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Communal microaerophilic–aerobic biodegradation of Amaranth by novel NAR-2 bacterial consortium

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ABSTRACT

A novel bacterial consortium, NAR-2 which consists of *Citrobacter freundii* A1, *Enterococcus casseliflavus* C1 and *Enterobacter cloacae* L17 was investigated for biodegradation of Amaranth azo dye under sequential microaerophilic–aerobic condition. The NAR-2 bacterial consortium with *E. casseliflavus* C1 as the dominant strain enhanced the decolorization process resulting in reduction of Amaranth in 30 min. Further aerobic biodegradation, which was dominated by *C. freundii* A1 and *E. cloacae* L17, allowed biotransformation of azo reduction intermediates and mineralization via metabolic pathways including benzoyl-CoA, protocatechuate, salicylate, gentisate, catechol and cinnamic acid. The presence of autoxidation products which could be metabolized to 2-oxopentenoate was elucidated. The biodegradation mechanism of Amaranth by NAR-2 bacterial consortium was predicted to follow the steps of azo reduction, deamination, desulfonation and aromatic ring cleavage. This is for the first time the comprehensive microaerophilic–aerobic biotransformation pathways of Amaranth dye intermediates by bacterial consortium are being proposed.

1. Introduction

In meeting the demand of the growing textile industry, many dye manufacturing industries produce various types of azo dyes which can eventually lead to problems of toxic polluted wastewaters. In recent years, biological treatment of this industrial pollutant has been favored since it could achieve complete mineralization without producing toxic sludge as by-products (Ali, 2010). In biological treatment, microorganisms are mainly used and these include bacteria, fungi, yeast and algae (Saratale et al., 2011). To date, the well-studied anaerobic bacterial decolorization of azo dyes due to azo reduction catalyzed by the action of azo reductases using NADPH is well-accepted. Unfortunately, azo reduction frequently generates aromatic amines that resist anaerobic degradation (Pandey et al., 2007). Such compounds were regarded as genotoxic and carcinogen, and the fate of aromatic amines under aerobic degradation could be varied (Pinheiro et al., 2004). In addition, microbial consortia generally demonstrated better decolorization ability compared to pure cultures (Chan et al., 2011; Joshi et al., 2008; Khehra et al., 2005). Functioning within microbial community, a higher degree of biodegradation and mineralization metabolic activities of mixed cultures can be expected. Some microbial consortia can complement each other and collectively carry out biodegradation that cannot be achieved using pure cultures (Pearce et al., 2003; Saratale et al., 2011). Hence, it is essential to investigate the fate of dye intermediates after biodegradation of azo dyes, particularly from sequential anaerobic–aerobic degradation carried out by bacterial consortia to determine if mineralization has really occurred.

In this study, a novel bacterial consortium, designated as NAR-2 consisting of *Citrobacter freundii* A1, *Enterococcus casseliflavus* C1 and *Enterobacter cloacae* L17 which demonstrated excellent decolorizing ability of a model azo dye under microaerophilic condition is reported for the first time. *C. freundii* A1 was isolated from the oxidation pond located in Universiti Teknologi Malaysia (Johor, Malaysia), whereas *E. casseliflavus* C1 and *E. cloacae* L17 were lab contaminants (Chan et al., 2011). Amaranth (C.I. Acid Red 27), known as trisodium salt of 1-(4-sulfo-1-naphthylazo)-2-naphthol-3,6-disulfonic acid was used as the model dye in this study. This is an acidic monosulfonated azo dye widely used in the textile industry (Jain et al., 2009). The main objective of this study was to explore the fate of Amaranth degradation by NAR-2 bacterial consortium under sequential microaerophilic–aerobic condition by liquid chromatography–mass spectrometry (LC–MS/MS). The biodegradation intermediates were deduced and biodegradation pathways which included reductive cleavage of azo bond that caused decolorization, followed by deamination and desulfonation

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that led to further biotransformation of dye intermediates are proposed here. Benzoyl-CoA metabolism and protocatechuate degradation could lead to mineralization of dye intermediates. In addition, metabolism of naphthalene by NAR-2 bacterial consortium leading to mineralization via TCA cycle was also explored. The knowledge of the fate of dye degradation is crucial prior to future application of NAR-2 bacterial consortium in pilot-scale bioremediation of textile wastewater process in order to ensure the treated wastewater is safe prior to release.

2. Methods

2.1. Microorganisms and inoculum preparation

Three local bacterial isolates belonging to the family *Enterobacteriaceae*, namely *C. freundii* strain A1, *E. casseliflavus* strain C1 and *E. cloacae* strain L17 were used in this study. Initially, *E. casseliflavus* C1 and *E. cloacae* L17 were used in combination and formulated as NAR-1 bacterial consortium (Chan et al., 2011). The addition of *C. freundii* A1 to NAR-1 formed a new bacterial consortium later designated NAR-2. The microorganisms were grown on nutrient agar at 37 °C. P5 medium containing K_2HPO_4 (35.3 g L^{-1}), KH_2PO_4 (20.9 g L^{-1}), NH_4Cl (2 g L^{-1}), glucose (10 g L^{-1}), nutrient broth (20 g L^{-1}) and trace elements was prepared according to the procedures described by Chan et al. (2011). To prepare the starter culture, a single bacterial colony was inoculated into a 250-mL conical flask containing 50 mL of P5 medium and incubated overnight at 37 °C with shaking at 200 rpm. Subsequently, 10 mL of the culture was transferred into a 250-mL conical flask containing 90 mL of P5 medium and incubated aerobically at 37 °C with shaking at 200 rpm until the exponential phase of bacterial growth was reached. Optical density (OD) of each bacterial culture was determined at 600 nm (Cary UV–Vis Spectrophotometer, USA). The

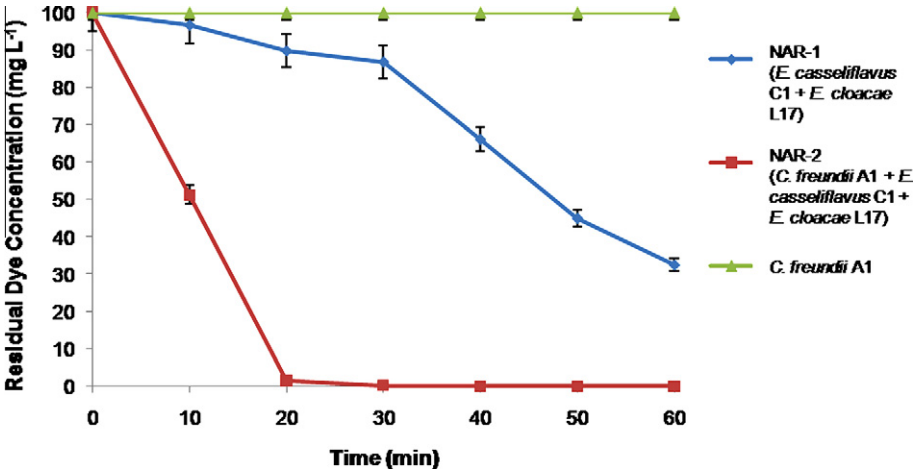

Fig. 1a. Comparison of decolorization profile of Amaranth by *C. freundii* A1, NAR-1 and NAR-2.

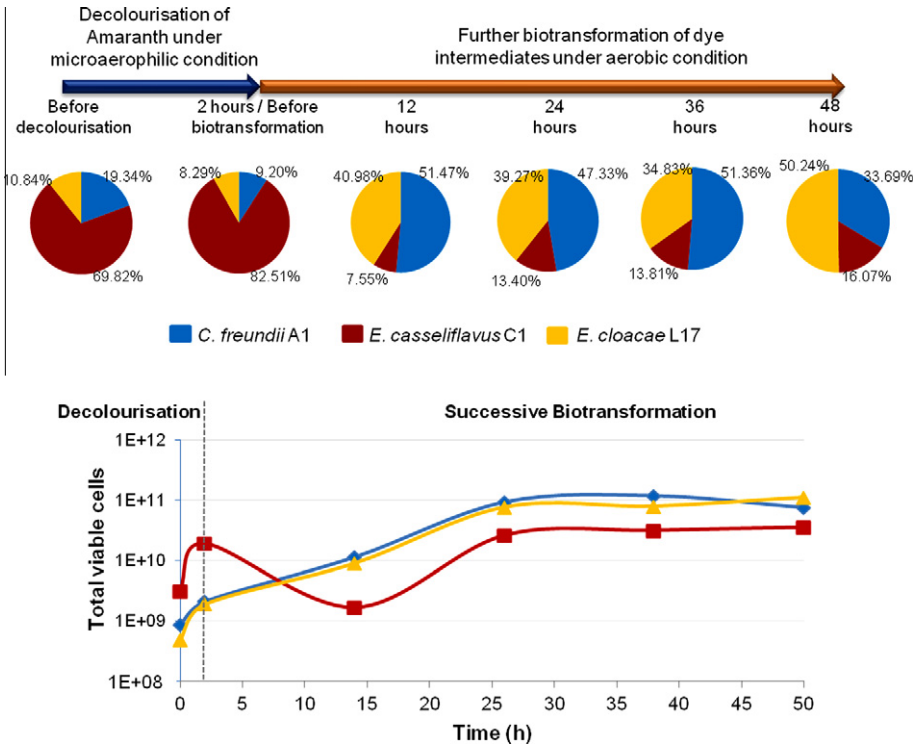

Fig. 1b. Population dynamics and cell density of NAR-2 bacterial consortium throughout Amaranth decolorization and successive biotransformation of dye intermediates.

