# Use of active consortia of constructed ternary bacterial cultures via mixture design for azo-dye decolorization enhancement

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## Abstract

This first-attempt study used constructed bacterial consortia containing *Escherichia coli* DH5α (a weak decolorizer) and its UV-irradiated mutants (*E. coli* UVT1 and UV68; strong decolorizers) via equilateral triangle diagram and mixture experimental design to assess color removal during species evolution. The results showed that although strain DH5α was not an effective decolorizer, its presence might still played a significant role in affecting optimal color removal capabilities of mixed consortia (e.g., *E. coli* DH5α, UVT1 and UV68) for two model azo dyes; namely, reactive red 22 (RR22) and reactive black 5 (RB5). Contour analysis of ternary systems also clearly showed that decolorization of RR22 and RB5 by DH5α-containing active mixed consortia was more effective than mono-cultures of the stronger decolorizer alone (e.g., UVT1). The optimal composition of the mixed consortium (UV68, UVT1, DH5α) achieving the highest specific decolorization rate was (13%:58%:29%) and (0%:74%:26%) for decolorization of RR22 and RB5, respectively, with initial total cell density fixed at $OD_{600} = 3.5 \pm 0.28$.
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## 1. Introduction

Azo dyes are the most commonly used synthetic dyes in textile, cosmetic, paper-making, and food industry [1–4]. However, effluents of dye-utilizing industries contain residual dyes, thereby remarkably deteriorating the water quality [5,6]. As a result, it is of great demand to develop effective means to treat dye-bearing wastewater. Bio-treatment usually has the advantage of low cost, high efficiency and no secondary pollution.

The biochemical basis of azo-dye decolorization has recently been a subject of debate. As indicated in Stolz [7], there are multiple reaction mechanisms involved for azo-dye decolorization under anaerobic conditions. For example, azo-dye decolorization is an almost ubiquitous property of bacteria under anaerobic or oxygen-limited or depleted conditions (e.g., static cultures). Russ et al. [8] reported that reduced flavins produced by cytosolic flavin-associated reductases were in charge for non-specific reduction of azo dyes via different genetic bases. Furthermore, if enzymatic reduction is involved, the enzymes are usually

not strictly specific azoreductases, but likely membrane-bound hydrogenases because of their relative non-specificity with respect to their electron acceptor. In addition, azo-dye reduction may also be due to a reaction with reduced enzyme cofactors (e.g., riboflavin). Decolorization at neutral pHs is expected to be particularly unspecific when low-molecular redox mediators are involved [7]. In addition, the presence of cometabolic carbon sources can enhance reduction rates via the formation of reduction equivalents used for azo bond breakdown.

This study is motivated to unlock the mysteries whether the predominance of the effective dye decolorizer(s) is one of indispensable criteria for optimal decolorization in practical treatment. In addition, this model system using genetically modified mutants originating from the parental strain DH5α tended to reveal whether dye-laden environments indeed favor the predominance of stains in higher decolorization capability for enhancement of color removal performance. Since the mechanisms responsible for azo-dye decolorization have still not been clearly revealed [9], random mutagenesis (i.e., UV irradiation) was employed on *E. coli* DH5α to develop mutants with a better decolorization capability. In addition, experimental design using mutants originating from the same parental strain DH5α was also

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done intentionally to exclude the confounding effects to affect this comparative analysis due to difference in non-growth associated metabolism in non-related species. In our recent work, two mutant strains (*E. coli* UVT1 and UV68) with excellent decolorization capabilities were screened [10]. It was indicated herein that using mixed consortia was more promising than using mono-cultures of the most effective decolorizer (e.g., UVT1) alone to achieve maximum decolorization.

