Journal of Hazardous Materials 163 (2009) 143–151

Contents lists available at ScienceDirect

Journal of Hazardous Materials

journal homepage: www.elsevier.com/locate/jhazmat

Toxicity assessment upon augmented biostimulation source to indigenous rhizobium *Cupriavidus taiwanensis*

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ARTICLE INFO

Article history:
Received 30 April 2008
Received in revised form 19 June 2008
Accepted 19 June 2008
Available online 4 July 2008

Keywords:
Cupriavidus taiwanensis
Chemostat pulse technique
Biostimulation
Phenol degradation

ABSTRACT

This novel-attempt study used chemostat pulse technique (CPT) and transient dynamics of dissolved oxygen (DO) in CSTR to quantify stimulating or inhibitory effects of augmented nutrient sources in the presence of phenol upon *Cupriavidus taiwanensis* R186. With injected augmented nutrients, phenol degradation performance of R186 was directly dependent on combined toxicity between phenol and the augmented substrate and the biodegradability of phenol. The findings indicated that although phenol was toxic to R186, all augmented nutrient sources still exhibited stimulating effects to bacterial growth of R186 in the presence of phenol. The simulating rankings of augmented nutrients were (1) at 200 mg/L, acetic acid > gluconic acid > yeast extract > glycerol ≫ phenol alone, (2) at 1000 mg/L, gluconic acid > acetic acid > glycerol > yeast extract ≫ phenol alone. This stimulating effect clearly suggested that this combined toxicity was antagonistic. It was also revealed that transient responses of DO seemed to be in parallel with the findings from CPT. It was thus concluded that substrate consumption patterns would play the most significant role in biostimulation to cultures with dual energy sources. This study can help uncover the mysteries of optimal biostimulation for phenol degradation as proposed in previous studies. In addition, this study directly provided a kinetic model to quantify the relative stimulation ranking of augmented nutrients in the presence of phenol for practical bioremediation.

1. Introduction

Among *Cupriavidus taiwanensis* strains isolated from southwest Taiwan, the *C. taiwanensis* R186 (R186 for short; original taxonomy of *Cupriavidus* genus was *Ralstonia* [1]) strain demonstrated an excellent capability to grow on a medium containing phenol or TCE as a carbon source [2,3]. Compared to other phenol biodegraders [3,4], R186 displayed a superior phenol biodegradability as well as tolerance (up to ca. 900 mg/L) in the phenol-bearing media. The dependence of phenol degradation described by Haldane's model also demonstrated that the optimal degradation rate was 61 μmol/mol/g cell occurring at a phenol concentration of 228 μM [3]. In addition, apparently the low saturation constant (K_S) of 5.46 μM and high inhibition constant of 9075 μM indicated a promising feasibility of R186 for practical bioremediation.

To determine the threshold criteria for process optimization of phenol degradation, previous studies [5,6] showed that phenol toxicity played a significant role to reduce the performance of phenol biodegradation. Thus, the strategy of biostimulation via augmentation of energy source to enhance phenol degradation was proposed [5]. Their findings [5,6] in batch cultures also revealed that optimal phenol degradation was observed in cultures with a dual carbon source of glycerol and phenol. The reasons to cause such an optimal degradation are straightforward. As the utilization of glycerol was at least partially associated with the metabolism of phenol degradation for simultaneous consumption, the monophasic growth instead of diauxic growth behavior in the cultures with a dual carbon source of glycerol and phenol was thus revealed [6]. Due to this non-diauxic growth characteristics and sufficient energy efficiently provided from glycerol consumption, R186 could degrade phenol much more effectively. However, to have overall optimization for biostimulation, some mysteries behind these findings still remained open to be uncovered. For example, whether combined interactions of phenol toxicity (e.g., syngerism or antagonism [7]) or substrate consumption patterns (e.g., diauxic or non-diauxic growth [8]) plays a crucial role to control the performance of phenol degradation in cultures containing a mixture of phenol and the augmented substrate. That is, this study is useful in finding plausible answers to mysteries of phenol toxicity not completely unlocked in previous studies [5,6].

To disclose these unsolved mysteries, our novel attempt of "chemostat pulse technique" (CPT [9]) was first carried out in pursuit of quantitative rankings of toxicity of augmented energy

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doi:10.1016/j.jhazmat.2008.06.107

Nomenclature

a	rate constant of phenol-loss due to the air flow in CSTR (h^{-1})
c_{in}	intracellular augmented nutrient concentration (mg/L)
c_{ex}	extracellular augmented nutrient concentration (mg/L)
C^*	saturated dissolved oxygen concentration (100% ≅ 6.7 ppm at 37 °C, 1 atm)
C_L	dissolved oxygen (DO) concentration (%)
D	dilution rate (h^{-1})
G	Gibbs free energy (joule)
k_La	oxygen transfer coefficient (h^{-1})
Δk	inhibitory (+) or stimulating (−) rate constant to bacterial growth ($L\,mg^{-1}\,h^{-1}$)
m	injected amount of tested phenol and/or augmented nutrient (mg)
q_{O_2}	specific oxygen uptake per unit cell concentration ($ppm\,h^{-1}\,L\,g^{-1}$)
S	augmented nutrient and/or phenol concentration (mg/L)
t_{lag}	lag time for growth second growth phase (h)
t_0	time delay in response to pulse injection (h)
X	cell concentration (g/L)

Greek letters

$\delta(t)$	Dirac delta function or the unit impulse function (i.e. $\delta(t) = \begin{cases} 0 & t \neq 0 \\ \infty & t = 0 \end{cases}$ and $\int_0^\infty \delta(t)dt = 1$)
μ	specific growth rate (h^{-1}; $\mu = d\ln X/dt$)
μ_{max}	maximal specific growth rate (h^{-1})
$\forall$	for all

sources in the presence of phenol. Although our first-attempt study [5] used the terms in toxicology (e.g., acute and chronic toxicity) to quantitatively asses all effects of energy sources in dual-substrate systems, these toxicity factors still could not be applicable in kinetic modeling for bioremediation. Thus, this study tended to seek for a novel attempt of feasibility assessment on biodegradation of toxic or inhibitory chemicals with the perspective of biochemical engineering. This approach (i.e., chemostat pulse technique (CPT)) could also be used in diverse applications in environment. As known, in a closed batch system the growth rate of cell population would tend towards zero either due to lack of essential nutrient(s) or gradual accumulation of inhibitory products/intermediates (i.e., factors for population saturation); hence such systems are always in transient states [10]. In contrast, in the open system (e.g., CSTR) there is the possibility that the rate of conversion of substrate to biomass will balance the rate of washout of the biomass. Thus, a steady state may be established (i.e., $\mu = D$ if $D < \mu_{max}$). With the steady-state behaviors in CSTR, the CPT via an instantaneous injection could be applied to identify the inhibitory or stimulating source(s) during biostimulation. This CPT not only can be applicable to define the toxic sources, but also quantify toxic level of suspected chemicals present in the culture. Thus, using CPT as a standard measure, quantitative rankings of toxicity of augmented energy sources to the growth of R186 in the presence of phenol could be obtained. After a steady-state growth in continuous culture was achieved, the process of testing substrate mixtures (i.e., augmented substrate and/or phenol herein) toxic (or inhibitory) to growth was conducted. As known, the growth rate in the exponential growth phase for batch cultures [9–12] was used as an equilibrium outcome of metabolic status in cells in response to a hostile environment (e.g., phenol-bearing wastewater). Such a core perspective could be extended herein to steady-state bacterial cultures in CSTR for toxicity assessment. First, phenol and/or the augmented substrate were intentionally injected into the culture broth individually as substrate(s) of either growth-stimulation or inhibition. After the impulse injection, allowing for a lag period of time to elapse the cell and dye concentration were continuously determined [11]. So long as the injected substrate stimulated the growth characteristics, an increase in injected substrate concentration should apparently yield a proportional increase in cell concentration. In contrast, if the injected source contained toxic or inhibitory compositions, an increase in injected source concentration should yield a marked decrease of cell concentration [11].

