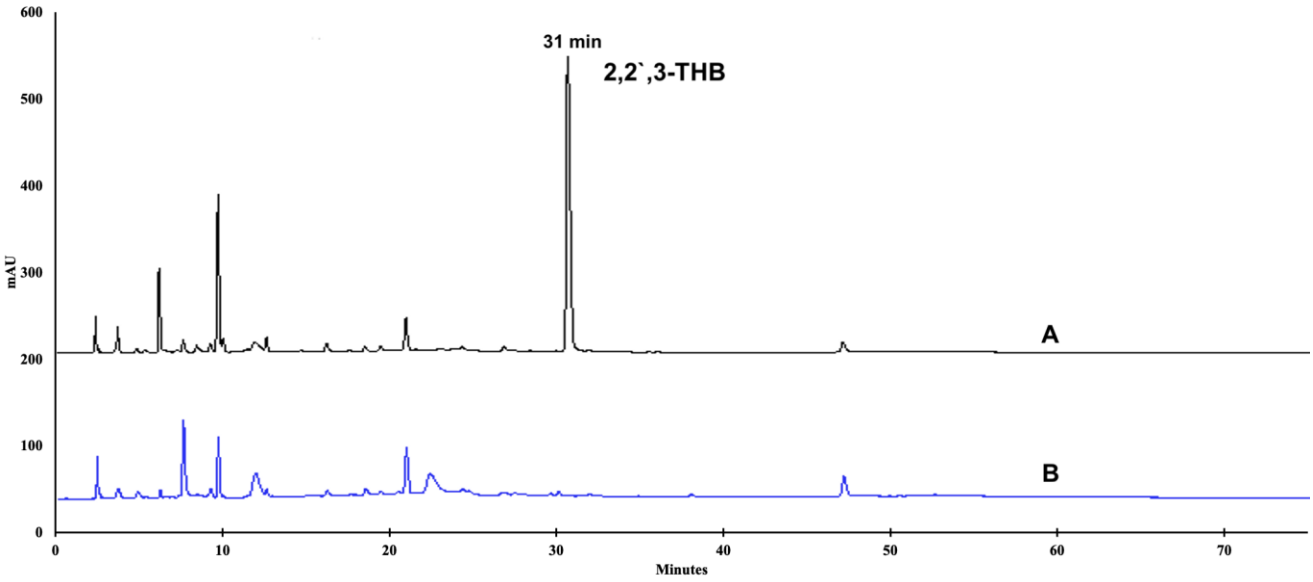

Figure 2. HPLC analysis of the cells supernatant on DF: A. Supernatant of the cells Bl21_pETD that contains only the angular dioxygenase (dxnA1A1_fdx3_redA2) indicating presence of 2,2´,3-THB peak at retention time of 31 min, B. Supernatants of the cells Bl21_pETDfB2 that contains the angular dioxygenase plus the ring cleavage dioxygenase (dxnA1A1_fdx3_redA2_dbfB2) that shows disappearance of the 2,2',3-THB from the supernatant

