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Energy Profiles of Activated Sludge Degradation of a Xenobiotic Compound

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Stable structure of xenobiotic requires a large amount of energy for the microorganisms to metabolize. In addition to the general hard-to-treat nature of a xenobiotic, the energy expense in breaking xenobiotic structure may add to the difficulty of xenobiotic degradation. This study was to determine the detail profiles of energy contents in activated sludge cells during the cells' degradation of a model xenobiotic compound 2,4-dichlorophenoxyacetic acid (2,4-D). Energy content in sludge cells was measured as Adenosine Triphosphate (ATP). Activated sludge degradation reactions of 2,4-D were performed with the sole feed of 2,4-D, and sucrose and/or peptone added to the 2,4-D degradation reactions to serve as external energy supply to the metabolism of the xenobiotic. Measurement results show that ATP content per unit mass of sludge biomass was lowered after sludge had degraded 2,4-D. Amount of sludge grown was inevitably lowered due to an energy deficit after 2,4-D catabolism. Biogenic addition indeed increased the average ATP content and yield higher cell mass. The time courses of 2,4-D degradation, cell mass growth, and ATP contained in activated sludge, are presented in this paper with discussion pertaining to the science of xenobiotic degradation and also applicability of ATP enrichment method to xenobiotic treatment bioprocess.

1. Introduction

Xenobiotic can be treated by chemical, physical and biological methods but biological method is most attractive as "green" remediation strategies for xenobiotic removal from aqueous environment (Tomei et al., 2009). However, xenobiotic organic compounds are hard-to-treat by microorganisms originated from nature, or are indigenous. The most obvious difficulty for indigenous activated sludge, when first exposed to a xenobiotic compound, is the lack of degradative genetic elements (Chong and Lin, 2007). Because the molecular structures of xenobiotics are different from natural microbial substrates, activated sludge cells must acquire specific metabolism pathways from evolution of degradative gene(s) before the cells can start to degrade their xenobiotic target. Indigenous microorganism can acquire a degradation capability for xenobiotic through a process called acclimation (Buitron et al., 1998). However, even after acclimation, xenobiotic organic compounds are hard to break-down due to their stable structure; in addition to the required metabolic function, a large amount of energy is required for the microorganisms to metabolize a xenobiotic compound. Structure breaking energy expense may add to the difficulty of xenobiotic degradation (Chong et al., 2010). As in metabolism of all organic substrates, the original substrate is broken down into smaller molecules. Parts of catabolism are oxidation processes and certain amount of energy is obtained by the cells from these substrate-level oxidations (Orhon and Artan, 1994). Since an oxidation process is coupled with a reduction. The precise biochemical design must not allow this reducing power to disappear for no reason. This reducing power is stored in the organic molecules of NADH (or NADPH), either pending short-term expenditure in syntheses of new cellular materials, or is turned into storage, from which most or all NADH is oxidized in the respiration reactions. The high degree of oxidation in respiration (oxidative level) produces a high amount of energy (Nelson and Cox, 2008). Energy from both the substrate- and oxidative-level productions is collectively stored in an organic molecule of ATP (Rittmann and McCarty, 2001).

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There are many questions to the energy flow, as can be accounted for by ATP flow, during metabolism of xenobiotic compounds. The energy flow measurements during xenobiotic degradation are important to understanding of the following questions: 1), to undergo the difficult catabolism steps, activated sludge must consume high amount of energy and as a result the flow of energy supply from catabolism may be disrupted, especially when the energy obtainable from metabolism may only be available at some later stages. As a consequence of energy consumption during the early stage of metabolism, the subsequent cell anabolism process may be hindered (Chong et al., 2010). Will xenobiotic metabolism result in serious energy shortage? 2), when xenobiotic is a sole carbon source, aerobic heterotrophic microorganisms must only obtain energy from oxidation of the xenobiotic. If there is an energy deficiency leading to difficulty of xenobiotic degradation, will it be possible to supply energy from an external source that could enhance xenobiotic degradation? It is commonly known that all cells, including microbial cells, are enriched with ATP when grown on a biogenic organic (Tsai et al., 1997). For xenobiotic degradation, Chong et al. (2012) reported that biogenic amendment helped shorten lag time during activated sludge acclimation to a xenobiotic. Oehmen et al. (2013) have also reported propionate addition enhanced herbicide propanil degradation rate.

