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An Application of Mixed Consortium in Microbial Degradation of Reactive Red: Effective Strategy of Bioaugmentaiton

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Abstract In this paper, replacement-series method and contour analysis were applied to investigate optimal bioaugmentation strategies for the treatment of a dye-contaminated aquatic system using a constructed mixed-community for biodecolorization of a model azo dye Reactive Red. The novelty emphasizes that a species without essential target functions in a mixed culture could still play a crucial role in influencing the treatment performance. That is, although non-decolorizers (i.e., *Escherichia coli* DH5α) were considered metabolically ‘‘dormant’’ in this model binarybiosystem, their presence still significantly enhanced decolorization performance of the decolorizers (i.e., *Pseudomonas spp*.). In aerobic growth conditions, *E. coli* DH5α possessed a growth advantage to out-compete *Pseudomonas spp*. due to preferential growth rate of DH5α. However, in static decolorization conditions, DH5α seemed to produce decolorization-stimulating extracellular metabolites to help the major decolorizer (*Pseudomonas spp*.) decompose the toxic pollutant (i.e., the azo dye) in a short term for the benefit of total survival in the environment. The experimental results show that the presence of *E. coli* DH5α increased the decolorization efficiency of *Pseudomonas spp*. even though DH5α was an inefficient decolorizer in this microbial community. Thus, addition of DH5α into a mixed culture containing *Pseudomonas spp*. as a major decolorizer may lead to a bioaugmentation effect on decolorization activity. The optimal population ratio for bioaugmentation was determined by the contour analysis. The results indicate that the optimal community species ecology for maximum overall decolorization rate almost maintained at a ratio of one viable *Pseudomonas spp*. (0.78×10^9 cells/mL) to one DH5α cell (0.70×10^9 cells/mL), representing a maximal diversity (i.e., Hmax $\cong$ 1.0).

Keywords: *Pseudomonas, reactive red, bioaugmentation, decolorization*

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1. Introduction

Since biotreatment of industrial wastewater had been developed, it is often designed to operate by open-system mixed consortia in an activated sludge treatment plant. Pollutant degradation is more frequently achieved by active consortia of organisms rather than a single ‘‘star’’ species at task, as the effective field application of this species has limited capacity to present maximal treatment performance due to competition ‘‘repression’’ in native populations which are well-acclimated to the existing environment [1]. Thus, the objective of this study is to get a grasp of species interactions (e.g., competition, mutualism) and to design an optimal microbial community for bioaugmentation of decolorization via ‘‘replacement-series method’’ and contour analysis. Obviously, lack of information on species interactions in the communities makes the applications for bioaugmentation unpredictable and unreliable for the on-site operation. This study is also to quantitatively emphasize that using mixed cultures instead of pure biodegraders as a bioaugmentation strategy is biologically favorable for microbial decolorization. To reach such perspective, understanding interspecific interactions in this mixed consortiumis ecologically significant and technologically imperative. As Chen [2] indicated, although Escherichia coli is not considered as an effective decolorizer, its presence still extensively enhances the decolorization activity of Pseudomonas luteola likely due to extracellular metabolites released as ‘‘stimulators/enhancers’’ [3]. The altruistic behavior of DH5α assists its ‘‘partner’’ species—P. luteola to implement decolorization more efficiently as both species are under a threat of dye ‘‘attack’’. It is anticipated that all species, irrespective of decolorizers or nondecolorizers, in the microbial community may make use of their metabolic activities to reach a goal of pollutant degradation or detoxification for total survival. Azo dyes are the largest chemical class of dyes frequently used in textile, food colorants, cosmetic, printing and pharmaceutical

industries [4,5]. Thus, these industrial effluents often contain residual dye, which deteriorates the water quality, and may become a threat to public health since certain azo dyes or their metabolites (e.g., aromatic amines) may be highly toxic and potentially mutagenic or carcinogenic [6]. Albeit not a direct growth substrate to most of bacteria, azo dye can be transformed or degraded via cometabolism of other energy sources by a variety of aerobic and anaerobic bacteria and fungi [1,7,8,9]. Bacterial degradation of azo dye is often initiated under anaerobic conditions by an enzymatic biotransformation step that involves cleavage of azo linkages with the aid of azoreductase utilizing NADH as the electron carrier [2,10,11,12,13]. The resulting intermediate metabolites (e.g., aromatic amines) are further degraded aerobically or anaerobically [14,15].

Fungal species (e.g., Phanerochaete chrysosporium) used lignin peroxidase to degrade azo dyes under aerobic conditions [16]. Pure fungal culture has been used to develop bioprocesses for mineralization of azo dyes [17,18,19]. However, a long growth cycle and moderate decolorization rate still limit the performance of the fungal decolorization system [1]. In contrast, bacterial decolorization is normally faster, but it may require a mixed community ecosystem to mineralize azo dyes completely through combined anaerobic-aerobic systems [13,20]. The biochemical basis of azo dye decolorization has recently been a subject of debate. As indicated in Stolz [21], 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/depleted conditions (i.e., static cultures). Russ et al. [22] 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 lowmolecular redox mediators are involved [21]. 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 employs a novel approach from an ecological engineering perspective to decouple mixed cultures for treatment of dye-bearing wastewater. This novelty emphasizes why a species carrying non-essential functions (i.e., nondecolorizer DH5α) still plays a crucial role to enhance color removal of decolorizers (i.e., *Pseudomonas spp.*). Moreover, the purposes of the study are to resolve certain problems of importance: (1) Is there any significant interference (e.g., competition, commensalism or mutualism; [23]) on the decolorizer due to the presence of non-decolorizer? (2) Does competitive exclusion or altruistic characteristics of species control either long or short-term species evolution as well as dye-decolorization performance? (3) Is there any feasible means to prevent the extinction of decolorizers due to the stress of non-decolorizers predominated in mixed consortia? (4) Is a monoculture of decolorizer more technically feasible than a mixed culture for bioaugmentation of decolorization activities? (5) Does there exist any ‘‘keystone species’’ [24] to enhance degradation capability of pollutant degraders?