Table 1
Aromatic intermediates detected by LC–MS/MS analyses after decolorization of Amaranth by NAR-2 bacterial consortium under microaerophilic condition for 2 h at 45 °C.

Molecular weight	*m*/*z* (amu)	Compound
535.5	536.587	Amaranth[a,b]
537.5	536.56	Hydrazo compound
317.3	318.278	1-Aminonaphthalene-2-hydroxy-3,6-disulfonic acid (RP2) [b,c,d]
318.28	319.343	3,4-Dihydroxynaphthalene-2,7-disulfonate [b,c]
255.22	256.332	5,6,7-Trihydroxynaphthalene-2-sulfonate [b,c,d]
176.17	175.2	Naphthalene-1,2,4-triol
128.17	127.04	Naphthalene

[a] Amaranth was detected at very low amount after extraction with ethyl acetate and 10 times concentration prior to LC–MS/MS.
[b] Compounds detected after decolorization of Amaranth by *C. freundii* A1.
[c] Compounds detected after decolorization of Amaranth by *E. casseliflavus* C1.
[d] Compounds detected after decolorization of Amaranth by *E. cloacae* L17.

inocula were ready to be used when the Abs_{600nm} reading reached 1.0 ± 0.2. For decolorization experiments carried out at 100-mL volume, NAR-1 bacterial consortium was used at a ratio 1:1 of OD_{600} corresponding to $3.0 \pm 0.2 \times 10^9$ cfu and $4.8 \pm 0.2 \times 10^8$ cfu of *E. casseliflavus* C1 and *E. cloacae* L17, respectively. NAR-2 bacterial consortium was prepared with equivalent cell density to the above for *E. casseliflavus* C1 and *E. cloacae* L17, but with the addition of *C. freundii* A1 at $8.6 \pm 0.2 \times 10^8$ cfu.

2.2. Biodegradation of Amaranth by NAR-2 bacterial consortium

Amaranth (C.I. Acid Red 27) was purchased from Sigma–Aldrich (USA) and was used as the model dye for decolorization experiments at a final concentration of 0.1 g L^{-1}. A master mix consisting of P5 medium with Amaranth (0.1 g L^{-1}) was inoculated with either pure culture of *C. freundii* A1 or in combination as a bacterial consortium (at the cell density mentioned in Section 2.1). The mix was then transferred into 100-mL bottles, filled up to the brim and capped tightly to create a microaerophilic condition. The samples were then incubated at 45 °C and sampling was done at 10-min interval. Each sample was centrifuged and the supernatant were analyzed at 521 nm (Cary UV–Vis Spectrophotometer, USA) to determine the level of decolorization. For analyses of degradation products, Amaranth decolorization by either single bacterial strains (*C. freundii* A1 or *E. casseliflavus* C1 or *E. cloacae* L17) or NAR-2 bacterial consortium was also carried out in a 100-mL volume. After complete decolorization, each of the decolorized samples was transferred to a 250-mL conical flask and then incubated aerobically at 37 °C with shaking at 200 rpm for 48 h. To determine the profile of dominant bacterial species for NAR-2 bacterial consortium, sampling for viable plate count was performed at both microaerophilic decolorization and consecutive aerobic biodegradation.

2.3. Liquid Chromatography-Mass Spectrometry analysis of biodegradation products

Decolorization under microaerophilic condition by NAR-2 bacterial consortium was carried out for 2 h, followed by successive biotransformation for 48 h under aerobic condition. Both decolorized and further biotransformed samples were centrifuged at 5000 rpm for 15 min. The supernatant was transferred to a separating funnel and extracted with an equal volume of ethyl acetate. The top layer was collected and concentrated to about 10 mL using a rotary evaporator. Samples were filtered through a 0.2-μm nylon filter prior to LC–MS/MS analyses. LC–MS/MS analyses of dye intermediates and metabolites were carried out using the analytical Acquity UPLC (Waters, USA) system coupled with an electrospray ionization (ESI) and a triple-quadrupole-linear ion trap of tandem mass spectrometer, 4000 Q TRAP (Applied Biosystems, Canada). The UPLC system was equipped with C18 reversed phase Acquity column (150 mm × 4.6 mm × 1.7 μm; Waters, USA) and mobile phase of binary solvent system consisting of 0.1% formic acid and acetonitrile. The UPLC gradient profile of screening was carried out at 0–5 min, 10% of acetonitrile; 5–15 min, 10–90% of acetoni-

Table 2
Metabolites detected by LC–MS/MS analyses after further biotransformation of Amaranth by NAR-2 bacterial consortium under aerobic condition for 48 h at 37 °C.

Molecular weight	*m*/*z* (amu)	Compound
271.22	272.205	(*E*)-2-carboxy-1-(2-carboxyphenyl)ethenesulfonate [a,b,c]
208.17	207.52	2-[(*E*)-2-carboxy-1-hydroxyethenyl]benzoic acid/2-(carboxyacetyl)benzoic acid
192.1	191.28	Naphthalene-1,2,3,6-tetrol
287.22	288.216	4-Carboxy-3-[(*E*)-2-carboxy-2-hydroxyethenyl]benzenesulfonate/4-carboxy-3-[(*Z*)-2-carboxy-2-hydroxyethenyl] benzenesulfonate [a,b,c]
238.15	237.293	6-(2-Carboxy-2-oxoethyl)-3,4-dioxocyclohexa-1,5-diene-1-carboxylic acid
315.9	314.96	1,2-Naphthoquinone-3,6-disulfonate (AP2 AHNDS) [b]
154.12	155.06	Protocatechuate
184.1	185.12	2-Oxo-2H-pyran-4,6-dicarboxylic acid
218.12	219.12	4-Carboxy-4-hydroxy-2-oxoadipate
87.05	88.032	Pyruvate
183.1	184.104	3-Carboxy-*cis*,*cis*-muconate/2-carboxy-5-oxo-2,5-dihydrofuran-2-acetate
158.11	159.028	3-Oxoadipate/(3E)-6,6-dihydroxy-2-oxohexa-3,5-dienoic acid
113.09	113.969	2-Oxopentenoate
110.11	109.2	Catechol
121.11	122.095	Benzoate
160.169	161.12	Naphthalene-1,2-diol [b]
122.121	121.44	2-Hydroxybenzaldehyde
137.12	136.4	Salicylate
154.11	155.06	Gentisate
184.104	185.12	3-Maleylpyruvate/3-fumarylpyruvate
114.057	113.04	Fumarate
148.159	149.104	Cinnamic acid

[a] Compounds detected after further biotransformation of Amaranth by *C. freundii* A1.
[b] Compounds detected after further biotransformation of Amaranth by *E. casseliflavus* C1.
[c] Compounds detected after further biotransformation of Amaranth by *E. cloacae* L17.

trile; 15–20 min, 90% of acetonitrile; 20–25 min, 90–10% of acetonitrile; 25–30 min, followed by 10% of acetonitrile for final washing and re-conditioning of the column for the next sample run. The flow rate was 0.25 mL min^{-1} and the injection volume was 5 μL. Full scan mass spectra from *m*/*z* 50–700 were measured using 20 ms for collection of the ions in the trap. Quantitative analysis was performed using Multiple Reaction Monitoring (MRM) based on two precursor-product ion transitions. The heated capillary and voltage of ESI source were maintained at 400 °C and −4.5 kV, respectively. Nitrogen was used as ion source gas for nebulizing, 40 psi; for drying solvent, 40 psi; curtain gas, 10 psi; collision gas, high; declustering potential, −40 V and collision exit energy, −10 V. The scan rate was 1000 amu s^{-1}. Data acquisition and processing was performed using Analyst 1.4.2. MS Fragmenter 12.0 (ACD/Labs, Canada) to predict compound fragmentation for both positive and negative ionization in data interpretation. The predicted degradation products from NAR-2 bacterial consortium were compared with products from individual bacterial strains of *C. freundii* A1, *E. casseliflavus* C1 and *E. cloacae* L17 following complete decolorization in 24 h and successive biotransformation for 48 h.

