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Title: Metabolic Uncoupling of *Shewanella oneidensis* MR-1, under the presence of excess substrate and 3,3',4',5 Tetrachlorosalicylanilide.

Abstract Approved:

Brian D. Wood

Dissociation between catabolism and anabolism, generically termed "metabolic uncoupling", has been studied for aerobic cultures of *Shewanella oneidensis* MR-1. Under conditions promoting metabolic uncoupling, the cell yield diminishes while the substrate utilization rate remains largely unchanged. The effects of excess substrate conditions and TCS addition on the metabolism of pyruvate by *S. oneidensis* MR-1 were examined and modeled. In the first phase, the observed cell yield (Y_{obs}) was successfully modeled as a function of relative substrate concentration (S_0/X_0), under excess substrate conditions. The degree of uncoupling was determined by uncoupling coefficient (E_u). Cellular yield was shown to decrease with increasing substrate concentrations. Preliminary experiments illustrated the effectiveness of TCS in reducing the cell concentration. In the later phase, an expression is proposed to model the effect of excess substrate as well as TCS addition, simultaneously. This model was verified by using the

experimental data obtained by introduction of three different TCS doses to cells grown under excess substrate conditions. The uncoupling coefficient was used to distinguish between the uncoupling effects of TCS addition and excess substrate conditions. In our experiments, it was observed that acetate accumulated as a metabolic intermediate. Average protein content of the cells was also found to increase with an increase in concentrations of either the substrate or the uncoupler. These could be the possible pathways for consumption of substrate for non-growth associated activities. Determining an optimum combination of substrate and uncoupler concentrations could be very useful in controlling the biomass growth in engineered microbial practices like wastewater treatment and bioremediation.

Metabolic Uncoupling of *Shewanella oneidensis* MR-1, under the presence of excess substrate and 3,3',4',5 Tetrachlorosalicylanilide

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I understand that my thesis will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my thesis to any reader upon request.

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CHAPTER 1

INTRODUCTION

The understanding of cellular metabolism is central to the success of many environmental engineering applications; important examples include wastewater treatment and sub-surface cleanup by bioremediation techniques. Metabolism consists of substrate breakdown along with energy release (catabolism) and biomass generation (anabolism). Apart from biomass production, energy generated from catabolism also fuels the cell maintenance operations (Pirt 1965). It has been observed by researchers that under some metabolic conditions, cells use an additional amount of energy that cannot be attributed to maintenance requirements (Senez 1962). This phenomenon is now known as *metabolic uncoupling*, and it is characterized by a reduced cellular yield with a relatively unaltered substrate utilization rate. Presence of excess substrate, chemical uncouplers, toxins, and some physico-chemical conditions can lead to uncoupling. A significant amount of the research on uncoupling has been conducted with the aim of reducing the sludge yield in the activated sludge process. Among the various uncouplers tested for this purpose 3, 3', 4', 5 tetrachlorosalicylanilide (TCS) has been found to be the most effective in terms of yield reduction and dose requirements (Ye et al. 2003; Chen et al. 2004; Chen et al. 2002; Strand et al. 1999; Ye and Li 2005).

Heavy metals and radionuclides are contaminants of concern at a large number of Department of Energy (DOE) sites. It has been proposed that some of these contaminants can be remediated by organisms capable of using the

contaminants as a terminal electron acceptor. The associated change of valence state alters the solubility of the metal or radionuclide, effecting a removal from the mobile fluid phase. *Shewanella oneidensis* MR-1, a facultative anaerobe, has been widely used as a model organism for a general class of microbes that are known as dissimilatory metal reducing bacteria (DMRB). In the study described here, the metabolic uncoupling of this bacterium was studied with the goal of providing control over biomass growth during microbial operations. The effect of excess substrate and TCS addition on its metabolism was examined and modeled.

Excess substrate conditions were brought about by varying the ratio of initial substrate to initial biomass (S_0/X_0). An existing model was used to verify the effect of excess-substrate conditions on cellular yield. Preliminary experiments were conducted to select an uncoupler, determine the optimal time for its addition and to illustrate its effect on the cell concentration. Uncoupling effect of TCS was studied by varying the ratio of uncoupler concentration (C_u) to the biomass (X). A comprehensive equation is proposed to model the effect of both these uncoupling mechanisms simultaneously. This model was verified by using different TCS doses in excess substrate cultures. In addition, degree of uncoupling, measured as a fractional decrease in maximum theoretical yield, was also determined.

