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APPENDICES

APPENDIX A

Standard Iodometric Method

Reagents

1. Potassium iodide solution (1% w/v): Dissolve 1.0 grams KI into 100 ml of RO water.

2. Ammonium molybdate solution: Dissolve 9 grams ammonium molybdate in 10 ml 6 N NH_4OH, add 24 grams NH_4NO_3 and dilute to 100 ml with RO water.

3. Sulfuric acid solution (1:4 H_2SO_4): Carefully add one part H_2SO_4 98 % to four parts RO water.

4. Starch indicator

5. Sodium thiosulfate (0.025 N Na_2SO_3. 5 H_2O) solution

Procedure

1. Sample was transfer to 250 ml Erlenmeyer flask.

2. Adding RO water to the Erlenmeyer flask until 50 ml. Next, 10ml of 1:4 sulfuric acid solution and 15 ml of 1% w/v of potassium iodide were added. Then 2 drops of ammonium molybdate was added.

3. Titrate with 0.025 N of sodium thiosulfate to faint yellow or straw color. Swirl or stir gentry during titration to minimize iodine loss.

4. Add about 2 ml starch indicator, and continue titration until the blue color just disappear.

5. Repeat steps 2-4 on a blank sample of water.

6. Note ml of 0.025 $Na_2SO_3.5H_2O$ for samples and blanks analysis.

Standardize

1. Weight out 2 grams of KI and transfer to 250 ml Erlenmeyer flask. Add RO water to 100 ml

2. Then, 10 ml of 0.025 N of $K_2Cr_2O_7$ and 10 ml of 1+ 9 H_2SO_4 were added. After that, keep the Erlenmeyer flask in dark place for 5 minutes.

3. Add RO water to the Erlenmeyer flask until 200 ml.

4. Tritrate with 0.025 N of sodium thiosulfate. And follow the procedure steps 3-4 as describe earlier.

5. Note ml of 0.025 $Na_2SO_3.5H_2O$ for standardize analysis.

Calculation

$$H_2O_2 \text{ (mg/l)} = \frac{(A-B) \times (\text{Normality of } Na_2SO_3) \times 17 \times 1,000}{\text{ml of sample}}$$

A = ml of Na_2SO_3 for sample

B = ml of Na_2SO_3 for blank

$$N = \text{Normality of } Na_2SO_3 = \frac{10 \times 0.025}{\text{ml of } Na_2SO_3 \text{ for standardize}}$$

APPENDIX B

Preliminary Study on biodegradability of formaldehyde using SBR biological treatment process

Procedure

Before start up the SBR biological treatment unit, the microorganism sludge from wastewater treatment plant (7,000 mg/l of SS) was raised to steady state(3,000 mg/l of SS) with glucose (1,500 mg/l COD). Then the sludge was divided to 9-liter reactor to investigate formaldehyde biodegradability at different initial concentrations using 7 reactors and a control reactor that use only glucose as energy source. The details of each SBR reactor are as shown in a table below.