In this study, the decolorization behavior of the mixed cultures containing parental strain *E. coli* DH5α and its mutants UVT1 and UV68 was examined to understand the interactive relationship (e.g., competition or mutualism) of these species in the azo dye containing environment. To understand the interactive behavior of the ternary-species system quantitatively, the concept of two-dimensional equilibrium diagram for ternary systems [11] was adopted to determine optimal allocation for maximal decolorization performance. Meanwhile, unbiased response surface mixture design was used to determine optimal composition of the constructed mixed consortia displaying the best decolorization performance. This study firstly provided an innovative means of analyzing biotreatment with mixed consortia by using the equilateral triangular diagram and mixture design. This suggests a novel approach (i.e., equilateral triangle diagram combined with mixture design) to transform three-dimensional (3D) design into 2D design for better realization in geometry. Compared to lower dimensional systems (e.g., conventional experimental design), higher dimensional system is not easily understood. With incorporation of operation performance as the third variable, 2D system could include the contour in response surfaces of the third variable to clearly disclose the optimal species compositions for maximal decolorization efficiency. Thus, clear geometric presentation in a lower-dimension domain is the most significant advantage for using this novel assessment. Another advantage of this study is that the population size for inspection was specified within admissible experimental conditions (e.g., non-saturated cultures and study domain in lower dimension) to prevent several local extrema in higher dimensional systems. Thus, lower-dimension systematic analysis converted from higher-dimension may simply remove several local optimal points supposed to be shown, thereby achieving the overall global optimum.

## 2. Materials and methods

### 2.1. Microorganisms and culture conditions

The strains used in this study were the parent strain *E. coli* DH5α and two isolated mutants *E. coli* UVT1 and *E. coli* UV68, which were screened among mutants for high decolorization potentials by random mutation of the original strain with UV light (maximum wavelength at ca. 253.7 nm, 15 W, 4-h exposure) and selected on azo-dye containing LB agar [10]. For single cultures, to obtain synchrony in the division of cultures for study, a loopful of bacterial seed strains (i.e., *E. coli* DH5α, UVT1 and UV68) taken from an isolated colony on a LB-streak plate was precultured in 50-ml Bacto LB broth ($10 \, \text{g} \, \text{l}^{-1}$ tryptone, $5.0 \, \text{g} \, \text{l}^{-1}$ yeast extract, $10 \, \text{g} \, \text{l}^{-1}$ NaCl)

for 10–12 h at 37 °C (optimal cell-growth temperature), pH 7.0, and 200 rpm. Note that pre-inoculated cultures of all strains were in the identical specific growth rate (data not shown), and thus all of precultured inocula could be reasonably achieved at the same time in the early stationary phase. Appropriate volume of cultured broth was then inoculated into fresh LB media to obtain an initial $OD_{600}$ of $3.5 \pm 0.17$ for cell cultures. For constructed mixed cultures (initial total cell density fixed at $OD_{600} = 3.5 \pm 0.28$), cells were precultured for 10–12 h at 37 °C, pH 7.0, and 200 rpm, then different mixed cultures were constructed at the cell density ($OD_{600}$) ratios of *x*:*y*:*z* for UV68 (*x*), UVT1 (*y*) and DH5α (*z*), respectively. Note that decolorization did not occur in sterile cell-free medium, suggesting the absence of abiotic decolorization activity.

### 2.2. Measurement of cell and dye concentrations

The cell concentration was determined by measuring the absorbance of the cell solution at a wavelength of 600 nm. The relation between the cell mass concentration and $OD_{600}$ was $1.0 \, OD_{600} \cong 0.291 \, \text{g} \, \text{l}^{-1}$ dry cell weight. The concentration of a single azo dye was also determined by monitoring the maximal absorbance ($\lambda_{max}$) at 595 and 510 nm for reactive black 5 (RB5) and reactive red 22 (RR22), respectively [12]. The relations between the dye concentration and $OD_{\lambda_{max}}$ were RR22 ($\text{mg} \, \text{l}^{-1}$) $\cong 40.79 \, OD_{510}$ and RB5 ($\text{mg} \, \text{l}^{-1}$) $\cong 31.25 \, OD_{595}$ within the linear ranges.