2. Materials and Methods

2.1. Microorganism and Culture Conditions

Pseudomonas spp. [10] isolated from activated sludge of a wastewater treatment plant in Ankleshwar, Gujarat,India was used as an indicator species to present decolorization performance. The model dye used in the study is C.I. Reactive Red (chemical structure as indicated in [2]). To demonstrate interspecies interactions between species, a fast-growing *E. coli* DH5α strain was chosen as an augmented non-decolorizer predominant in nature. A loopful of *Pseudomonas spp.* seed taken from an isolated colony in YG-streak plate was precultured in 50-mLYG medium (0.3% Difco yeast extract and 0.125% glucose) for 24 h at 28 8C, pH 7.0 and 200 rpm. Note that Bacto-agar (1.5%) was added for agar plate use. The 5% (v/v) cultured broth was then inoculated to fresh YG media and *Pseudomonas spp.* was harvested at early-stationary phase (ca. 8 h). A loopful of *E. coli* DH5α seed taken from an isolated colony in LB-streak plate was precultured in 50-mL Luria-Bertani medium (LB broth, Miller, Difco) for 12–24 h under same conditions. Cultured broth 5% (v/v) was inoculated into fresh LB media and then *E. coli* DH5α was harvested at 12 h. For study uses, harvested cells were centrifuged at 4°C, 10,130 x g for 10 min. After supernatants were removed, cell precipitates were mixed with 15 mL PBS (phosphate-buffered saline; 8 g/L NaCl, 0.2 g/L KCl, 1.15 g/L Na_2HPO4, 0.2 g/L KH_2PO_4) solutions and then stirred to obtain homogenized solutions. To prevent possible interference of residual metabolites or inhibitors present in harvested cultures, the centrifugation procedure was repeated. After supernatant was discarded, the cell paste was rinsed and mixed well with 2.5 mL PBS solutions. Cell mixtures were then ready for decolorization and replacement-series study uses. Aerobic growth (AG) and static decolorization (SD) experiments were conducted in mixed-consortia batch cultures designed at approximately constant initial total viable cell (ca. (1.0 ± 0.3) x 107 and (1.0 ± 0.3) x 109 cell/mL for AG and SD, respectively) invarious ratios of *Pseudomonas spp.* To DH5α (e.g., 1.0/0.0, 0.9/ 0.1, 0.8/0.2, 0.7/0.3, 0.6/0.4, 0.5/0.5, 0.4/0.6, 0.3/0.7, 0.2/0.8, 0.1/0.9, 0.0/1.0). The cultures containing 0.5%yeast extract-bearing media supplemented with various dye concentrations were incubated at 28 8C, pH 7.0 and 200 rpm (Figure 1 – Figure 4). Since the SD process would inhibit cell growth [2] due to insufficient oxygen supply, higher initial total cell densities (i.e., (1.0 ± 0.3) x 10^9 cell/mL) were used for decolorization experiments to guarantee color removal in significant levels. The ‘‘static decolorization’’ means ‘‘color removal in the presence of trace dissolved oxygen (ca. <3 mg/L)’’ in our previous report [11].

2.2. Replacement-series Method

Implementation of replacement-series method marked a milestone to understand species interactions occurring in mixed communities. In particular, there has been a marked

rise in the numbers of ecologists who use replacement-series evaluation as an index of interspecific competition [25,26,27]. Replacement-series method provides information of transient characteristics of species evolution to indicate the likelihood of one species (i.e., competitor species) to out-compete another (i.e., target species) to conclude operation stability for long-term degradation in different community compositions. In these designs, bacterial species are grown in monocultures and mixed cultures of a given population. In addition, bacteria are grown at a constant initial total population density whereas proportions of bacteria in mixtures are varied. In this study, considering criteria of resource exhaustion, contour analysis upon decolorization performances in various community compositions not only revealed the mixed consortium for a maximal degradation, but also concluded a guideline for optimization of bioaugmentation agents in practical applications. Supplementation of this decisive bioaugmentation agent at optimal community compositions provides the most scientifically preferential strategy to resume degradation capability in a short-term, particularly to solve ''shock-loading'' operations for stability maintenance in practical wastewater treatment. To clearly reveal competition and replacement trends of species i in the presence of non-sibling species j (i # j), relative CFU (colony forming unit or cell) is defined as the CFU of species i in the presence of species j divided by the CFU of pure species i in the absence of species j at the same time. This normalized term—relative CFU is to present the growth characteristics of species i in mixed cultures compared to that in pure cultures of species i at the same time in dye-laden environments. For example, relative CFU less or greater than unity indicated that the population size of species i is suppressed or enhanced due to the presence of the species j, respectively. Therefore, as Mark and Lindow [27] mentioned, a replacement-series curve located below and above the theoretical line (i.e., the line predicted by pure culture of species i) indicates that the population size of species i is repressed and stimuated in the presence of species j, respectively.

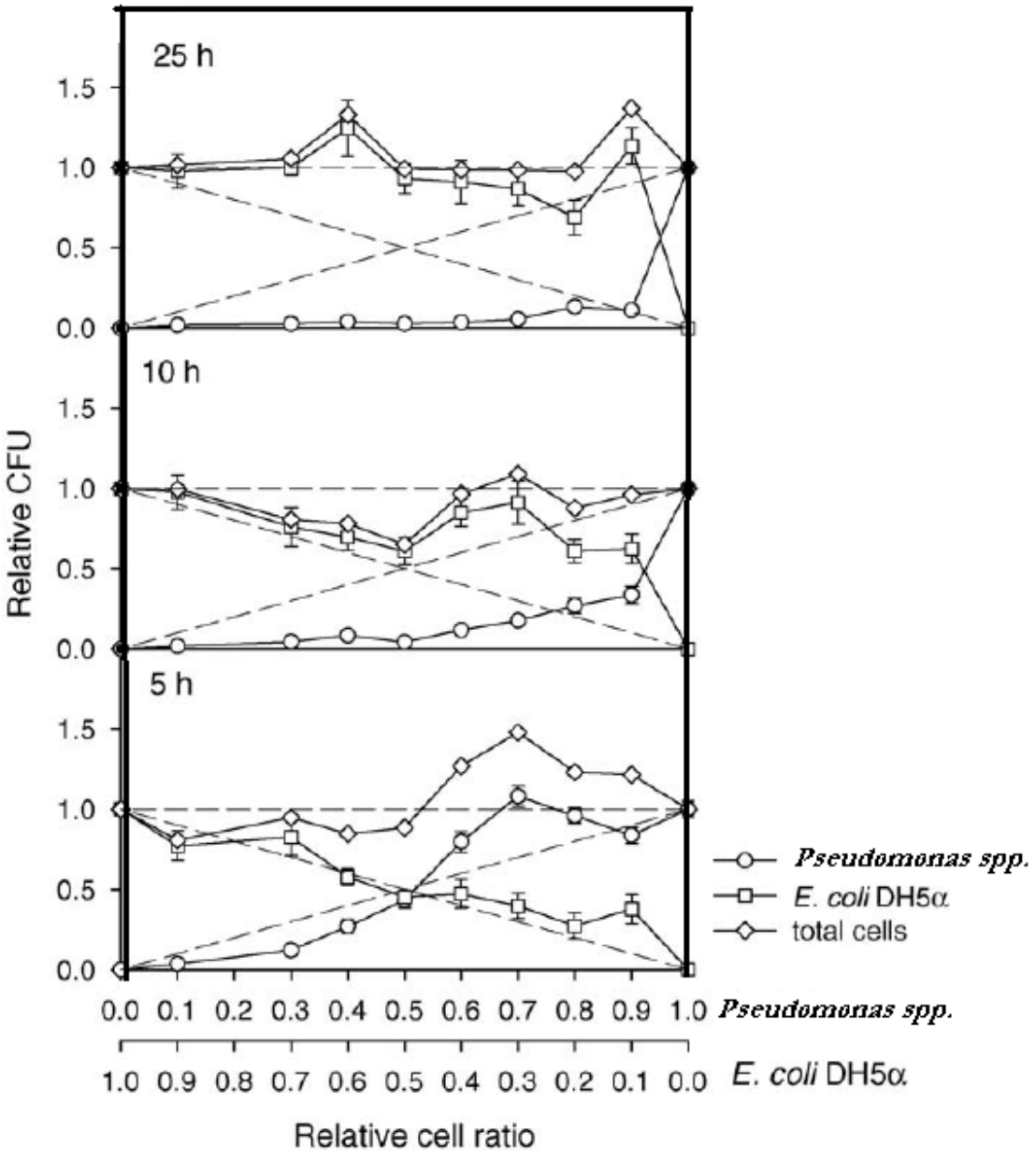

Figure 1. Aerobic growth of replacement experiments between two species in dye-free cultures at various initial population ratios for cultivation time (t) = 5, 10, 25 h. The dashed lines indicated theoretical population sizes of *E. coli* DH5$_{\alpha}$, *Pseudomonas spp.* and total population. Theoretical lines were obtained from monocultures without interspecific interactions of any other species

