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Optimal biostimulation strategy for phenol degradation with indigenous rhizobium *Ralstonia taiwanensis*

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Abstract

This study provides a first attempt from a perspective of Gaden's classification of fermentation and phase–plane to put forward phenol degradation using various augmented nutrient media for biostimulation. It aimed to identify the most promising nutrient source(s) to attenuate synergistic interactions with phenol for optimal phenol degradation. Therefore, the growth association of phenol degradation using various nutrient media in place of combined toxic interactions was established via Gaden's classification scheme of fermentation and phase–plane analysis. In cultures grown on medium bearing dual carbon sources (glycerol and phenol) or phenol alone, phenol was found to be firstly biodegraded for microbial growth (i.e., growth-associated degradation). In contrast, when yeast extract or acetate was supplemented, a diauxic growth behavior was observed as the augmented nutrient was primarily utilized while phenol degradation was repressed. Moreover, using glycerol as the nutrient source, phenol degradation seemed to be enhanced simultaneously during the consumption of glycerol for cellular growth after ca. 2 h response lag in growth. Although gluconic acid could enhance cell growth as well as phenol degradation, the phenol degradation performance was still not as good as that of glycerol. Thus, biostimulation with glycerol appeared to show the most favorable metabolic characteristics against phenol toxicity on *Ralstonia taiwanensis*, leading to better degradation efficiency of the toxic pollutant. Phase–plane trajectories also clearly confirmed that glycerol was the optimal biostimulating nutrient source for phenol degradation.

Keywords: *Ralstonia taiwanensis*; Phenol biodegradation; Combined toxicity; Growth association; Biostimulation

1. Introduction

Among *Ralstonia taiwanensis* strains isolated from the environment in southern Taiwan, the *R. taiwanensis* R186 (R186 for short) strain demonstrated an excellent capability to grow on a medium containing phenol or TCE as a carbon source [1,2]. Compared to other phenol biodegraders [2,3], R186 displayed a superior phenol biodegradability as well as tolerance (up to ca. 900 mg/L) in the phenol-laden 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 [2]. In addition, the low saturation constant (K_s) of 5.46 μM and high inhibition constant of 9075 μM suggested a promising feasibility of R186 for practical bioremediation.

As conservation of wild-species in local environment is always a concern, indigenous rather than foreign microbiota should be first considered for *in situ* or *ex situ* biotreatment. In the previous studies, the indigenous rhizobium *R. taiwanensis* R186 was used for bioremediation of phenol-contaminated soil and freshwater [2,4]. In addition, to reveal promising feasibility of using strain R186 for industrial applications, operation criteria (e.g., combined toxic thresholds in the presence of augmented nutrient sources) were also considered [4]. It illustrated a striking effect of combined toxicity of supplemented nutrients on the performance in phenol degradation. It is also noted that phenol degradation of R186 is biomineralization (i.e., complete biodegradation to CO_2) instead of

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biotransformation (i.e., incomplete degradation to organic intermediates). Clearly, lack of adequate combined toxicity figures of augmented nutrient–phenol media to cell population for biostimulation makes phenol degradation unpredictable and unreliable for on-site operations in bioremediation.

Although our previous work [4] presented an attempt in pursuit of a perspective in toxicology for biostimulation, a straightforward approach to conclude optimal strategy was not clearly uncovered. It also showed that almost all supplemental nutrients synergistically interacted with chronic toxicity of phenol to the phenol-degrading isolate *R. taiwanensis* R186. Thus, this follow-up study emphasized how the Gaden's classification scheme of fermentation [5,6] and phase–plane analysis [7] could be applied to disclose the relationship of such toxicity interactions with growth association, allowing a clear identification of optimal biostimulation strategy and bioreactor operation for more efficient phenol degradation.

2. Materials and methods

2.1. Bacterial strains and culture conditions

R. taiwanensis R186 originally isolated from root nodules of *Mimosa pudica* in southern Taiwan was routinely grown at 28 °C on yeast extract-mannitol (YEM) agar plates [1,2]. To grow strain R186 in phenol-containing medium, a single colony grown on YEM agar plate was inoculated to 50 ml of basal salt (BS) medium supplemented with phenol (Merck). The BS medium consisted of (g/L) Na_2HPO_4, 7.0; KH_2PO_4, 3.0; NaCl, 0.5; NH_4Cl, 1.0. The pH was adjusted to pH 6.8 ± 0.2. Cells of R186 were grown at 28 °C with shaking at 125 rpm for 7 days. Culture media were monitored daily by absorbance at 600 nm (OD_{600}). The concentration of phenol in the medium was determined by using a colorimetric assay [7] with a detection limit of ± 2.5 μM. Colormetric reagent contained pH 10 buffer (HBr 3.0, KCl 3.73, NaOH 1.76 g/L), 4-aminoantipyrine and potassium hexacyanoferrate.