As phenol is a well-known toxic pollutant, it is crucial to investigate the critical condition of its biodegradability and address the scientific feasibility of bioremediation prior to practical applications on site. Thus, this study is prioritized to explore whether there exists a noteworthy change in combined toxicity of phenol with augmented energy source to R186. The combined toxicity may be either increased (synergism) or decreased (antagonism) [7,12]. Synergistic toxic effects are defined as those in which the toxicity observed in the presence of two or more toxicants exceeds that predicted from the sum of the toxic effects of each chemical acting alone (e.g., 2 + 2 = 5 > 4). In contrast, when two chemicals act antagonistically, the combined toxicity is cloaked to be less than what could be predicted from individual toxicities (e.g., 2 + 2 = 3 < 4). Thus, the objective of this study was to provide a first attempt from a toxicological perspective to put forward, in significant terms and quantitative evaluations, to the simulating or inhibitory effect of augmented energy source to the growth of R186 using the CPT. In addition, this feasibility study is being conducted to develop an operation guideline that would give on-site professionals a promising chance to examine practical biotreatment before they launch globally. Moreover, we are already running pilot tests and some are linked into national grids for bioremediation according to Taiwan Environmental Protection Agency and Ministry of Economic Affairs. Previous study [6] suggested that the performance of phenol degradation was likely directly dependent upon the characteristics of biochemical reactivity and combined biotoxicity of phenol and augmented energy source towards phenol degradation. However, lack of adequate quantitative assessment upon these sources makes the biotreatment unreliable for on-site applications. This study thus tended to clearly reveal a promising feasibility of CPT as well as transient-response assessment upon dissolved oxygen for combined toxicity of augmented sources to R186. The purposes of this research are also to resolve the unsolved questions raised in previous studies [5,6]: (1) Will the augmentation of energy substrate (e.g., glycerol, acetic acid, gluconic acid, yeast extract) for biostimulation interact with phenol toxicity? (2) Can second nutrient source act antagonistically (if possible) with phenol as a "biostimulator" instead of an inhibitor to improve phenol degradation? (3) What is the performance-determining factor to control overall phenol treatment (e.g., combined interactions of phenol toxicity [7,12] or substrate consumption patterns [8])?

2. Materials and methods

2.1. Microorganism and culture conditions

C. taiwanensis R186 originally isolated from root nodules of *Mimosa pudica* in southwest Taiwan was routinely grown at 28 °C on yeast extract-mannitol (YEM) agar plates [2,3]. For study, a loopful

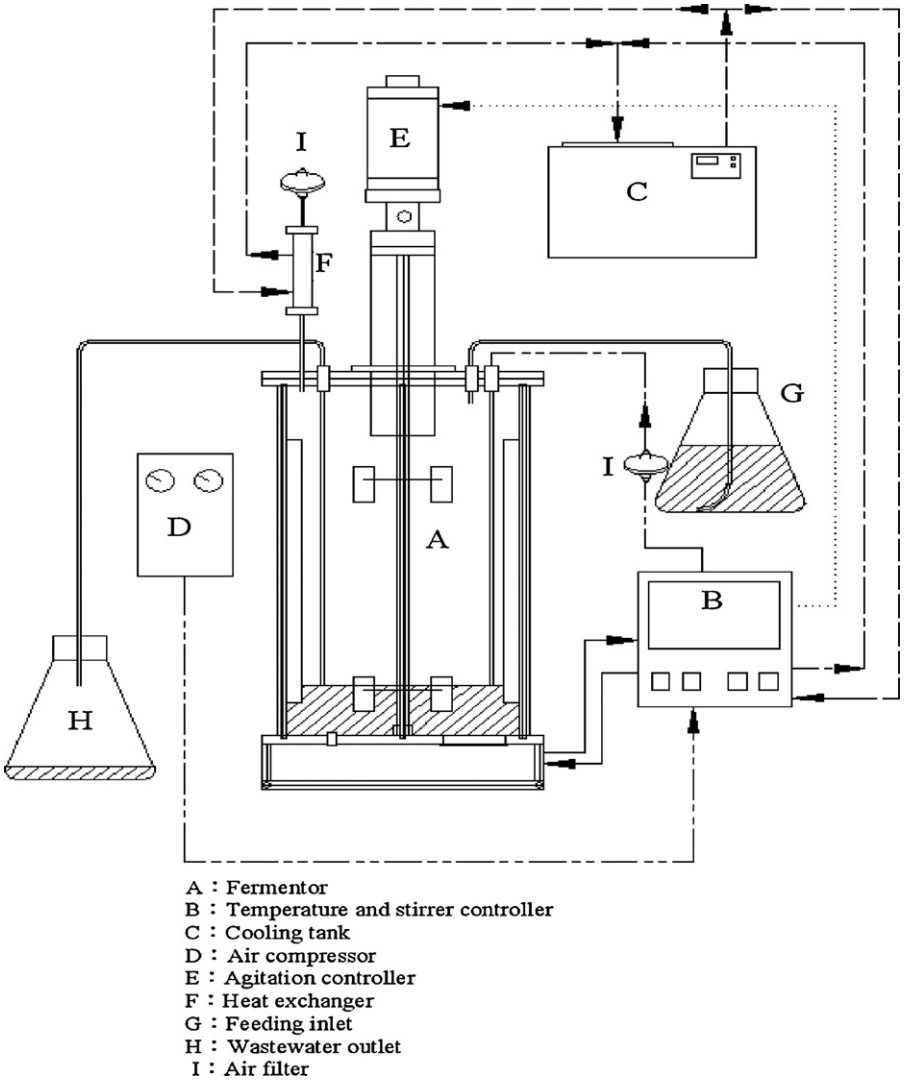

Fig. 1. Experimental setup of fermentor systems for chemostat pulse technique. Lines connected (B), (C) and (F) denoted PID control loop for temperature, and lines connected (B), (D) and (E) are manual/automatic on–off control loop for aeration [9].