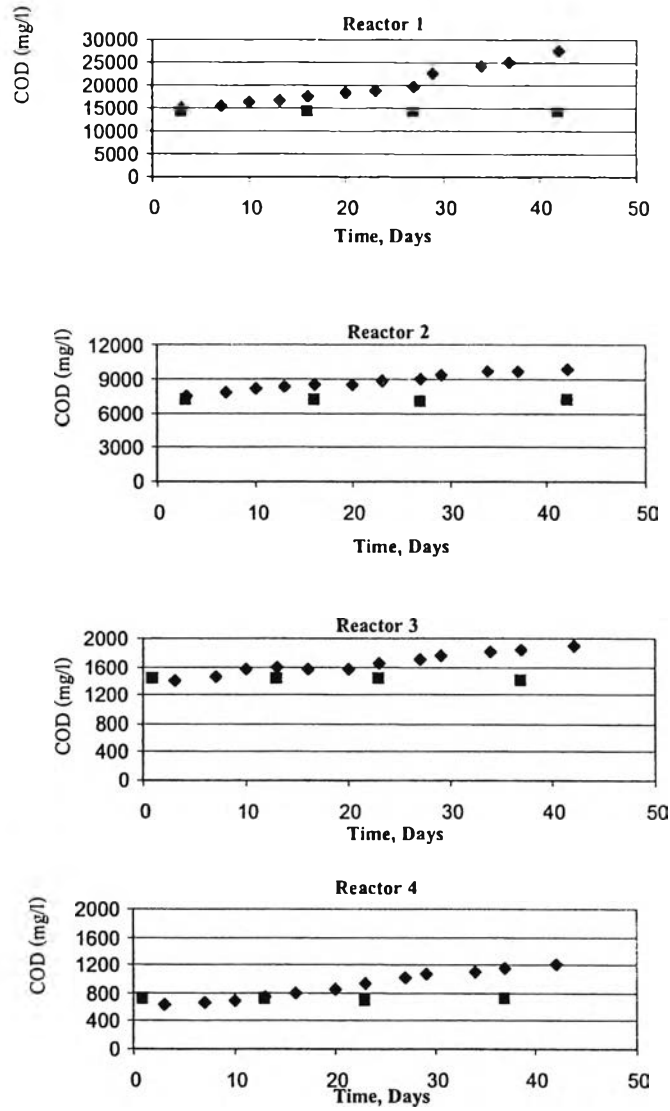| Reactor	Formaldehyde concentration		COD (mg/l)
	mg/l	molar	
1	10,000	0.333	14,000
2	5,000	0.165	7,000
3	1,000	3.33×10^{-2}	1,400
4	500	1.65×10^{-3}	700
5	100	3.33×10^{-3}	140
6	50	1.65×10^{-4}	70
7	10	3.3×10^{-4}	14
8	0	0	1,500

Figure B-1 Comparison of COD in influent and Effluent at different initial concentration of formaldehyde
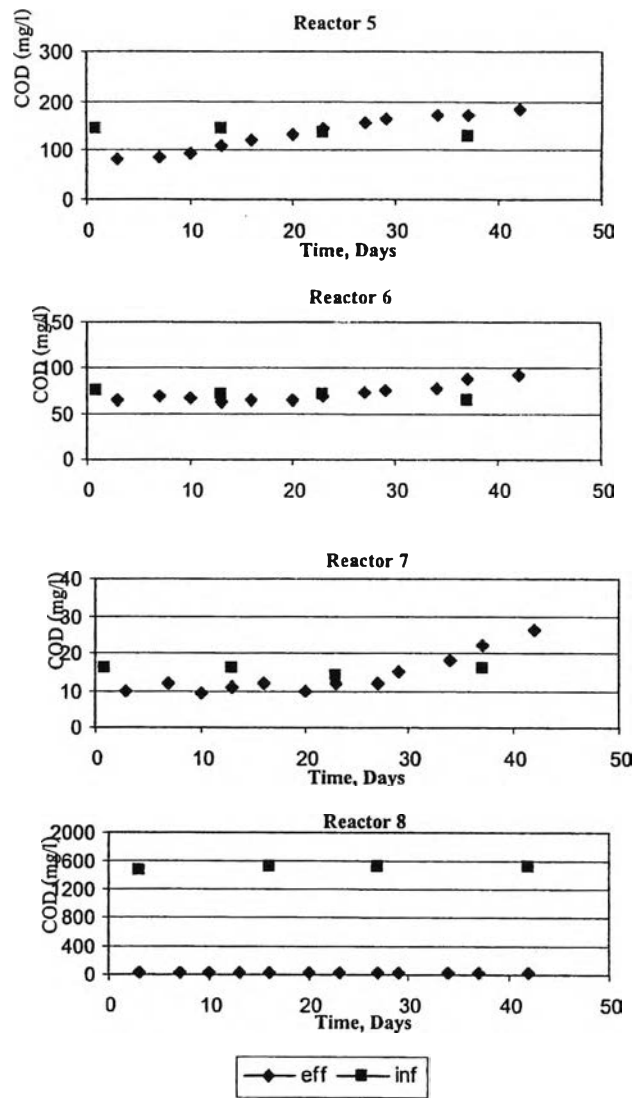
Figure B-1 (cont.) Comparison of COD in influent and Effluent at different initial concentration of formaldehyde
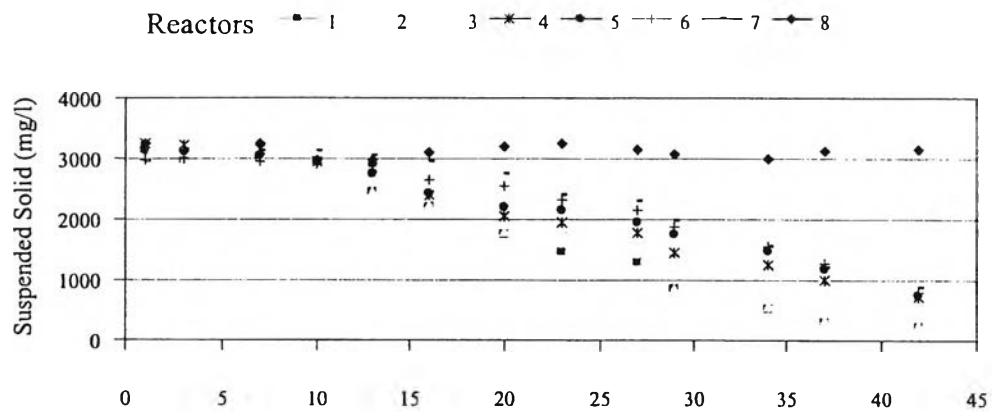
Figure B-2 Comparison of SS at different initial concentration of formaldehyde