Although this phenomenon has been known for some time, the exact mechanism of metabolic uncoupling is still not completely understood. Some of the known mechanisms for metabolic uncoupling include changes in cell membrane resistance (Cook and Russell 1994; Mathews and vanHolde 1990), the accumulation of the metabolites (Russell and Cook 1995), the presence of specific

uncoupling proteins (Klaus et al. 2005; Ricquier 2005), and inhibitory effect of specific chemical uncouplers (Weigel and Englund 1977). A better understanding of metabolic uncoupling may provide a method for controlling biomass growth during engineered microbial processes, and could have useful applications in wastewater treatment and in-situ bioremediation activities.

CHAPTER 2

Metabolic uncoupling of *Shewanella oneidensis* MR-1 under the presence of excess substrate growth conditions

Saini, Gaurav and Brian D. Wood

ABSTRACT

It has been observed that, under the presence of excess substrate, activated sludge and other microbial cultures exhibit uncoupled metabolism. The occurrence of metabolic uncoupling lead to reduced cellular yield and partial dissipation of growth energy. *Shewanella oneidensis* MR-1 is a facultative bacterium capable of reducing several metals and radionuclides. In this work, the effect of relative initial substrate concentration (S_0/X_0) on the yield coefficient for this organism has been demonstrated and a descriptive model is presented for quantifying this effect. Further, the degree of uncoupling has been computed as a fraction of decrease in theoretical maximum yield. Increase in average cellular protein content is shown to be a possible pathway of substrate consumption for non-growth activities. Strategic application of excess substrate conditions could potentially provide means for controlling the biomass generation during microbial processes.

Keywords: Metabolic uncoupling, *Shewanella oneidensis* MR-1, yield, model, protein.

INTRODUCTION

Microbial processes form the crux of some of the most common environmental engineering applications. Examples include the Activated Sludge process and the bioremediation of subsurface contaminants. Microbial processes generate biomass; although biomass generation is desirable in many processes (e.g., where biomass is desired end-product), excessive biomass production is undesirable in others. For example, the highly organic sludge waste from wastewater treatment poses a significant disposal problem (Liu and Tay 2001; Mayhew and Stephenson 1997; Liu 2003). Similarly, the production of excessive biomass during subsurface bioremediation can lead to loss of process control or process failure (Anderson et al. 2003). Thus, the success of these processes is contingent upon the control over biomass growth.

It has been observed by a number of researchers that, under certain growth conditions, there can be a drastic drop in cellular yield. Senez (Senez 1962) was the first one to point out this anomaly, and observed roughly 50% drop in yield of *Desulfovibrio desulfuricans* (strain Berre S). It is now known that presence of excess substrate, addition of organic protonophores, toxins such as heavy metals, and other thermal or physico-chemical processes can lead to dissociation between catabolism and anabolism. This process is generically termed as *metabolic uncoupling*, and is characterized by a decrease in the amount of cell mass produced per unit substrate processed (i.e., the observed cell yield).

Much of the applied research on metabolic uncoupling has been focused on reducing the biomass production during activated sludge treatment. The

motivation for this has been the high costs of sludge disposal and increasing legislative restrictions. Studies have illustrated that a higher food per microbe (f/m) ratio, which is same as the ratio of initial substrate concentration to initial biomass concentration (S_0/X_0), leads to a reduction in cellular yield (Liu 1998; Liu 1996). In other words, a high strength waste would result in reduced biomass production and disposal cost. This effect has been attributed to incomplete oxidization of substrates, excretion of metabolites, futile cycles, energy spilling etc (Zeng and Deckwer 1995; Liu et al. 1998).

Most metabolic uncoupling studies have been restricted to mixed cultures. These include development of mathematical models for quantification of cell yield and extent of uncoupling (Liu 1998; Pirt 1982; Zeng and Deckwer 1995; Liu and Chen 1997; Tsai and Lee 1990; Liu et. al 1998). There is a lack of literature on metabolic uncoupling of pure cultures, although many of them have been shown to be very useful for *insitu* remediation activities. A large number of Department of Energy (DOE) sites are contaminated with heavy metals like uranium, cadmium and chlorinated solvents like TCE and PCE. It has been proposed that some metals and radionuclides might be bioremediated at these sites by promoting the growth of microorganisms that can use contaminant metals and radionuclides as terminal electron acceptors. *Shewanella oneidensis* MR-1 is a facultative DMRB that has been widely used as a model organism for metal reduction. In application, generation of excess biomass in the subsurface during *insitu* remediation activities poses problems. It could lead to changes in composition of subsurface microbial population and possible failure of bioremediation scheme (Anderson et al. 2003).

Controlling cellular metabolism by promoting metabolic uncoupling could provide a means for controlling biomass growth, leading to successful application of microbial processes in engineered systems.

