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Characterization of a novel pathway for xanthene degradation by the engineered strain *Sphingobium yanoikuyae* B1DR

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Abstract:

Polyaromatic hydrocarbons (PAHs) are a group of aromatic compounds that contain at least two rings. These compounds are found naturally in petroleum products and are considered the most prevalent pollutants in the environment. The lack of microorganism capable of degrading some PAHs led to their accumulation in the environment which usually causes major health problems as many of these compounds are known carcinogens. Xanthene is one of the small PAHs which has three rings. Many xanthene derivatives are useful dyes that are used for dyeing wood and cosmetic articles. However, several studies have illustrated that these compounds have toxic and carcinogenic effects. The first step of the bacterial degradation of xanthene is conducted by dioxygenase enzymes that introduces two oxygen atoms in the structure of the aromatic rings. In this study we focused on the bacterial bioremediation of xanthene via *Sphingobium yanoikuyae* B1DR, an engineered strain carrying the dioxin angular dioxygenase from *Sphingomonas wittichii* RW1. HPLC analysis of supernatant from resting cells of *S. yanoikuyae* B1DR grown on xanthene and succinate showed the ability of this strain to transform xanthene to 2-hydroxyphenylacetate that was not produced by the wild type of *Sphingobium yanoikuyae* B1. Production of 2-hydroxyphenylacetate was confirmed by GC-MS. Our results show the importance of this strain in reducing the toxic effects of xanthene in the environment and showed for the first time that ring-hydroxylation enzymes and hydrolases for biphenyl degradation in *S. yanoikuyae* B1 may function on metabolites generated from the degradation pathway of xanthene. By analyzing our results we were able to draw a novel pathway for xanthene degradation in *S. yanoikuyae* B1DR.

Keywords: Biodegradation**,** Polyaromatic hydrocarbons**,** *Sphingobium yanoikuyae***,** Xanthene, Genetic engineered bacteria.

Introduction:

Polyaromatic hydrocarbons (PAHs) are a group of organic compounds that are biochemically stable and contain at least two aromatic rings. The major sources of PAHs are petroleum products and the incomplete combustion of fossil fuels[1]. Also, PAHs have five emission sources; agricultural, industrial, mobile, domestic, and natural. These compounds are considered as widespread pollutants in the environment and some of them are known carcinogens, where many studies on rabbits showed that exposure to PAH-containing compounds can cause tumors. However, PAHs are still used in the manufacture of plastic, pesticides, and dyes[2,3]. The accumulation of these compounds in the environment is due to the difficulty of microorganism to degrade them and to the fact that most PAHs are insoluble in water. Each compound of PAHs has a unique UV spectrum which can be used for their identification[4]. PAHs are classified based on their size into small simple compounds, which contain up to six rings, and complex large compounds, which contain more than six rings[5]. Xanthene is an example of small PAHs as it has three aromatic rings with the formula $CH_2[C_6H_4]_2O$. Many xanthene derivatives are useful dyes such as rhodamines, eosins, rose bengal and fluorescein. These dyes are used for dyeing wood, cosmetic articles, and fountain pen inks, therefore, are

unintentionally excluded to the environment[6-8]. Several studies have tested the toxicity and the carcinogenicity of xanthene dyes, for instance: acridine red, rhodamine 6G, fluorescein sodium, and eosine yellowish. Multiple studies have illustrated that most of these dyes, especially those containing o-carboxy-phenyl group, caused sarcoma in rats[9].

Several physical and chemical methods were used to reduce the hazardous effects of PAHs including xanthene dyes from the environment[10]. However, bioremediation, the treatment of pollutants by the use of microorganisms, has been found to be the most effective as it is less expensive and has low impacts on the environment[11,12]. The microorganisms that can degrade PAHs have special enzymes called dioxygenases which initiate the degradation process by introducing the oxygen atoms into the aromatic rings. The rate of the PAHs degradation depends on the molecular weight of these compounds and the type of the microorganisms that involved in the process. PAHs with low molecular weight are degraded faster and easier than the PAHs that have high molecular weight. The reason for that is the low molecular weight PAHs has high water solubility which increases the accessibility to the degrading microorganisms. Also, there are other factors that affect the PAHs bioremediation such as oxygen availability, moisture, soil type, temperature, pH, and PAH concentration[13,14].