### 2.3. Batch decolorization experiments

Cell growth was conducted aerobically, while decolorization was carried out under static cultures (i.e., low dissolved oxygen condition) [13]. Typical procedures for batch decolorization of single dye (i.e., RR22 or RB5 (i.e., RBB) [12]) are as follows: an appropriate amount of filtered sterilized dye (RR22 or RB5) and 0.5% (v/v) of yeast extract (Difco) was added into early-stationary-phase cultures (ca. 10–12 h) of the three strains after aerobic growth in LB to reach an initial dye concentration of $200 \, \text{mg} \, \text{l}^{-1}$. The cultures were then incubated at 28 °C for decolorization without agitation (i.e., static incubation) and samples were taken at designated time intervals to measure the concentration of cells and residual dye. For experiments of constructed mixed community $MC_{xyz}$ at a fixed total population density of $OD_{600} = 3.5 \pm 0.28$, similar decolorization procedures were carried out on cultures consisting of a cell density ratio of UV68:UVT1:DH5α = *x*:*y*:*z*.

### 2.4. Determination of biodecolorization performance

The performance of decolorization was evaluated by specific decolorization rate (SDR). The SDR represents the decolorization activity per cell, taking into account the performance of the cells within the reactor. The specific decolorization rate (SDR) was calculated according to the equation $SDR = -(1/X)(\text{d[Dye]}/\text{d}t)$, where *X* denotes cell concentration ($\text{g} \, \text{l}^{-1}$ dry cell weight), [Dye] the residual dye concentration ($\text{mg} \, \text{l}^{-1}$), and *t* denotes the incubation time (h). As decolorization

performance is apparently time-dependent due to non-steady state characteristics in batch cultures, the SDR is termed as a maximal value of $-(1/X)(\mathrm{d}[\mathrm{Dye}]/\mathrm{d}t)$ similar to the definition for specific growth rate.

## 2.5. Mixture design of experiments

Response surface method (RSM) for experimental designs is regularly built to fit a curved surface (quadratic) to continuous factors so that the factor values for the minimum or maximum response can be sought. However, the three- or two-factor RSM design cannot simply be used in our systems as all of the factor levels were located between 0 and 1, and the sum of the factor levels in all design points was normalized to unity. Due to the constraints (i.e., $\sum x_i = x + y + z = 100\%$ and a constant initial total population size), we reduce the degree of freedom from 3 to 2. Thus, mixed cultures assigned in different species compositions could be conducted by combining "mixture design" (i.e., augmented simplex design with center point) [14] and RSM for non-biased experimentation as indicated in an equilateral-triangle graphical presentation (Fig. 1A). A three-factor rotatable central composite design (RCCD) of RSM was used to build up the whole experiment list. In this RCCD design, to ensure all the experiment data were large than 0, the center point was set as (1.6818, 1.6818, 1.6818). Every factor in each experimental run was normalized by dividing the total amount of the three factors and presented as a relative ratio. Table 1 shows the code of RSM and the values of related factor. The number of runs listed in Table 1 was also shown in Fig. 1A. The collocated points in this mixture design were chosen to reveal optimal conditions (e.g., compositions in the microbial community) in non-biased and equally weighted domain (i.e., assumed data present in a normal distribution). All the experimental points were selected in symmetrical to "center of mass" in the defined triangular domain. For example, point # (10, 12, and 14), points #(2, 3, and 5) are triplet points symmetric to center of mass (i.e., point 1). Points 1, 8, and 15 were triplicated trials for inspection of variation within the same sampled point. The purpose of using this "response surface mixture design" is to consider an equal distribution of all experimental tasks over the response surface for this ternary system, obtaining a non-biased optimal composition for an active consortium for maximal decolorization capability.