2.4. Prediction of biodegradation pathways and metabolites

In order to analyze the mass of compounds generated from LC–MS/MS, the biodegradation pathways and metabolites were hypothesized with reference to pathways described in MetaCyc database at http://metacyc.org/ (Caspi et al., 2010) and KEGG Pathway database at http://www.genome.jp/kegg/pathway.html. The predicted metabolites were drawn and named using Symyx Draw 3.3 and ACD/ChemSketch (Version 12.01), and queried using ChemSpider at http://www.chemspider.com/ to obtain further references. The presence of a few key enzymes involved in azo reduction, deamination, desulfonation and benzoyl-CoA metabolism in

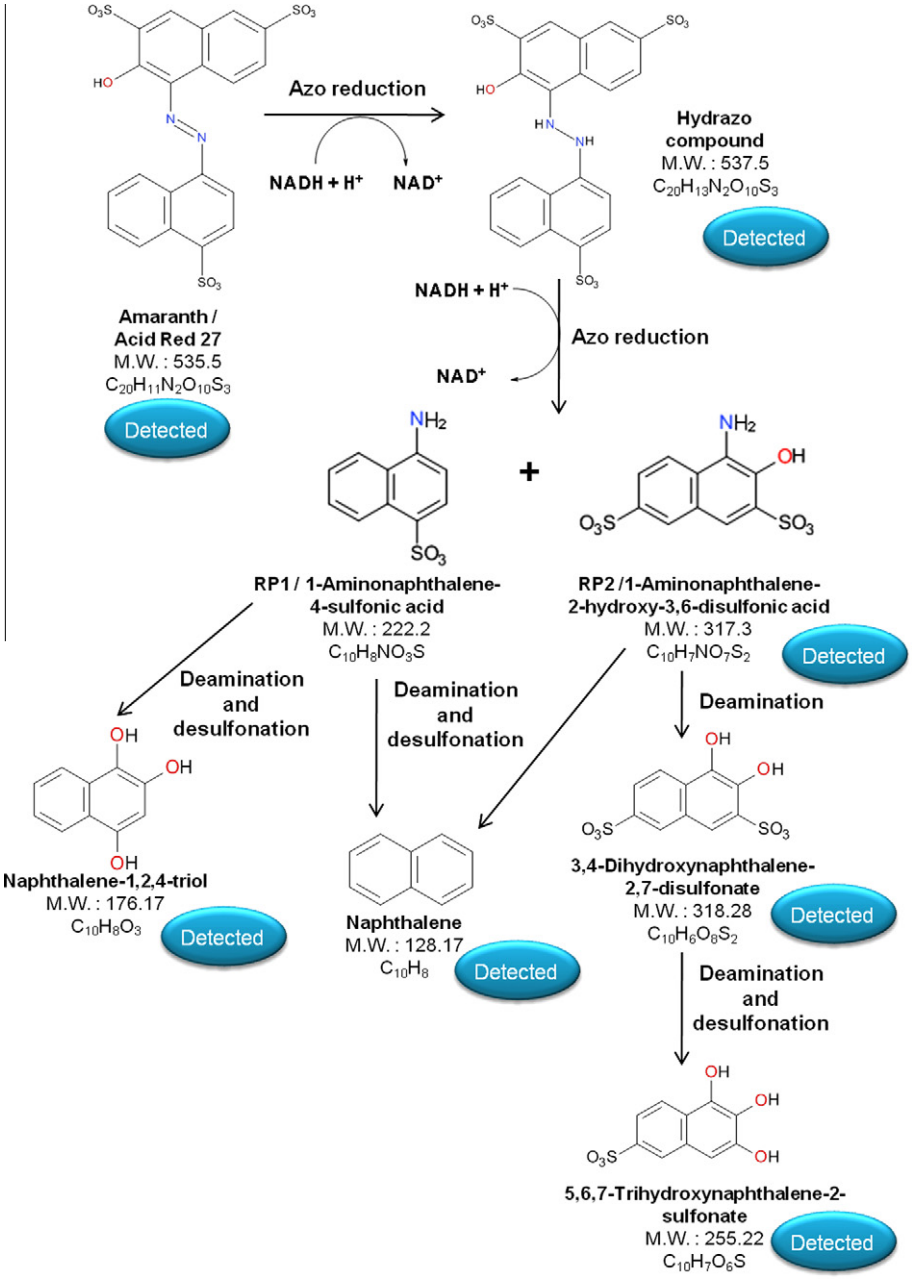

Fig. 2. Proposed reductive degradation pathway of Amaranth azo dye by NAR-2 bacterial consortium under microaerophilic condition.

NAR-2 bacterial strains was probed by PCR of the predicted genes that coded for the enzymes. The amplified genes of degradation enzymes (i.e. flavin reductase, sulfatase, transaminase and fatty acid-CoA ligase) from *C. freundii* A1, *E. casseliflavus* C1 and *E. cloacae* L17 were analyzed based on DNA sequences available in GenBank database (Nasiri, 2011).

3. Results and discussion

3.1. Decolorization of Amaranth under microaerophilic condition

Decolorization of Amaranth at 0.1 g L^{-1} in P5 medium at 45 °C under microaerophilic condition by *C. freundii* A1 alone, NAR-1 and NAR-2 was compared. Decrease in dye concentration was observed from a peak at absorbance 521 nm and the result is shown in Fig. 1a. NAR-2 bacterial consortium exhibited the highest decolorization rate with complete color removal in 30 min. To the best of our knowledge, novel NAR-2 bacterial consortium exhibited one of the shortest times of 30 min in decolorization of Amaranth. As comparison, Handayani et al. (2007) reported on complete decolorization of Amaranth by *Enterococcus faecalis* within 3 h. Meanwhile, Hong et al. (2007) achieved decolorization of Amaranth to near completion after 36 h of incubation using *Shewanella decolorationis* S12.

Earlier on, we reported on the increased decolorization rate of Orange II by NAR-1 bacterial consortium compared to pure cultures of *E. casseliflavus* C1 and *E. cloacae* L17 (Chan et al., 2011). However, during the screening for dye decolorization efficiency by NAR-1 and NAR-2 bacterial consortia, NAR-2 was observed to be more efficient in decolorizing Amaranth than NAR-1. Hence, as shown in Fig. 1a, the decolorization rate of Amaranth was significantly improved by the addition of *C. freundii* A1. The bacterial strain could probably result in a unique bacterial interaction that balances up the bacterial population dynamic which in return enhances the rate of Amaranth decolorization. The population dynamics of these bacterial strains during microaerophilic decolorization followed by consecutive aerobic biotransformation were investigated and the findings are shown in Fig. 1b. It was observed that the cell density of NAR-2 bacterial strains varied significantly with the level of oxygen present under two different culture conditions. Under microaerophilic condition, *E. casseliflavus* C1

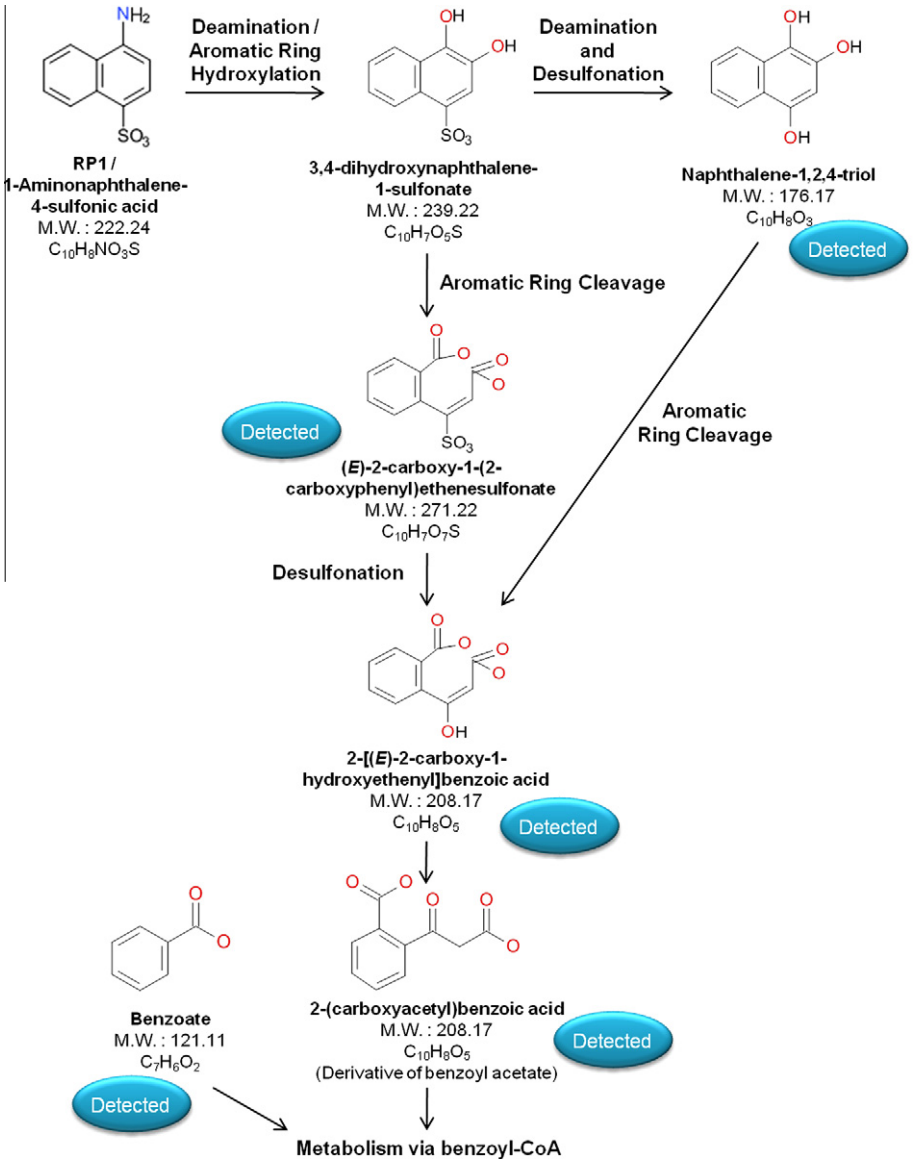

Fig. 3a. Proposed oxidative degradation pathway of RP1 by NAR-2 bacterial consortium.