2.2. Determination of phenol degradation characteristics

R. taiwanensis R186 strain from −80 °C stock was cultured overnight in Luria-Bertani medium and then 1.0 ml of the culture was inoculated to 50 ml of BS amended by various concentrations of phenol. The cultures were incubated at 37 °C and 200 rpm. To assess whether phenol toxicity would be synergistic or antagonistic due to the presence of other nutrient sources, the R186 cells were grown on phenol (400 mg/L)-containing BS medium supplemented with 0.1% (w/v) of the specified nutrient source (e.g., glycerol, yeast extract, acetate and gluconic acid). After inoculation, the cell concentration and residual phenol concentration were determined with respect to time. The specific growth rate (μ) and specific phenol degradation rate (q_p) were determined through the relationships of $d \ln X/dt$ and $(-1/X)\, d[Ph]/dt$, respectively; where X denotes cell concentration, [Ph] denotes phenol concentration, and t denotes incubation time.

2.3. Construction of phase–plane portraits

According to Perlmutter [6,7], the (bio)chemical reactor models can be in terms of the form:

$$-\frac{1}{X}\frac{d[Ph]}{dt} = f_1(X, [Ph], S) = q_P \tag{1a}$$

$$\frac{1}{X}\frac{dX}{dt} = f_2(X, [Ph], S) = \mu \tag{1b}$$

where X, [Ph], S denoted the concentration of cell, phenol and augmented nutrient substrate, respectively. Such models lend themselves to a graphical analysis in which the dependent variables (or state variables) are plotted against one another on what is called a phase–plane. For phase–plane analysis, it is postulated that the contribution of other factors (e.g., augmented substrate, dissolved oxygen) were relatively negligible or insignificant compared to two major state variables (i.e., phenol and biomass concentration). As the system evolves continuously over time through a series of states from its initial to final condition, its mapping on the (X, [Ph]) plane will trace a trajectory to be a biological fingerprint specific to the system. To combine this perspective with Gaden's classification scheme of fermentation [8], the phase–plane was modified to disclose on the (μ, q_P) plane since the instantaneous slope of the curve in the (X, [Ph]) plane and in the (μ, q_P) plane [9] are theoretically identical. For example, the instantaneous slope m on the (μ, q_P) plane can be obtained by

$$\frac{q_P}{\mu} = \frac{-\frac{1}{X}\frac{d[Ph]}{dt}}{\frac{1}{X}\frac{dX}{dt}} = -\frac{d[Ph]}{dX} = \frac{f_1(X, [Ph], S)}{f_2(X, [Ph], S)} = m \tag{2}$$

and the entire trajectory can be drawn as a sequence of small steps using the computed slope at each step to move on to the next (i.e., exploited in the trajectory direction to show time frame on the phase–plane). This is so-called a set of auxiliary isoclines in the (X, [Ph]) plane:

$$f_1 = m f_2 \tag{3}$$

which are the loci of points where the trajectories have slope m. As Perlmutter [5] indicated, for systems of the form (1), the trajectory from any point will be unique when the f_1 and f_2 functions have continuous first partial derivatives (e.g., the cases of biochemical reactor model shown herein). In light of these, no trajectories can ever cross, except at singular points where the derivatives dX/dt and $d[Ph]/dt$ are both zero (i.e., lag phases in growth and phenol degradation curves should be excluded). Thus, it is feasible to apply this phase–plane analysis to reveal growth-dependent phenomena of fermentation in this study.