Although there are many microorganisms that were biochemically and genetically characterized for their ability to degraded PAHs such as sphingomonads and pseudomonads, yet to our knowledge no bacterium has been isolated for its ability to degrade xanthenes[15,16]. Through our search in literature we were only able to detect a unique white rot fungus (*Coriolus versicolor*) that has the ability to degrade xanthene and its derivatives[17]. Xanthone is another simple polyaromatic hydrocarbon that is similar to xanthene in structure. Our search found a single mutant bacteria (*Arthrobacter* GFB100) that was capable of degrading xanthone and use it as a sole source for carbon and energy[18]. We predicted that their steps for degradation would also be similar so that we can use the degradation pathway for xanthone to predict how xanthene is degraded. Xanthone degradation pathway by *Arthrobacter* GFB100 is similar to the degradation pathways of some polyaromatic heterocyclic compounds such as biphenyl, DBF, and DD[19-22]. The first step begins with the oxidation of xanthone to 3,4-dihydroxyxanthone (DHX) via a dihydroxylating dioxygenase complex. The second step is catalyzed by (DHX) dioxygenase which stimulates an aromatic ring cleavage reaction and forms a yellow metabolite known as 4-hydroxy-3-(2'-oxo-3-trans-butenoic acid)-coumarin (RFM). The third step is catalyzed by an NADPH dependent reductase which reduces the RFM to gentisic acid. The last step involves a dioxygenase that catalyzes the cleavage of gentisate which goes through sequential intermediates and ultimately forms fumarate and pyruvate that enters the TCA cycle to generate energy[18].

Sphingobium yanoikuyae B1 was first isolated from polluted stream by Gibson in 1973 and was known as Beijerinckia species[19]. *Sphingobium yanoikuyae* is gram negative, obligate aerobes, nonmotile, nonsporulating, rod-shaped bacteria that produce deep-yellow colonies. The species that belong to sphingomonas has a unique outer membrane which contain glycosphingolipids instead of lipopolysaccharides[20]. The wild type *S. yanoikuyae* B1 is a biphenyl degrader that is incapable of degrading xanthene, DD, or DBF probably due to lack of an angular dioxygenase. However, previous studies showed that replacing its lateral dioxygenase with the angular dioxygenase of *S. wittichii* RW1 led to its growth on biphenyl and DD. Due to the fact that the angular dioxygenase of RW1 is capable of oxygenating xanthene at an angular position[23]. We predicted that this strain will be capable of metabolizing the further downstream intermediates formed. In this study, we have tested the ability of the engineered strain *S. yanoikuyae* B1DR to degraded xanthene, identified the intermediates generated using GC-MS, and were able to draw a proposed pathway for the degradation of xanthene in this strain.

Materials and methods:

Bacterial strains and cultivation conditions

Sphingobium yanoikuyae B1DR is an engineered strain constructed from the substitution of the biphenyl lateral dioxygenase of *S. yanoikuyae* B1 with the dioxin angular dioxygenase from *S. wittichii* RW1. The wild type *S. yanoikuyae* B1 strain was kindly donated by Dr. Gerben J. Zylstra (Rutgers University, USA) and used as a control. *S. yanoikuyae* B1DR was grown on Luria Bertani (LB) media or Mineral Salts Basal (MSB) minimal media containing 50µg/ml kanamycin and an appropriate carbon source when needed and incubated at 30ºC for 24-48 hours. *E. coli* BL21/ pET_DBFDOS is an expression vector used as a positive control to produce THDM, this vector contains the dioxygenase system of *S. wittichii* RW1 and was grown on LB media containing 50µg/ml kanamycin at 37ºC for 24 hours[23].