## 2.6. Ternary plot and contour analysis for optimal active consortia

To assess constructed ternary systems for bioaugmentation, the equilateral triangle method, which is originally used to plot two-dimensional phase equilibrium diagrams for ternary systems, has been proposed (Fig. 1A and B). Note that the initial total population size was controlled at a constant level (i.e., $OD_{600} = 3.5 \pm 0.28$) for comparative purpose to exclude confounding interferences of total cell population. Each apex of the triangle is taken as 100% of the species with which it is designated (e.g., A ($y = 100$), B ($z = 100$), C ($x = 100$) in Fig. 1A). To plot any point on the diagram (Fig. 1B) such as D, having $x\%$ $x$, $y\%$ $y$ and $z\%$ $z$, we locate first $x\%$ line DE, and next the $y\%$ line DF. Then, the intersection of two lines yields the desired point. Aware that the relationships in geometry (Fig. 1B) hold as follows

$$z = 100 - x - y, \tag{1}$$

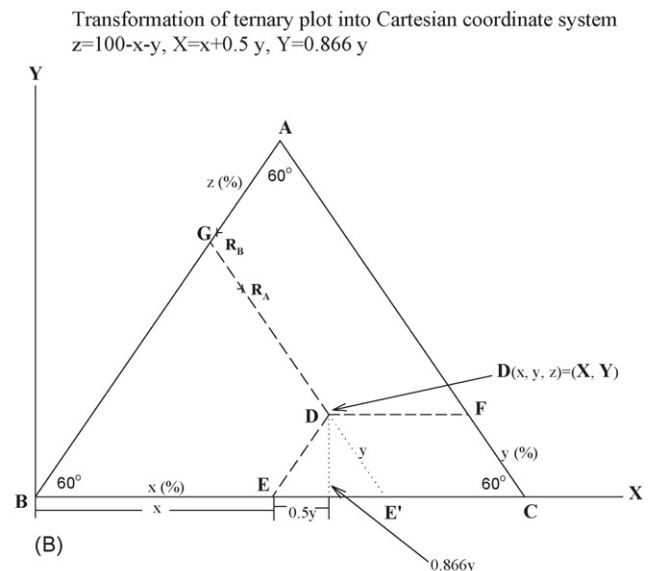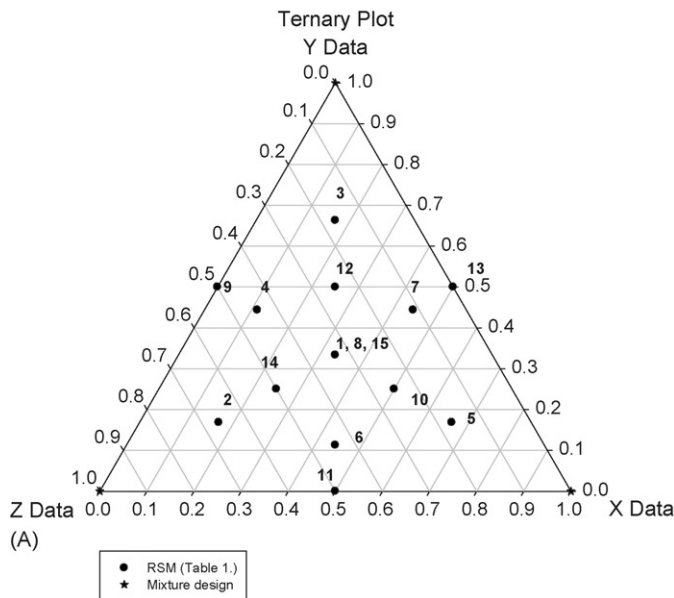$$\mathrm{CF} = \mathrm{DE}' = y, \tag{2}$$



Fig. 1. (A) Geometric design of experiments via response surface methodology (RSM) and mixture designs. (B) Transformation of ternary plot in equilateral triangle diagram into Cartesian coordinate system ($z = 100 - x - y$, $X = x + 0.5y$, $Y = 0.866y$). Symbols $x$, $y$, $z$ represent *Escherichia coli* UV68, UVT1 and DH5α, respectively. (A) Design I (mixture design): (1) number of factors = 3; (2) type design of "central composite". Design II (response surface method): (1) number of factors = 3; (2) type design of "central composite".