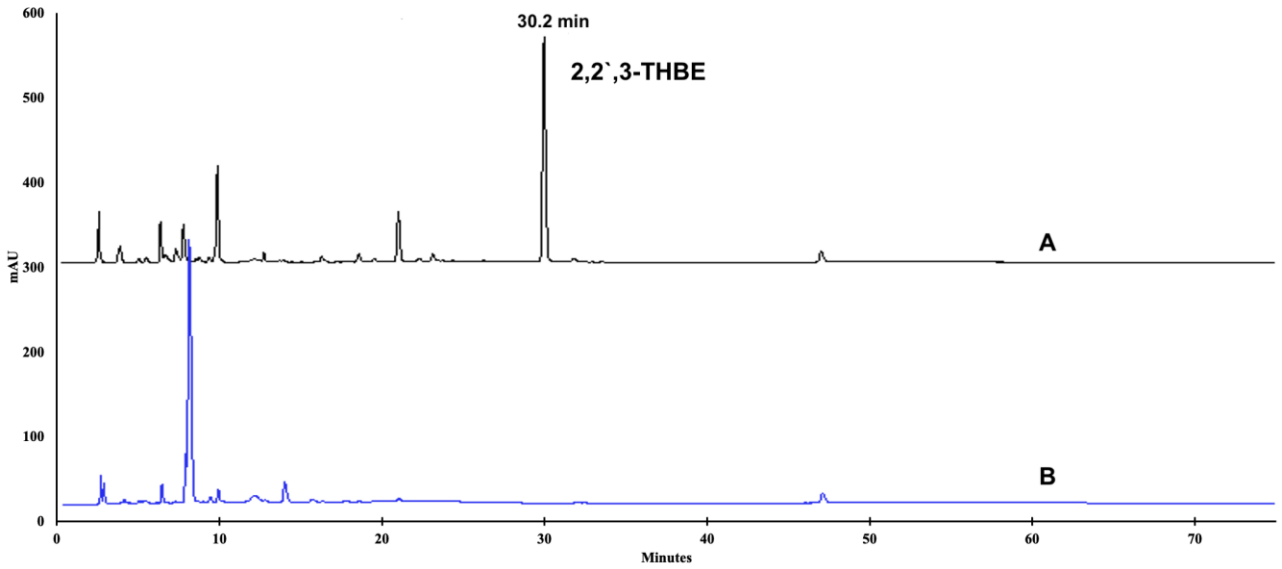

Figure 3. HPLC analysis of the cells supernatant on DD: A. Supernatant of the cells Bl21_pETD that contains only the angular dioxygenase (dxnA1A1_fdx3_redA2) indicating presence of 2,2´,3-THBE peak at retention time of 30.2, B. Supernatants of the cells Bl21_pETDfB2 that contains the angular dioxygenase plus the ring cleavage dioxygenase (dxnA1A1_fdx3_redA2_dbfB2) that shows disappearance of the 2,2',3-THBE from the supernatant

Microorganisms such as bacteria and fungi are more effective in degrading polyaromatic hydrocarbons and contaminants removal from the environment (Sari et al. 2019; Cao et al. 2020). Microbial-based bioremediation is one of the most recent strategies due to the incredible metabolic ability to metabolize a wide variety of organic materials and their ability to resist harsh environmental conditions (Novianty et al. 2022; Yamini and Rajeswari 2023). Several studies have tried to isolate new bacterial strains to degrade such toxic chemicals. However, the efficiency of degradation of those isolates is either very low or they can only metabolize non dioxins compounds. Recently, engineered *E. coli* was used to metabolize several aromatic compounds (Yetti et al. 2016; Thacharodi et al. 2023). Moreover, using bioinformatics and genetic engineering techniques could help in enhancing the bioremediation of toxic hydrocarbons in contaminated environments (Tan and Kusuma 2021; Vasandani and Tan 2022). Wang et al. (2019) showed that using engineered *E. coli* can metabolize phenol completely through introducing two cassettes that transform phenol to catechol and catechol into the TCA cycle. *Escherichia coli* is widely used as a host for several genetic manipulations.