The current work aims to determine and model the effect of excess-substrate conditions on the metabolism of *S. oneidensis* MR-1. Excess-substrate conditions, as defined in this study, refer to growth conditions (measured in terms of S_0/X_0) that lead to an observed cell yield which is less than the theoretical maximum value. Batch experiments have been conducted using pure culture of wild type *S. oneidensis* MR-1 grown with pyruvate as the sole energy/carbon substrate. The cellular yield is modeled as a function of relative initial substrate concentration (S_0/X_0). The degree of uncoupling is quantified by using an uncoupling coefficient, defined as the ratio of fractional reduction in cell yield to the theoretical maximum yield.

The present work is limited to the understanding of metabolic uncoupling induced by relative substrate levels (S_0/X_0) and its modeling. Future experiments are planned to study the additional metabolic uncoupling caused by a specific class of chemicals known as protonophores or uncouplers. The combined results of these studies would enable the development of a comprehensive model capable of quantifying the uncoupling effects of both excess substrate and uncouplers. This work is expected to contribute significantly to a better understanding of metabolic uncoupling and could lead to development of strategies for control of microbial processes.

MATERIAL AND METHODS

Bacterial Cultures and Cultivation Methods

A pure culture of *S. oneidensis* MR-1 wild type was used. This facultative anaerobe was first isolated from anaerobic sediments obtained from Lake Oneida, NY in 1988 (Myers and Nealson 1988). Frozen stock of this bacterium was obtained from Pacific Northwest National Laboratory (PNNL), Washington. For the purpose of experimentation, the frozen stock was prepared by growing cells in 10% Tryptic Soy Broth (TSB) solution in distilled water. These cells were then harvested and stored with media and glycerol in a 2:1 ratio at -75ºC.

Cells were grown in a modified version of a minimal media suggested by Kostka (Kostka and Nealson 1998), with pyruvate as the sole carbon and energy substrate. The pH of the media was maintained at 7.0 using a PIPES buffer (Mallinckrodt Baker, NJ). The inoculum was grown in the minimal media with 100 mM pyruvate in sterile 50 ml conical centrifuge tubes for 24 hours. For experiments, the inoculum was diluted in 1:100 ratio in batches containing 250 ml minimal media (in 500 ml volume Kimax® glass bottles). All the cultures were shaken constantly in an incubator (Barnstead Max^Q 4000) maintained at 30 ºC. Resazurin was added at a concentration of 1 ppm to detect the presence of dissolved oxygen in the batch cultures. Aeration was applied using lab-grade compressed breathable air. Samples were taken at pre-selected intervals and were divided into three parts. One part of the samples was filter sterilized using 0.1 μm Acrodisc® syringe filters (Pall Corporation, MI) for determining the substrate

consumption by High Performance Liquid Chromatography (HPLC). The second portion was stained by 4', 6-diamidino-2-phenylindole (DAPI) and used for direct cell enumeration by Epi-fluorescence microscopy (Olympus, US) at a resolution of 1000X using an Olympus$^{\circledR}$ UPLFL 100X objective. Black polycarbonate filters 25 mm in diameter with a 0.22 μm pore size (Osmonics Inc.) were used for slide preparation. The third part of samples was used to determine the total protein content of cultures using Micro BCA^{TM} Protein Assay Kits (Pierce Chemicals, IL). Aseptic techniques were used for cell cultivation, sampling and analysis.

Excess Substrate induced uncoupling Test

Batch experimentation to observe the effect of substrate concentration on cellular yield was done by varying the initial amount of pyruvate (S_0) available for growth, while keeping the initial cell concentration (X_0) constant. Cells were grown in minimal media containing 5, 20, 50 and 100mM pyruvate in 500 ml volume Kimax$^{\circledR}$ bottles. The experiment was carried out in duplicate and samples were taken at 0, 24, 36 and 48 hours.

The results obtained for biomass growth (determined by direct cell count) and substrate utilization (determined by HPLC) were used to model the kinetics of *S. oneidensis* MR-1 growing on pyruvate. Similar analysis was done by using the protein content, determined by Micro BCA^{TM} kit, as a measure of biomass. The biomass was measured as cells per ml of culture or total protein content (ppm) and

the observed biomass yield (Y_{obs}) was determined as the ratio of biomass produced at the end of 48 hours to the corresponding amount of pyruvate utilized.