The purpose of this study, therefore, was to determine the detail profiles of ATP that is contained in activated sludge cells during the cells' degradation of a model xenobiotic compound 2,4-dichlorophenoxyacetic acid (2,4-D). In hope to enhance 2,4-D degradation, biogenic substrates of sucrose and also peptone were added to the 2,4-D degradation reactions. Overall, ATP contained in the cells of activated sludge that was used for degradation of 2,4-D were measured in four (4) types of reactions: 1) feed of sole 2,4-D, 2) feed of 2,4-D and sucrose, 3), feed of 2,4-D with peptone, and 4) feed of 2,4-D with sucrose and peptone. The measurement results are presented in time courses of 2,4-D degradation, cell mass growth, and ATP contained in the cell. Indications of the ATP time courses in xenobiotic degradation bioenergetics are discussed, with implication to applicability of this ATP enrichment method to xenobiotic treatment bioprocess.

2. Material and Methodology

2.1 Xenobiotic and Activated Sludge

The target xenobiotic was the herbicide 2,4-dichlorophenoxyacetic acid (2,4-D).The initial activated sludge seeds were obtained from a soil that did not have any record of 2,4-D nor metal contamination. This indigenous activated sludge seed was grown in a fed-batch reactor that was re-fed once daily with a fresh medium containing biogenic substrates (120 mg/L sucrose and 50 mg/L peptone) and minerals: $FeCl_3$ 1.0 mg/L, NH_4Cl 30.0 mg/L, K_2HPO_4 200.0 mg/L, KH_2PO_4 156.0 mg/L, $MgSO_4.7H_2O$ 65.0 mg/L.

Acclimated sludge was prepared (or cultivated), each time before its degradation use, by subjecting the fed-batch indigenous sludge to reactions with 2,4-D. Acclimation of activated sludge was achieved after the sludge had completely degraded 2,4-D three (3) consecutive times, in batch reactions (shake-flasks). Lag time for degrading 2,4-D was totally eliminated after the third-time acclimation reaction and a fully acclimated sludge was obtained that is highly degradative of 2,4-D in reactions subsequent to acclimation.

2.2 Degradation experiments

Degradation of 2,4-D were conducted in shake-flasks, shaken 100 rpm orbital, at room temperature (25 ± 2 °C). The starting acclimated activated sludge concentration was targeted at 100 mg/L. Media in degradation reactors contained 100 mg/L of 2,4-D, minerals listed above and in the biogenic-added cases, biogenic substrates at designated concentrations.

A 2,4-D degradation test with the presence biogenic substrate(s) was conducted with the additions of sucrose (technical grade) or peptone (technical grade) or their combine, to a 2,4-D degradation reactor at the start of reaction. The concentrations of sucrose and peptone added were preliminarily tested to find ones that were most beneficial in term of a fastest 2,4-D degradation rate resulting wherefrom: Sucrose was added at 50 mg/L, peptone was added at 80 mg/L, and sucrose and peptone at 20 mg/L and 40 mg/L.

From a degradation test, a 2,4-D course was obtained from measurements of 2,4-D concentrations in the reactor liquid (filtered), a biomass growth course was obtained from measurements of sludge cell mass in the reactor suspension, and a ATP-in-cell course was obtained from measurements of ATP contained in the cells in the reactor suspensions. Reactor suspensions were harvested for these measurement purposes at regular intervals (typically hourly). All tests were performed at least in duplicates.

2.3 Measurements

Soluble 2,4-D concentrations in the degradation reactor suspensions were measured with HPLC (Shimadzu 20AT). Concentrations of activated sludge were measured as the dried weight (SS) of the sludge biomasses from the filterable (Whatman GF/C) portion of reactor suspensions. SS measurement

was performed following the standard methods of SM2540-D (filtered and dried at 103 – 105 °C) (APHA, 1998).