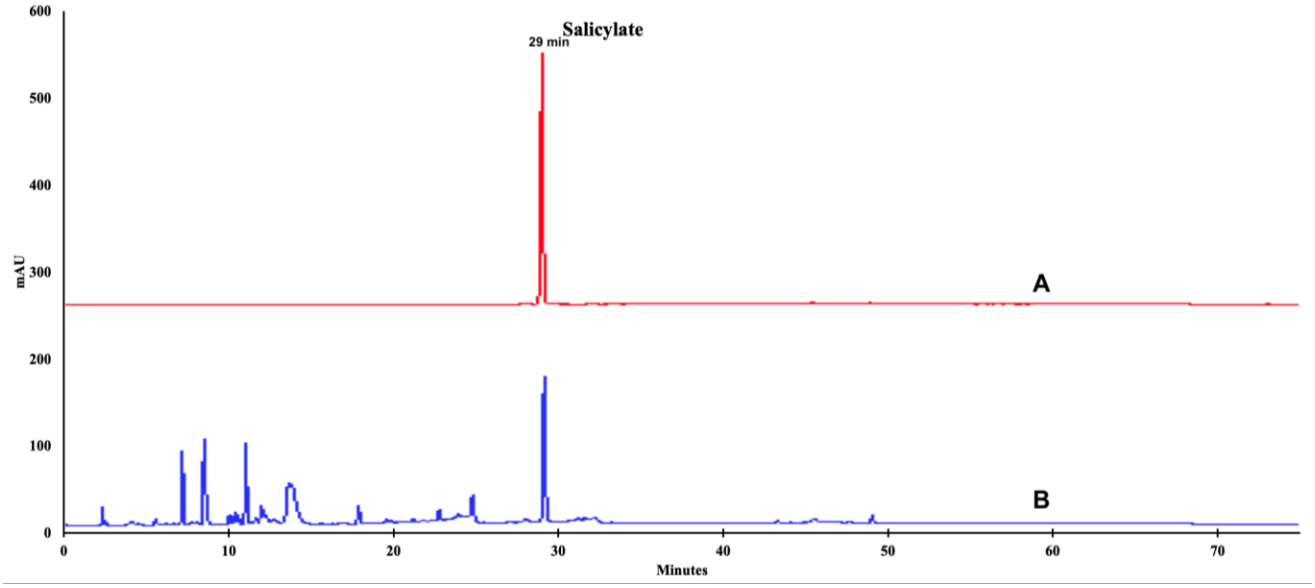

Figure 4. HPLC analysis of the cells supernatant on DF: A. Standard salicylate (1 mM), B. Supernatant of the cells Bl21_pETDfB2xB1that contains the angular dioxygenase, the ring cleavage dioxygenase, and the hydrolase *dxnB1* which shows the salicylate as an intermediate at retention time of 29 min

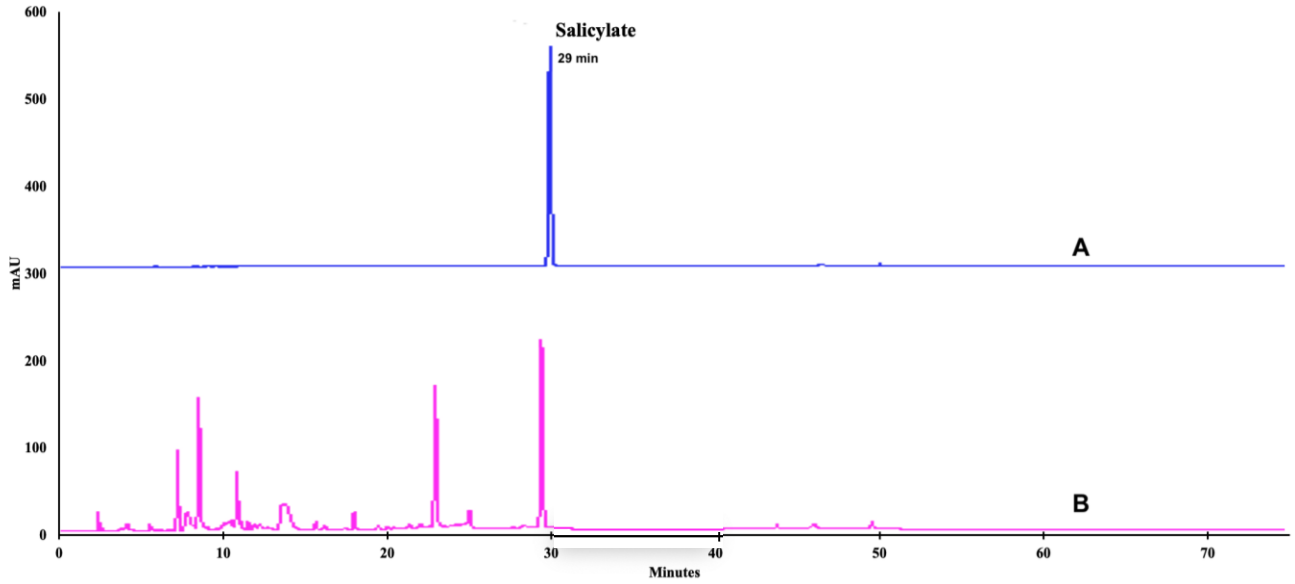

Figure 5. HPLC analysis of the cells supernatant on DF: A. Standard salicylate (1 mM), B. Supernatant of the cells Bl21_pETDfB2xB2 that contains the angular dioxygenase, the ring cleavage dioxygenase, and the hydrolase *dxnB2* which shows the salicylate as an intermediate at retention time of 29 min