Preparation of resting cells

B1DR stain was cultured on LB plates containing 50 μg/ml kanamycin. The growing colonies were subcultured into 100 ml LB broth and incubated for 16 hours. Then, 2ml of this culture was used to inoculate 100 ml of LB broth which was incubated at 37°C with shaking (180 rpm). After reaching an OD_{600} of 1, the cells were pelleted and washed twice with phosphate buffer (50 mM sodium-potassium phosphate buffer) at pH 6.8. They were resuspended in phosphate buffer and 3mM of the xanthene plus 20mM succinate were added to the flasks and incubated at 30°C for 24 hours, the supernatant was then collected and analyzed by HPLC[24]. The supernatant from *S. yanoikuyae* B1DR strain grown on succinate alone and *E. coli* BL21/ pET_DBFDOS were used as controls were processed in a similar method. Supernatants were then filtered and analyzed with a Beckman (CA, USA) liquid chromatography system.

HPLC analysis

In order to examine the oxygenated intermediates, a reverse phase C18 column (4.6mm by 25mm) and a gradient of 0-50% methanol in water were used under acidic conditions (0.1% acetic acid) and a flow rate of 1ml/min. Peaks detection were monitored at both 254nm and 280nm wavelength[25].

Analysis by GC-MS

The water-soluble products of the samples that showed positive results on the HPLC were extracted from the aqueous phase of the bacterial suspension by ethyl acetate as previously described[26]. Then, a mixture that contained 1ml of each extracted sample with 50μl of Sylon BFT reagent (contained trimethylsilyl (TMS) used for derivitization) was made followed by incubation for 1 hour at 80°C.

GC-MS analysis was done using a Varian 3400 GC machine with a capillary column (30 meter × 0.32 mm) Guardian-ZB-5MS (Phenomonex) (5% phenyl, poly - dimethylphenyl-siloxane) with a 0.25μm film thickness. One micro-liter was the injection volume at 300°C. The temperature of the GC column was set to start at 50°C for 3min and then rised up to 320°C at a rate of 10°C /min with a 10 min hold at the upper limit. The transfer line temperature of the GC-MS was 320°C. EI mode (70 eV) scanning masses 35-750 m/z was used to operate the TSQ-7000 mass spectrometer once each second. In order to have data, peak list, and area integration values on spreadsheet, total ion current (TIC) chromatograms were integrated with the system software.

Results and Discussion:

Testing growth on xanthene and resting cells experiments

S. yanoikuyae B1DR lacked the ability to grow on MSB plates supplemented with xanthene, however, the culture produced a brown intermediate when incubated for 48 hours on this substrate unlike the *S. yanoikuyae* B1 wild type. Therefore, we hypothesized that the presence of the dioxin dioxygenase of RW1 in this strain is being involved in metabolizing xanthene to a further extent. To determine the pathway that this organism is taking to degrade xanthene, two different supernatants from resting cells of *S. yanoikuyae* B1DR growing on succinate+xanthene and another growing on succinate alone (negative control), were collected and analyzed by Hplc and compared to a supernatant from *E.coli* pET_DBFDOS grown with xanthene (positive control). As illustrated in Fig. 1, our results detected a huge peak at the minute 33 from the supernatant of *E.coli* pET_DBFDOS grown with xanthene that represents the initial intermediate formed from the oxygenation of xanthene, 2,2'3- trihydroxydiphenylmethane (THDM). A previous study proved that this intermediate was 2,2'3- trihydroxydiphenylmethane after GC-MS, 1H NMR, and gCOSY analysis[23]. Interestingly, the peak for THDM disappeared from the supernatant of *S. yanoikuyae* B1DR even though this organism still contains the same dioxygenase system found in *E.coli* pET_DBFDOS (originally the *S. wittichii* RW1 dioxygenase). In addition, *S. yanoikuyae* B1DR growing on succinate+xanthene contained extra peaks in the Hplc spectra that were not found neither in *S. yanoikuyae* B1DR growing on succinate (negative control) nor *E.coli* pET_DBFDOS grown with xanthene (positive control). This indicates the ability of *S. yanoikuyae* B1DR to further metabolize xanthene to simpler intermediates that follow THDM. Detection of degradation pathways depending on the structure of intermediates generated has been used to study the degradation of fluoranthene in Celeribacter baekdonensis B30[27].