of R186 seed taken from an isolated colony on a LB-streak plate was precultured in 50-mL Bacto LB broth, Miller (Luria-Bertani) (per liter; 10 g Bacto tryptone, 5 g Bacto yeast extract, 10 g sodium chloride) for 12 h at 37 °C, pH 7.2 ± 0.2, 125 rpm using a water bath shaker (SHINKWANG, SKW-12). Approximately 200 mL precultured broth was then inoculated into 1300 mL fresh 1/5× LB broth (per liter; 2.0 g Bacto tryptone, 1.0 g Bacto yeast extract, 10 g sodium chloride) containing ca. 30 μL/L antifoam 204 (Sigma) for aerobic continuous culture (2 vvm, 200 rpm, 37 °C) at $t = -60$ h. Note that using 1/5× LB instead of LB as the culture medium was to shorten culture time in cell growth due to rich compositions in LB borth prior to applications of CPT to the chemostat culture. In addition, it was to signify the crucial interactive effect of augmented nutrient(s) when CPT was conducted. After 12 h batch cultivation (i.e., $t = -48$ h; late exponential growth phase), the fresh 1/5× LB broth stream was introduced in 80 mL/h (i.e., $D \approx 0.053 \pm 0.002\ h^{-1}$) to the fermentor in order to maintain constant working volume at 1500 mL for continuous cultures (refer to Fig. 1 for experimental setup). Once the steady state (i.e., $X_0 = 0.789 \pm 0.099$ g/L; data not shown) was achieved at 48 h cultivation (i.e., $t = 0$), appropriate amounts of augmented nutrient source-bearing phenol solutions (i.e., test or control cases) were injected into the fermentor (ca. 200 or 1000 mg/L augmented nutrient and 2800 mg/L phenol) using sterile syringe. Note that as 1/5× LB was a favorable growth nutrient to R186, the toxic response exhibited in marked decrease in cell growth via CPT was thus solely due to the toxicity of phenol [5,6]. That is, the degradation of less preferential substrate—phenol was temporarily repressed in the presence of other favorable substrate (e.g., yeast extract or LB media) due to the "diauxie" growth phenomenon on multiple substrates. The augmented nutrient-free control was simply the case with injected phenol alone. In addition, phenol was intentionally introduced in chemostat runs with the augmented nutrient sources to reveal whether the combined toxicity of phenol and augmented nutrient source could still be disclosed by the proposed CPT. In these continuous cultures, the pH was not controlled to simulate on-site or *in situ* practical situations.

2.2. Analytical methods

The chemicals used for study were phenol (Crystal, 99.1% J.T. Baker A.C.S. reagent), yeast extract (Difco-Becton Dickinson), gluconic acid (45–50 wt% Sigma–Aldrich), glycerol (99% Riedel-deHaën), acetic acid (Glacial, 99.7% Nihon Shiyaku Extra Pure

Reagent). Prior to experiments, phenol was sterilized by filtration (sterile Millipore Millex®-GS 0.22 μm filter unit) and augmented nutrient sources were disinfected by moist-heat sterilization. The concentration of biomass was determined using an UV–vis spectrophotometer (HITACHI Spectrophotometer, model UV-2001). To exclude the confounding readings in optical density (OD) due to factors other than cell concentration, the supernatant of the sample was also taken for measurement after centrifugation for 2 min at 700 × *g* (HSIANGTAI Centrifuge MCD-2000). A sterile cell-free medium was chosen as the control. As all samples contained biomass and a mixture of phenol and augmented nutrient source, concentrations of biomass (*X*) was evaluated as follows:

(1) OD_{600nm} of sample mixtures (mix) without centrifugation: $OD^{X+mix}_{600\,nm} = OD^{mix}_{600\,nm} + OD^{X}_{600\,nm}$,
(2) OD_{600nm} of sample supernatant (sup) after centrifugation: $OD^{sup}_{600\,nm} = OD^{mix}_{600\,nm}$.

Samples were diluted to an optical density of less than 0.6 when absorbance was not within the linear range (0.1–0.7). The relationship between the cell concentration and OD_{600nm} (ODU) is 1.0 ODU ≅ 0.34 g/L dry cell weight.

The concentration of phenol in the medium was determined by using a colorimetric assay [5] with a detection limit of ±2.5 μM. The colormetric reagent contained pH 10 buffer (HBr 3.0, KCl 3.73, NaOH 1.76 g/L), 2% 4-aminoantipyrine (4-AA) and 8% potassium hexacyanoferrate (PHF). First, the 30 μL supernatant of ca. 1.0 mL cell-culture sample was taken after centrifugation for 5 min at 700 × *g* (HSIANGTAI Centrifuge MCD-2000). Meanwhile, a sterile cell-free medium was chosen as the control. The supernatant was then well mixed with 2.97 mL pH 10 buffer solution as a "test-tube vial" (TTV). The TTV was then added in series with 30 μL 2% 4-AA, 30 μL 8% PHF. The reaction mixture was well mixed via vortex mixer (Model VM-100) for 15 s. Colorimetric reaction was then taking place at room temperature in 15 min static incubation for completion. The reaction mixture was then measured at λ_{max} = 500 nm by Spectrophotometer (Thermo Spectronic 20D+) to determine phenol concentration via the calibration curve.

3. Results and discussion

3.1. Batch culture containing phenol and LB broth

As shown in Fig. 2, the diauxic growth of R186 was observed in batch cultures initially containing LB broth and phenol in various levels (0, 25, 50, 500 mg/L). Multiple lag phases in diauxic growth were taking place due to situations with multi-substrate limitation. Diauxic growth was caused by a shift of metabolic patterns in the midst of growth. The cells passed through at least two transitions for growth; two growth phases would result each with a successively decreasing growth rate. Apparently, compared to phenol, LB broth was a more preferential nutrient source for cell growth of R186. After essential nutrients in LB broth were exhausted, the cell diverted its energies from growth to utilization for next available nutrient (i.e., phenol). However, as phenol was toxic to R186 [5,6], with increasing level phenol cells must consume more energy sources to maintain cellular tolerance/defense mechanism (e.g., highly selective diffusion) effectively to avoid cell death from occurring. That was why, in the second growth phase, longer periods of time in growth lag (t_{lag}) were observed in the cultures of higher concentrations of phenol (i.e., t_{lag}(500 mg/L) ≅ 54 h > t_{lag}(50 mg/L) ≅ 16 h > t_{lag}(25 mg/L) ≅ 10 h > t_{lag}(0 mg/L) ≅ 0 h). However, trace amounts (e.g., 25 and 50 mg/L) of phenol in cultures might not significantly affect specific growth

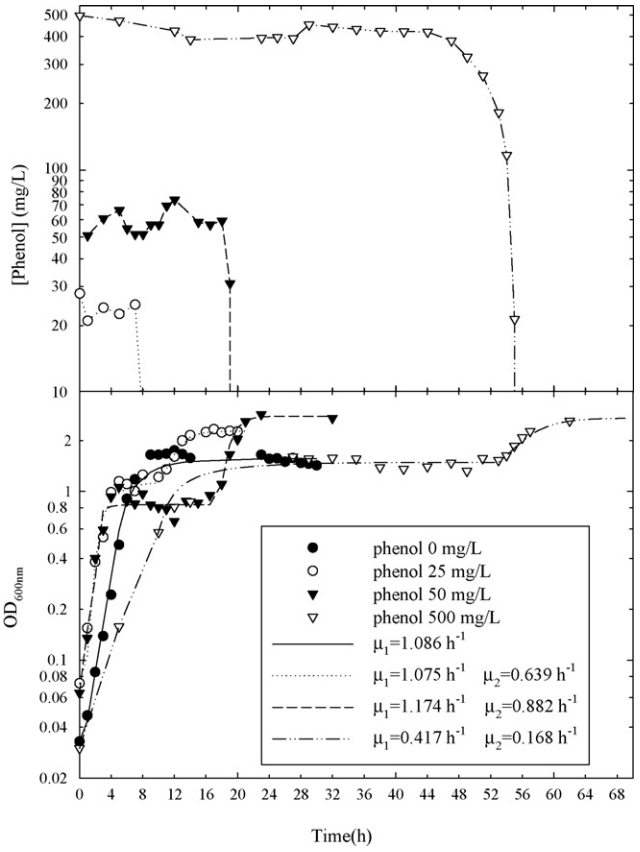

Fig. 2. Comparison on batch cultures of R186 initially containing LB broth and various concentrations phenol.

rate of first growth phase. In contrast, due to phenol toxicity and substrate inhibition, a high-level phenol stress (500 mg/L) apparently lowered growth rate in first phase (μ_1 = 0.417 h^{-1}) and second phase (μ_2 = 0.168 h^{-1}), respectively.