dominated the population with an increased to almost 82.5% during decolorization. On the contrary, during the shift from microaerophilic to aerobic condition, the profile changed where *E. cloacae* L17 and *C. freundii* A1 increased favorably to a higher cell density and eventually dominated the population during the successive aerobic biodegradation of the reduced dye intermediates. Hence, the ratio of bacterial population could affect the dye intermediates produced, and consecutively influence the biodegradation pathways involved.

Due to the enhanced decolorization rate demonstrated by the defined mixed culture in this study, it is proposed that synergetic interaction may have occurred among the bacterial strains which perhaps complement each other in their degradation capability. This finding corroborates with the report by Phugare et al. (2011) who found that there was an improved decolorization rate by SDS bacterial consortium, consisting of *Pseudomonas aeruginosa* strain BCH and *Providencia* sp. due to coordinated metabolic interaction. Saratale et al. (2009) also reported on high decolorization performance of azo dye Scarlet R by consortium-GR, consisting of *Proteus vulgaris* NCIM-2027 (PV) and *Micrococcus glutamicus* NCIM-2168 (MG), which could mineralize the dye to near completion within 3 h.

3.2. Analyses of azo reduction dye intermediates

LC–MS/MS analysis was carried out to investigate the dye intermediates formed after decolorization of Amaranth and further biotransformation of dye intermediates by NAR-2 bacterial consortium as well as the individual bacterial strains of *C. freundii* A1, *E. casseliflavus* C1 and *E. cloacae* L17. The compounds and its respective mass interpreted from both positive and negative ionization mass spectra are listed in Table 1 and Table 2. The compounds produced by individual bacterial strains are indicated in Table 1 and Table 2 (footnotes). Based on the products detected

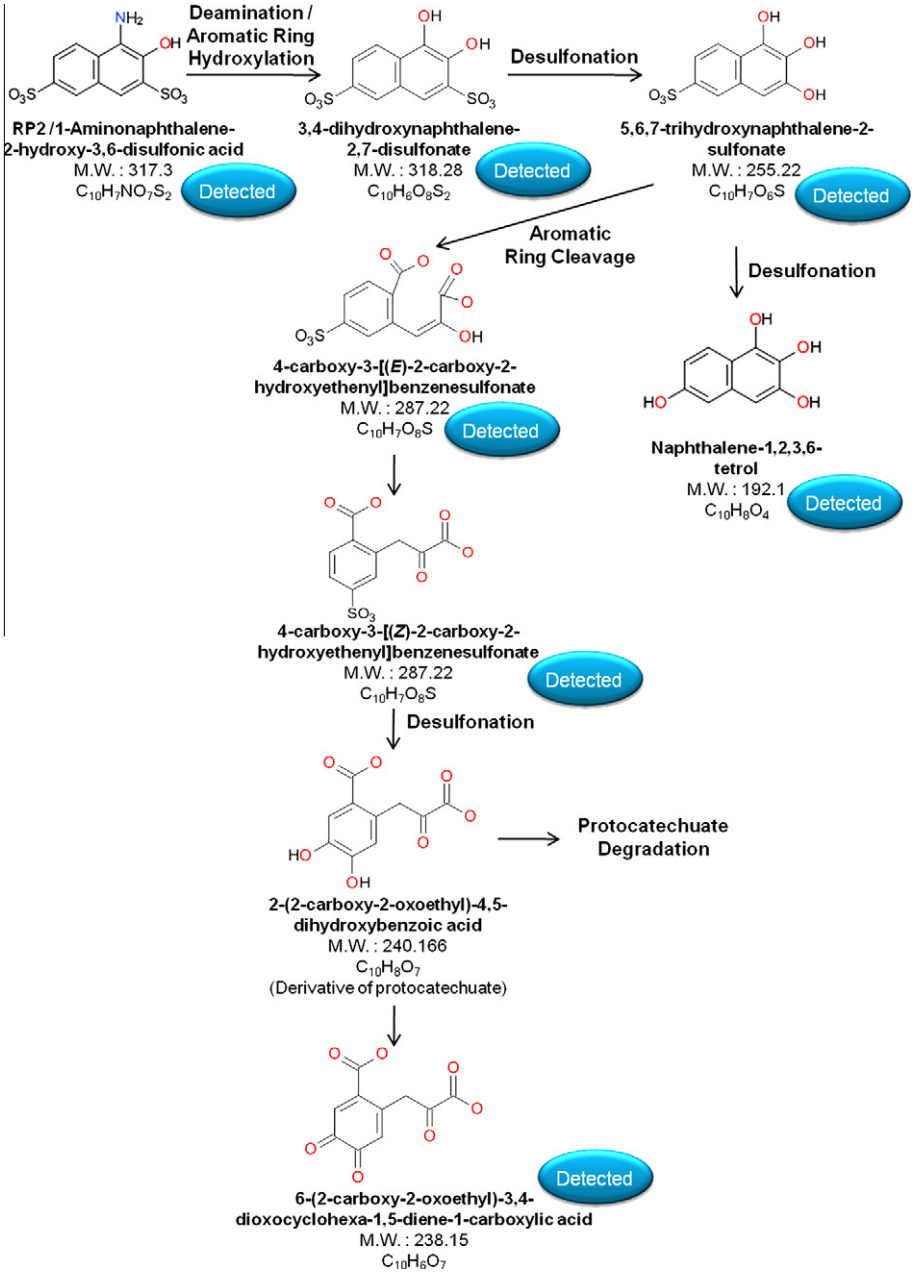

Fig. 3b. Proposed oxidative degradation pathway of RP2 by NAR-2 bacterial consortium.

by LC–MS/MS, individual bacterial strains of *C. freundii* A1, *E. casseliflavus* C1 and *E. cloacae* L17 showed great potential in azo reduction, deamination, desulfonation and aromatic ring cleavage, but lacked the ability to perform successive biotransformation of the degradation intermediates. Not only *E. casseliflavus* C1 was the dominant strain in decolorization which probably played an important role in azo reduction, this species could also biodegrade further, up to desulfonation of the reduced dye intermediates. On the other hand, NAR-2 bacterial consortium demonstrated better potential as compared to individual bacterial strains in consecutive aerobic biodegradation of dye intermediates, as shown in both Table 1 and Table 2 that listed additional biotransformation products.

Decolorization of Amaranth by NAR-2 bacterial consortium was carried out under microaerophilic condition suggesting that reductive degradation of Amaranth was more likely to happen during the preliminary stage of biodegradation (Fig. 2). As indicated in Fig. 2, Amaranth initially underwent partial cleavage of the azo bond to produce hydrazo intermediate followed by symmetric reductive cleavage to produce sulfonated aromatic amines, namely 1-aminonaphthalene-4-sulfonic acid and 1-aminonaphthalene-2-hydroxy-3,6-disulfonic acid, designated as reduction products, RP1 and RP2, respectively. Hong et al. (2007) revealed the presence of similar sulfonated aromatic amines, RP1 and RP2 as the first products of Amaranth degradation by *S. decolorationis* S12. The presence of hydrazo compound had been demonstrated by Ramya et al. (2010) during azo reduction of Acid Red 37 by *Acinetobacter radioresistens*, which was further reduced to aromatic amines. In addition, hydrazo-intermediate was also traced by Bin et al. (2004), and this provides convincing evidence to the assumption that azo dyes were degraded via azo reduction as the first step in biodegradation. Microbial degradation of azo dyes via the reduction of azo bonds catalyzed by azo reductases have been extensively reported (as reviewed by Saratale et al., 2011; Pandey et al., 2007). In a previous study done in this lab, it was found that the crude cell-free extract of *E. casseliflavus* C1 exhibited azo reductase activity in the presence of NADH and riboflavin (Lee, 2003). In another study, flavin reductase was proven to be the azo reductase from *C. freundii* A1 responsible for the reduction of azo dyes (Wahab, 2007). Hence, azo reduction can be elucidated as the first step in biodegradation of azo dyes by NAR-2 bacterial consortium.