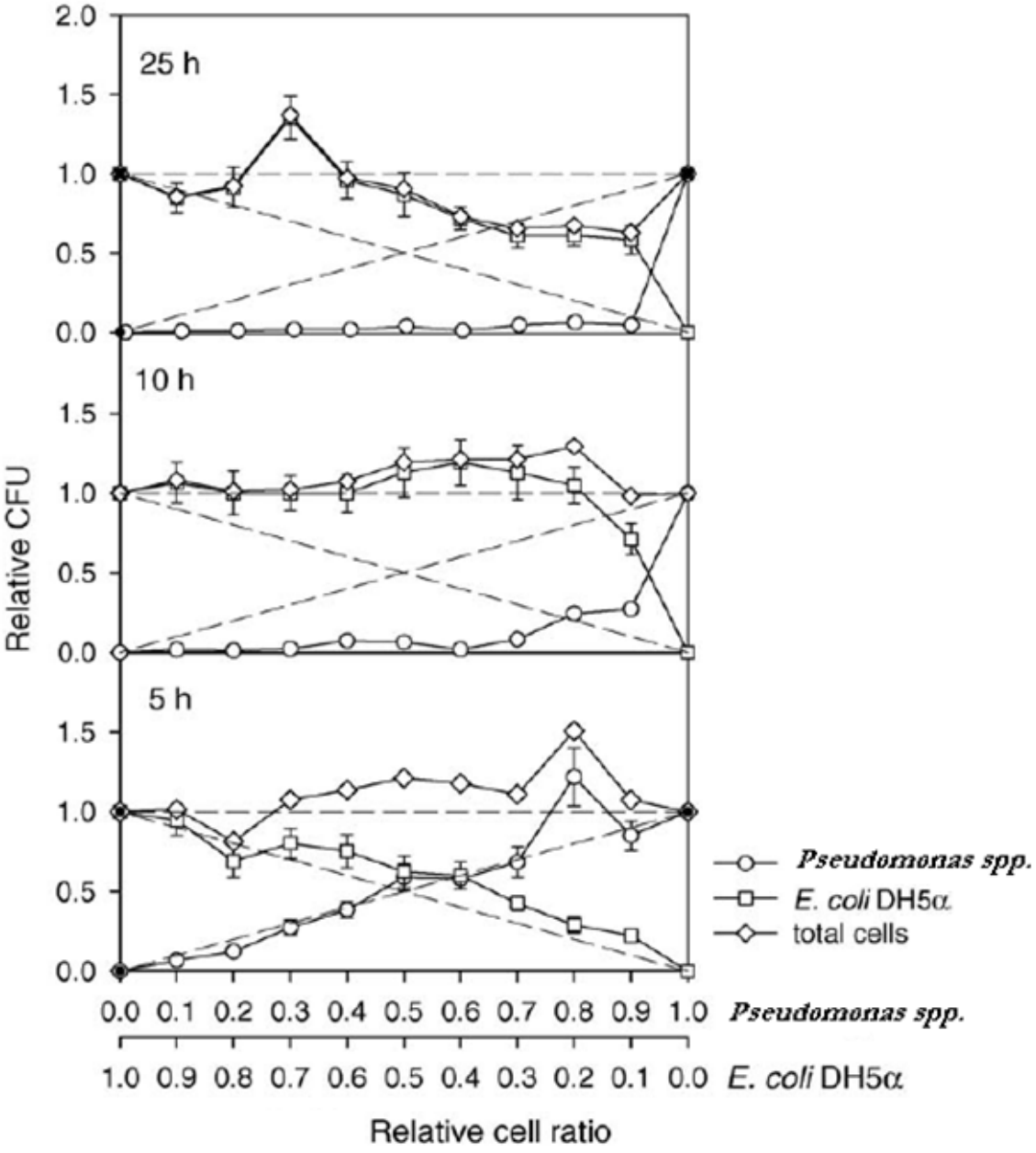

Figure 2. Aerobic cultures of replacement experiments at dye concentration of 1000 mg/L at various initial population ratios (cultivation time t = 5, 10, 25 h). The dashed lines indicated theoretical population sizes of E. coli DH5$_\alpha$, *Pseudomonas spp.* and total population in the absence of interspecific interactions of any other species

This analysis can be considered as a graphical representation of pair wise interactions in dynamic microbial populations [28]. Note that replacement series (Figure 1 – Figure 4) designated to select at t = 5, 10, 25 h disclose the competition trends at the early exponential, mid exponential and stationary growth phase, respectively. Moreover, as maximal biodecolorization was reached when cell growth had nearly stopped at the stationary phase [2], color removal at t = 25 h may indicate overall decolorization performance of the community.

2.3. Contour Analysis

To grasp overall scheme in decolorization for binary mixed-consortia, contour analysis upon overall decolorization rate (ODR) and specific decolorization rate (SDR) in variably spaced grids of species populations was conducted. Contoured data contained a two-dimensional (2-D) array of values (i.e., x, y for species population), and contoured outcome z (e.g., ODR or SDR). Theoretically, a contour line is formed by connected successive contour points at same-level outcome z with straight lines. Many contour points can form a complete branch. Linear interpolation can be used to predict the location of a contour point within a branch. In addition, the contoured branch in any z can be interpolated between branches in z + δz and z - δz.With this analysis, one may find a complete contour diagram for various z and a global optimization of z occurring in the 2-D species space (x and y) to be studied. Here, we postulate response-surface models for various contours of ODR and SDR as follows:

$$ODR = -\frac{d[dye]}{dt} = g_1(x_1, x_2)$$

$$and \quad SDR = \frac{1}{X}\frac{d[dye]}{dt} = g_1(x_1, x_2),$$

Where X, [dye] and t denote total viable cell population (cells/mL), Reactive Red (mg/L or ppm) and time (h),

respectively. To reveal the characteristics of contoured profiles in response surfaces, we acquire data to fit empirical second order polynomial models (shown below) which pass through (0,0) using a software tool MATLAB1 (1994–2003 Math Works, Inc.):

$$f(x_1, x_2) = \sum_{i=0}^{2} \sum_{j=0}^{2} b_{ij} x_1^i x_2^j.$$

The optimum responses and corresponding community compositions can then be determined.