3.2. Repetitive injection effect of phenol

Although *C. taiwanensis* is a strain with high phenol degradation capability and could express a short-term tolerance (or acute tolerance [12]) up to 900 mg/L in phenol-laden media, long-term adverse effects of phenol in different levels (e.g., chronic toxicity) still needed to be explored prior to practical applications in bioremediation. To disclose the tolerance figures of R186, repetitive pulse injections were thus applied to CSTR for tests. When first low-level pulse injection at 250 mg/L phenol was applied (i.e. Phase (I) for 250 mg/L → 1800 mg/L in Fig. 3), dissolved oxygen (DO) was slightly increased to ca. 80% initially and then resumed normal oxygen uptake within 2 h (i.e., DO ≈ 0). However, cell concentration was almost still stayed invariant, indicating that the provoking toxicity was insignificant to cells at this level. After nearly complete elution of residual phenol (ca. 40 h; Fig. 3), second injection in high-level phenol (ca. 1800 mg/L) was employed (i.e., Phase (II)a; Fig. 3), leading to a dramatic decrease in cell concentration ($k = 3.81 \times 10^{-5}$ L mg^{-1} h^{-1}; refer to Appendix A for parameter estimation). In addition, DO noticeably increased up to ca. 90% saturation as the cells must extract its energy for maintenance of cell viability due to this provoked toxicity. Once phenol concentration decreased to ca. 800 mg/L (ca. 50 h; Fig. 3), cells resumed the cell viability and metabolic functions for aerobic growth and cellular respiration (i.e., decreases in DO). In contrast, when repetitive injections were all applied

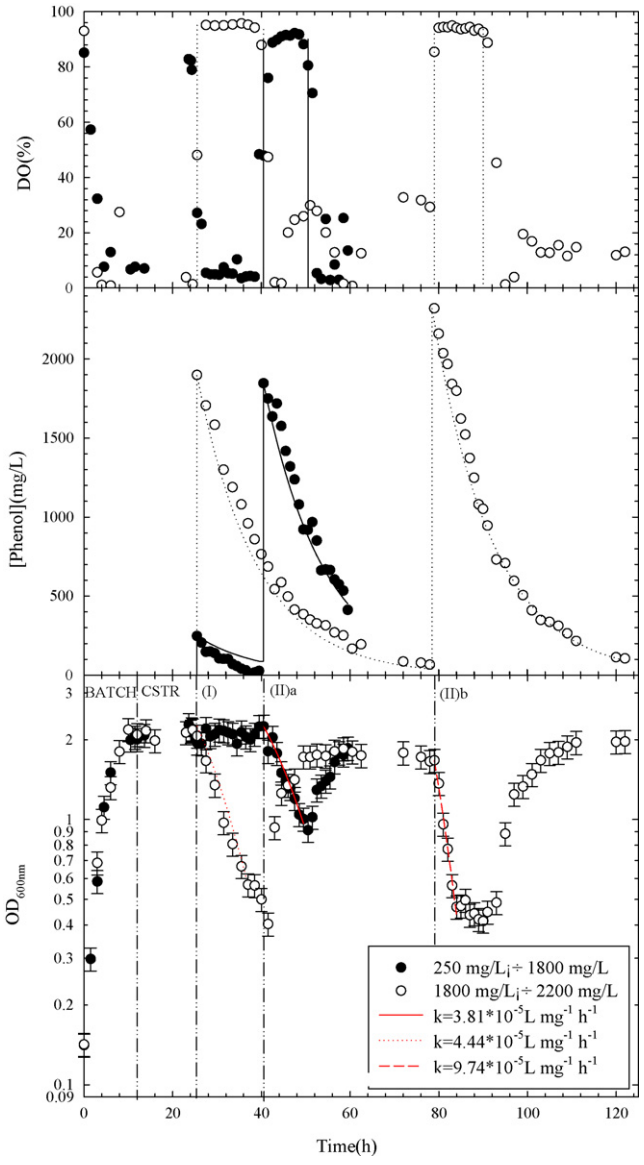

Fig. 3. Time courses of cell, DO and phenol concentrations in continuous cultures containing phenol in different concentrations using repetitive pulse injection techniques. The predicted curve of phenol was $[PhOH]_t = [PhOH]_{t=0} \times \exp[-(D+a)t]$, where the parameter a is rate constant of phenol-loss due to the air flow in CSTR.

in high-levels (i.e., 1800 mg/L → 2200 mg/L; Fig. 3), noteworthy toxic responses after first and second injection were observed as $k = 4.44 \times 10^{-5}$ L mg^{-1} h^{-1} and $k = 9.74 \times 10^{-5}$ L mg^{-1} h^{-1}, respectively. In comparison with these two injection tests, evidently first injection at low-level phenol (e.g., 250 mg/L) markably induced the effective tolerance to phenol in a shorter period of time (i.e., the duration for cell recovery decreased from 16 h (1800 → 2200 mg/L) to 6 h (250 → 1800 mg/L)). For repetitive high-level injections (1800 → 2200 mg/L), the longer period of time for cell tolerance/adaptation was required to resumed the original steady-state cell concentration at $t = 0$ (ca. 15 h (250 → 1800 mg/L) extended to 24 h (1800 → 2200 mg/L); Fig. 3). This comparison simply suggested that the acclimation of low-level phenol might provide the highest possible tolerance/adaptation for cell survival and growth. The high tolerance after acclimation of low-level phenol injection was simply because the cells that were affected had some effective "defense" mechanism against phenol and had such an ability to repair or compensate for damage that took place intracellularly. In contrast, after high-level injection the cell culture had much less ability to feedback this viability loss due to the inefficiency of the defense mechanism against phenol.

3.3. Low-level effect of augmented nutrients

As shown in time courses of cell growth characteristics (Figs. 4 and 5), there were no significant time delays ($t_0 \cong 0$ h) to respond changes in cell concentrations after pulse injection. This explained that phenol seemed to be relatively toxic to R186, compared to less toxic azo dye to *Pseudomonas luteola* ($t_0 = 1/3$ mean residence time [9]). The stable cell concentration in the culture in the absence of additions of phenol or augmented nutrients (i.e., control without any injection) was stably maintained at $X \approx X_0 = 0.789 \pm 0.099$ g/L (data not shown), indicating that the steady state of the chemostat culture of R186 was actually achieved. For 200 mg/L of augmented nutrient sources (i.e., glycerol, yeast extract, gluconic acid, acetic acid) in the presence of 2800 mg/L phenol (Fig. 4), the estimated kinetic parameters Δk ($=k - k_0$; refer to Appendix A for parameter estimation) for augmented nutrient-free control (i.e., phenol alone), acetic acid,