More precisely, *S. yanoikuyae* B1DR should contain a meta cleavage dioxygenase that is capable of metabolizing THDM. Genetic studies have diagnosed two important ring cleavage dioxygenases in *S. yanoikuyae* B1 designated BphC (2,3 dihydroxybiphenyl 1,2 dioxygenase) and xylE (catechol 2,3 dioxygenase) that are involved in biphenyl and xylene degradation, respectively[28]. Gene knockout experiments of *bphC* have showed that the second ring cleavage dioxygenase, *xylE*, can take place *bphC* in 2,3 dihydroxybiphenyl degradation thus complement growth on biphenyl

when *bphC* is missing. Even though the growth was slower, however *xylE* was successfully capable of complementing *bphC* mutants[29]. Therefore, we suggest that one of these enzymes, more likely BphC, is involved in THDM metabolism as this enzyme was found to be more specific towards 2,3 dihydroxybiphenyl which is somehow structurally similar to THDM unlike XylE which was more specific for single ring aromatics particularly catechol degradation.

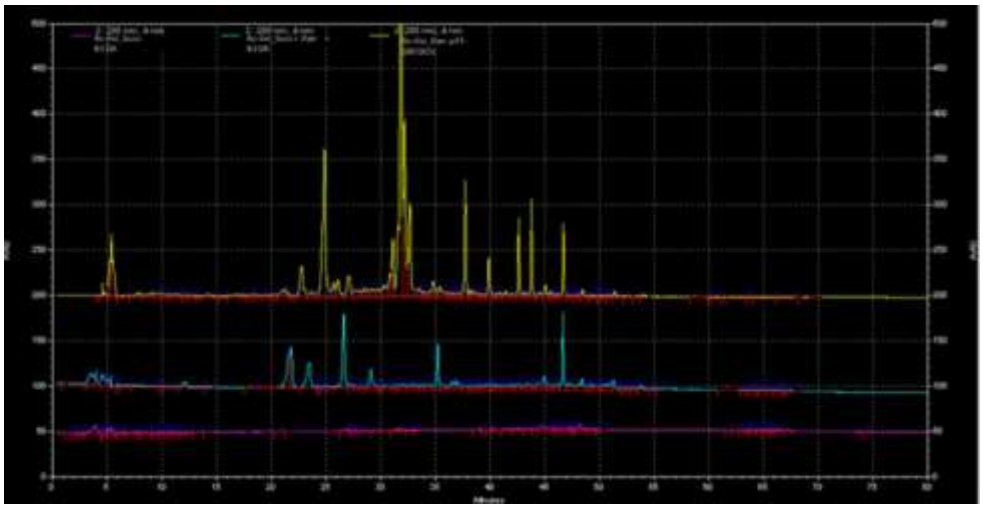

Figure 1. HPLC analysis of the supernatant of *S. yanoikuyae* B1DR growing on succinate+xanthene (Blue spectrum), *S. yanoikuyae* B1DR growing on succinate alone (negative control) (Purple spectrum), and *E.coli* pET_DBFDOS grown with xanthene (positive control) (Yellow spectrum).

GS-MS analysis

Ring cleavage products formed from the degradation pathways of aromatic hydrocarbons are known to have a yellow color[30]. Thus, if *S. yanoikuyae* B1DR contained an enzyme that cleaves one of the rings in THDM, a yellow intermediate should accumulate. However, this was not what we noticed with our accumulated metabolite from xanthene degradation. This led us to hypothesize that the ring cleavage product for xanthene can be further metabolized by *S. yanoikuyae* B1DR to a simpler compound. For this purpose, the supernatant from *S. yanoikuyae* B1DR growing on xanthene and succinate was extracted and analyzed by GC-MS to identify the molecular weight of the accumulated product. Results from GC-MS analysis detected a product that carried a molecular weight of 296 which is equivalent to the molecular weight of hydroxyphenyl acetate (152) with two TMS molecules attached (total of 144) as shown in (Fig. 2).