APPENDIX C

CellTiter 96® Aqueous Non-Radioactive Cell Proliferation Assay

Description

The CellTiter 96® AQueous Non-Radioactive Cell Proliferation Assay is a colorimetric method for determining the number of viable cells in proliferation or chemosensitivity assays. The CellTiter 96® Aqueous Assay is composed of solutions of a novel tetrazoliurn compound (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; MTS[a]) and an electron coupling reagent (phenazine methosulfate; PMS). MTS is bioreduced by cells into a formazan product that is soluble in tissue culture medium. The absorbance of the formazan at 490 nm can be measured directly from 96 well assay plates without additional processing. The conversion of MTS into aqueous, soluble formazan is accomplished by dehydrogenase enzymes found in metabolically active cells (Figure II-1). The quantity of formazan product as measured by the amount of 490 nm absorbance is directly proportional to the number of living cells in culture (see Figure II-1).
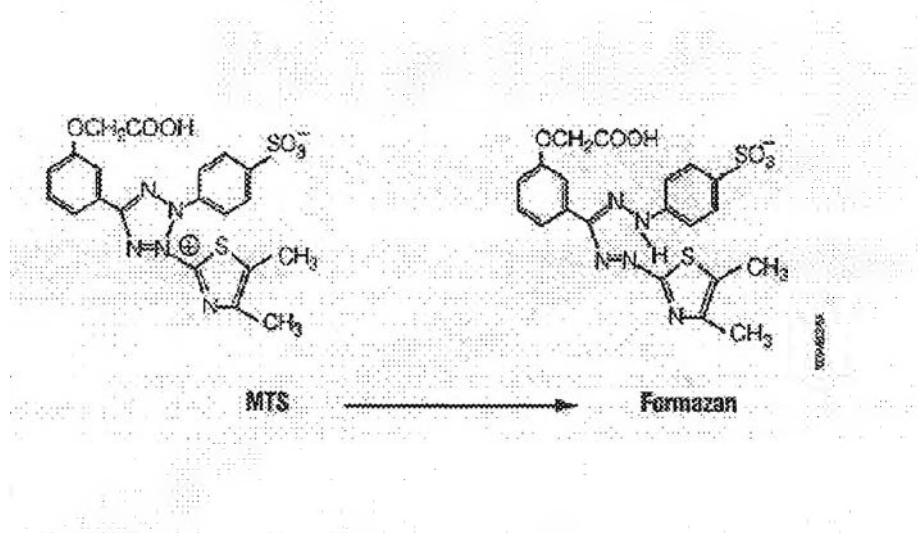