ATP (Adenosine triphosphate) measurement: ATP contained in microbial cells of activated sludge was measured along the courses of 2,4-D degradation by the sludge. ATP was extracted from an aliquot of sludge (η mL, with κ mg/L SS). Activated sludge (4 mL) was harvested from the shake flask to a Falcol tube into which 10 mL of 0.005 % Sodium Dodecyl Sulfate (SDS) was added, and centrifugation for 10 min at 5000 rpm at 4 °C followed. The supernatant was discarded and the pellet was resuspended in 10 mL deionnized water, followed by centrifugation for 10 min at 5,000 rpm at 4 °C. The supernatant was discarded and the pellet resuspended in 2 mL 0.02 M Tris (hydroxymethyl) aminomethane (Tris buffer, pH 7.8) that contained 0.5 mL 10 % of trichloroacetic acid (TCA) to a final concentration of 2 % TCA. The final Falcol tube content and 0.5 mL Tris buffer for washing the tube wall were transferred to a 7 mL polypropylene bead blasting, and was bead blasted with 0.5 g of 0.5 mm diameter sterile glass beads at 4,000 rpm using Minilys (Bertin technologies, France), for 30 s followed by 1 min incubation on ice and another 30 s of bead blasting. The mixture of extract and glass beads was transferred to a 15 mL Falcon tube, with 1 mL Tris buffer from washing of bead blasting tube wall and cap. The Falcon tube was then centrifuged at 5,000 rpm for 5 min. The supernatant, containing ATP extracted from the cells was then filtered through a 0.22 µm membrane filter (Millex-GS) to remove suspended particles (and stored if necessary, at 4 °C for less than 3 h) for ATP measurement by HPLC.

ATP concentrations in the supernatants of each and every extraction of all tests were measured using HPLC (Shimadzu - 20AT; Column: Phenomenex Luna 00G-4252-E0, reverse phase C18, length 250 mm and ø 4.6 mm, with particle size 5 µm). The column was maintained at room temperature (25 ± 2 ^{o}C). Mobile phase A consisted of 150 mM KH_2PO_4 and 150 mM KCl (pH adjusted to 6.0 with 0.1 M KOH). Mobile phase B consisted of 85 % mobile phase A and 15 % acetonitrile. The continuous gradient elution scheme was, at time 0, 0.28, 9.72, 13.89, 19.44, 33.0 and 40.0 min, 0 %, 3 %, 9 %, 100 %, 100 %, 0 % and 0 % of mobile phase B was injected. Total flow-rate was 0.54 mL/min. Sample injection volume was 20 µL (loop injection volume); a total retention time of 40 min was provided for the injection. ATP was detected by a UV detector at 254 nm.

An ATP concentration (χ µg-ATP/mL) was determined from the chromatogram peak area compared with that of the external standard. ATP contained in the sludge was calculated as µg-ATP/mg-SS with the equation (χ µg-ATP/mL×4.0 mL)/(η×10^{-3} L× κ mg-SS/L), where χ is ATP concentration in an extract; κ and η, SS concentration and volume (mL) of activated sludge sample from which ATP was extracted; and 4.0 mL is final volume of supernatant (in Falcon tube) containing ATP extracted from the cells.

2.4 Calculations

From measured ATP values between the control and biogenic-added reactions, the amount of added ATP obtainable from biogenic substrates were calculated as follows:

Added ATP = turn-around point ATP – ATP of control below this turn-around point ('base point'). A maximum deficit of ATP during 2,4-D degradation was:

$$\frac{Initial\ \mathrm{ATP} - Final\ \mathrm{ATP}}{Initial\ \mathrm{2,4\text{-}D}}\left(\frac{\mathrm{M\ ATP}}{\mathrm{M\ 2,4\text{-}D}}\right) \quad (1)$$

where initial ATP and final ATP, were amount of ATP in activated sludge cells at first and the respective end point of 2,4-D degradation course.