3. Results and discussion

3.1. Feasible nutrients for the growth of R186

To understand the available nutrient source(s) for R186, treatability study was carried out with BS medium supplemented by various nutrient sources at a concentration of 1000 mg/L (0.1%). After 3-day cultivation, significant growth was observed

Table 1
List of maximal specific growth rates (μ_{max}; h^{-1}) and yield coefficients ($Y_{X/S}$; g DCW/g nutrient substrate) for *Ralstonia taiwanensis* R186 grown on phenol-containing media supplemented with various nutrient sources

	GA	YE	PA	Gly	AA
μ_{max} (h^{-1})	0.43	0.72	0.61	0.080	0.50
$Y_{X/S}$ (g/g)	0.515	0.234	0.531	0.406	0.347

Phenol concentration: 400 mg/L; nutrient concentration: 1000 mg/L. GA: gluconic acid; YE: yeast extract; PA: pyruvic acid; Gly: glycerol; AA: acetate.

for media containing gluconic acid (GA), yeast extract (YE), pyruvic acid (PA), glycerol (Gly), acetate (AA) and phenol alone. In contrast, R186 cells did not grow on media containing a sole carbon source of glucose, lactose, sucrose, ethanol, galactose, mannitol, galacturonic acid, raffinose, dulcitol, oxalic acid or malic acid. In addition, the specific growth rates and yield coefficients determined through batch cultures using BS medium supplemented with 0.10% nutrient sources were chosen as a comparative basis (Table 1), which will be used later to illustrate combined toxic interaction and phenol degradation in phenol-bearing cultures.

3.2. Growth of R186 with phenol alone

As shown in Figs. 1 and 2, maximal growth rate lagged behind maximal phenol degradation rate by ca. 2 h (e.g., 8–10 h; Fig. 2), indicating that there was a response lag while converting phenol degradation for cell growth. The relationship of a constant stoichiometric relationship between phenol consumption and cell growth rate (ca. 0.0513 $d[Ph]/dt \cong dX/dt$) in the

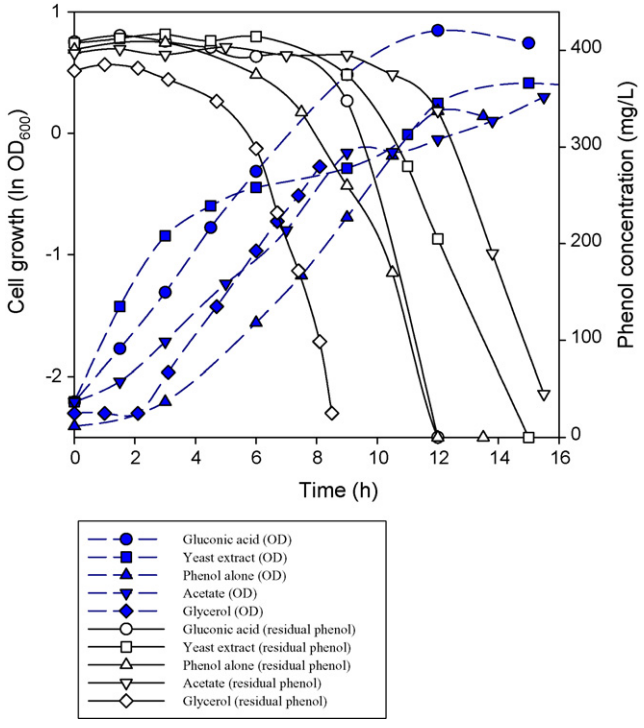

Fig. 1. Time courses of cell growth, phenol removal characteristics in the BS culture initially contained 400 mg/L phenol and 0.1% augmented nutrient source.