In this study, the presence of RP1 could not be detected from LC–MS/MS analysis done on decolorization products from NAR-2 bacterial consortium as well as from individual bacterial strains. The presence of RP1 was perhaps very transient as it may have undergone rapid biodegradation to naphthalene and naphthalene-1,2,4-triol as these intermediates were actually detected by LC–MS/MS analysis done on NAR-2 decolorization products (Table 1). On the other hand, RP2 was detected and it was further biodegraded to 3,4-dihydroxynaphthalene-2,7-disulfonate and 5,6,7-trihydroxynaphthalene-2-sulfonate (Table 1). Fig. 2 shows

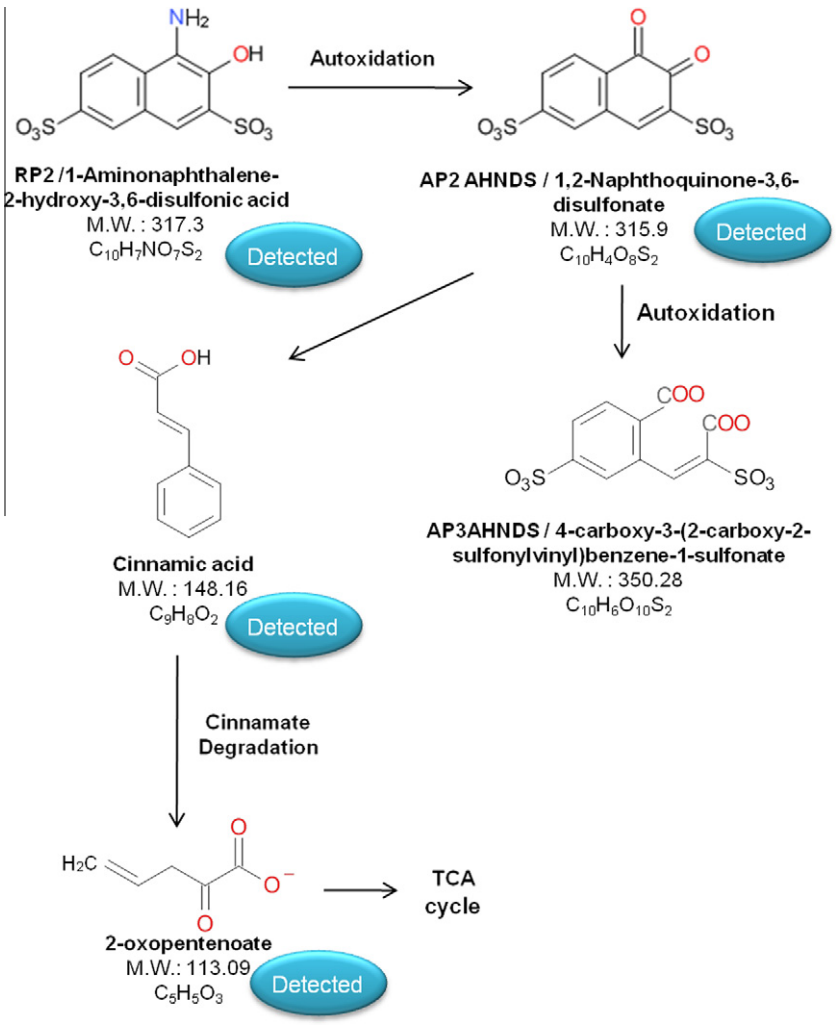

Fig. 3c. Autoxidation of RP2 under microaerophilic condition.

the proposed biodegradation pathway for RP1 and RP2 by NAR-2 bacterial consortium. Further reductive biotransformation of RP1 and RP2 was observed under microaerophilic condition and this could involve reductive deamination and desulfonation pathways. In analyzing the communal action of consortium PMB11 consisting of *Bacillus odysseyi* SUK3, *Morganella morganii* SUK5 and *Proteus* sp. SUK7 for Red HE3B azo dye degradation, Patil et al. (2010) proposed deamination and desulfonation as the degradation mechanisms for biotransformation of 5-aminonaphthal sulfate to naphthalene. Interestingly, even though deamination and desulfonation was mostly reported as oxidative biodegradation steps, the results obtained here suggest that NAR-2 bacterial consortium could possibly utilize deamination and desulfonation steps during microaerophilic biodegradation of sulfonated aromatic dye intermediates following azo reduction.

Following azo reduction, the step prior to desulfonation could possibly be deamination by transaminase enzyme that removed the amine group. In a preliminary study done, PCR was used to amplify partial genes from *C. freundii* A1 and *E. cloacae* L17 which were shown to possess homology to transaminase genes (Nasiri, 2011). Hence, the bacterial strains *C. freundii* A1 and *E. cloacae* L17 from the NAR-2 bacterial consortium could possibly function in the deamination of the reduced dye intermediates. Savelieva et al. (2004) have demonstrated that deamination of aminoaromatic acid by *C. freundii* strain WA1 was possible under anoxic condition. Simultaneous deamination by *C. freundii* strain WA1 was observed to reduce 5-aminosalicylate to the respective alcohol during growth on pyruvate, glucose, or serine as electron donor (Savelieva et al., 2004). Reductive deamination was also proposed as a new initial step in anaerobic biodegradation of halogenated anilines

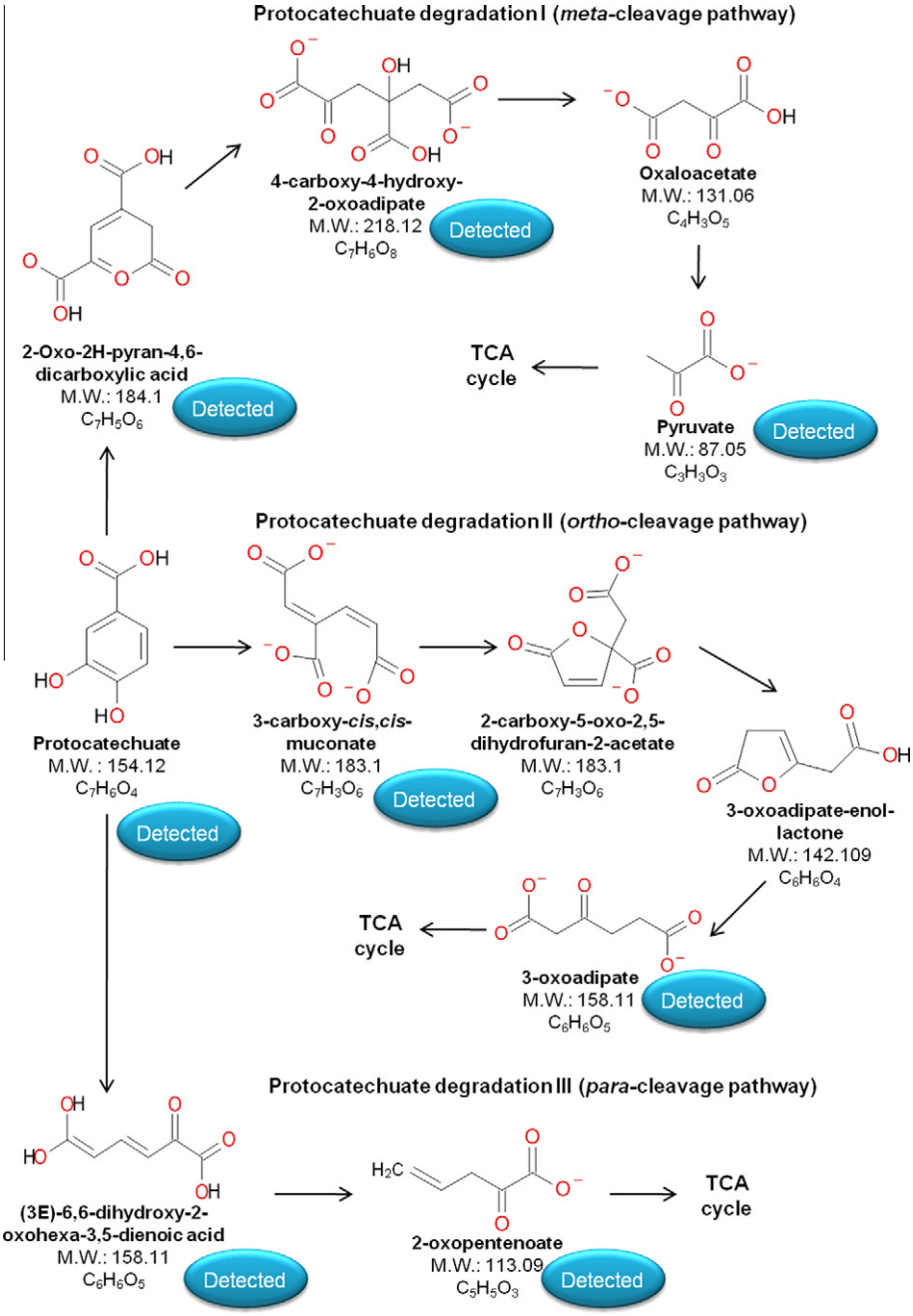

Fig. 4. Proposed protocatechuate degradation pathways by NAR-2 bacterial consortium.