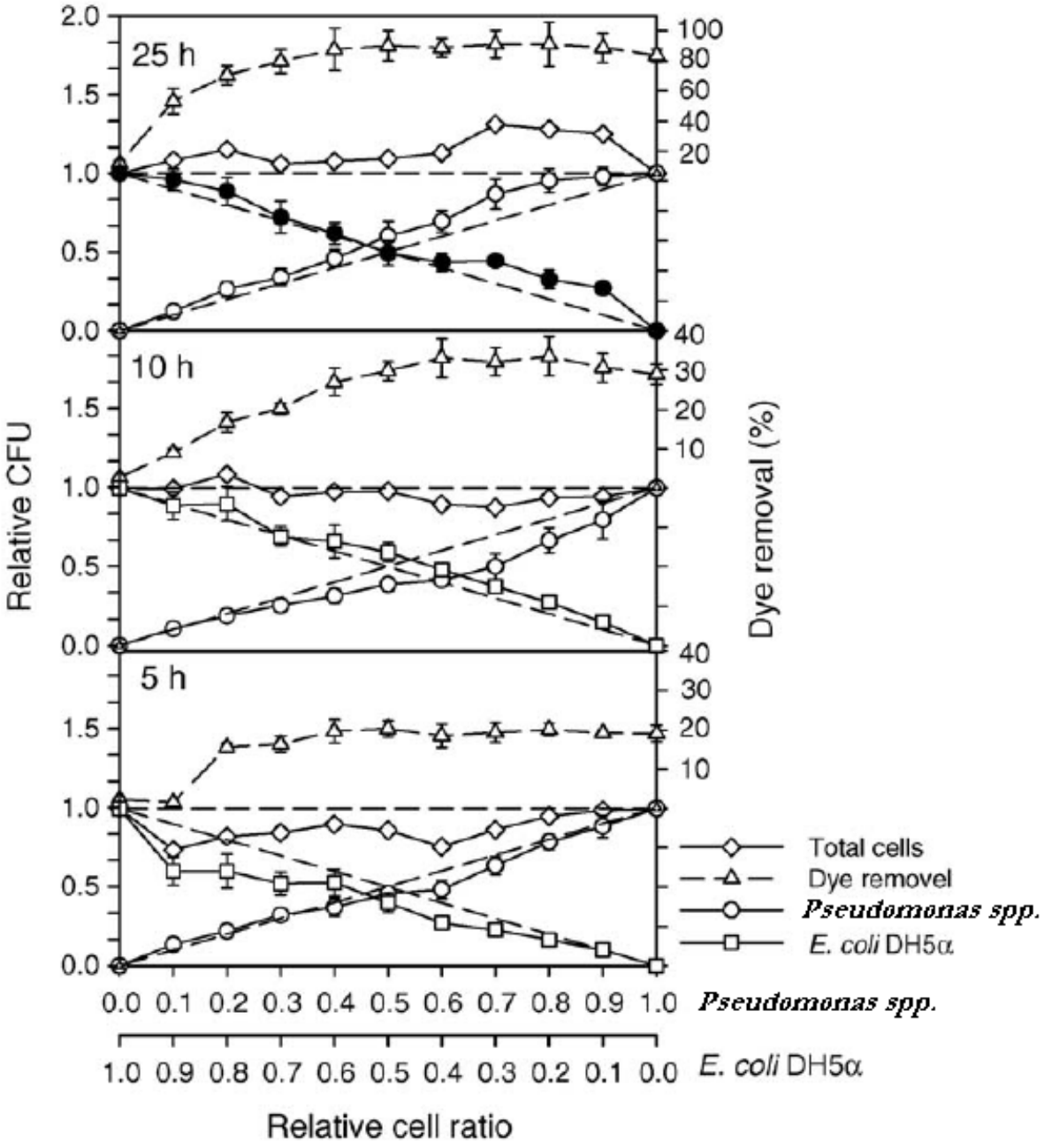

Figure 3. Static-incubation culture of replacement experiments for decolorization phase at initial dye concentration of 100 mg/L in a variety of population ratios (t = 5, 10, 25 h). Dashed lines indicated normalized theoretical population densities in the absence of inter specific interactions

2.4. Analytical Methods

The model azo-dye used for decolorization was C.I. Reactive Red obtained from local chemical industry (India). With appropriate calibrations, concentrations of biomass and reactive red were determined from absorbance (ODλ) at 600 and 510 nm, respectively, using an UV–Vis spectrophotometer (Shimadzu UV-1800, Japan). The concentration of reactive red was primarily determined by measuring the optical density (OD_{510nm}) of the sample. To understand interspecific competition phenomenon of bacteria *Pseudomonas spp*. and *E. coli* DH5α during decolorization, standard plate count in YG medium was carried out as follows: cultures were serially diluted with sterile PBS immediately after sampling, and then appropriate volumes were spread onto agar Petri plates. It is assumed that all metabolically viable cells are culturable on agar plates [29]. The different strains in the microbial community were distinguished on the agar plates by colony morphology, size and color [30,31]. Solid agar medium contained 0.125% glucose, 0.3% yeast extract, 1.0% tryptone and 15.0 g/L Bacto-agar. After plates were directly incubated at 38 8C for appropriate time (ca. 16–24 h), yellow and white colonies with

different radii (i.e., different growth rates) obtained on plates represent *Pseudomonas spp*. & *E. coli* DH5α cell counts (i.e., colony forming units, cfu), respectively. Serial dilution-agar plating procedures were carried out in duplicate to guarantee viable cell counts for greater accuracy. Only plates containing 30–300 colonies were chosen for enumerating. The cell count was determined by averaging the number obtained from both plates to indicate statistical significance. Experiments to confirm that extracellular metabolites of DH5α enhance dye decolorization efficiency of *Pseudomonas spp*. were performed as follows: *E. coli* DH5α was first cultivated in LB medium for 12–14 h at 37 8C using a water-bath incubator shaker at 200 rpm. DH5α cells obtained from designated culture were then separated by centrifugation (10,130 x g, 8 min). After removal of cell precipitate, supernatants containing metabolites of DH5α were collected by filtration through 0.2 mm filters to completely remove DH5α cells. This collected filtrate contained metabolites of DH5α produced in the exponential growth phase (EGP). The effects of DH5α a metabolites upon decolorization of *Pseudomonas spp*. were undertaken by adding various volumes of the filtrates into culture media prior to static incubation.

3. Results and Discussion

3.1. Competition of Aerobic Cell Growth in a Binary Culture of *Pseudomonas spp*. and E. coli DH5α

To get an evident grasp of competition phenomena between the two bacterial species (*Pseudomonas spp*. and E. coli DH5α), replacement-series experimental designs [26,27] were carried out. The possible outcomes (Figure 1 – Figure 4) of a competition experiment via replacement-series plots are anticipated as follows: Data points located on the theoretical line indicated that this species grows equally well with another as it does in monoculture. On the other hand, experimental points located under and above the line revealed that this species grows worse and better in the binary culture, respectively. Since the maximum specific growth rate (μ_{max}) of pure *Pseudomonas spp*. and DH5α alone in dye-free batch cultures were 0.652 ± 0.013 and 0.775 ± 0.004 h^{-1}, respectively (data not shown), E. coli DH5α gradually eliminated *Pseudomonas spp*. entirely in aerobic mixed cultures as time went by according to replacement series plots (Figure 1 and Figure 2) and competitive exclusion principle [28]. It is known that under aerobic conditions, intracellular azoreductase activity of *Pseudomonas spp*. for decolorization is repressed [2], hence all species simply behave as ‘‘non-decolorizers’’ in both dye laden and dye-free environments. This implies that in aerobic conditions azo-dyes still remained intact even though decolorizer *Pseudomonas spp*. was present. When both bacteria were cultivated in mixed cultures, *Pseudomonas spp*. population is restricted or vanished due to the preferential growth rate of DH5α in the absence or presence of dye (Figure 1 and Figure 2). As higher population propagation of DH5α may enhance either exhaustion of an essential nutrient or accumulation of toxic products to *Pseudomonas spp*., a declined growth phase of *Pseudomonas spp*. is evolved (e.g., t = 10, 25 h in Figure 1 and Figure 2). This is why in aerobic conditions DH5α always won in competition and ‘‘extinction’’ of *Pseudomonas spp*. was anticipated (e.g., t = 5 h in Figure 1 and Figure 2) [28].