Error Analysis

In order to account for the error in the measurement of various parameters, repeated measurements of the same samples were carried out so that 95% confidence levels could be determined. These confidence levels correspond to an uncertainty of 11.7% in determination of both cell yield and S_0/X_0 using cell count as the measure of biomass. A corresponding uncertainty level of 9.3% was observed for the case where protein was used as the measure of biomass. Since the uncoupling coefficient (E_u) is dependent on the observed yield (Y_{obs}), the same uncertainty levels were used for plotting it.

MODEL DEVELOPMENT

Duclaux (1898) was the first to distinguish between energy requirements for growth and maintenance, and he proposed an expression that accounted for the amount of substrate required for maintenance per unit biomass per unit time. Later Pirt (Pirt 1965) proposed a model describing the relationship between cell yield (Y) and maintenance coefficient (m), specific growth rate (μ) and true or theoretical maximum yield (Y_G) as

$$\frac{1}{Y} = \frac{m}{\mu} + \frac{1}{Y_G} \qquad (1)$$

This model works well for substrate-limited cultures where there is little carbon and energy to be wasted. However, the model fails to explain energy uncoupling in cultures having a higher ratio of S_0/X_0, or under excess-substrate conditions.

It was first observed by Senez (Senez 1962) that catabolism and anabolism could be dissociated under some conditions, such as elevated temperature of culture or presence of specific chemical compounds. Under such circumstances, it was noticed that the observed growth yield drops while the rate of substrate utilization remains same (Senez 1962). He termed this phenomenon as *uncoupling* and it was first observed in a nitrogen fixing strain of *D. desulfuricans*.

It has been hypothesized that in presence of excess-substrate conditions, a part of energy generated from substrate utilization is lost through one or more energy wasting processes. Possible processes that have been proposed to explain energy over-expenditure include the induction of futile cycles, the formation of intermediate products, and metabolic shifts that create changes in end products and ATP production (Russell and Cook 1995).

A model has been proposed by Liu (Liu 1996) to describe the relationship between cell yield (Y) and the ratio of the initial substrate concentration (S_0) to initial biomass concentration (X_0) as follows

$$\frac{1}{Y_{obs}} = \frac{1}{Y_{obs}'} + \frac{1}{(Y_w)_{min}} \times \frac{(S_0/X_0)}{(S_0/X_0) + K_{s/x}} \tag{2}$$

Here Y_{obs} is the observed growth yield, Y_{obs}' is the observed growth yield under substrate-limited conditions (same as the left hand side of Pirt's model,

Eq.1), $(Y_w)_{min}$ is the minimal energy spilling related growth yield, and $K_{s/x}$ is a saturation constant related to S_0/X_0 ratio (Liu 1996). This model works well for substrate-sufficient cultures, where growth is limited by something other than the growth substrate. Under substrate-limited conditions (growth limited only by substrate availability), the observed growth yield (Y_{obs}) approaches the theoretical maximum yield Y_{obs}'. The constant Y_{obs}' is determined as the intercept of curve drawn between $(1/Y_{obs})$ and S_0/X_0. Similarly, a graph between $1/[1/Y_{obs} - 1/Y_{obs}']$ and $1/(S_0/X_0)$ would yield $(Y_w)_{min}$ as the slope and the product $(K_{s/x})(Y_w)_{min}$ as the intercept. In this way all the three constants, Y_{obs}', $(Y_w)_{min}$, and $K_{s/x}$ can be determined.

A dimensionless energy uncoupling coefficient (E_u) is often used as a measure of the extent of uncoupling. It has been defined as the change in biomass yield due to uncoupling as a fraction of maximum possible yield (Liu and Chen 1997; Liu et al. 1998). Mathematically, it can be expressed by

$$E_u = \frac{Y_{obs}' - Y_{obs}}{Y_{obs}'} \tag{3}$$

This parameter quantifies the fraction of substrate that is wasted as a result of metabolic uncoupling. Further, it can be used to distinguish between the uncoupling effects caused by the occurrence of excess-substrate growth as opposed to the addition of a chemical uncoupler.