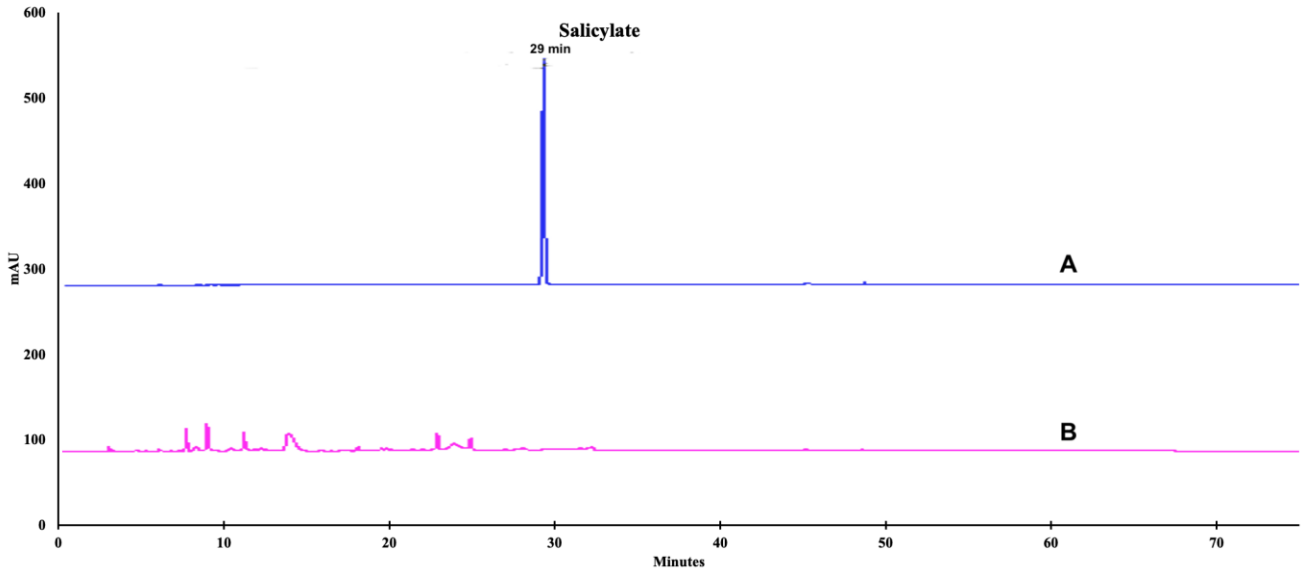

Figure 6. HPLC analysis of the cells supernatant on DF: A. Standard salicylate (1 mM), B. Supernatant of the cells Bl21_pETDfB2xB3 that contains the angular dioxygenase, the ring cleavage dioxygenase, and the hydrolase *dxnB3* which shows that salicylate is not formed using this construct (no salicylate peak)

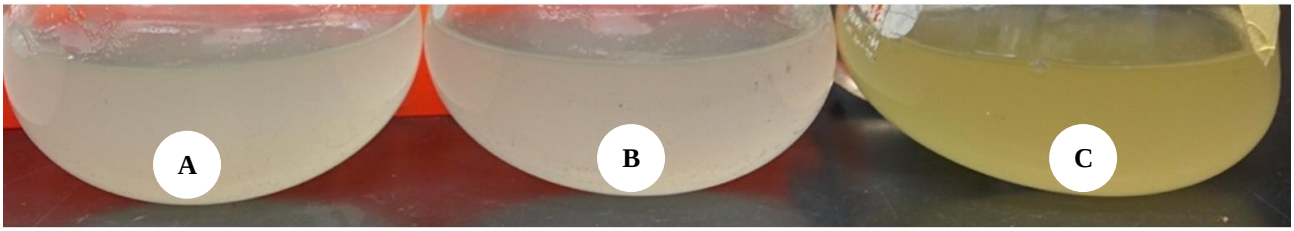

Figure 7. Supernatants of the cells harboring: A. pETDfB2xB1, B. pETDfB2xB2, and C. pETDfB2xB3 on DF