3.2. The Augmentation Effect of E. coli DH5α for Azo-dye Decolorization

As Chen [2] indicated, bacterial decolorization is considered non-growth-associated. Under static decolorization conditions (Figure 3 and Figure 4), *Pseudomonas spp*. gains a survival advantage via decolorization to compensate the cost of competition exclusion due to the expression of intracellular azo-reductase activity. This is why coexistence of both species instead of singlespecies predominance was observed in Figure 3 and Figure 4. How can both species of seemingly conflicting interests be reconciled? The reasons to enforce DH5α to surrender such growth predominance for total coexistence are straightforward. Note that azo-dye may be highly toxic to bacteria [6], hence decolorization is a merely feasible means to reduce dye toxicity for total survival [2,32]. Chen et al. [32] also showed that a significant loss in cellular viability of DH5α at higher dosage of reactive red (i.e., 1400, 1800 mg/L) was observed. To guarantee a successful survival and propagation under such a dye threat, *E. coli* as a non-decolorizer may not engage in a direct conflict to decolorization of *Pseudomonas spp*. through conducting a noncooperative strategy to gain its growth advantage. Otherwise DH5α turns out to trade in part of its growth advantage as a cost to maintain basic metabolism for tolerance in dye-laden environments. These all imply that both species cannot arouse a deep interest in interspecies competition, since removal of the existing pollutant (i.e., azo-dye) is a top-priority task to be accomplished for survival. Hence, ‘‘immediate action’’ to lower the toxicity present in the living habitat must be made, although a longer ‘‘action’’ lag may be required in response to higher concentration of pollutants [33]. To investigate the existence of such ‘‘immediate move’’, biostimulation experiments using DH5α metabolits were conducted. Figure 5 indicates that lack of extracellular metabolites (EM) of DH5α led to a prolonged lag in reaching a maximal decolorization performance of *Pseudomonas spp*.. In addition, addition of more DH5α EM resulted in better decolorization performance. Note that unlike metabolites of DH5α, extracellular metabolites of *Pseudomonas spp*. showed insignificant effect on decolorization (data not shown). The metabolites expressed by E. coli may be ‘‘precursors’’ or act as a redox mediator or reducing agent [34] to stimulate decolorization activity of *Pseudomonas spp*., since dye decolorization is significantly affected by the oxidation–reduction potential (ORP; representing the abundance of NADH) as Keck et al. [3] pointed out. Chang et al. [35] mentioned that addition of *E. coli* strain generated a negative ORP (or a more reduced) environment for decolorization. Thus, DH5α, as an inefficient decolorizer, still offered the ‘‘altruistic act’’ (e.g., metabolite biostimulation) to help its partner species (i.e, *Pseudomonas spp*.) employ dye decolorization more efficiently. The goal of mutual interest in decolorization could be to create a more viable (e.g., less toxic or

unpolluted) environment for species fitted to survive. Since intracellular azoreductase activity of *Pseudomonas spp*. was induced in static incubation to remove reactive red accumulated in intracellular compartments (e.g., cytoplasm; [2]), *Pseudomonas spp*. might gain certain benefits (e.g., dye removal and toxicity reduction) for resuming its metabolic activity to compete with DH5α. This is very likely why complete predominance of DH5α as time went by (e.g., t = 5 < 10 < 25 h; Figure 1 and Figure 2) turned out to be total coexistence of both species (e.g., t = 5, 10, 25 h; Figure 3 and Figure 4). This phenomenon is similar to a co evolutionary result of host–parasite relationship due to skill improvement of hosts and predator reduction of parasites [36]. Figure 3 and Figure 4 indicate that there had been a marked rise in dye removal when the decolorizer *Pseudomonas spp*. became more predominant in the population, but DH5α lacked the ability to deal with dye removal. Furthermore, the community showed a less removal performance at higher dye concentration (i.e., 1000 mg/L) (Figure 3 and Figure 4) likely due to dye inhibition or toxicity [2,32]. More significantly, there exists an optimal community composition (around 70% *Pseudomonas spp*. and 30% DH5α) for maximal dye removal, especially at a higher dye concentration (e.g., 1000 mg/L) (Figure 4). This implies that the presence of a proper amount of the inefficient decolorizer (DH5α) could still enhance the overall decolorization activity due primarily to the production of decoloriziation stimulators by the DH5α strain. With these observations, contour analysis was adopted to specify the optimal population size and ratio in community for maximal decolorization over feasible population domains (discussed later in Figure 6).