RESULTS

The data obtained from batch experiments with 5, 20, 50 and 100 mM pyruvate media have been plotted. All the results have been plotted as a function of relative substrate concentration (S_0/X_0). Figures 1 and 2 illustrate the variations in observed cellular yield using cell count and total protein as measures of biomass, respectively.
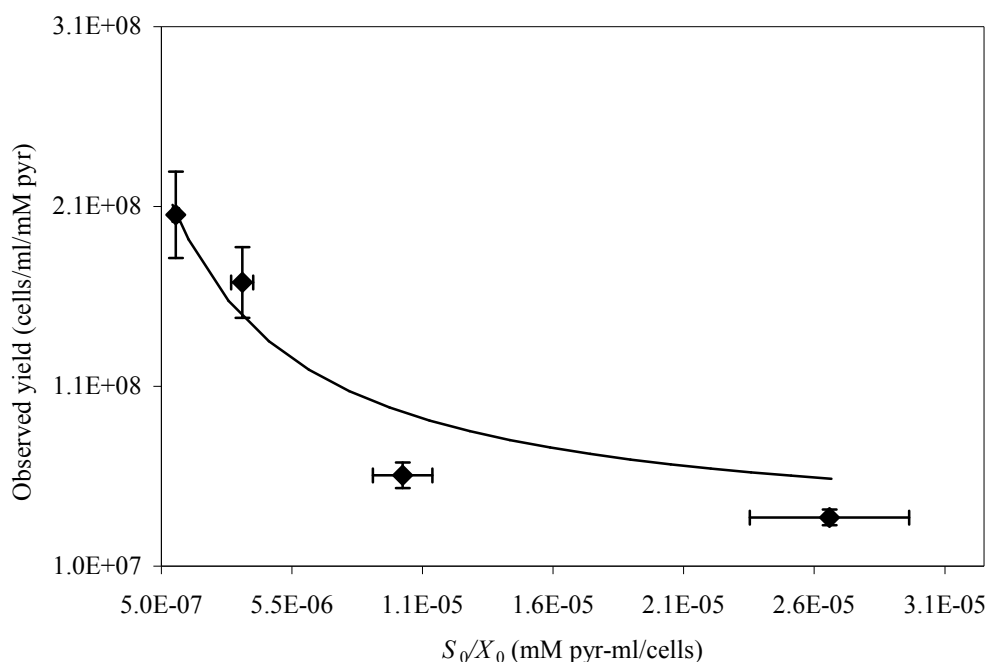
Figure 1. Effect of relative substrate concentration (S_0/X_0) on cellular yield, measured by using direct cell count as a measure of biomass. The model parameters are $Y_{obs}' = 2.5 \times 10^8$ cells/ml/mM pyruvate, $(Y_w)_{min} = 3 \times 10^7$ cells/ml/mM pyruvate, and $K_{s/x} = 4.06 \times 10^{-5}$ mM pyruvate (cells/ml)$^{-1}$. ◆ represents observed cell yield and model predictions are shown by the solid line. The error bars show 95% confidence intervals.
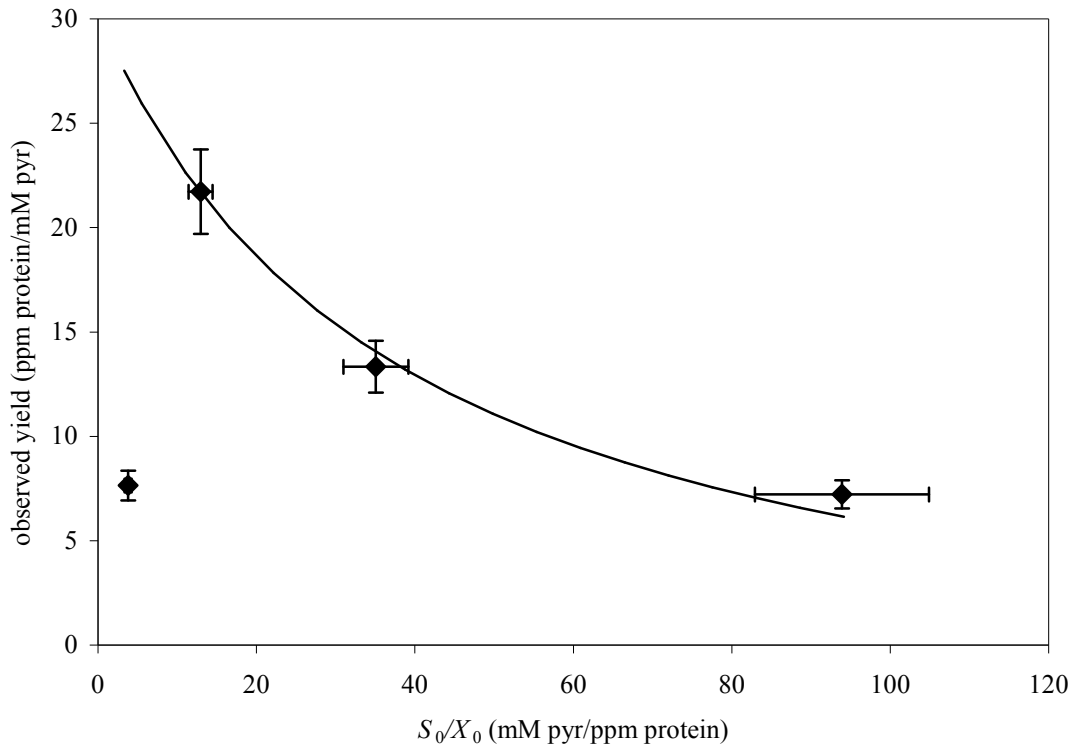
Figure 2. Effect of relative substrate concentration (S_0/X_0) on cellular yield measured by using total protein content as a measure of biomass. The model parameters are Y_{obs}' = 30.21 ppm protein/mM pyruvate, $(Y_w)_{min}$ = -3.27 ppm protein/mM pyruvate, and $K_{s/x}$ = -316.74 mM pyruvate (ppm protein)$^{-1}$. The symbol (◆) represents observed cell yield and model predictions are shown by the solid line. The error bars show 95% confidence intervals.