The net -loss of ATP was: initial ATP – ATP at the last hour tested.

3. Results and discussion

Figures 1, 2, and 3 show the courses of 2,4-D degradation, the growth of sludge, and ATP contents of activated sludge cells in the control and biogenic added cases. All biogenic added cases had enhancements of 2,4-D degradation rates compared to the control. Concentrations of biogenic substrates of sucrose, peptone and their combine were tested to find the optimum in 2,4-D degradation rate enhancement for each biogenic, before ATP measurements.

From the ATP course of the control (2,4-D as the sole carbon and energy source), it is shown that ATP content of activated sludge biomass continuously decreased corresponding to the disappearance of 2,4-D. This trend shows that xenobiotic (2,4-D in this case) degradation is indeed an ATP consuming reaction. Each cell in the biomass must suffer a deficit in its energy reserve. ATP had a maximum deficit of 0.006 M-ATP/M-2,4-D compared with the ATP contained in the original activated sludge. A regain of ATP occurred at the time when 2,4-D was completely degraded due to metabolism of the lower intermediates that are biogenic (Chong et al, 2010). In all cases including biogenic added, this regain of ATP was slow, if

possible, to reach the initial level, thus in the time span over 2,4-D degradation, there was a net loss of sludge's ATP content.

The ways that biogenic additions help 2,4-D degradation can be seen from ATP changes in all sucrose, peptone and combined sucrose-and-peptone tests. From Figure 1 of the sucrose-added test, ATP content of cells increased in the first hour. This energy increase coincided with a rapid utilization of sucrose (from COD data, not shown due to lack of space); a turn-around point shows that there is an amount of supplemented ATP from sucrose metabolism. Due to the added ATP from sucrose, ATP in the subsequent 2,4-D degradation enjoyed a relief, although the curve of cellular ATP decline after this increment was parallel to that of the control. The maximum deficit was 0.0037 M-ATP/M-2,4-D, approximately 37 % lower than that of the control.

From Figure 2 of the peptone-added test, ATP content of cells did not increased in the initial hour, coinciding with the fact that peptone was utilized even slower than 2,4-D in the initial stage (from COD data not shown), but peptone also added an amount of ATP to activated sludge cells earlier on (at about the 3rd hour). A maximum deficit was 0.004 M-ATP/M-2,4-D, approximately 33 % lower than that of the control.

From Figure 3, combined sucrose-and-peptone addition increased sludge's ATP in a much similar way compared to the combined effects of individual biogenic addition (Figures 1 and 2); ATP contribution by sucrose and peptone occurred at the 1st and the 3rd hour. Maximum deficit of ATP was 0.0038 M-ATP/M 2,4-D, approximately 36 % lower than that of the control.

Trends of change of ATP contents in activated sludge biomass of reactors with additional biogenic feeds can be examined as follows:

(1) 2,4-D is a persistent organic compound, difficult to degrade and requiring high amount of energy for metabolism. When 2,4-D was the sole substrate, the degrading activated sludge has to spend ATP from the cells' reserve, thus causing a deficit at the end of 2,4-D degradation. When adding biogenic substrates at concentrations that made optimal increases in 2,4-D degradation rate, sludge cells metabolized the biogenic faster (sucrose) or simultaneously (peptone), with the effect that ATP was added to the cells' reserve earlier on. While this extra ATP was similarly consumed in subsequent, especially the later part of 2,4-D degradation, the rate of 2,4-D degradation did enjoy an enhancement (see part (a) of all figures).

(2) Xenobiotic degradation requires high amount of energy. For practical application consideration, external energy sources, such as those from the metabolism of easy-to-use organics, can become a beneficial factor in improving 2,4-D degradation. Energy enrichment subsidize for energy, which may further produce a higher yield of degrader cells thus multiply to the advantage of improving the overall xenobiotic degradation rate and concomitantly the overall xenobiotic treatment performance.