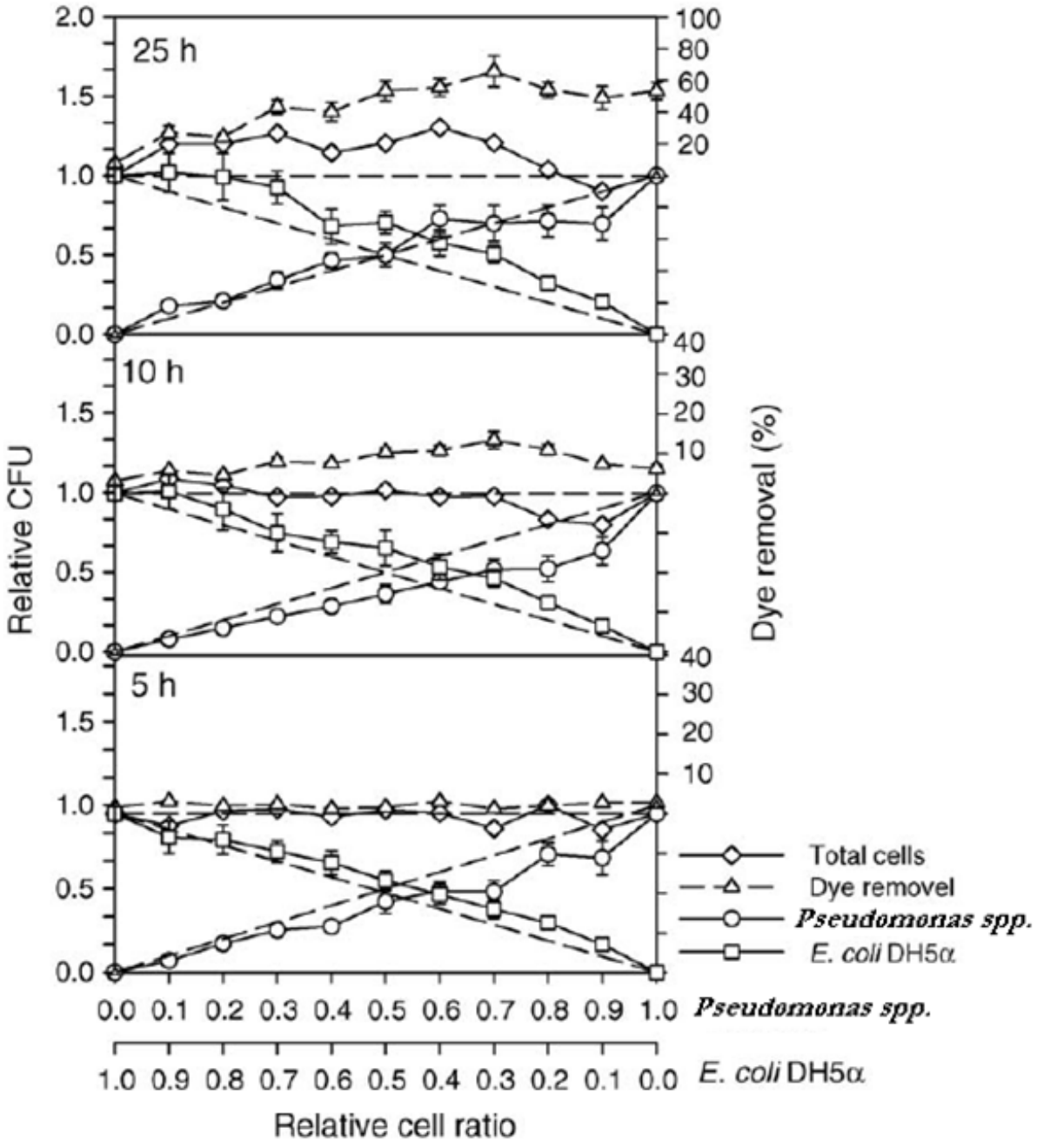

Figure 4. Static-incubation culture of replacement experiments for decolorization phase at initial dye concentration of 1000 mg/L in a variety of population ratios (t = 5, 10, 25 h). Dashed lines indicated normalized theoretical population densities in the absence of inter specific interactions

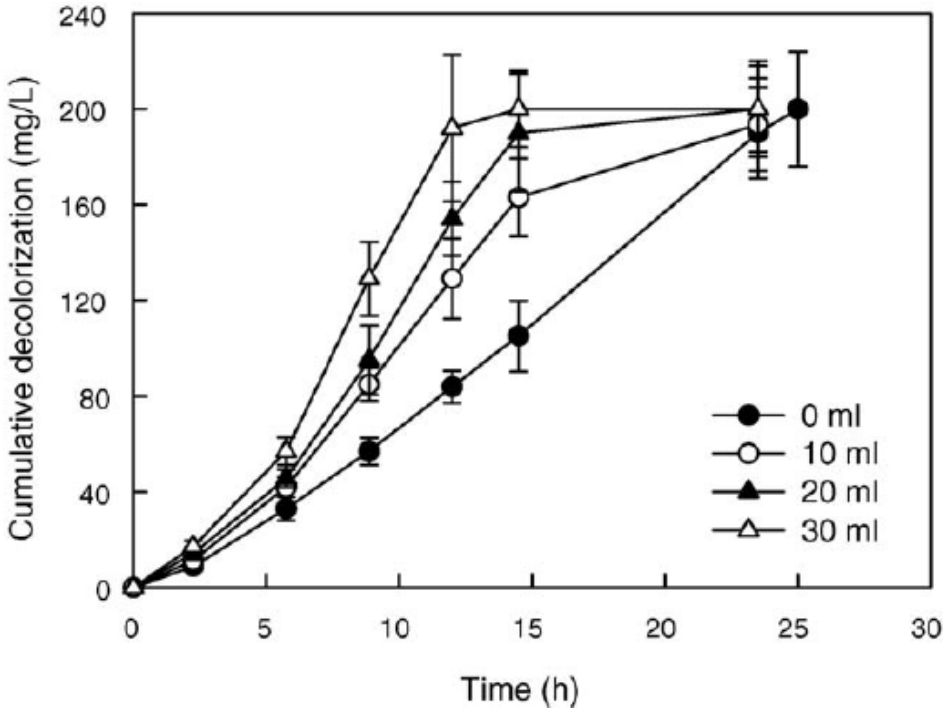

Figure 5. Cumulative decolorization of pure *Pseudomonas spp.* cultures containing various volumes of decolorization metabolites of DH5a obtained in the exponential growth phase

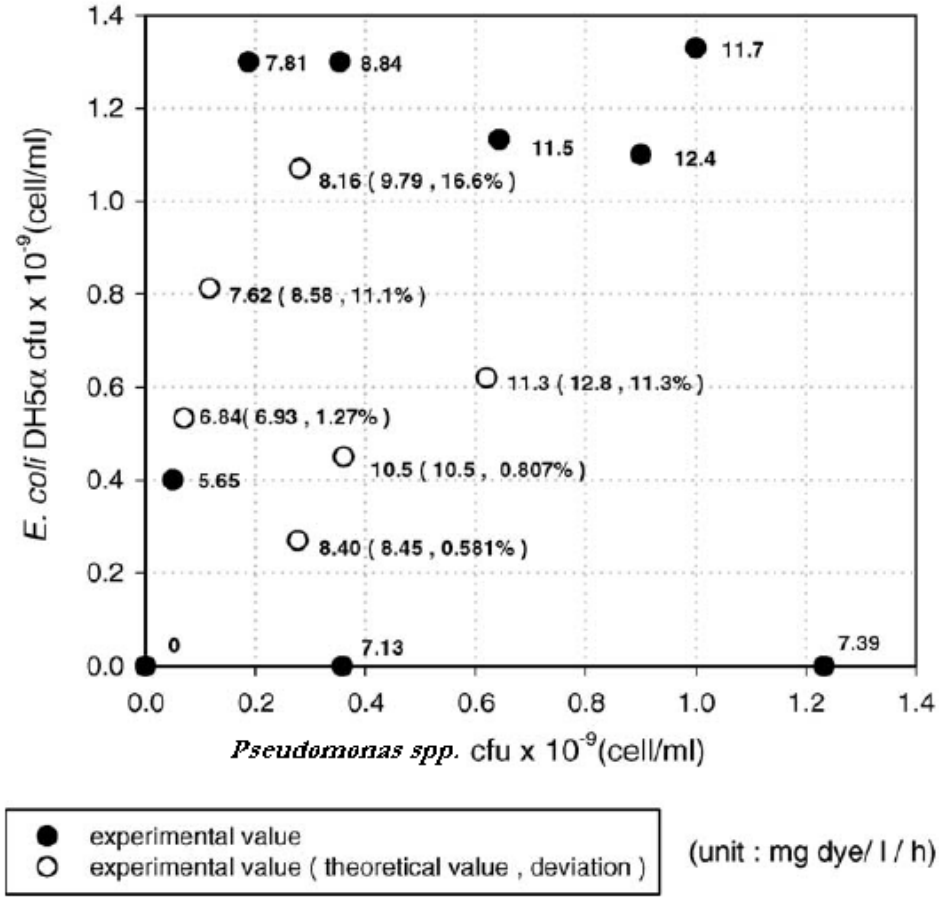

Figure 6. Three-dimensional contour diagram to present overall decolorization rates (ODR) at different binary-species communities. The optimal community occurred at 0.78 x 10^9 and 0.70 x 10^9 cells/mL for *Pseudomonas spp.* and *E. coli* $DH5_{\alpha}$, respectively