by *Rhodococcus* sp. (Travkin et al., 2002). Magony (2004) found that a locus containing genes encoding transaminase and ring hydroxylating dioxygenase enzymes, which probably participated in the biotransformation of *p*-amino-benzenesulfonate (which is a typical representative of aromatic sulfonated amines) to sulfocatechol. Furthermore, *in silico* analysis of partial transaminase genes from both *C. freundii* A1 and *E. cloacae* L17 revealed the presence of the upstream gene which codes for GntR-family transcriptional regulator. This regulator is a transcriptional repressor of pathway that controls the degradation of aromatic compounds (Tropel and van der Meer, 2004). In the absence of pathway substrate, the regulator regulates by repression. Tropel and van der Meer (2004) explained that in the presence of pathway substrate, repression is released by interaction of the regulator with aromatic compounds or one of its metabolites.

Besides, *E. casseliflavus* C1 from NAR-2 bacterial consortium was found to possess a gene which codes for sulfatase domain protein (Nasiri, 2011). Therefore, this could be the candidate in the NAR-2 bacterial consortium which contributed to the desulfonation process of sulfonated dye intermediates. According to a review by Hanson et al. (2004), sulfatase enzyme was involved in hydrolytic desulfonation of sulfate esters (CO–S) and sulfamates (CN–S) and this enzyme was discovered in *E. faecalis*. More biological functions of this enzyme from prokaryotic sources have yet to be discovered and explored. Desulfonation was previously reported as a step in biodegradation of other sulfonated aromatic compounds, including substituted naphthalene sulfonates and benzene sulfonates (Zurrer et al., 1987; Haug et al., 1991). Anoxic desulfonation of aromatic sulfonates by *Clostridium* sp. was established by Denger et al. (1996). Hence, NAR-2 bacterial consortium may possess comparable multi-component dioxygenases with broad substrate specificity which cleave C–S bond in bacterial desulfonation of aromatic compounds.

The metabolites formed during the oxidative biodegradation process of RP1 and RP2 were analyzed and the biodegradation pathways are described in Fig. 3. Fig. 3a shows the proposed pathway for the biodegradation of RP1 by NAR-2 bacterial consortium under aerobic condition. The mass of intermediates including (*E*)-2-carboxy-1-(2-carboxyphenyl)ethenesulfonate, 2-[(*E*)-2-carboxy-1-hydroxyethenyl]benzoic acid and 2-(carboxyacetyl)benzoic acid were observed after further biotransformation of RP1 (Table 2). The degradation mechanism probably involved oxygenases which function in aromatic ring cleavage. The 2-(carboxyacetyl)benzoic acid intermediate formed was hypothesized to be further biodegraded via benzoyl-CoA metabolism. Bacterial components in NAR-2 consortium could probably biodegrade RP1 via benzoyl-CoA. From the MetaCyc database, *C. freundii*, *E. casseliflavus* and *E. cloacae* were found to possess both aerobic and anaerobic benzoyl-CoA degradation pathways. Interestingly, in our preliminary study, *C. freundii* A1 and *E. cloacae* L17 were also found to possess genes which code for long chain fatty acid-CoA ligase (Nasiri, 2011). According to Egland et al. (1995), several fatty acid-CoA ligases had significant level of identity with benzoate-CoA ligase,

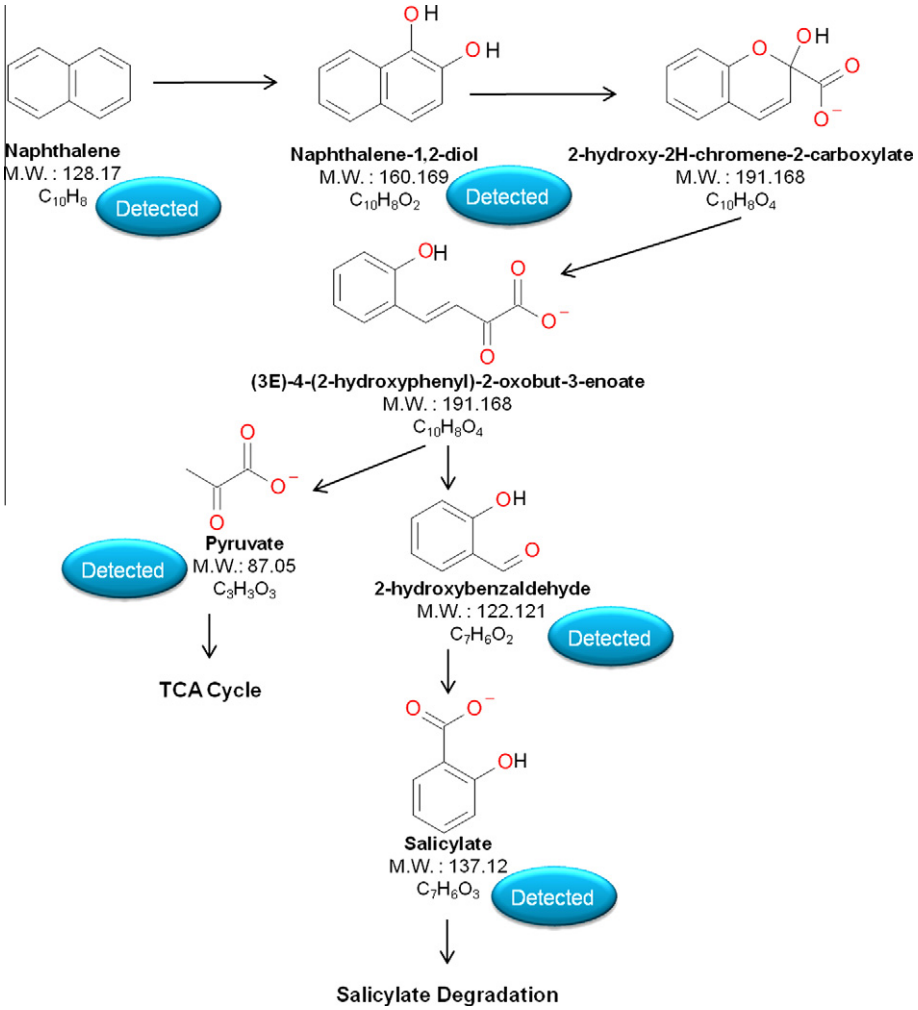

Fig. 5. Proposed naphthalene degradation pathway by NAR-2 bacterial consortium.

which is the first enzyme in metabolism involving benzoate, benzoyl acetate and other related derivatives.

Similar degradation mechanism on RP2 is also proposed, which may involve the function of oxygenases for aromatic ring cleavage (Fig. 3b). The oxidative degradation by NAR-2 bacterial consortium resulted in the observation of mass of intermediates including 4-carboxy-3-[(*E*)-2-carboxy-2-hydroxyethenyl]benzenesulfonate, 4-carboxy-3-[(*Z*)-2-carboxy-2-hydroxyethenyl]benzenesulfonate, and 6-(2-carboxy-2-oxoethyl)-3,4-dioxocyclohexa-1,5-diene-1-carboxylic acid (Table 2). Desulfonation most likely occurred to remove the sulfonates. It is possible to hypothesize that the 2-(2-carboxy-2-oxoethyl)-4,5-dihydroxybenzoic acid formed undergone successive biotransformation and mineralization via protocatechuate degradation pathways (as discussed later in Section 3.3).