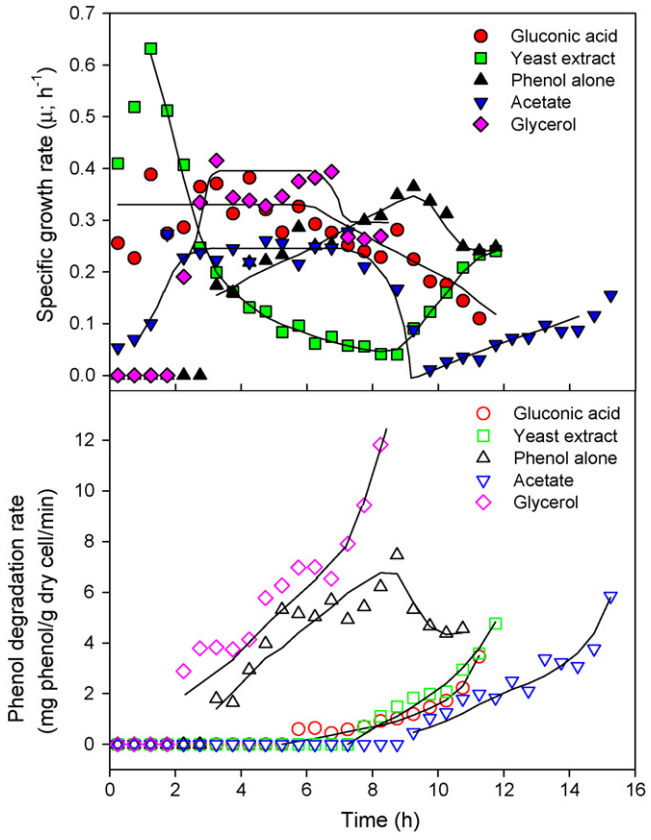

Fig. 2. Time courses of specific growth rate and phenol degradation rate in the BS culture initially contained 400 mg/L phenol and 0.1% augmented nutrient source.

exponential growth phase clearly exhibited the characteristics of type I fermentation [5,10] (i.e., cells as catabolic products). This growth-associated behavior directly implies that phenol was primarily used as the carbon source for direct oxidation to primary energy metabolism. The response lag (ca. 2 h) in growth was observed as phenol could be completely converted to catechol via catabolic routes, and then dissimilation of catechol took place via the pathways involving *meta*-cleavage or *ortho*-cleavage [11] for conversion to cell mass.

3.3. Growth of R186 with a dual carbon source of glycerol and phenol

Figs. 1 and 2 clearly reveal that the specific growth rate at 3–6 h was ca. 0.35–0.38 h^{-1}, indicating that phenol rather than glycerol was firstly consumed since μ_{max} values for phenol and glycerol were 0.40 and 0.08 h^{-1}, respectively (Table 1). After ca. 2 h lag to adjust cellular metabolism for adaptation (Fig. 2), phenol was gradually utilized for cell growth (also shown in Fig. 1 for phenol degradation). Once phenol was almost exhausted, metabolic routes were switched to glycerol utilization and growth rate progressively decreased (i.e., 7–8 h; Fig. 2). Compared to all other nutrient sources (Figs. 1 and 2), glycerol was the most economically feasible to enhance cell growth as well as phenol degradation. Although there was a ca. 2 h response lag for cell growth (Fig. 1), the maximal phenol

degradation rate and growth rate (Fig. 2) still indicate the most promising role of glycerol as an energy and carbon source for biostimulation.

3.4. Growth of R186 with a dual carbon source of yeast extract and phenol

As indicated in Fig. 1, within the first 7 h, cell growth took place, but phenol was not degraded. This points out that yeast extract was first exploited as a primary nutrient substrate as it contains rich essential vitamins and other nutritious compositions. Once yeast extract was completely depleted (i.e., nearly zero growth at ca. 8 h), the expression of phenol-metabolizing enzymes (e.g., phenol hydroxylase enzymes [12,13]) was induced to degrade phenol. Therefore, diauxic growth characteristics was observed (i.e., biphasic stepwise growth pattern as shown in Figs. 1 and 2). Cell growth activities reached maximum early (ca. 1–2 h); at later stages phenol degradation became more significantly (ca. >7 h). According to Deindoerfer's classification of fermentation patterns [6], this diauxic growth simply suggested that the presence of yeast extract significantly repressed the inducible expression of phenol-metabolizing enzymes. Exhaustion of yeast extract resulted in induced synthesis of these enzymatic activities for phenol degradation (ca. 2 h lag phase at 6–8 h for expression). Due to the biphasic growth, apparently complete repression of phenol degradation at the first growth phase significantly decreased overall biodegradation performance. This suggests that the appearance of biphasic growth might give rise to poor phenol degradation.