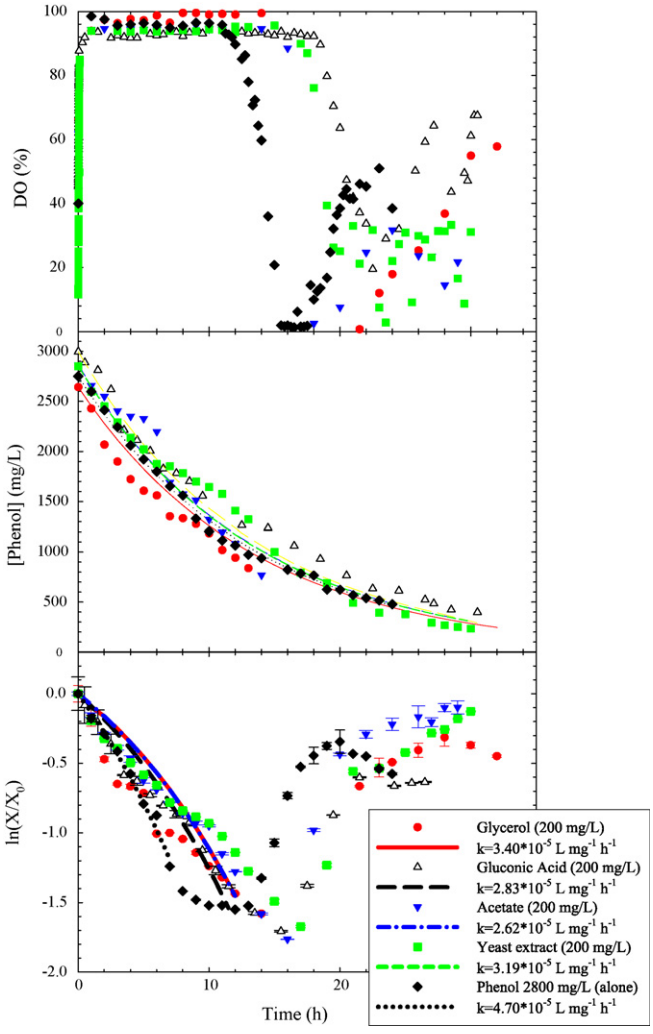

Fig. 4. Time courses of cell, DO and phenol concentrations in continuous cultures containing different augmented nutrient sources at 200 mg/L in the presence of phenol (2800 mg/L) via CPT. The predicted curve of phenol was $[PhOH]_t = [PhOH]_{t=0} \times \exp[-(D+a)t]$, where the parameter a is rate constant of phenol-loss due to the air flow in CSTR.

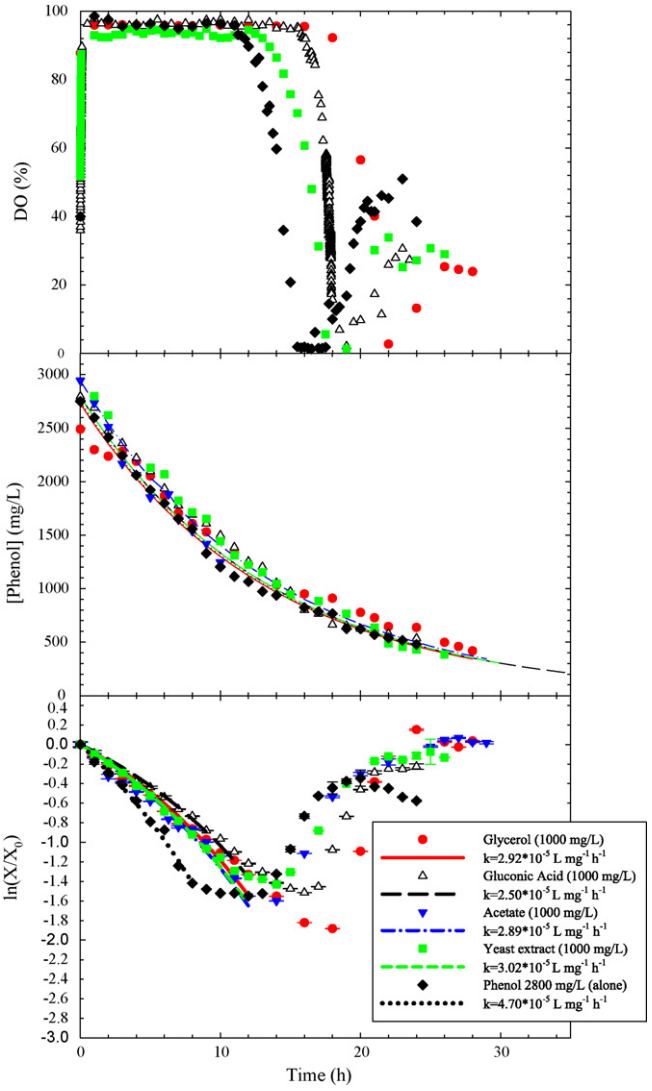

Fig. 5. Time courses of cell and phenol concentrations in continuous cultures containing different augmented nutrient sources at 1000 mg/L in the presence of phenol (2800 mg/L) via CPT. The predicted curve of phenol was $[PhOH]_t = [PhOH]_{t=0} \times \exp[-(D+a)t]$, where the parameter a is rate constant of phenol-loss due to the air flow in CSTR.

gluconic acid, yeast extract, glycerol were obtained to be 0.0, -2.08×10^{-5}, -1.87×10^{-5}, -1.51×10^{-5} and -1.30×10^{-5} L/mg h, respectively. Note that the kinetic parameter k of phenol alone (i.e., augmented nutrient-free control) was 4.70×10^{-5} L/mg h, clearly exhibiting the toxic characteristic of phenol to R186. In addition, all of the negative Δk values simply implied that all augmented nutrient sources were stimulating to bacterial growth of R186. This implied that the strategy of using augmented nutrient sources for biostimulation to *in situ* or on-site phenol degradation should be feasible. Moreover, the relative stimulation ranking of augmented nutrients to the growth of R186 (units in L/mg h) was acetic acid (-2.08×10^{-5}) > gluconic acid (-1.87×10^{-5}) > yeast extract (-1.51×10^{-5}) > glycerol (-1.30×10^{-5}) > phenol alone (0).

3.4. High-level effect of augmented nutrients

According to Fig. 5, in the presence of phenol at 2800 mg/L gluconic acid, acetic acid, glycerol and yeast extract at 1000 mg/L also showed stimulating characteristics to the growth of R186 as the differences of kinetic parameters Δk were -2.18×10^{-5}, -1.81×10^{-5}, -1.78×10^{-5} and -1.68×10^{-5} L/mg h, respectively. Thus, the relative ranking for stimulation of augmented nutrients to the growth of R186 was gluconic acid > acetic acid > glycerol > yeast extract > phenol alone. Compared these high-level effects to low-level effects of augmented nutrients, apparently more supplementation of energy nutrients seemed not considerably improve the stimulating effects to the growth of R186.

Compared to low-level augmented nutrients (200 mg/L; refer to Section 3.3), relatively higher concentrations of augmented nutrients (1000 mg/L) might be easier moved across the membrane from the region of high concentration (i.e., extracellular phase) to low concentration (i.e., intracellular compartment) via passive diffusion [13] due to relatively higher concentration gradient as a driving force. According to thermodynamics (i.e., the free energy change $\Delta G^\circ = RT \times \ln(c_{in}/c_{ex})$), passive diffusion is spontaneous (i.e., $\Delta G^\circ < 0$), where c_{in} and c_{ex} denoted intracellular and extracellular augmented nutrient concentration, respectively. Moreover, the difference of these two terms of Gibbs free energy $\Delta G^\circ|_{1000ppm} \cong \Delta G^\circ|_{200ppm} - RT$ ln 5 simply suggested the larger equilibrium constant at 1000 mg/L due to the more negative ΔG° at 1000 mg/L. This point implied that augmented nutrients at 1000 mg/L are more favored to move across the membrane than at 200 mg/L. However, even the driving force was more favorable to transport augmented nutrients in high-level, the enzyme activity to proceed the metabolic activity for phenol tolerance and cell growth might be saturated. This might be the reason why supplementation of high concentration energy nutrients failed to considerably improve the stimulating effects for cell growth of R186.