Table 1
The experiment design list in which the data of the mixture design were transformed from central composite design of response surface methodology (RSM)

| No. | Code for RSM design | | | Transform to mixture design | | |
|---|---|---|---|---|---|---|
| Two level points | | | | | | |
| 1 | 0.6818 | 0.6818 | 0.6818 | 0.3333 | 0.3333 | 0.3333 |
| 2 | 0.6818 | 0.6818 | 2.6818 | 0.1685 | 0.1685 | 0.6629 |
| 3 | 0.6818 | 2.6818 | 0.6818 | 0.1685 | 0.6629 | 0.1685 |
| 4 | 0.6818 | 2.6818 | 2.6818 | 0.1128 | 0.4436 | 0.4436 |
| 5 | 2.6818 | 0.6818 | 0.6818 | 0.6629 | 0.1685 | 0.1685 |
| 6 | 2.6818 | 0.6818 | 2.6818 | 0.4436 | 0.1128 | 0.4436 |
| 7 | 2.6818 | 2.6818 | 0.6818 | 0.4436 | 0.4436 | 0.1128 |
| 8 | 2.6818 | 2.6818 | 2.6818 | 0.3333 | 0.3333 | 0.3333 |
| Axial points | | | | | | |
| 9 | 0.0000 | 1.6818 | 1.6818 | 0.0000 | 0.5000 | 0.5000 |
| 10 | 3.3636 | 1.6818 | 1.6818 | 0.5000 | 0.2500 | 0.2500 |
| 11 | 1.6818 | 0.0000 | 1.6818 | 0.5000 | 0.0000 | 0.5000 |
| 12 | 1.6818 | 3.3636 | 1.6818 | 0.2500 | 0.5000 | 0.2500 |
| 13 | 1.6818 | 1.6818 | 0.0000 | 0.5000 | 0.5000 | 0.0000 |
| 14 | 1.6818 | 1.6818 | 3.3636 | 0.2500 | 0.2500 | 0.5000 |
| Center | | | | | | |
| 15 | 1.6818 | 1.6818 | 1.6818 | 0.3333 | 0.3333 | 0.3333 |

$$E'G = x + y = BG,  \qquad (3)$$

$$AB = AG + BG = x + y + z = 100. \qquad (4)$$

To carry out contour analysis for species allocation of RSM, we then transform this ternary plot into Cartesian coordinate system via geometric relationships:

$$X = x + \frac{y}{2} = x + 0.5y, \qquad (5)$$

$$Y = \sqrt{\frac{3}{2}}y = 0.866y. \qquad (6)$$

The transformed data $(X, Y)$ is then acquired to fit an empirical polynomial model:

$$SDR = f(X, Y) = \sum_i \sum_j b_{ij} X^i Y^j. \qquad (7)$$

According to Taylor's expansion, polynomial model may be feasible to describe kinetic model in the domain near operation conditions. Here, the function (e.g., specific decolorization rate SDR) may be written as a relationship:

$$
\begin{aligned}
f(X, Y) &= f(X_s + \delta X, Y_s + \delta Y) \\
&\cong f(X_s, Y_s) + \left.\frac{\partial f}{\partial X}\right|_{(X_s, Y_s)} \delta X + \left.\frac{\partial f}{\partial X}\right|_{(X_s, Y_s)} \delta Y \\
&\quad + \frac{1}{2}\left.\frac{\partial^2 f}{\partial X^2}\right|_{(X_s, Y_s)} (\delta X)^2 + \left.\frac{\partial^2 f}{\partial X \partial Y}\right|_{(X_s, Y_s)} (\delta X)(\delta Y) \\
&\quad + \left.\frac{\partial^2 f}{\partial Y^2}\right|_{(X_s, Y_s)} (\delta Y)^2 + O(\varepsilon^2) \\
&\cong a + b(X - X_s) + c(Y - Y_s) + d(X - X_s)^2 \\
&\quad + e(X - X_s)(Y - Y_s) + f(Y - Y_s)^2 + \cdots
\end{aligned}
$$