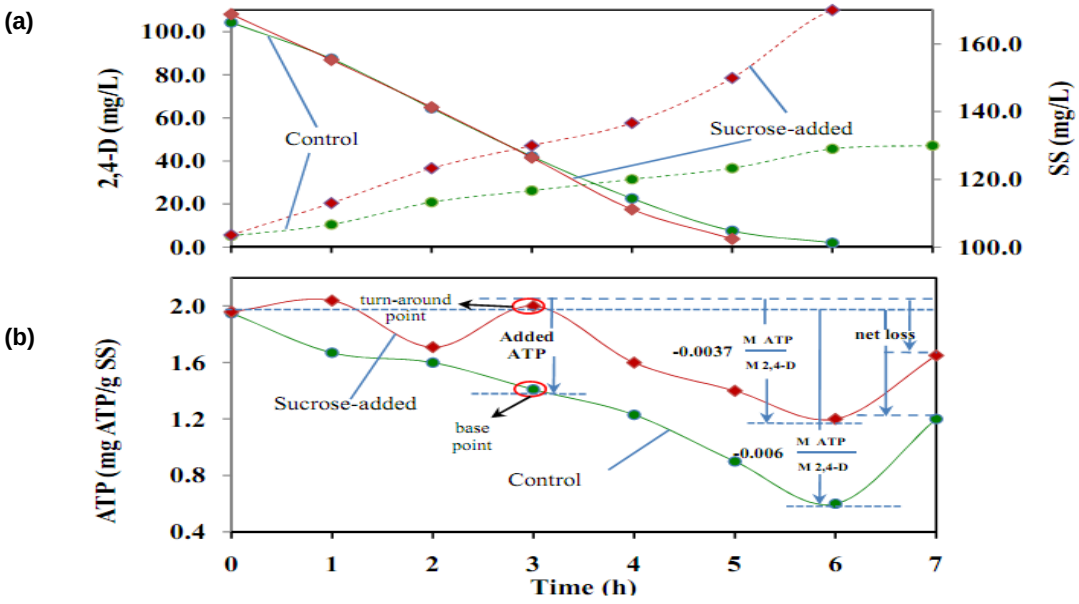

Figure 1: Time courses of 2,4-D, SS growth and ATP contents of activated sludge biomasses in control and sucrose-added reactions