3.5. Recovery response analysis upon oxygen uptake

Effects of injected substrates on transient dynamics of cell concentration via CPT apparently revealed the stimulation or inhibition characteristics of the injected chemical(s) to R186 ([9] and Sections 3.2–3.4). Moreover, the recovery profiles of cellular oxygen uptake (i.e., changes in dissolved oxygen concentration) also provided a direct indicator for cell tolerance to phenol toxicity. For cellular respiration, a series of electron-carrying molecules that transfers energy-rich electrons and protons to O_2 via cell membrane (prokaryotes) or mitochondria (eukaryotes). Energy is then transferred and conserved in the form of ATP [14]. Oxygen uptake simply suggested that the energy-driven capability in cells (i.e., essential conditions for cellular growth and tolerance) is taking place. That is, in a single-stage chemostat culture after an impulse injection of toxic chemical(s) (e.g., phenol-laden medium), aerobic microorganisms would lose oxygen respiration for assimilating energy in a short period of time (i.e., immediate escalating from 40–60% to ca. 95–100% DO concentration; Figs. 4 and 5). Due to this significant loss of cellular viability for growth, the rate of wash out of biomass would exceed the cell growth rate so that the cell concentration decreased (i.e., $D > \mu$; Figs. 4–5). Besides, phenol concentration approximately decreased in first-order kinetics over time simply due to washout in CSTR (refer to the cases of phenol in Figs. 4–5). Once phenol concentration declined to a "threshold" concentration (i.e., a concentration below which no toxic response could be detected [7,15]), the recovery and tolerance mechanism of cell populations could thus handle phenol in a manner that apparently resumed the cellular viability and metabolic functions for survival as well as growth. That is, oxygen acted again as the ultimate electron or hydrogen acceptor by mans of oxygenase enzyme(s) to incorporate into nutrient substrates in their catabolism for growth [10]. Since cellular oxygen uptake rate constant q_{O_2} (Appendix B) could be directly determined from the transient response of DO

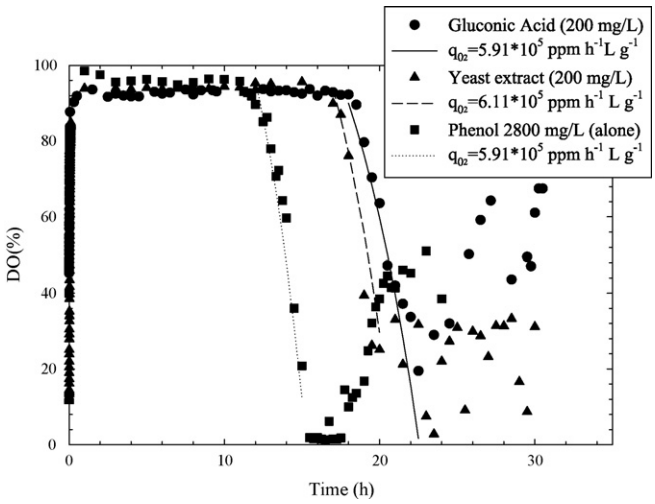

Fig. 6. Transient dynamics of dissolved oxygen concentrations in continuous cultures containing different augmented nutrient sources at 200 mg/L in the presence of phenol (2800 mg/L) via CPT. The 100% DO denoted 100% saturated ≈ 6.7 ppm for sparging air at 37 °C, 1 atm.

concentration, the q_{O_2} values apparently disclosed a measure of the recovery phenomena of cell culture against phenol stress. As shown in Fig. 6, for 200 mg/L of augmented nutrient source (e.g., gluconic acid, yeast extract) in the presence of 2800 mg/L phenol, the q_{O_2} values were almost identical (ca. 5.91–6.11 × 10^5 ppm h^{-1} L g^{-1}), indicating that an augmentation of low-level nutrient source induced an insignificant capability to defense phenol toxicity for cell recovery. In contrast, for 1000 mg/L of augmented nutrient source (e.g., gluconic acid, yeast extract, glycerol) in the presence of 2800 mg/L phenol, the q_{O_2} values suggested that gluconic acid seemed to have the most significant effect (7.88 × 10^5 ppm h^{-1} L g^{-1}; Fig. 7) to stimulate cell recovery. The relative ranking for this stimulation were gluconic acid (7.88 × 10^5 ppm h^{-1} L g^{-1}) > glycerol (5.91 × 10^5 ppm h^{-1} L g^{-1}) ≅ phenol alone (5.91 × 10^5 ppm h^{-1} L g^{-1}; Fig. 6) >≅ yeast extract (5.71 × 10^5 ppm h^{-1} L g^{-1}). This ranking seemed to be in parallel with the findings from CPT.

According to toxicology [7], chronic toxicity refers to the systemic effects produced by long-term, low-level exposure to chemicals (e.g., augmented nutrient sources and phenol herein).

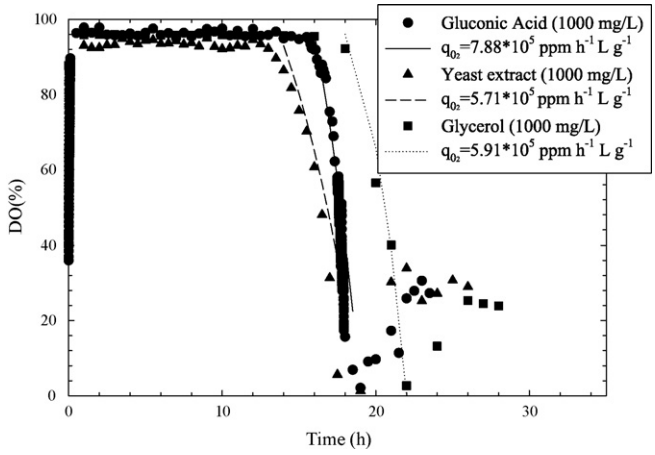

Fig. 7. Transient dynamics of dissolved oxygen concentrations in continuous cultures containing different augmented nutrient sources at 1000 mg/L in the presence of phenol (2800 mg/L) via CPT. The 100% DO denoted 100% saturated ≈ 6.7 ppm for sparging air at 37 °C, 1 atm.

The "symptoms" (i.e., recovery responses in DO concentration) of these mild augmentations might be slow to develop; thus, the connection between exposure and effect might be obscured (e.g., q_{O_2} values in Fig. 6). In contrast, high-level exposures (e.g., 1000 mg/L augmented nutrients) might signify the recovery responses in DO (e.g., the case of gluconic acid) concentration. However, perhaps because chronic toxicity is much more complex and subtle in its manifestations, the relative ranking of the q_{O_2} values for the stimulation at 1000 mg/L was not so clear revealed as predicted from CPT.