Even though certain azo dyes can be mineralized by the anaerobic–aerobic treatment systems, this strategy also has serious drawbacks. Most amines formed during the anaerobic reduction of the azo dyes are unstable under aerobic conditions as the compounds could undergo autoxidation to produce recalcitrant intermediates (Stolz, 2001). Under microaerophilic condition, 1-aminonaphthalene-2-hydroxy-3,6-disulfonic acid (RP2) was autoxidized to 1,2-naphthoquinone-3,6-disulfonate (AP2 AHNDS), traced in samples from Amaranth decolorization (Table 2). AP2 AHNDS was also observed from decolorized sample of *E. casseliflavus* C1 (Table 2). The biotransformation pathway is illustrated in Fig. 3c. The RP2 intermediate contains a hydroxy group in *ortho*-position to the amino group which could undergo autoxidation in the presence of oxygen and subsequently reacted to form cinnamic acid and 2-oxopentenoate. The LC–MS/MS data further suggests the presence of cinnamic acid and 2-oxopentenoate. Further metabolism of cinnamic acid could have happened very slowly as a significant intensity of the mass for this compound was detected under aerobic condition during consecutive biotransformation. Besides that, according to Kudlich et al. (1999), AP2 AHNDS could be further autoxidized to AP3 AHNDS (4-carboxy-3-(2-carboxy-2-sulfonylvinyl) benzene-1-sulfonate). However, AP3 AHNDS could not be detected here and there is a possibility that biotransformation from AP2 AHNDS to AP3 AHNDS did not occur. This may be due to the presence of catalase as both *C. freundii* A1 and *E. cloacae* L17 are catalase-positive. Kudlich et al. (1999) reported a similar observation on the absence of AP3 AHNDS in the presence of 50U catalase. AP2 AHNDS could be subsequently reacted to form a disulfonated cinnamic acid derivative (Kudlich et al., 1999).

3.3. Analyses of successive biotransformation pathways

Our LC–MS/MS data further suggests the involvement of three protocatechuate degradation pathways, as shown in Fig. 4. From the MetaCyc database, *C. freundii*, *E. casseliflavus* and *E. cloacae* were known to possess three protocatechuate degradation pathways. The *meta*-cleavage pathway could result in detection of

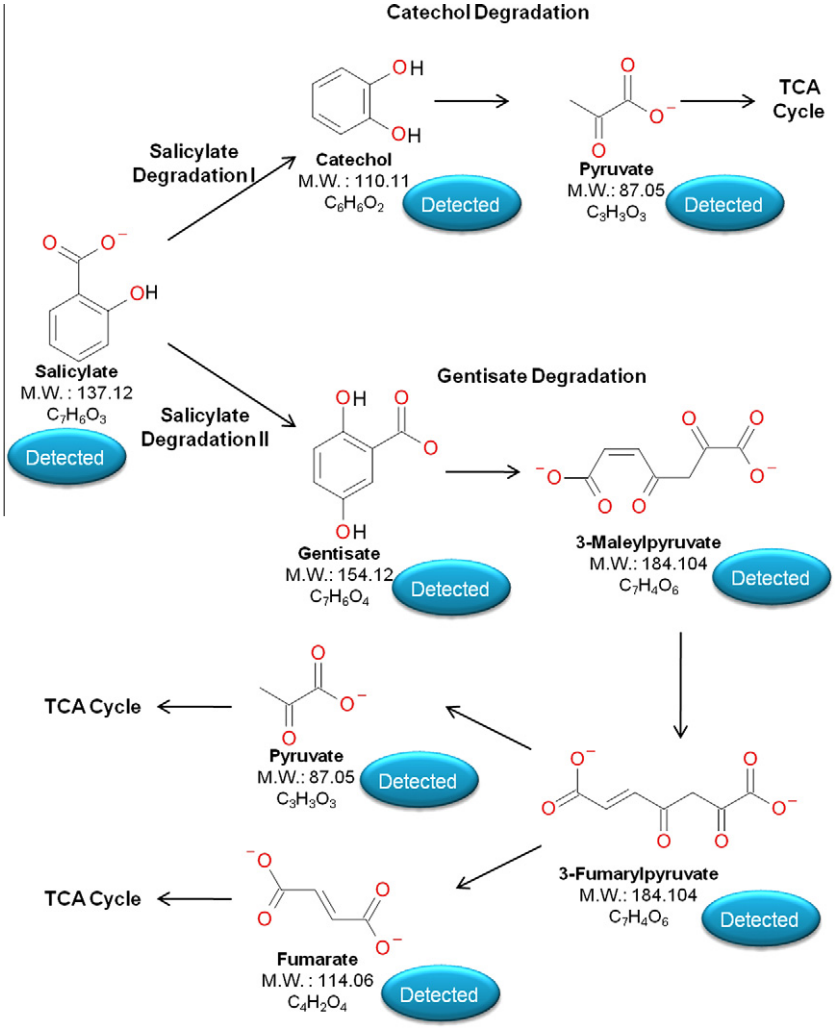

Fig. 6. Proposed salicylate degradation pathways by NAR-2 bacterial consortium.

metabolites including 2-oxo-2H-pyran-4,6-dicarboxylic acid, 4-carboxy-4-hydroxy-2-oxoadipate and pyruvate (Table 2), which eventually enter the TCA cycle (Fig. 4). The mass of metabolites including 3-carboxy-*cis*,*cis*-muconate, 3-oxoadipate, (3*E*)-6,6-dihydroxy-2-oxohexa-3,5-dienoic acid and 2-oxopentenoate were observed (as listed in Table 2) and this suggests the involvement of both *ortho*-cleavage and *para*-cleavage pathways (Fig. 4). The further biotransformed metabolites would then enter the TCA cycle. These observations demonstrated that there is a high possibility for mineralization to occur under aerobic degradation of dye products, RP1 and RP2 by NAR-2 bacterial consortium.

In addition, naphthalene could be detected after microaerophilic degradation by NAR-2 consortium (Table 1, Fig. 2). So far, two main routes to activate the anaerobic degradation of naphthalene had been proposed, including carboxylation (Zhang et al., 2000) or methylation followed by fumarate addition (Safinowski et al., 2006). In the carboxylation pathway proposed by Zhang et al. (2000), intermediates including 2-naphthoate, dihydro-2-naphthoic acid, 5,6,7,8-tetrahydro-2-naphthoic acid, hexahydro-2-naphthoic acid, octahydro-2-naphthoic acid and decalin-2-carboxylate were produced. On the other hand, intermediates from the methylation and fumarate addition pathway include 2-methylnaphthalene, naphthyl-2-methyl-succinic acid, naphthyl-2-methyl-succinyl-CoA and naphthyl-2-methylene-succinyl-CoA (Safinowski et al., 2006; Meckenstock and Mouttaki, 2011). However, none of the mass for these intermediates was observed in our LC–MS/MS data.

Hence, a different degradation pattern of naphthalene under aerobic condition is proposed here, and metabolites observed including naphthalene-1,2-diol, 2-hydroxybenzaldehyde, salicylate and pyruvate (Table 1 and Table 2). Fig. 5 shows the proposed naphthalene degradation pathway of NAR-2 bacterial consortium. From the MetaCyc database, *C. freundii*, *E. casseliflavus* and *E. cloacae* were identified to possess naphthalene degradation pathway that converts naphthalene to salicylate. Interestingly, salicylate degradation produces catechol and gentisate which could be mineralized. The metabolites for the two degradation pathways of salicylate are described in Fig. 6. Until now, there is no report on naphthalene degradation by bacteria from *C. freundii*, *E. casseliflavus* and *E. cloacae*. Recently, Selvakumaran et al. (2011) reported on the genetic diversity of aromatic ring-hydroxylating dioxygenase (ARHD) genes from *Citrobacter* spp. which code for dioxygenase enzymes. The variability of these dioxygenases would be reflected upon their functional diversity and their ability to completely degrade of benzoate, hydroxybenzoic acid and phenol (Selvakumaran et al., 2011).

4. Conclusions

Enhanced decolorization rate by novel NAR-2 bacterial consortium which could decolorise Amaranth in 30 min is reported here. The fate of azo dye intermediates which is a major concern in textile wastewater treatment is addressed here through LC–MS/MS analyses. The detection of mass for benzoyl-CoA, protocatechuate, salicylate, gentisate, catechol, cinnamic acid and 2-oxopentenoate points to the great potential of NAR-2 bacterial consortium to mineralize dye intermediates as these metabolites could be metabolized via TCA cycle. Novel NAR-2 consortium could function sequentially under two diversed conditions; microaerophilic condition for decolorization of azo dyes and aerobic condition for mineralization of reduced dye intermediates – suggesting the flexibility of their metabolic capabilities.

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References

Ali, H., 2010. Biodegradation of synthetic dyes – a review. Water Air Soil Pollut. 213, 251–273. doi:10.1007/s11270-010-0382-4.

Bin, Y., Jiti, Z., Jing, W., Cuihong, D., Hongman, H., Zhiyong, S., Yongming, B., 2004. Expression and characteristics of the gene encoding azo reductase from *Rhodobacter sphaeroides* AS1.1737. FEMS Microbiol. Lett. 236, 129–136. doi:10.1111/j.1574-6968.2004.tb09638.x.

Caspi, R., Altman, T., Dale, J.M., Dreher, K., Fulcher, C.A., Gilham, F., Kaipa, P., Karthikeyan, A.S., Kothari, A., Krummenacker, M., Latendresse, M., Mueller, L.A., Paley, S., Popescu, L., Pujar, A., Shearer, A.G., Zhang, P., Karp, P.D., 2010. The MetaCyc database of metabolic pathways and enzymes and the BioCyc collection of pathway/genome databases. Nucleic Acid Res. 38, D473–D479. doi:10.1093/nar/gkp875.