3.5. Growth of R186 with a dual carbon source of acetate and phenol

Similar to yeast extract, acetate utilization and phenol degradation were also mutually exclusive for cell growth (Figs. 1 and 2). This diauxic growth behavior depicted that once the growth rate reached approximately zero at ca. 9 h phenol was then gradually degraded for cell growth (Fig. 2). In addition, the specific growth rate at first growth stage (ca. 0.25 h^{-1} at time <8 h) was only half of the maximal growth rate (i.e., 0.50 h^{-1}; Table 1) of acetate-bearing culture. Thus, the decrease in growth performance was very likely due to synergistic interactions between acetate and phenol [4]. This may suggest that a significant portion of acetate was exploited to express defense mechanism (e.g., maintenance via membrane transport proteins) in cells to handle this threat in a manner to prevent combined toxic responses (e.g., loss in metabolic functions) from occurring. Evidently, since phenol degradation was completely repressed at the first growth phase, a marked decrease in biodegradation efficiency would be observed as mentioned earlier.

3.6. Growth of R186 with a dual carbon source of gluconic acid and phenol

As indicated in Figs. 1 and 2, gluconic acid was not a favorable nutrient source for phenol degradation when compared to the results from phenol alone. Although cell growth was significantly enhanced due to addition of gluconic acid, phenol degradation was still low. It is very likely that due to combined toxic potency of phenol, the specific growth rate during gluconic acid consumption (ca. 0.33 h^{-1}) was slightly lower than that in the phenol-free culture (i.e., 0.43 h^{-1}; Table 1). As shown in Figs. 1 and 2, nutrients selectively converted to cells in preferential order perhaps due to mutually exclusive metabolic pathways for cellular growth. Similar to yeast extract or acetate, phenol degradation began immediately when the primary nutrient source gluconic acid was completely exhausted (i.e., stepwise or diauxic growth). However, a new lag phase was not clearly observed while the cell activated the expression of phenol-metabolizing enzymes and a second exponential phase apparently was not revealed (Figs. 1 and 2). Although gluconic acid could enhance cell growth as well as phenol degradation, the phenol degradation performance was less efficient than that for glycerol. The insignificant diauxic growth phenomenon for gluconic acid suggests that some of the enzymes responsible for gluconic acid utilization might be also crucial to phenol degradation. Since the enzymic expression for phenol degradation still indirectly arised from energy metabolism of gluconic acid, phenol degradation was thus termed partially related to utilization of gluconic acid.

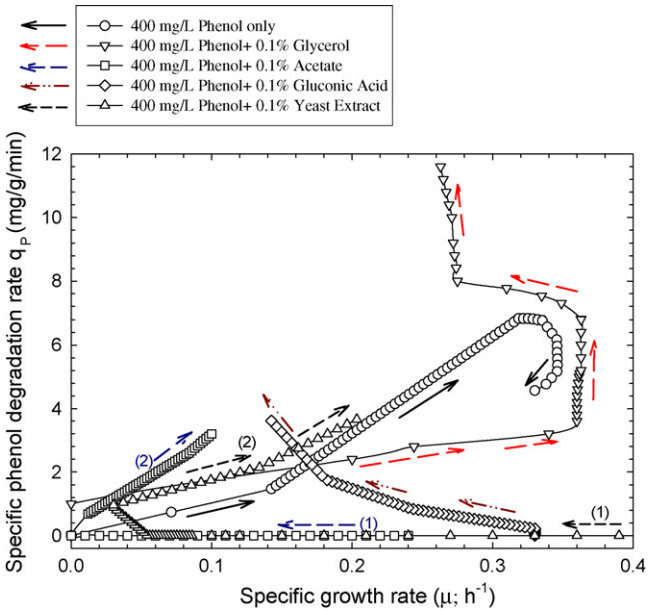

Fig. 3. Phase–plane profiles for the batch cultures using various nutrient media. Trajectories with arrows (1) and (2) denoted the characteristics of diauxic (or biphasic) growth. The arrows indicated the orientation of all trajectories of profiles as time went by.

3.7. Phase–plane analysis

To obtain comparative figures for various nutrient media, plane-curve profiles of q_P versus μ at various times to depict the trajectories of phenol degradation (Fig. 3). In the cases of using acetate (AA) and yeast extract (YE) as augmented nutrient sources, when times increased the orientation of both profiles

started at certain point (e.g., 0.24 and 0.6 h^{-1} for AA and YE, respectively) in $+x$-axis (i.e., $+\mu$-axis or phenol degradation-independent axis) and headed to negative x-direction to the origin and then turned into quadrant I (i.e., growth-dependent domain). These trajectories clearly state that acetate and yeast extract were utilized preferentially prior to phenol degradation (i.e., biphasic growth phenomena). Thus, the consumption of these favorable substrates for growth was metabolism-independent of phenol degradation, implying that phenol toxicity still remained intact and could not be attenuated in the first growth phase. Specific growth rates (ca. <0.1 and <0.2 h^{-1} for AA and YE, respectively) in second growth phase (i.e., arrows with (2) in Fig. 3) were relatively lower than that in the culture using phenol as the sole carbon source ($\mu \sim 0.3\ h^{-1}$), indicating that the persistent phenol toxicity still strongly inhibited the growth of R186 [13].