4. Conclusions

This feasibility study clearly indicated the stimulating rankings of augmented nutrients to R186 in terms of kinetic models from a toxicological perspective. In addition, it quantitatively showed inhibitory characteristics of phenol in continuous cultures, suggesting technical viability of using CPT for toxicity assessment upon R186. Although all augmented nutrients illustrated stimulating nature to the growth of R186 and antagonism to phenol toxicity (refer to the method and theory in [7,16,17]), the poor performance of phenol degradation was still observed in cultures with a dual energy source (e.g., acetate and phenol, yeast extract and phenol [6]). This discrepancy simply suggested that substrate consumption patterns would play the most significant role in biostimulation to phenol degradation as proposed before [5,6]. That is, the occurrence of diauxic growth would fail the effectiveness of biostimulation to cell growth and phenol degradation for R186. Although almost parallel trends of CPT and transient responses in DO concentration were shown, the slight difference in kinetics might be due to the microbial characteristics in response to chronic (CPT) and acute (DO) toxicity [7,9,12,15]. Thus, this study revealed a feasibility of CPT as well as transient dynamics of DO for toxicity assessment of augmented nutrients to R186. Follow-up studies will focus on how and what the mechanism(s) and the kinetics of substrate consumption patterns affect the efficiency of biostimulation to phenol degradation for conclusive remarks. In addition, using CPT results shown herein we will propose the optimal biostimulation strategy to use more appropriate augmented nutrient substrates for *in situ* or on-site phenol degradation (Appendix C).

Acknowledgements

Financial supports (NSC 94-2214-E-197-003, NSC 95-2221-E-197-005, NSC 96-2815-C-197-001-E and NSC 96-2221-E-197-012) from National Science Council, Taiwan, R.O.C. for this research and seeding grants for Biochemical Engineering Laboratory^{sdg} of National I-Lan University (NIU) from the Ministry of Education, Taiwan, ROC are very much appreciated. Part of the crucial concept in the study has been accepted to be presented at the sixth International Conference for Remediation of Chlorinated and Recalcitrant Compounds to be held in Monterey California, May 19–22, 2008. The authors gratefully acknowledge Chih-Yung Huang, Chi-Chin Kuo, Ming-Yun Huang, Ya-Ting Shu (NIU) for experimental data analysis for the work. The authors also extend sincere appreciation to Professor Jo-Shu Chang (Department of Chemical Engineering, National Cheng Kung University) and Professor Wen-Ming Chen (Department of Seafood Science, National Kaohsiung Marine University) for their valuable suggestions. This work is also dedicated to the memory of Dolloff F. Bishop who passed away on July 4, 1999 when the prime author was on duty under his mentorship in US EPA.

Appendix A. Mathematical model for CPT

In continuous operation, the transient dynamics of cell (X), phenol and augmented nutrient source (S) concentrations could be represented as follows:

$$\frac{dX}{dt} = (\mu - D)X; \quad X(0) = X_0 \tag{A1}$$

$$\frac{dS}{dt} = -D \cdot S + \frac{m}{V}\delta(t); \quad S(0) = S_0 \tag{A2}$$

Note that kinetic parameters to indicate biostimulation or inhibition of individual substrate via CPT was determined under the assumption of linear combinations of kinetic parameters of augmented substrate and phenol by neglecting effects of two factors. Moreover, the limitation of the CPT assessment is that the true response time to reflect inhibitory/stimulating effects of substrate(s) to be tested will be strongly dependent upon the characteristics of the microorganism and substrate(s). That is why steady-state conditions should be ensured prior to test to guarantee data validity of CPT. After the steady state was achieved at $\forall t > 0^+$, chemostat pulse injection was carried out as follows:

$$m\int_0^{+\infty} \delta(t)dt = m, \tag{A3}$$

where $\delta(t)$ is Dirac delta function or the unit impulse function (i.e., $\delta(t) = \begin{cases} 0 & t \neq 0 \\ \infty & t = 0 \end{cases}$ and $\int_0^{\infty} \delta(t)dt = 1\ \forall t \in R^+$). Eq. (A2) can be reformulated via Laplace transform to obtain $S(t) = (m/V)e^{-Dt} = S_0 e^{-Dt}$, where m and D denoted amount of injected phenol (PhOH) and/or augmented nutrient source (ANS) (mg) and dilution rate (h^{-1}). Suppose that the initial specific growth rate can be approximated by a linear approximation of $\mu \cong \mu^*(S_0) + d\mu/dS\big|_{S=S_0}(S - S_0) = \mu_0^* - k(S - S_0) \cong \mu_0 - k \cdot S \cong D - k \cdot S$, since a steady state was reached (i.e., $\mu_0 = D$). Since injected phenol and ANS-containing solutions are assumed to be toxic, positive and negative value of k directly suggested the inhibitory and stimulating characteristics to overall bacterial growth, respectively. In the initial short period of time, Eq. (A1) can thus be written as

$$\frac{d\ln X}{dt} = -kS_0 e^{-Dt}. \tag{A1'}$$

After integration, one may obtain

$$\ln\left(\frac{X}{X_0}\right) = \frac{kS_0}{D}(e^{-Dt} - e^{-Dt_0}), \tag{A4}$$

where t_0 indicated the time delay in response to injection as suggested by Goldberg and Er-el [11]; and X and X_0 denoted cell concentrations at $t = t$ and $t = t_0$, respectively. Note that although R186 is a phenol biodegrader, phenol still seemed to be toxic to R186 and thus this time delay was negligible ($t_0 \cong 0$) (also refer to R&D). Using Eq. (A4), one may obtain the kinetic parameter k to quantify whether the introduced chemical(s) are either a toxicant or stimulant to overall bacterial growth of *C. taiwanensis*. It was also assumed that the difference of kinetic parameter k of studied case and kinetic parameter of ANS-free control k_0 (i.e., $\Delta k = k - k_0$ or $\Delta k = \Delta k|_{ANS} = k|_{ANS+PhOH} - k_0|_{PhOH} = k - k_0$) quantitatively indicated the stimulating (−) and inhibitory (+) effect of augmented nutrient source to *C. taiwanensis*.

Appendix B. Dynamic measurement for oxygen uptake rate constant

Considering the oxygen uptake rate (OUR) and oxygen transfer rate (OTR) in terms of dissolved oxygen concentration, we might perform a material balance on DO during the operation as follows:

$$\frac{dC_L}{dt} = k_L a(C^* - C_L) - q_{O_2}X; \quad C_L(0) = C^*, \tag{B1}$$

where q_{O_2} is specific oxygen uptake rate per unit cell concentration (ppm h^{-1} L g^{-1}), X denotes cell density and C_L, C^* are concentrations of DO (%) and saturated DO (100% saturated ≈ 6.7 ppm for sparging air at 37 °C, 1 atm), respectively. Note that for OUR zero time (i.e., $t = 0$) is defined here as the time cell population resumed oxygen uptake for cell growth.

When cells adapted from a phenol-laden culture to resume cell growth, in a short period of time the transient dynamic can be considered as

$$\frac{dX}{dt} = kX; \quad X(0) = X_0, \tag{B2}$$

where k denoted the specific growth rate constant for cell recovery. The solutions could be resulted in the following equations:

$$X = X_0 e^{kt}, \tag{B3}$$

$$C_L = C^* + \frac{q_{O_2}X_0}{k_L a + k}(1 - e^{kt}). \tag{B4}$$

Prior to the determination of rate constant k for comparison, oxygen transfer coefficient was evaluated from the blank experiment in cell-free media at operating conditions (working volume 1.5 L, 37 °C, 200 rpm, air flow rate 2 vvm). In the cell-free solution, the material balance on DO concentration could be obtained as

$$\frac{dC_L}{dt} = k_L a(C^* - C_L); \quad C_L(0) \cong 0, \tag{B1'}$$

where the initial condition was achieved via pure nitrogen sparging to remove all residual DO. The solution of (B1′) can be obtained as

$$\ln\left(\frac{C^* - C_L}{C^*}\right) = -k_L a \cdot t. \tag{B5}$$

From the linear regression of least squared error for $\ln(C^* - C_L/C^*)$ versus t, one might obtain the slope of oxygen transfer coefficient $k_L a$ (ca. 208.5 h^{-1}). With this oxygen transfer coefficient, we might determine q_{O_2} value using transient dynamic of DO concentration in cultures to from Eq. (B4) via non-linear random search technique for the best fit curve.