The data was fitted to Eq. (2) and different parameters were determined. The model fit is shown by continuous lines. The error bars represent 95% confidence levels. Energy uncoupling coefficient (E_u) values were also determined using the observed growth yield using both direct cell counts and total protein as biomass measures. Observed yield values were used to compute the values of observed E_u using Eq. (3). While model predicted yield values were used for predicting E_u values. The observed and predicted values of E_u are shown in Figs. 3 and 4.
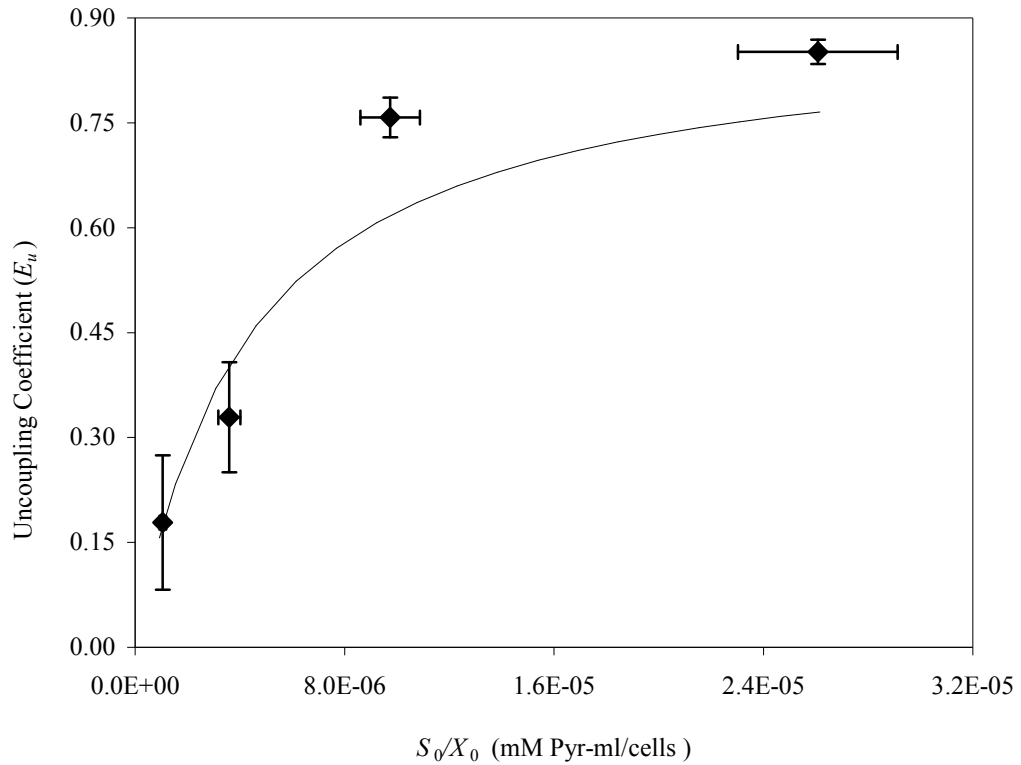
Figure 3. Uncoupling coefficient in excess-substrate cultures, using direct cell count as a measure of biomass. Model parameters are $Y_{obs}' = 2.5 \times 10^8$ cells/ml/mM pyruvate, $(Y_w)_{min} = 3 \times 10^7$ cells/ml/mM pyruvate, and $K_{s/x} = 4.06 \times 10^{-5}$ mM pyruvate (cells/ml)$^{-1}$. ◆ denotes the observed values of E_u and model predictions are represented by the solid line. The error bars show 95% confidence intervals.