Figure C-1 Structures of MTS tetrazolium salt and its formazan product.
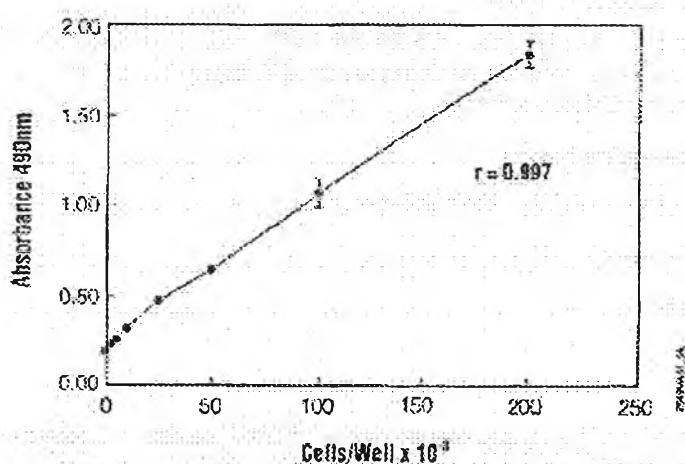
Figure C-2 Effect of cell number on absorbance at 490 nm measured using the CellTiter 96® Aqueous Assay.

Various numbers of K562 (human chronic myelogenous leukemia) cells in RPMI supplemented with 5% fetal bovine serum (FBS) were added to the wells of a 96 well plate. The medium was allowed to equilibrate for 1 hour, then 20 ul/well of combined MTS/PMS solution were added. After1 hour at 37°C in a humidified, 5% CO_2 atmosphere, the absorbance at 490 nm was recorded using an ELISA plate reader. Each point represents the mean ± SD of 4 replicates. The correlation coefficient of the line was 0.997, indicating that there was a linear response between cell number and absorbance at 490nm. The background absorbance shown at zero cells/well was not subtracted from these data.

Advantages of the CellTiter 96® AQueous Assay Include:

- **Easy to Use:** Combine MTS and PMS solutions, add combined reacent to cells, incubate and read.

- **Fast:** Assay is performed in a 96 well plate with no washing or cell harvesting. Solubilization steps eliminated because the MTS formazan product is soluble in tissue culture medium.

- **Safe:** Requires no volatile organic solvent to solubilize the formazan product (unlike MTT).

- **Convenient:** Supplied as ready-to-use, stable, frozen sterile solutions (unlike XTT).

- **Flexible:** Plates can be read and returned to incubator for further color development (unlike MTT).

- **Non-Radioactive:** Requires no scintillation cocktail or radioactive waste disposal.

XTT, commonly used in cell proliferation assays, is a tetrazolium compound similar to MTS, which is bioreduced into an aqueous soluble formazan product. Unlike MTS, XTT has limited solubility and is not stable in solution. Procedures using XTT require daily preparation of fresh solutions using prewarmed (37°C) or hot (60°C) culture medium to produce a 1 mg/ml solution. In contrast, the components of the CellTiter 96® Aqueous Assay are supplied as filter-sterilized solutions in physiological buffered saline ready for use.

Because the MTS formazan product is soluble in tissue culture medium, the CellTiter 96® AQueous Assay requires fewer steps than procedures that use tetrazolium compounds such as MTT (supplied in Promega's original CellTiter 96® Assay) or INT. The formazan product of MTT reduction is a crystalline precipitate that requires an additional step in the procedure to dissolve the crystals before recording absorbance readings at 570 nm.

If you currently use a [^{3}H]thymidine incorporation assay, addition of the combined MTS/PMS solution can be substituted for [^{3}H]thymidine at the time point in the assay when the pulse of radioactive thymidine is usually added. Data from proliferation bioassays comparing the CellTiter 96® Aqueous Assay and [^{3}H] thymidine incorporation show similar results. This is in agreement with similar radioactivity incorporation studies performed using Promega's original CellTiter 96® Assay.