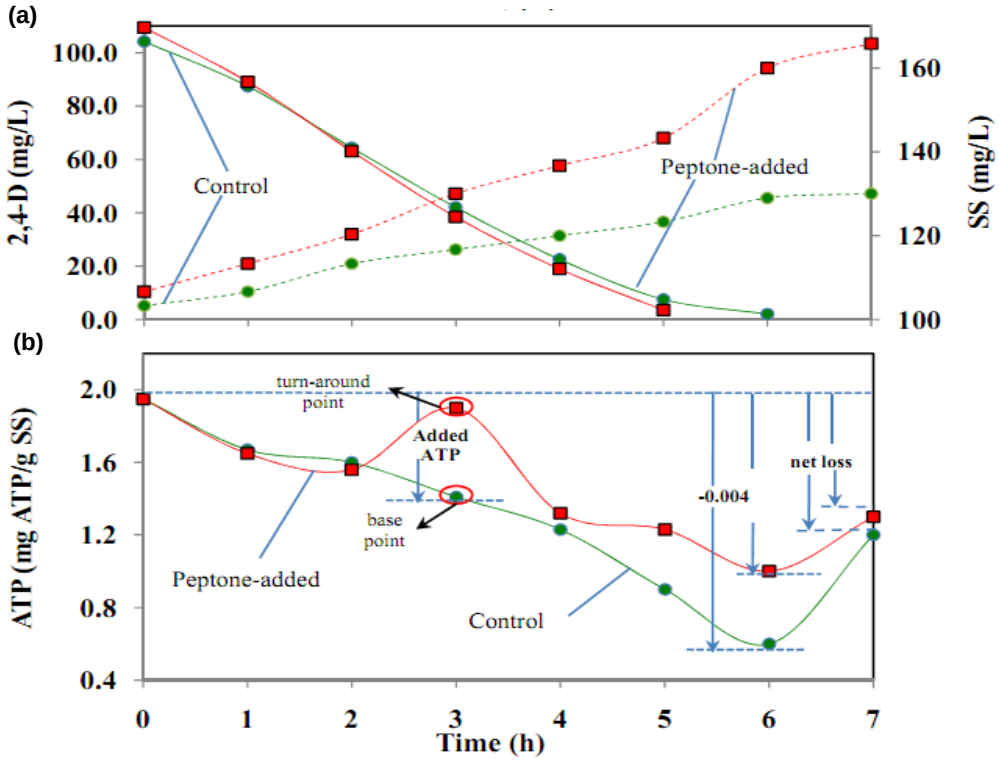

Figure 2: Time courses of 2,4-D, SS growth and ATP contents of activated sludge biomasses in control and peptone-added reactions

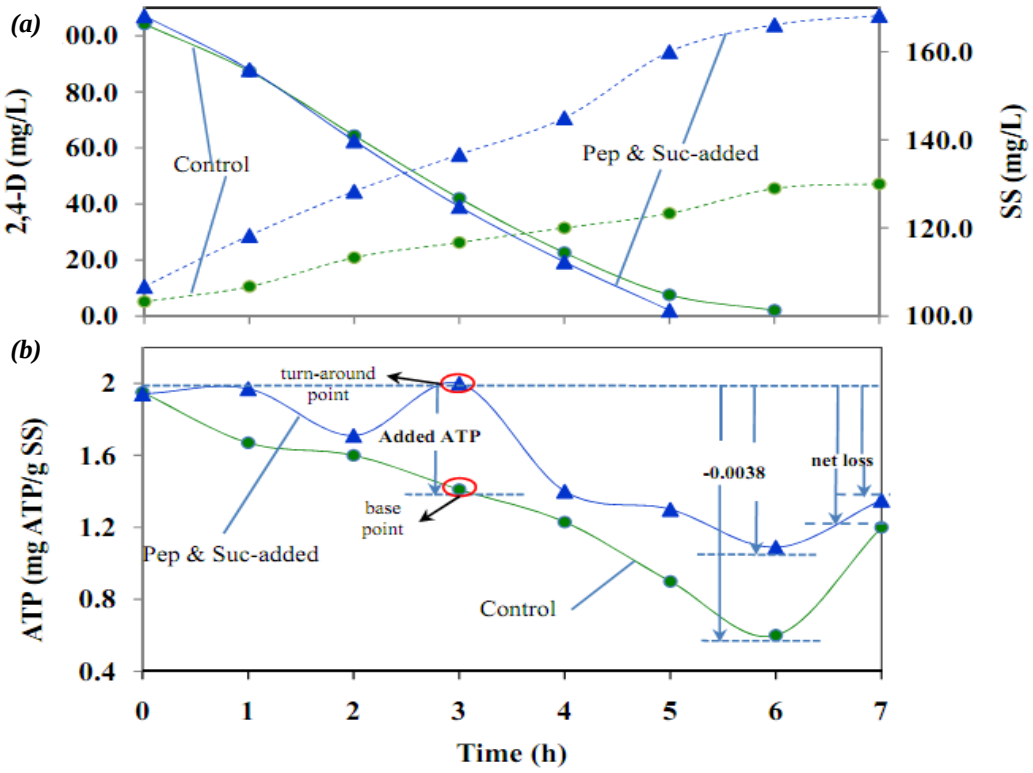

Figure 3: Time courses of 2,4-D, SS growth and ATP contents of activated sludge biomasses in control and sucrose-and-peptone-added reactions

4. Conclusion

Activated sludge degradation of a xenobiotic, exemplified with 2,4-D, consumed a large amount of energy that caused energy deficit of activated sludge cells during the degradation course. By supplementing some optimal amounts of biogenic substrates, extra ATP was invested into the cells and this energy was useful in increasing or compensating part of the ATP that may be spent in metabolism of the xenobiotic. This benefit is useful for practical application to xenobiotic treatment bioprocess.

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