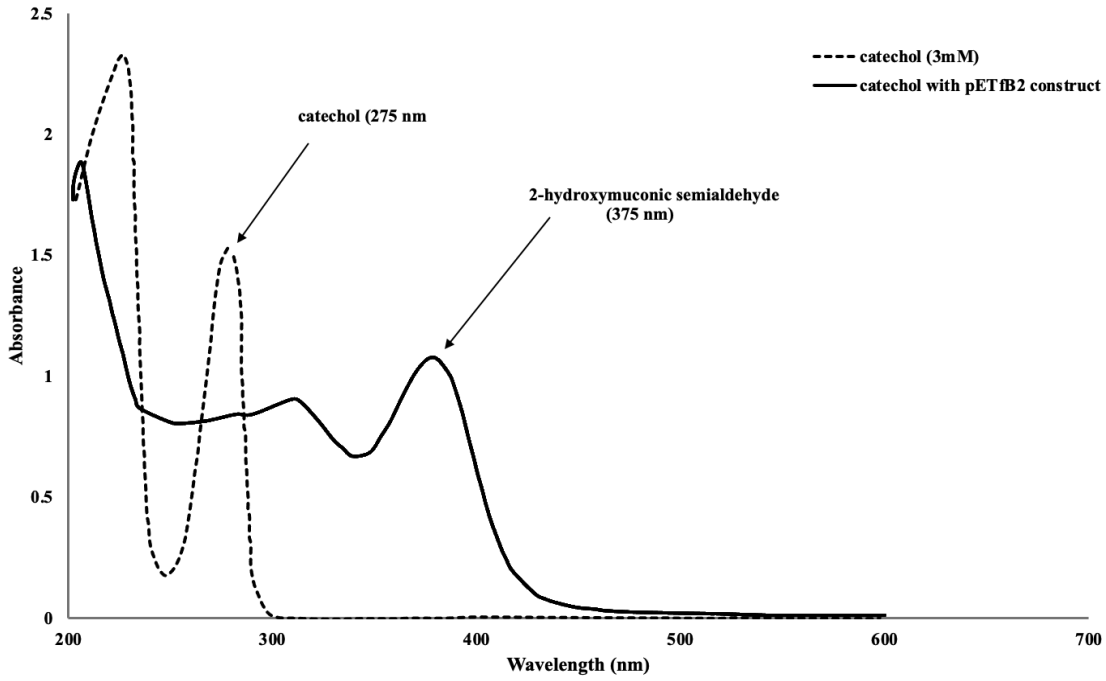

Figure 8. UV spectra: (----) absorbance of catechol at 275 nm, (——) cells supernatant on catechol containing the construct pETfB2 that contains only the ring cleavage dioxygenase dbfB2 showing 2-hydroxymuconic semialdehyde at 375 nm

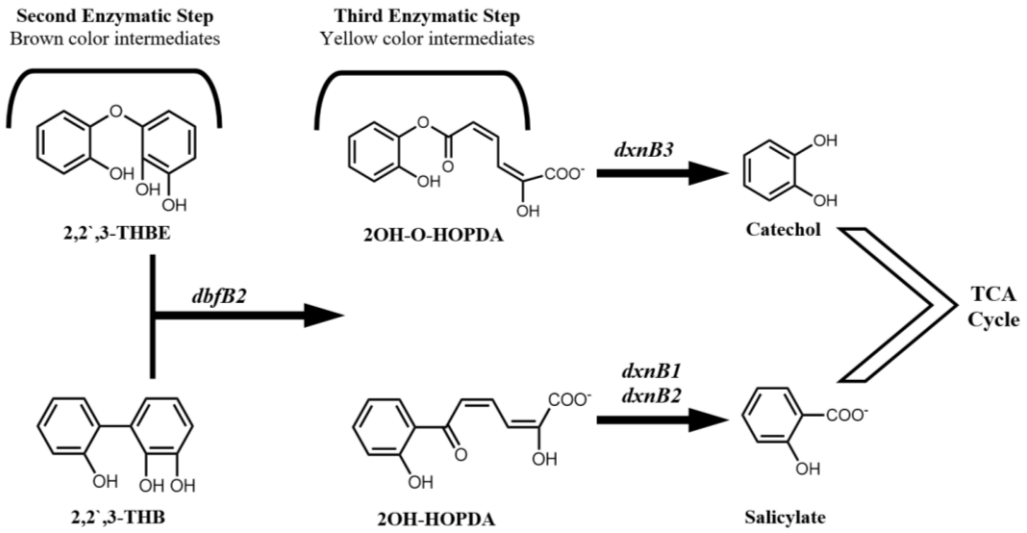

Figure 9. Genes responsible for the second and third enzymatic steps in dibenzo-*p*-dioxin and dibenzofuran pathways in *R. wittichii* RW1

Also, it was used as a consortium with *Pseudomonas* for phenanthrene removal from the environment (Jia et al. 2019). The study clarifies and answer the question regarding the rare ability of other organisms that can degrade dibenzofuran but can't degrade dibenzo-*p*-dioxin. The work findings confirmed and introduced extra experimental evidence that both substrates are metabolized in RW1 by two different sets of hydrolases and that newly identified ring cleavage dioxygenase (DbfB2) can attack both intermediates from DD and DF pathways (Figure 9).

In conclusion, three different cassettes for the upper pathway of RW1 genes of dioxins degradation were assembled in *E. coli* and tested for transformation of dibenzofuran and dibenzo-*p*-dioxin into salicylate and catechol, respectively. The study gave additional experimental evidence for the exact enzymes involved in dioxins degradation in the RW1 strain. This study provides a promising experimental technique to engineer *E. coli* for fast degradation through introducing the lower pathway (salicylate pathway) along with the dioxins upper pathway. The results confirmed that the newly identified *meta* cleavage dioxygenase in RW1 (*dbfB2*) can transform both intermediates 2,2',3-THB and 2,2',3-THBE from DF and DD; respectively. Also, the results confirmed the involvement of DxnB1 and DxnB2 in DF metabolism and that newly identified hydrolase DxnB3 is involved only in DD metabolism.

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