Preparation of MTS Solution from CellTiter 96® Aqueous MTS Reagent Powder

The following protocol is recommended for the preparation of 21 ml of MTS Solution (sufficient for ten 96 well plates).

1. Select a light-protected container or wrap a container with foil.

2. Add 21ml of DPBS to the container.

3. Weigh out 42 mg of MTS Reagent Powder and add to DPBS.

4. Mix at moderate speed on a magnetic stir plate for 15 minutes or until the MTS is completely dissolved.

5. Measure the pH of the MTS Solution. The optimum pH is between pH 6.0-6.5. If the solution is above pH 6.5, adjust to pH 6.5 with 1 N HCl.

6. Filter-sterilize the MTS Solution through a 0.2 um filter into a sterile, light-protected container.

7. Store the MTS Solution at –20 °C protected from light.

General Protocol for Use of CellTiter 96® Aqueous Assay Reagents

The following recommendations are for the preparation of reagents sufficient for one 96 well plate containing cells cultured in a 10 volume.

1. Thaw the MTS Solution and the PMS Solution. It should take approximately 90 minutes at room temperature or 10 minutes in a 37 °C water bath to completely thaw the 20 ml size of MTS Solution.

2. Remove 2.0ml of MTS Solution from the amber reagent bottle using aseptic technique and transfer to a test tube.

3. Add 100 µl of PMS Solution to the 2.0ml of MTS Solution immediately before addition to the culture plate containing cells.

4. Gently swirl the tube to ensure complete mixing of the combined MTS/PMS solution.

5. Pipette 20 µl of the combined MTS/PMS solution into each well of the 96 well assay plate containing 100 µl of cells in culture medium. Note: We recommend repeating pipettes, electronic digital pipettes or multichannel pipettes for addition of the combined MTS/PMS solution to wells of the 96 well plate. It may be

convenient to dilute the combined MTS/PMS solution into a sufficient volume of culture medium to allow for the extra non recoverable volume required when using reagent reservoirs and multichannel pipettes.

6. Incubate the plate for 1-4 hours at 37 °C in a humidified, 5% CO_2 atmosphere.

Note: To measure the amount of soluble formazan produced by cellular reduction of the MTS, proceed immediately to Step 7. Alternatively, to measure the absorbance at a later time, add 25 µl of 10% SDS to each well to stop the reaction. Store SDS-treated plates protected from light in a humidified chamber at room temperature for up to 18 hours. Proceed to Step 7.

7. Record the absorbance at 490 nm using an ELISA plate reader.

Note: The recommended concentrations of MTS Solution and PMS Solution have been optimized for a wide variety of cell lines cultured in 96 well plates containing100 µl of medium per well. If different volumes of medium are used in a standard operating protocol for an assay in your laboratory, adjust the volume of the combined MTS/PMS solution to maintain a ratio of 20 µl combined MTS/PMS solution per 100 µl culture medium. This results in final concentrations in the assay of 333 µg/ml MTS and 25µl MPMS. If sensitivity in your assay (adequate 490nm absorbance readings) is not a limiting factor, 20 µl of the combined MTS/PMS solution may be adequate for use with volumes as large as 200 µl/well, but this will reduce the MTS and PMS to suboptimal concentrations. At suboptimal concentrations of MTS and at high cell numbers (50,000-200,000 K562 cells/well), we have observed that the absorbance at 490nm is not linear with cell number.

General Considerations

Light Sensitivity

The PMS and MTS Solutions and MTS Reagent Powder are light sensitive and are supplied in amber containers. Slight discoloration may occur if solutions are stored at 4°C for several days to weeks. This discoloration may cause higher background 490 nm absorbance readings, but it should not affect the performance of the CellTiter 96® AQueous Assay.

Safety

MTS: Because the toxicological properties of MTS have not been thoroughly investigated, caution should be used. Other tetrazolium compounds are generally classified as irritants.

PMS: PMS is classified as a carcinogen; however, according to 29 CFR (U.S. Code of Federal Regulations) 1910.1200, a mixture that contains less than 0.1% by weight or volume of a carcinogenic, hazardous component is not considered hazardous, unless there is evidence to the contrary. Suitable precautions should be taken in the use and disposal of this product.