Figure 5 shows the average protein content (protein/cell) of the cells at different time instants. The average cell protein content has been determined as a ratio of total protein content (ppm protein) to cell concentration (cells/ml) at any given time instant.
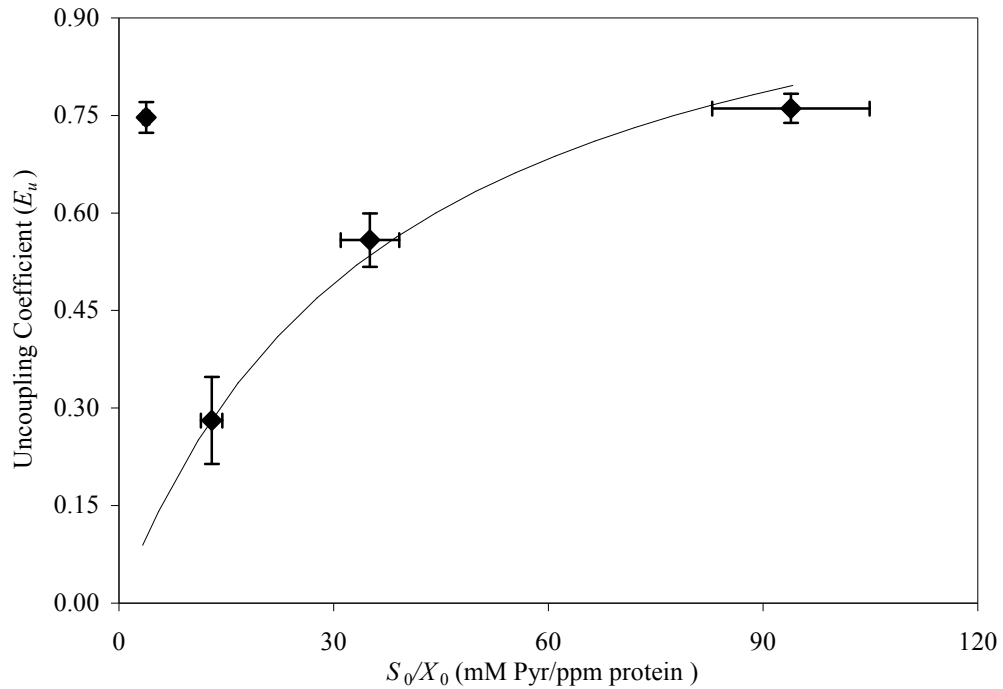
Figure 4. Uncoupling coefficient in excess-substrate cultures, determined by using total protein content as a measure of biomass. Model parameters are $Y_{obs}' = 30.21$ ppm protein/mM pyruvate, $(Y_w)_{min} = -3.27$ ppm protein/mM pyruvate, and $K_{s/x} = -316.74$ mM pyruvate (ppm protein)$^{-1}$. ◆ represents observed E_u and model predictions are shown by the solid line. The error bars show 95% confidence intervals.

DISCUSSION

Batch experiments with different substrate concentrations demonstrated that metabolic uncoupling could be observed with an increasing ratio of S_0/X_0. This effect can be seen in Figs. 1 and 2, where the observed yield falls rapidly as the S_0/X_0 ratio increases. The observed biomass yield (in cells/ml/mM pyruvate) decreased by about 82% as the substrate concentration was increased from 5 mM to 100 mM pyruvate (Fig. 1).
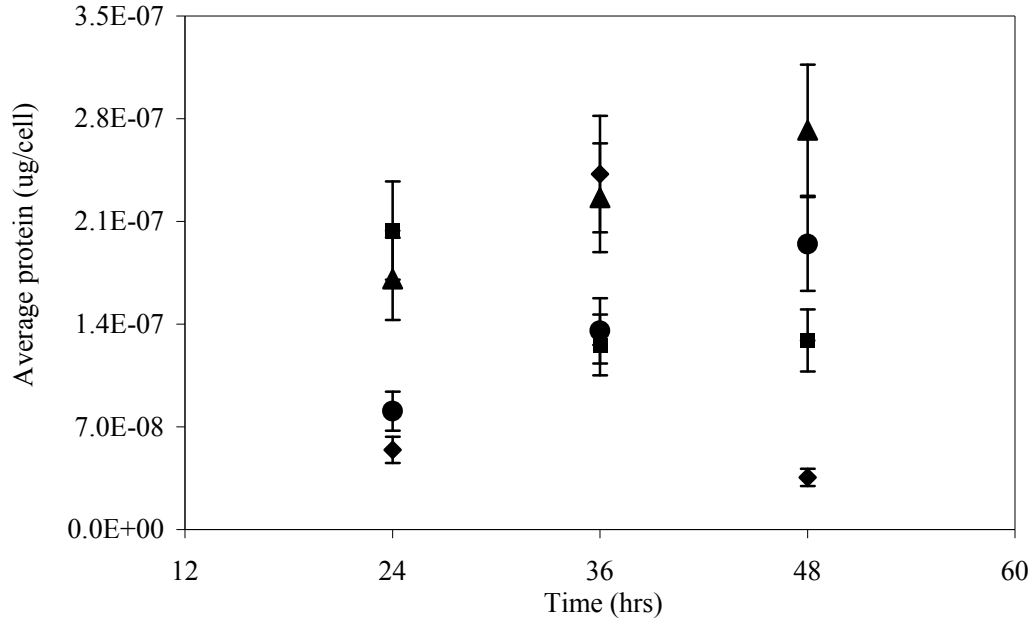
Figure 5. Average protein content of the cells under excess substrate conditions. Cultures grown in 5 mM (♦), 20 mM (■), 50 mM (▲) and 100 mM (●) pyruvate media are shown.