$$\cong a^* + b^* X + c^* Y + d^* X^2 + e^* XY + f^* Y^2 + \cdots, \qquad (7')$$

where $\delta X$ and $\delta Y$ denoted the deviations of variables $X$ and $Y$ from the set point $(X_s, Y_s)$, respectively. That is why empirical polynomial model (i.e., contour analysis) is technically feasible to describe the system behavior (not simply curve-fitting). Aware that one may use the equilateral triangle method for the ternary systems to reduce a quadratic response in "three-dimensional contour surface":

$$
\begin{aligned}
g(x, y, z) &= ax^2 + by^2 + cz^2 + dxy + exz + fyz + gx \\
&\quad + hy + lz + j
\end{aligned} \qquad (8)
$$

to three-dimensional response surface:

$$f(X, Y) = \alpha X^2 + \beta Y^2 + \delta XY + \phi X + \gamma Y + \eta \qquad (9)$$

By introducing constraint relationship $z = 100 - x - y$ and linear transform of (5) and (6), the relative extrema of $f(X, Y)$ can then be determined by

$$f_X(a, b) = 0 \quad \text{and} \quad f_Y(a, b) = 0. \qquad (10)$$

To test for relative extrema of a function of two independent variables $f(X, Y)$, one may consider the quantity:

$$d = f_{XX}(a, b) f_{YY}(a, b) - [f_{XY}(a, b)]^2 \qquad (11)$$

(1) If $d > 0$ and $f_{XX}(a, b) < 0$, then $f$ has a relative maximum at $(a, b)$.
(2) If $d < 0$, then $(a, b, f(a, b))$ is a saddle point (i.e., neither relative maximum nor minimum).

In addition, according to Calculus [15], global extrema (i.e., optimal points) on a closed boundary only occur at relative extrema or endpoints on boundary. That implies if there exists no

relative optimal points within the considered range, the extreme point will be simply located on endpoints on boundary domain (e.g., side AB, BC, CA or binary systems, see Fig. 1B) [15]. Once optimal point(s) have been obtained, one may thus perform inverse transform:

$$x = X - \frac{Y}{\sqrt{3}}, \tag{12}$$

$$y = \frac{2Y}{\sqrt{3}}, \tag{13}$$

and then use Eq. (1) to obtain species composition in this consortium as anticipated.

## 3. Results and discussion

To get a grasp of whether dye decolorization is more effectively accomplished by active mixed consortia than a single "hero decolorizer", contour analysis of ternary species systems was carried out. For mono-azo dye RR22, the SDR response in an empirical polynomial model is obtained as $f(X, Y) = -0.0007359X^2 - 0.001002Y^2 - 0.0002041XY + 0.11Y + 3.211$. The optimal composition of maximal active consortium can be obtained at $(X, Y) = (42.31, 50.59)$ due to holding relationships of relative maximum (i.e., $d > 0$ and $f_{XX} < 0$). Inverse transform of $(X, Y) = (42.31, 50.59)$ gives the point $R_A$ (see Fig. 1B) in Cartesian coordinate system (UV68%:UVT1%:DH5α%) = (13.11:58.41:28.48), indicating the optimal composition of mixed consortia for the maximal RR22 decolorization activity. This optimum can also be observed in Fig. 2 (i.e., contour diagram).

For diazo-dye RB5, the response in an empirical polynomial model can be expressed as $f(X, Y) = 0.0003367X^2 -$