Background Absorbance

A slight amount of spontaneous 490 nm absorbance occurs in culture medium incubated with combined MTS/PMS solution. The type of culture medium used, type of serum, pH and length of exposure to light are variables that may contribute to the background 490 nm absorbance. Background absorbance is typically 0.1-0.2 absorbance units after 4 hours of culture. If background 490nm absorbance is significant using your experimental conditions, correct for it as follows. Prepare a triplicate set of control wells (without cells) containing the same volumes of culture medium and combined MTS/PMS solution as in the experimental wells. Subtract the average 490 nm absorbance from these "no cell" control wells from all other absorbance values to yield corrected absorbances.

Optional Wavelengths to Record Data

Figure II-3 shows an absorbance spectrum of MTS/formazan after bioreduction by K562 cells. The spectrum indicates an absorbance peak at 490 nm. We recommend recording data at 490 nm; however, if the ELISA reader available does not have a 490 nm filter, data can be recorded at wavelengths anywhere between 450-540 nm. Absorbance may be recorded at other wavelengths if necessary, but loss of sensitivity will result. For example, data recorded at 570 nm results in absorbance readings of approximately one-third of the readings obtained at 490nm. A reference wavelength between 630-700 nm may be used. Use of a reference wavelength

eliminates background contributed by cell debris, fingerprints and other nonspecific absorbance.

Lymphocyte Assays

Lymphocytes may produce less formazan than other cell types. To achieve significant absorbance changes with Iymphocytes, increase the number of cells per well to approximately 2.5-10 x 10^4, and incubate the plate with MTS/PMS solution for the entire 4-hour period.
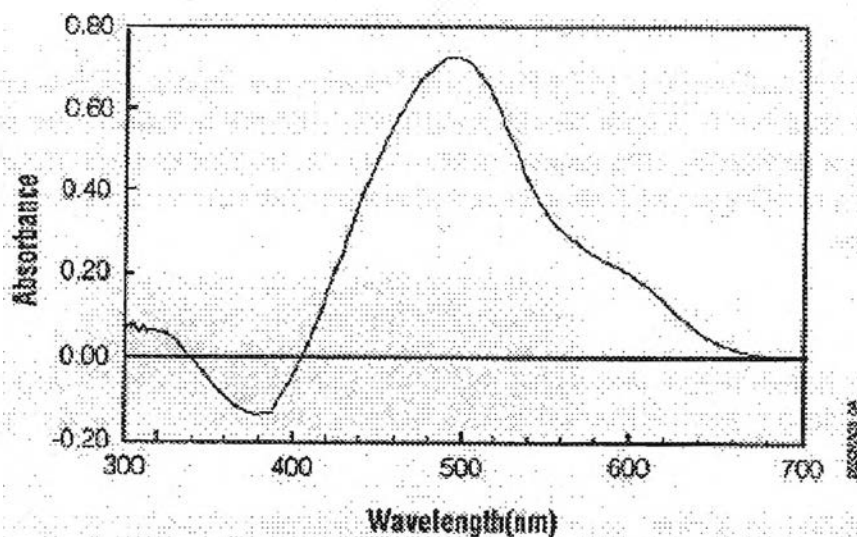

Figure C-3 Absorbance spectrum of MTS/formazan after bioreduction by K562 cells.

The K562 cells were cultured in RPMI1640 supplemented with 10% FBS. The blank used to generate this absorbance spectrum was culture medium containing MTS that was not bioreduced by cells. The negative absorbance values (382nm) correspond to the disappearance of MTS converted into formazan.

BIOGHAPHY

Miss Aditsuda Jamroensan was born on November 21, 1979 in Kalasin province, Thailand. She attended Anukoolnaree School and graduated in 1997. She received her Bachelor's degree in Environmental Engineering, Faculty of Environmental Engineering, King Mongkut's University of Technology Thonburi in 2002. After that, she pursued her Master Degree studying in the International Postgraduate Program in Environmental Management (Hazardous Waste Management), Inter-Department of Environmental Management Chulalongkorn University, Bangkok, Thailand on April, 2002.