3.3. Contour Analysis for Optimal Biodecolorization Consortium Structure

To reveal the optimal community composition for maximal decolorization capacity, designs of experiments (denoted as DOX) at approximately ‘‘two-levels’’ (ca. 0.0, 1.2 x 10^9 cfu/ mL; Figure 7) and randomized levels (Figure 8) of DH5α in various *Pseudomonas spp*. concentrations were conducted. To elucidate this optimum from ecological perspective, a ‘‘chess board’’ of DOX is designed to exclude boundary constraints of stationary

population level of both species based on nutrient exhaustion. Since a gradual depletion of decolorization performance continues with respect to an increase in population size, color removal is expected to level off eventually, perhaps at stationary population levels (i.e., boundary constraints). Note that initial inoculum sizes of individual species in the community were not likely to be precisely controlled, since they could only be determined afterwards. This DOX is used to acquire data to fit the empirical polynomial model which passes through (0,0) using MATLAB®:

$$f(x_1, x_2) = \sum_{i=0}^{2}\sum_{j=0}^{2} b_{ij} x_1^i x_2^j,$$

Where $f(x_1, x_2)$ is either ODR (overall decolorization rate, unit: mg dye/L/h) or SDR (specific decolorization rate; mg dye/cell/ h); and x_1, x_2 denotes DH5α and P. luteola (unit: x 10^9 cfu/mL), respectively. The application of response surface methodology offers a contour analysis for empirical relationships between ODR, SDR values and the tested variables (x^i; i = 1, 2) through the following regression equations:

$$ODR = 4.137x_1^2x_2^2 - 1.330x_1^2x_2 - 7.372x_1^2 + 5.041x_1x_2^2 - 12.33x_1x_2 - 14.60x_1 - 15.58x_2^2 + 25.59x_2.$$

$$SDR = -90.48x_1^2x_2^2 + 142.4x_1^2x_2 - 52.56x_1^2 + 107.4x_1x_2^2 - 195.1x_1x_2 + 89.91x_1 - 23.55x_2^2 + 31.27x_2.$$

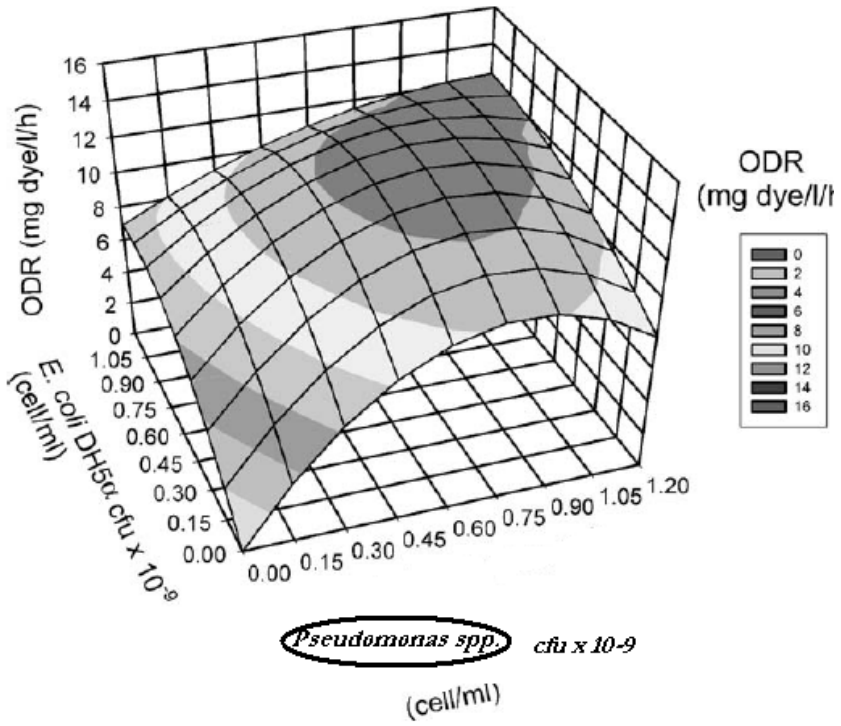

Figure 7. Experimental design at approximately "two-levels" of DH5α to determine overall decolorization rates (ODR) at various binary-species communities. Experimental data pionts (●) were used for model simulation to predict results (◯) located within the studied region

Figure 6 evidently indicates the circular characteristic of 3-D contour plots [37]. This study of contour plots also indicates optimal population sizes for *Pseudomonas spp*. and DH5α to achieve maximal overall decolorization at 0.78 x 10^9 and 0.70 x 10^9 cfu/mL respectively. This maximum does not occur either at points located on x- or y-axis (i.e., pure cultures), simply indicating that *Pseudomonas spp*. alone cannot present a maximal capacity for decolorization. This optimum reveals a decisive condition of population abundance (i.e., total population) and relative richness (i.e., species diversity) for maximal decolorization capability [23]. If Shannon's information entropy [38– 40] $H = -\Sigma^2_{J=I} P_j \log p_j$ is used as an index to indicate community diversity for this optimal community, almost the maximum entropy in this binary microbial system (i.e.,

$$H \cong H_{\max} = -\left[(1/2)\log_2(1/2) + (1/2)\log_2(1/2)\right] = \log_2 2 = 1$$

bit) would be obtained. It indicates that there was an approximately equal distribution of DH5α and *Pseudomonas spp*. in this optimal culture for decolorization such that the probability of finding either would be the same. This also supported that DH5α surrendered its growth advantage to choose total coexistence to tackle the threat to its survival. Moreover, maximal SDR (ca. 38 x 10^{-9} mg dye/cell/h) occurred at the community theoretically approached pure culture of DH5α (ca. 0.85 x 10^9 cfu/mL; Figure 9). This implies that the presence of non-decolorizer DH5α is still of great importance to decolorization enhancement as SDR is a differential property for color removal. In addition, all species, irrespective of decolorizers or nondecolorizers, in the microbial community may make use of their metabolic activities to reach a goal of pollutant degradation or at least detoxification for total survival Williams [41] indicated that ''the optimum population size of each species will be that with the greatest excess of benefit, (e.g., from higher reduction in dye toxicity), over the cost, from decreased nutrition.'' As P. luteola plays a crucial role in decolorization for pollutant reduction, this may indirectly alter DH5α to be more ''altruistic/cooperative'' rather than ''egotistic/ conflicted'' toward survival of

Pseudomonas spp.. Dover [42] mentioned that ‘‘altruism is when an organism (i.e., DH5α) helps another unrelated individual (i.e., ‘‘non-siblings’’; *Pseudomonas spp*.) survive to the detriment of its own Darwinian reproductive success (or ‘fitness’).’’ Relations between decolorizers and nondecolorizers are characterized by a complex array of cooperation, conflict, and compromise. In dye-free environments DH5α owns a growth advantage (i.e., higher growth rate) over *Pseudomonas spp*. (Figure 1 and Figure 2), however, in decolorization conditions DH5α pays a cost to lose certain competitive benefit (i.e., metabolite stimulation) to assist *Pseudomonas spp*. for decolorization (Figure 3 and Figure 4). This is why total coexistence of both species is resulted, since the persistence of decolorization capability is the only alternative to establish a more feasible environment for both species fitted to survive.