$0.0008731Y^2 - 0.0008006XY - 0.0105X + 0.1634Y + 3.4831$. Although the relative extreme of maximal active consortium can be obtained at $(X, Y) = (82.09, 55.94)$, it is unfortunately a saddle point due to the holding relationship of $d = f_{XX}(a, b)f_{YY}(a, b) - [f_{XY}(a, b)]^2 < 0$ as indicated in Fig. 3. In addition, it is obviously located outside of the equilateral domain $\triangle ABC$ (Fig. 1B) defined for our ternary systems (i.e., $(x, y, z) = (49.79, 64.59, -14.38)$). According to Calculus [15], the optimal extrema should thus be located on the boundaries. From graphical presentation in Fig. 1B, if it is on side AB (i.e., $x = 0$ and $Y = \sqrt{3}X$), one may obtain $f(X, Y) = -0.0036863X^2 + 0.272517X + 3.4831$ and the maximal value of 8.53 occurs at $(X, Y) = (37.12, 64.27)$. If it is on side BC (i.e., $y = 0$, $X = x$ and $Y = 0$), one may obtain $f(X, Y) = 0.003367X^2 - 0.0105X + 3.4831$ and a minimum occurs at $X = 15.59$. On the other hand, if it is on side CA (i.e., $z = 0$, $x + y = 100$, $X = 50 - 0.5x$, and $Y = 86.6 - 0.866x$), one may simply obtain a monotonically decreasing $f(X, Y)$ (i.e., $df/dX < 0$) without an optimal value. Thus, it is concluded that the maximal biodecolorization should lie on boundary line AB (i.e., $x = 0$) as shown in Figs. 1B and 3. The optimal composition of maximal active consortium can be evaluated at $(X, Y) = (37.12, 64.29)$ using relationships of $X = Y/\sqrt{3}$, $y = 2Y/\sqrt{3}$ and $\partial f/\partial Y = 0 \wedge \partial^2 f/\partial Y^2 < 0$. This result implies that to have a maximal decolorization of RB5, UV68 should be excluded to construct an UVT1-dominant binary consortium. This point $R_B$ (i.e., $R_B$ in Fig. 1B) was (UV68%:UVT1%:DH5α%) = (0.00:74.27:25.73). These results clearly support that mother species DH5α that was considered metabolically dormant in decolorization still played a significant role to assist its daughter species (e.g., UVT1) to achieve effective decolorization [16]. In contrast to DH5α, UV68 (a sibling of UVT1 and an active decolorizing mutant)
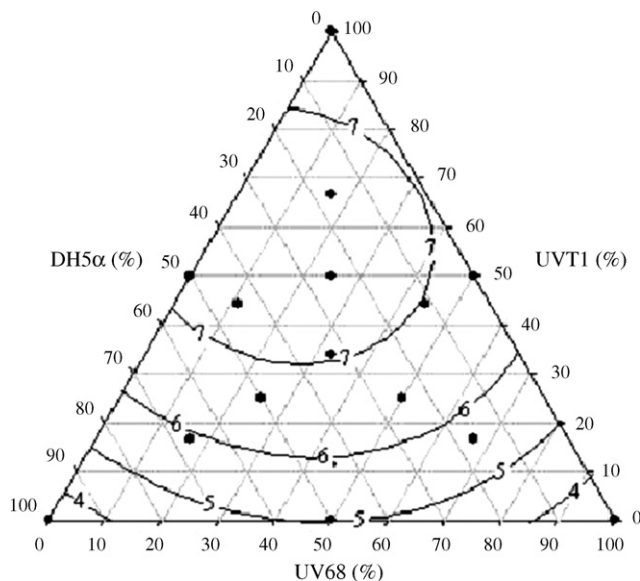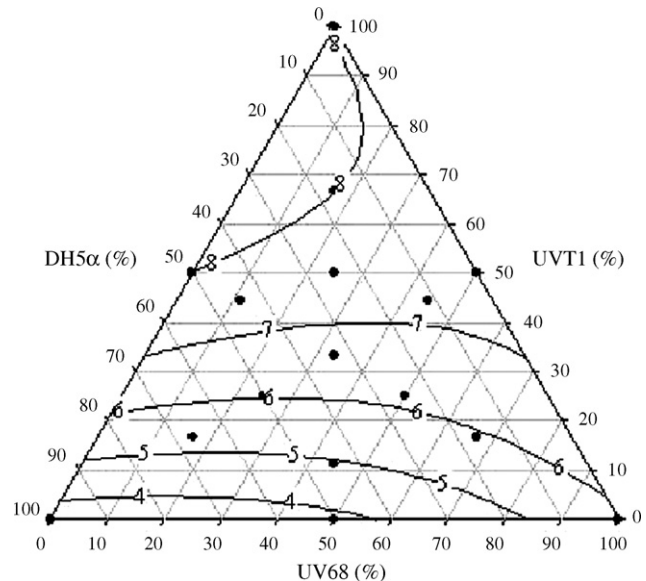