By contrast, if the utilization of augmented nutrient was at least partially relevant to phenol degradation, monophasic growth phenomenon would be observed. For instance, as glycerol was not a preferential substrate to R186 compared to phenol, phenol was primarily utilized and then glycerol was consumed afterwards. This point can be confirmed by the early trajectory for glycerol (Fig. 3) nearly convergent to the spiraling convergent point (i.e., focus) for phenol as the sole carbon medium. However, the biphasic growth phenomenon was not clearly perceived for glycerol, most likely due to partial association of glycerol utilization with phenol degradation. The trajectories for phenol alone and glycerol–phenol (Fig. 3) might also suggest that early reduction of phenol toxicity may be crucial to maximize phenol degradation capability. Phase–plane trajectories clearly indicate that the highest phenol degradation and growth rate was obtained for the growth with a dual carbon source of glycerol and phenol. This suggests that glycerol should be a feasible nutrient source providing better microbial growth and efficiently reducing phenol toxicity. Therefore, glycerol is conclusively the most promising nutrient source for biostimulation. In addition, to prevent significant toxicity of phenol to cellular growth, the m value in Eq. (2) for dual-substrate cases should be greater than the m value (denoted as m_{cr}) of phenol degradation in the absence of augmented nutrient source (i.e., phenol alone). As shown in Fig. 3, apparently nearly all m values for dual-substrate cases were less than m_{cr}, indicating that the second nutrient source seemed to synergistically inhibit phenol degradation (either loss of growth or degradation capability). The marked rise in combined toxicity might indicate that the utilization of second nutrient could by-pass certain portion of energy requirement in phenol degradation. Moreover, residual metabolic intermediates of phenol degradation might be significantly increased to inhibit phenol degradation via feedback repression [14]. However, after certain period of time (see Fig. 3), glycerol might still significantly enhance intracellular tolerance mechanism to phenol degradation as well as cellular growth, leading to the optimal performance of phenol degradation. As indicated in Fig. 3, it is concluded that the optimal biostimulation strategy should be to direct the m value to be greater than m_{cr} as quick as possible (e.g., adding glycerol).

In summary, regarding nutrient sources of "diauxic growth type", separation of growth stimulation from phenol degradation via a two-stage operation is inevitable. For "parallel-reaction type" nutrients (e.g., gluconic acid and glycerol), fed-batch (semi-batch) mode of operation will be the most feasible to maximize phenol degradation and cell growth concurrently. As phenol might still be inhibitory to metabolically functioning cells, to optimize degradation performance, phenol should be fed granually (e.g., dropwise feeding) instead of fully provided at the very beginning [15] (e.g., fed-batch dropwise feeding strategy).

4. Conclusions

This study shows that whether diauxic growth characteristics exists or not plays a crucial role in biostimulation strategy. The results show that glycerol was most feasible to be used for biostimulation as cells could efficiently utilize glycerol as an energy source to "antagonize" phenol toxicity, to propagate cell population, and to degrade phenol concurrently. In contrast, the diauxic growth characteristics in yeast extract and acetate-containing cultures simply implies the preferential utilization of the augmented nutrients and complete repression upon phenol degradation, while phenol toxicity cannot be attenuated during utilization of the supplemented nutrients. According to Fogler [15], for phenol degradation of the "diauxic growth type" nutrients, the two-stage operation mode would be the favorable selection for operation to prevent the occurrence of biphasic growth. That is, cells should be propagated in phenol-free cultures at the maximal specific growth rate in the first stage to achieve optimal cell density, then the phenol-bearing medium is fed in dropwise after depletion of the nutrients for an efficient phenol degradation. In the cultures grown on glycerol–phenol medium, phenol degradation was significantly enhanced in parallel with cell growth leading to the optimal biostimulation effect (Fig. 3).

Acknowledgments

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