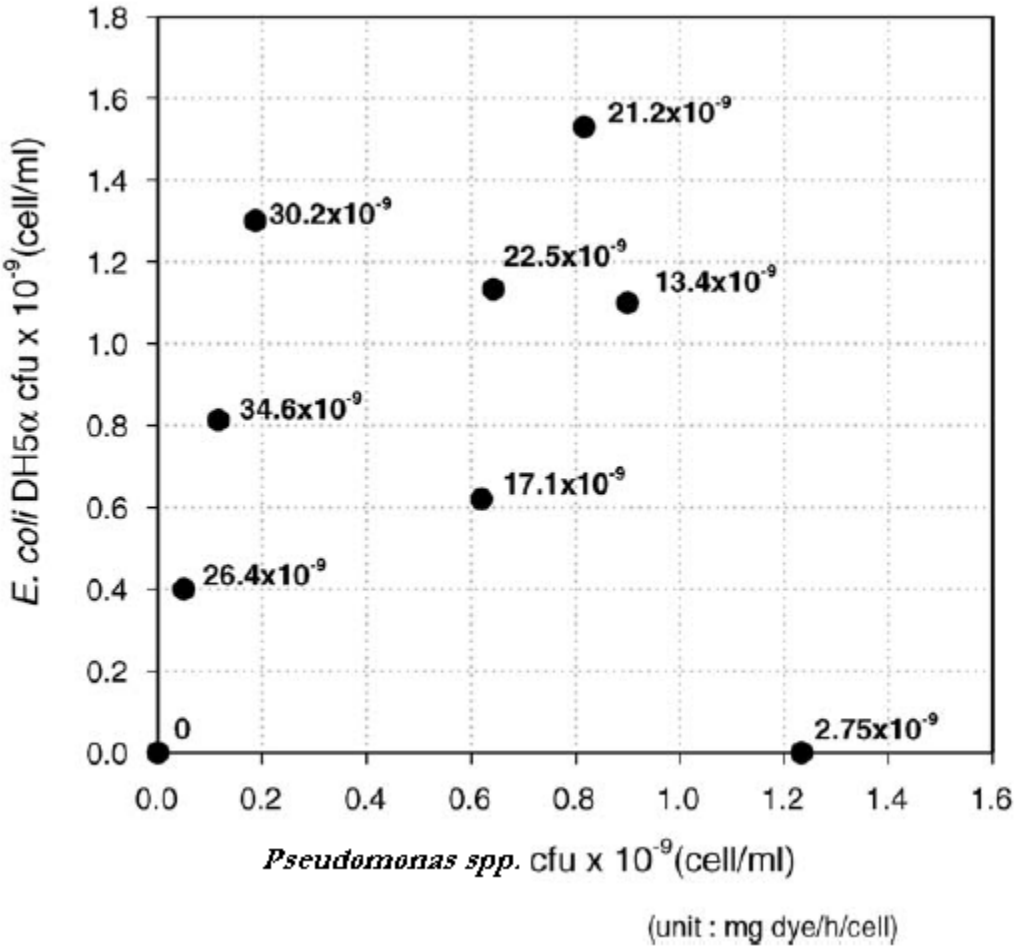

Figure 8. Experimental design at randomized levels of DH5α (●) to determine specific decolorization rates at various binary-species communities

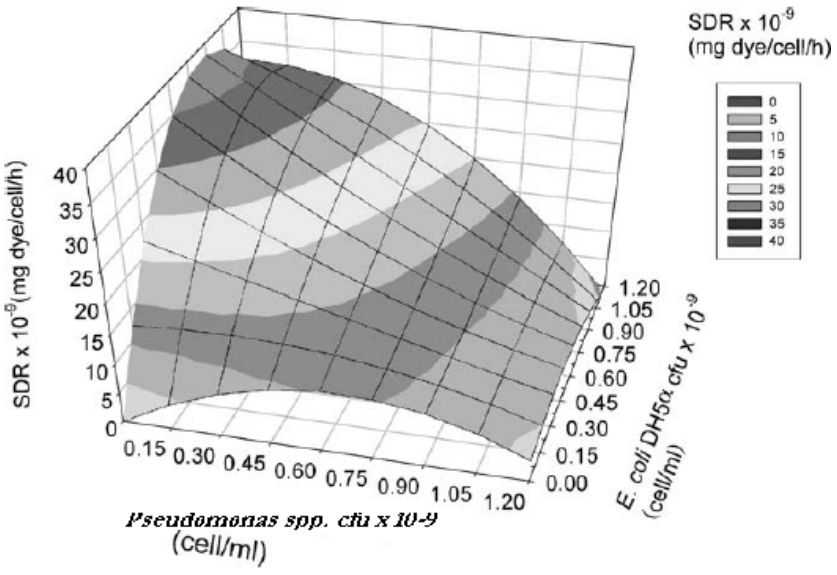

Figure 9. Three-dimensional contour diagram to present specific decolorization rates (SDR) at different binary-species communities. The optimal community for maximal SDR theoretically occurred at a monoculture of *E. coli* DH5α (0.85×10^9 cfu/mL)

4. Conclusions

This study reveal the interactive correlations between an azo-dye decolorizer (*Pseudomonas spp*.) and an inefficient decolorizer (*E. coli* DH5α) in terms of cell growth and decolorization performance. The replacement-series analysis shows that DH5α dominated the culture under aerobic growth conditions, while DH5α and *Pseudomonas spp*. coexisted in the binary culture under static (anaerobic) decolorization conditions. The results also show that although *Pseudomonas spp*. was more effective in decolorization, the presence of DH5α also played a crucial role in producing decolorization stimulating metabolites, markedly enhancing the overall decolorization efficiency in the mixed culture. Therefore, it seems feasible to supplement DH5α as an available option for bioaugmentation to enhance decolorization in practical on-site wastewater treatment. Contour analysis indicates that optimal species ecology for maximal overall decolorization almost occurred at a ratio of one viable *Pseudomonas spp*. (0.78×10^9 cells/mL) to one DH5α cell (0.70×10^9 cells/mL). SDR contour analysis depicts the presence of DH5α as a significant role in enhancement of reactive red decolorization. Mutualism between two species is very likely as a driving force directing species evolution to achieve decolorization capability and maintain total survival of all species. This study clearly indicates that a mixed culture at the optimal population ratio provided a maximal decolorization capability for bioaugmentation. This also states that mixed consortia regularly used in wastewater treatments do tie in well with a consideration of community ecology.

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