Chan, G.F., Rashid, N.A.A., Koay, L.L., Chang, S.Y., Tan, W.L., 2011. Identification and optimization of novel NAR-1 bacterial consortium for the biodegradation of Orange II. Insight Biotechnol. 1, 7–16. doi:10.5567/IBIOT-IK.2011.7.16.

Denger, K., Kertesz, M.A., Vock, E.H., Schon, R., Magli, A., Cook, A.M., 1996. Anaerobic desulfonation of 4-tolysulfonate and 2-(4-sulfophenyl) butyrate by a *Clostridium* sp. Appl. Environ. Microbiol. 62, 1526–1530.

Egland, P.G., Gibson, J., Harwood, C.S., 1995. Benzoate-coenzyme A ligase, encoded by *badA*, is one of three ligases able to catalyze benzoyl-coenzyme A formation during anaerobic growth of *Rhodopseudomonas palustris* on benzoate. J. Bacteriol. 177, 6545–6551.

Handayani, W., Meitiniarti, V.I., Timotius, K.H., 2007. Decolorization of acid red 27 and reactive red 2 by *Enterococcus faecalis* under a batch system. World J. Microbiol. Biotechnol. 23, 1239–1244. doi:10.1007/s11274-007-9355-1.

Hanson, S.R., Best, M.D., Wong, C.-H., 2004. Sulfatases: structure, mechanism, biological activity, inhibition, and synthetic utility. Angew. Chem. Int. Ed. 43, 5736–5763. doi:10.1002/anie.200300632.

Haug, W., Schmidt, A., Nortemann, B., Hempel, D.C., Stolz, A., Knackmuss, H.-J., 1991. Mineralization of the sulfonated azo dye mordant yellow 3 by a 6-aminonaphthalene-2-sulfonate-degrading bacterial consortium. Appl. Environ. Microbiol. 57, 3144–3149.

Hong, Y., Guo, J., Zhicheng, X., Cuiyun, M., Meiying, X., Guoping, S., 2007. Reduction and partial degradation mechanisms of naphthylaminesulfonic azo dye Amaranth by *Shewanella decolorationis* S12. Appl. Microbiol. Biotechnol. 75, 647–654. doi:10.1007/s00253-007-0838-7.

Jain, R., Sharma, N., Radhapyari, K., 2009. Electrochemical treatment of pharmaceutical azo dye Amaranth from waste water. J. Appl. Electrochem. 39, 577–582. doi:10.1007/s10800-008-9695-z.

Joshi, T., Iyengar, L., Singh, K., Garg, S., 2008. Isolation, identification and application of novel bacterial consortium TJ-1 for the decolorization of structurally different azo dyes. Bioresour. Technol. 99, 7115–7121. doi:10.1016/j.biortech.2007.12.074.

Khehra, M.S., Saini, H.S., Sharma, D.K., Chadha, B.S., Chimni, S.S., 2005. Comparative studies on potential of consortium and constituent pure bacterial isolates to decolorize azo dyes. Water Res. 39, 5135–5141. doi:10.1016/j.watres.2005.09.033.

Kudlich, M., Hetheridge, M.J., Knackmuss, H.-J., Stolz, A., 1999. Autoxidation reactions of different aromatic *o*-aminohydroxynaphthalenes that are formed during the anaerobic reduction of sulfonated azo dyes. Environ. Sci. Technol. 33, 896–901.

Lee, S.Z., 2003. Development of an enzyme assay for decolorization of azo dyes by bacterium C1. Dissertation Universiti Teknologi Malaysia.

Magony, M., 2004. Characterization of the biodegradation pathways of sulfanilic acid and catechol derivatives in *Sphingomonas subarctica*. Acta Biologica Szegediensis. 48, 65.

Meckenstock, R.U., Mouttaki, H., 2011. Anaerobic degradation of non-substituted aromatic hydrocarbons. Curr. Opin. Biotech. 22, 406–414. doi:10.1016/j.copbio.2011.02.009.

Nasiri, R., 2011. Molecular identification of biodegradation related genes from dye-degrading microbes. Dissertation Universiti Teknologi Malaysia.

Pandey, A., Singh, P., Iyengar, L., 2007. Bacterial decolorization and degradation of azo dyes. Int. Biodeterior. Biodegrad. 59, 73–84. doi:10.1016/j.ibiod.2006.08.006.

Patil, P.S., Phugare, S.S., Jadhav, S.B., Jadhav, J.P., 2010. Communal action of microbial cultures for Red HE3B degradation. J. Hazard. Mater. 181, 263–270. doi:10.1016/j.jhazmat.2010.05.006.

Pearce, C.I., Lloyd, J.R., Guthrie, J.T., 2003. The removal of color from textile wastewater using whole bacterial cells: A review. Dyes Pigm. 58, 179–196. doi:10.1016/S0143-7208(03)00064-0.

Phugare, S.S., Kalyani, D.C., Surwase, S.N., Jadhav, J.P., 2011. Ecofriendly degradation, decolorization and detoxification of textile effluent by a developed bacterial consortium. Ecotoxicol. Environ. Safe. 74, 1288–1296. doi:10.1016/j.ecoenv.2011.03.003.

Pinheiro, H.M., Touraud, E., Thomas, O., 2004. Aromatic amines from azo dye reduction: status review with emphasis on direct UV spectrophotometric detection in textile industry wastewaters. Dyes Pigm. 61, 121–139. doi:10.1016/j.dyepig.2003.10.009.

Ramya, M., Iyappan, S., Manju, A., Jiffe, J.S., 2010. Biodegradation and decolorization of Acid Red by *Acinetobacter radioresistens*. J. Bioremed. Biodegrad. 1, 105. doi:10.4172/2155-6199.1000105.

Safinowski, M., Griebler, C., Meckenstock, R.U., 2006. Anaerobic cometabolic transformation of polycyclic and heterocyclic aromatic hydrocarbons:

evidence from laboratory and field studies. Environ. Sci. Technol. 40, 4165–4173. doi:10.1021/es0525410.

Saratale, R.G., Saratale, G.D., Chang, J.S., Govindvar, S.P., 2011. Bacterial decolorization and degradation of azo dyes: a review. J. Taiwan Inst. Chem. E 42, 138–157. doi:10.1016/j.jtice.2010.06.006.

Saratale, R.G., Saratale, G.D., Kalyani, D.C., Chang, J.S., Govindwar, S.P., 2009. Enhanced decolorization and biodegradation of textile azo dye Scarlet R by using developed microbial consortium-GR. Bioresout. Technol. 100, 2493–2500. doi:10.1016/j.biortech.2008.12.013.

Savelieva, O., Kotova, I., Roelofsen, W., Stams, A.J.M., Netrusov, A., 2004. Utilization of aminoaromatic acids by a methanogenic enrichment culture and by a novel *Citrobacter freundii* strain. Arch. Microbiol. 181, 163–170. doi:10.1007/s00203-003-0645-1.

Selvakumaran, S., Kapley, A., Kashyap, S.M., Daginawala, H.F., Kalia, V.C., Purohit, H.J., 2011. Diversity of aromatic ring-hydroxylating dioxygenase gene in *Citrobacter*. Bioresour. Technol. 102, 4600–4609. doi:10.1016/j.biortech.2011.01.011.

Stolz, A., 2001. Basic and applied aspects in the microbial degradation of azo dyes. Appl. Microbiol. Biotechnol. 56, 69–80.

Travkin, V., Baskunov, B.P., Golovlev, E.L., Boersma, M.G., Boeren, S., Vervoort, J., van Berkel, W.J.H., Rietjens, I.M.C.M., Golovleva, L.A., 2002. Reductive deamination as a new step in the anaerobic microbial degradation of halogenated anilines. FEMS Microbiol. Lett. 209, 307–312. doi:10.1111/j.1574-6968.2002.tb11149.x.

Tropel, D., van der Meer, J.R., 2004. Bacterial transcriptional regulators for degradation pathways of aromatic compounds. Microbiol. Mol. Biol. R. 68, 474–500. doi:10.1128/MMBR.68.3.474-500.2004.

Wahab, M.F.A., 2007. Analysis of *Citrobacter freundii* A1 whole-cell and its recombinant flavin reductase biodegradation of azo dyes using spectrophotometric and voltammetric techniques. Dissertation Universiti Teknologi Malaysia.

Zhang, X., Sullivan, E.R., Young, L.Y., 2000. Evidence for aromatic ring reduction in the biodegradation pathway of carboxylated naphthalene by a sulfate reducing consortium. Biodegradation 11, 117–124. doi:10.1023/A:1011128109670.

Zurrer, D., Cook, A.M., Leisinger, T., 1987. Microbial desulfonation of substituted naphthalenesulfonic acids and benzenesulfonic acids. Appl. Environ. Microbiol. 53, 1459–1463.