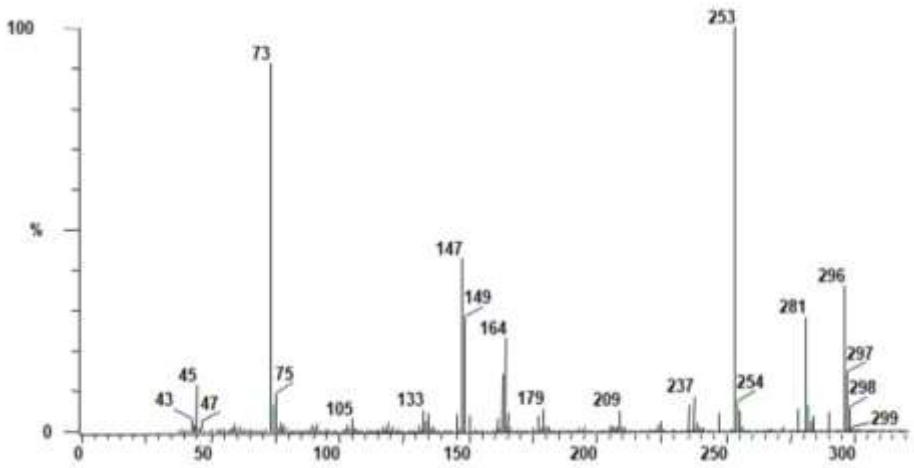

Figure 2. GC-MS spectrum of hydroxyphenyl acetate. The x axis represents the retention time and the y axis is the relative abundance.

The formation of hydroxyphenyl acetate indicates the presence of a hydrolase enzyme in *S. yanoikuyae* B1DR that can hydrolyze the ring cleavage product formed (2-hydroxy-8-(2-hydroxy

phenyl)-6-oxo-2,4-octadieneoic acid) to 2-hydroxyphenyl acetate and 2-hydroxypenta 2,4-dienoate (Fig. 3). The latter was not seen in the extract of *S. yanoikuyae* B1DR growing on xanthene and succinate which is probably due to its conversion to simpler products. The best candidate we believe is involved in this hydrolysis step is the biphenyl hydrolase, BphD, as this enzyme has been proven to function on ring cleavage products formed from the biphenyl and the dibenzo-dioxin pathway, therefore we believe it may be the enzyme functioning on this step due to the similarities of the substrates from these pathways[23]. To our knowledge, we expect the degradation pathway for xanthene in *S. yanoikuyae* B1DR to follow the proposed pathway shown in Fig. 3.

Although 2-hydroxyphenylacetate has a lower level of toxicity than xanthene, yet it can cause eye and skin irritation. However, there have been evidence for the presence of many bacterial strains that are known for their ability to degrade 2-hydroxyphenylacetate, the best example is *Geobacillus thermodenitrificans* NG80-2[31]. On the other hand, 2-hydroxyphenylacetate was found to be one of the precursors of benzylpenicillin production via filamentous fungi (e.g. *Penicillium rubens* and *Aspergillus nidulans*), thus these fungal species may help to remove this byproduct from the environment thus removing the toxicity of xanthenes in the environent[32].

Organic synthetic dyes, including xanthenes, have been used extensively as colors in paper, plastic, cosmetics, and ink. During the synthesis and processing of these dyes, about 15% are lost in effluent. Some of these dyes are toxic compounds and can cause organic pollution as well as they are very recalcitrant against biodegradable processes [17,33].

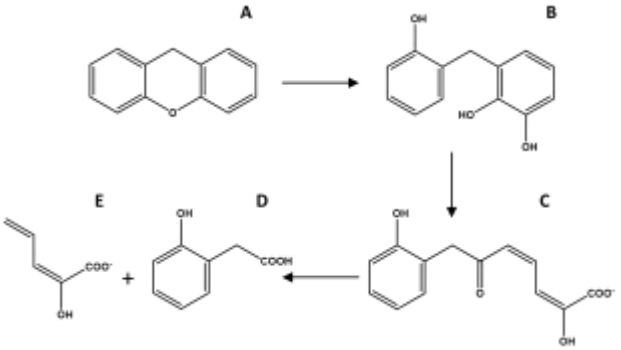

Figure 3. A proposed xanthene degradation pathway in *S. yanoikuyae* B1DR showing the chemical structures of the intermediates formed. Xanthene (A); Trihydroxydiphenylmethane (B); 2-hydroxy-8-(2-hydroxy phenyl)-6-oxo-2,4-octadieneoic acid (C); 2-hydroxyphenylacetate (D); 2-hydroxypenta 2,4-dienoate (E).