Appendix C. Theoretical analysis on monophasic or diauxic growth patterns

For possible competing reactions of phenol degradation, we may choose parallel reactions

$$ANS + PhOH + X \xrightarrow{k_X} 2X \quad \text{(desired)},$$

$$ANS + PhOH + X \xrightarrow{k_U} U \quad \text{(undesired)},$$

where PhOH, X, ANS and U denote phenol, biomass, augmented nutrient substrate and undesired by-product, respectively [18]. The rate law are

$$r_X \cong k_X[PhOH]^{a_1}[ANS]^{b_1}[X],$$

$$r_U \cong k_U[PhOH]^{a_2}[ANS]^{b_2}[X],$$

where a_1, a_2, b_1, b_2 are positive reaction orders. The rate of disappearance of phenol for this reaction sequence is the sum of the rates of formation of X and U:

$$-r_{PhOH} \cong r_X + r_U = k_X[PhOH]^{a_1}[ANS]^{b_1}[X] + k_U[PhOH]^{a_2}[ANS]^{b_2}[X].$$

Taking the ratio of these rates, we obtain a rate of selectivity (ROS), which is to be maximized:

$$S_{X/U} \cong \frac{r_X}{r_U} = \frac{k_X}{k_U}[\mathrm{PhOH}]^{a_2-a_2}[\mathrm{ANS}]^{b_2-b_2}.$$

Assuming $a_1 - a_2 = a$ and $b_1 - b_2 = b$, we can carry out order-of-magnitude analysis on initial ROS to obtain

(a) for diauxic growth cases, complete exclusion for phenol degradation will be resulted (i.e., $a_1 \to -\infty$)),
(b) for non-diauxic growth cases, a competition on the utilization of two substrates will be taking place (i.e., $+\infty > a_1 > 0$),
(c) for monophasic growth situations, the order relation between a and b will be bounded in a neighborhood of the same order of magnitude (i.e., $+\infty > a \approx O(1) \approx b > 0$ [19]).

To increase $S_{X/U}$ for diauxic growth systems (i.e., $a \to -\infty$), the only non-trivial solution for this operation will be $[\mathrm{ANS}] \cong 0$ (i.e., failure biostimulation). That is, the necessary condition for possible biostimulation using augmented substrate is to guarantee a finite value of rate parameter a (i.e., $a \in (-\infty,+\infty)$) present in the dual-substrate systems. We also set some operation criteria to inspect whether the biostimulation strategy will be successful as follows:

(d) if $a - b \in (-\infty,0)$ (i.e., negative real numbers), the degradation will be favorable to utilization of ANS),
(e) if $a - b \in (0,+\infty)$ (i.e., positive real numbers), the degradation will be favorable to phenol degradation),
(f) the condition of possible successful biostimulation will be $a \approx b$.

References

[1] P. Vandamme, T. Coenye, Taxonomy of the genus *Cupriavidus*: A tale of lost and found, Int. J. Syst. Evol. Microbiol. 54 (2004) 2285–2289.
[2] W.M. Chen, S. Laevens, T.M. Lee, P. De Vos, M. Mergeay, P. Vandamme, *Ralstonia taiwanensis* sp. nov. isolated from root nodules of *Mimosa* species and sputum of a cystic fibrosis patient, Int. J. Syst. Evol. Microbiol. 51 (2001) 1729–1735.
[3] W.M. Chen, J.S. Chang, C.H. Wu, S.C. Chang, Characteristization of phenol and trichloroethene degradation by the rhizobium *Ralstonia taiwanensis*, Res. Microbiol. 155 (2004) 672–680.
[4] B.R. Folsom, P.J. Chapman, P.H. Prichard, Phenol and trichloroethylene degradation by *Pseudomonas cepacia* G4: Kinetics and interactions between substrates, Appl. Environ. Microbiol. 56 (1990) 1279–1285.
[5] B.Y. Chen, J.S. Chang, Phenol degradation and toxicity assessment upon biostimulation to an indigenous rhizobium *Ralstonia taiwanensis*, Biotechnol. Prog. 21 (2005) 1085–1092.
[6] B.Y. Chen, W.M. Chen, J.S. Chang, Optimal biostimulation strategy for phenol degradation with indigenous rhizobium *Ralstonia taiwanensis*, J. Hazard. Mater. B139 (2007) 232–237.
[7] M.A. Ottoboni, Chronic toxicity, in: The Dose Makes the Poison, 2nd ed., Int'l Thomson Pub, New York, 1997 (Chapter 8).
[8] A. Narang, S.S. Pilyugin, Bacterial gene regulation in diauxic and non-diauxic growth, J. Theoret. Biol. 244 (2007) 326–348.
[9] B.Y. Chen, Toxicity assessment of aromatic amines to *Pseudomonas luteola*: chemostat pulse technique and dose-response analysis, Process. Biochem. 41 (7) (2006) 1529–1538.
[10] S.J. Pirt, Chapter 11 Effects of oxygen on microbial cultures. in: Principles of microbe and cell cultivation, in: A Halsted Press Book, Wiley, New York, 1975.
[11] I. Goldberg, Z. Er-el, The Chemostat—an efficient technique for medium optimization, Proc. Biochem. (October/November) (1981) 2–8.
[12] B.-Y. Chen, H.-L. Liu, Y.-W. Chen, Y.-C. Cheng, Dose–response assessment of metal toxicity upon indigenous *Thiobacillus thiooxidans* BC1, Proc. Biochem. 39 (2004) 735–745.
[13] J.E. Bailey, D.F. Ollis, Metabolic stoichiometry and energetics, in: Biochemical Engineering Fundamentals, 2nd ed., McGraw-Hill, Inc., New York, 1986 (Chapter 5).
[14] K.P. Talaro, A. Talaro, Fundations in Microbiology, 4th ed., McGraw Hill, Boston, 2002.
[15] US Environmental Protection Agency, Toxicology Handbook, 4th ed., Government Institutes, Inc., Rockville, MD, 1989.
[16] B.Y. Chen, H.L. Liu, Y.W. Chen, Y.C. Cheng, Synergistic interactions of binary metal toxicity to Indigenous *Acidithiobacillus thiooxidans*, J. Chin. Inst. Chem. Eng. 37 (3) (2006) 217–225.
[17] B.-Y. Chen, C.-H. Wu, J.-S. Chang, An assessment of the toxicity of metals to *Pseudomonas aeruginosa* PU21 (Rip64), Biores. Technol. 97 (15) (2006) 1880–1886.
[18] H.S. Fogler, Multiple reactions, in: Elements of Chemical Reaction Engineering, 4th ed., Pearson International Ed., 2006 (Chapter 6).
[19] J.D. Logan, Perturbation methods, in: Applied Mathematics, 3rd ed., Wiley-Interscience, John Wiley & Sons, Inc., New York, 2006 (Chapter 2).