Fig. 2. Specific decolorization rate (SDR) response curve and experimental design of ternary species systems for decolorization of RR22 in equilateral triangle contour plot. Projected contour profiles indicated the existence of response maximum at (UV68:UVT1:DH5α) = (13.1%:58.4%:28.5%) and "volcano" characteristics of responses (i.e., point $R_A$ in Fig. 1B). Numbers shown on the contours indicated the level values of SDR.



Fig. 3. Specific decolorization rate (SDR) response curve and experimental design of ternary species systems for decolorization of RB5 in equilateral triangle contour plot. Projected contour profiles indicated the presence of the critical extreme at the DH5α side of triangle and "saddle" characteristics of responses (i.e., point $R_B$ in Fig. 1B). Numbers shown on the contours indicated the level values of SDR.

seemed to be relatively ineffective in assisting UVT1 for decolorization enhancement [16,17]. The reasons to cause this difference in species compositions of mixed consortia for optimal dye decolorizations are straightforward. As known, different cells are effective at controlling gene expression and translation to accommodate their specific metabolic functions for optimal decolorization. Thus, although the mutants were all derived from DH5α, all genes are not transcribed and translated at the same rate among mutants very likely due to random mutation upon the regulation and control for gene expression in decolorization. For example, at any given time in a mutant, only certain genes are "turned-on" or expressed to produce proteins (e.g., azoreductase), while some other mutant genes are silenced or repressed. These alterations in gene regulation after mutation significantly affected relationships among mutants in decolorization. In addition, this change would direct some species with nonessential functions in mixed cultures to play a crucial role as a "keystone species" to influence treatment performance [17,18]. This novel ternary-plot analysis using equilateral triangle method provides a useful assessment model to construct feasible mixed consortia as bioagents to bioaugmentation for on-site or *in situ* applications. Follow-up study to use optimal constructed consortia for bioaugmentation will be carried out in practical field applications. In addition, molecular-level understanding of the causes of ineffective decolorization capability of UV68 will also be revealed.

## 4. Conclusions

In summary, this study suggested that (1) decolorization by mono and constructed mixed cultures showed that *E. coli* UVT1 mutant accounted for the majority of decolorization activity for biotreatment of dye pollutants; (2) in the absence of less efficient decolorizers (i.e., DH5α and UV68) the optimal decolorization performance of the whole bacterial population might not be obtained; (3) response surfaces of mixture design in ternary systems clearly indicated that the presence of less effective decolorizers might still play a crucial role to enhance overall azo-dye decolorization. The discrepancy of optimal species composition for decolorization in these two different dyes might also suggest the presence of a most feasible treatment of combined interactions among species to decolorize the specific dye likely due to different chemicals structures of dyes required diverse optimal metabolic functions for cooperation. The contour analysis shows that maximal decolorization of monoazo RR22 and diazo RB5 took place at active mixed consortia rather than the single cultures. Due to interspecies interactions (e.g., competition or cooperation) in decolorization of various dyes, certain species might express higher color removal capability toward one dye than another. It might suggest why optimal consortia were not always identical to various dyes due to different combined interactions for treatment. In addition, this study only suggested some bioaugmentation strategy for color removal in a short-term (i.e., batch culture) rather than long-term basis (e.g., CSTR), as the outcome variables were all based on transient dynamics in batch data. From an ecological perspective, the

species evolution will be considered in the follow-up studies and to determine stable consortia for decolorization in a long-run. Moreover, the detailed molecular mechanism will be disclosed regarding why the specific constructed consortium possessed optimal decolorization efficiency.

## Acknowledgements

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