Conclusion:

In this study we have shown the ability of the engineered strain *S. yanoikuyae* B1DR to transform xanthene to the less toxic compounds, 2-hydroxyphenylacetate and 2-hydroxypenta 2,4-dienoate. These end products are easily degraded by other environmental organisms[31]. In addition, this study is successfully able to prove that *S. yanoikuyae* B1DR contains a ring cleavage enzyme and a hydrolase that metabolize THDM formed from the dioxygenation of xanthene. By analyzing our results, we are able to draw a proposed pathway for xanthene degradation in this strain. Our results identify for the first time a novel pathway in *S. yanoikuyae* B1DR that is used to degrade xanthene to fewer toxic products. This strain can be used as a bacterial candidate to eliminate xanthene from contaminated sites.

Authors' declaration:

- Conflicts of Interest: None.
- We hereby confirm that all the Figures and Tables in the manuscript are mine ours. Besides, the Figures and images, which are not mine ours, have been given the permission for re-publication attached with the manuscript.
- Ethical Clearance: The project was approved by the local ethical committee in University of Mosul.

Authors' contributions statement:

F. R. designed, analyzed, and conducted the research. Al. A. did the Hplc and GC-MS work and collected all required references. Both F. R. and AL. A. wrote the paper. Both authors have read and approved the final manuscript.

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توصيف مخطط ايضي جديد لتحليل مادة الزانثين بواسطة السلالة البكتيرية المعدلة وراثيا *Sphingobium yanoikuyae* B1DR

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الخلاصة:

الهيدروكربونات متعددة الحلقات (PAHs) هي مجموعة من المركبات الاروماتية التي تحتوي على حلقتين على الأقل. مصدر هذه المركبات هي المنتجات البترولية وتعتبر من أكثر الملوثات انتشارًا في البيئة. تراكم هذه المركبات في البيئة هو نتيجة لصعوبة تحللها بواسطة الاحياء المجهرية. عدم معالجة هذه المركبات قد يتسبب في مشكلة صحية خطيرة حيث يعتبر البعض منها مواد مسرطنة. الزانثين هو أحد هذه المركبات اذا تحتوي على ثلاث حلقات اروماتية. العديد من مشتقات الزانثين عبارة عن أصباغ مفيدة تستخدم في صباغة الأخشاب ومستحضرات التجميل. ومع ذلك. أوضحت العديد من الدراسات أن هذه المركبات لها تأثيرات سامة ومسرطنة. تتم الخطوة الأولى من تحلل هذه المركبات بما في ذلك الزانثين عن طريق الانزيم البكتيري ديوكسجيناز التي تدخل ذرات الأكسجين في الحلقات الاروماتية. في هذه الدراسة ، ركزنا على المعالجة الحيوية البكتيرية للزانثين عبر Sphingobium yanoikuyae B1DR التي تعتبر سلالة معدلة وراثيا تحمل انزيم الديوكسين ديوكسجيناز الزاوي الخاص بالسلالة البكتيرية *Sphingomonas wittichii* RW1. حيث أظهرت نتائج التحليل باستخدام جهازHPLC لمستخلص الوسط الزرعي لخلايا *S. yanoikuyae* B1DR النامية على مادة xanthene و succinate قدرة هذه السلالة على تحويل الزانثين إلى 2-hydroxyphenyl acetate والتي لم يتم إنتاجها من قبل النمط البري لسلالات*Sphingobium yanoikuyae* B1. تم التأكد من إنتاج 2-hydroxyphenyl acetate بواسطة جهاز GC-MS. حيث تظهر نتائجنا أهمية هذه السلالة في الحد من التأثيرات السامة للمركب الاورماتي الـ xanthene في البيئة وأن إنزيمات الهيدروكسيل الحلقية والهيدروكسيل المساهمة في تحلل مادة الـ biphenyl في *S. yanoikuyae* B1 قد تعمل على المركبات الوسطية الناتجة من المسار الايضي لتحلل الـ xanthene.

الكلمات المفتاحية: التحلل البيولوجي, الهيدروكربونات متعددة الحلقات, *Sphingobium yanoikuyae* , الزانثين، البكتريا المهندسة وراثيا.