It can be seen that the model provides a good fit between 20mM and 100mM pyruvate cultures, when total protein was used as a measure of biomass (Fig. 2). On the other hand, the model describes the yield trend satisfactorily when the direct cell count is used as the measure of biomass (Fig. 1). It is not clear why for an S_0 value of 5 mM pyruvate the observed yield, expressed in terms of protein, is not well represented by the model.

It can be seen that as the substrate concentration increases from 5 mM to 100mM pyruvate, the observed uncoupling coefficient increases from 0.33 to 0.85 (Fig. 3). The model predictions also follow a similar trend. When total protein was used to measure biomass, the model shows good agreement with observed values

of uncoupling coefficient for substrates levels between 20 and 100mM of pyruvate (Fig. 4).

It was observed that total biomass increases with an increase in the substrate concentration (S_0/X_0). However, the increase in cell concentration is not proportional to the increase in protein concentration. This could possibly mean that when a higher concentration of substrate is present, a greater amount of protein is produced per cell. Figure 5 confirms this hypothesis and it can be seen that the average protein content of the cells increases with increase in substrate concentrations. This rise in protein content could partly explain the enhanced consumption of substrate under excess substrate conditions.

CONCLUSIONS

The effect of presence of excess substrate conditions on the yield of *S. oneidensis* MR-1 is shown and the degree of uncoupling was also determined. Both direct cell count and the total protein concentration were used as measures of biomass. The observed growth behavior is used to validate a model relating observed cell yield and relative substrate concentration (Eq. 2). The equation successfully explained the observed behavior of cells, under different S_0/X_0 values. It is clearly shown that catabolism seriously dissociates from anabolism at higher S_0/X_0 values. Consequently, the cell yield is greatly reduced. The model is capable of quantitative interpretation of the observed growth yield and metabolic uncoupling in terms of relative substrate concentration. Buildup of cellular protein

was observed and is proposed to be a potential pathway of substrate utilization for

non-growth associated activities.

Notation

S_0 :   initial substrate concentration (mM pyruvate)

X_0 :   initial biomass concentration (cells/ml or ppm protein)

Y :   cell yield (cells/ml/mM pyruvate or ppm protein/mM pyruvate)

m :   maintenance coefficient

μ :   specific growth rate

Y_G :   true or theoretical maximum yield

Y_{obs} :   observed growth yield (cells/ml/mM pyruvate or ppm protein/mM pyruvate)

$Y_{obs}{}'$:   observed growth yield under substrate-limited conditions (cells/ml/mM pyruvate or ppm protein/mM pyruvate)

$(Y_w)_{min}$:   minimal energy-spilling related growth yield (cells/ml/mM pyruvate or ppm protein/mM pyruvate)

$K_{s/x}$:   saturation constant related to S_0/X_0 (mM pyruvate/(cells/ml) or mM pyruvate/ppm protein)

E_u :   uncoupling coefficient

$(Y_{obs})_{max}$: (same as $Y_{obs}{}'$)

REFERENCES

Anderson RT, Vrionis HA, Ortiz-Bernad I, Resch CT, Long PE, Dayvault R, Karp K, Marutzky S, Metzler DR, Peacock A and others. 2003. Stimulating the in situ activity of Geobacter species to remove uranium from the groundwater of a uranium-contaminated aquifer. Applied & Environmental Microbiology 69(10):5884-5891.

Cook GM, Russell JB. 1994. Energy-spilling reactions of Streptococcus bovis and resistance of its membrane to proton conductance. Applied & Environmental Microbiology 60(6):1942-1948.

Kostka JE, Nealson KH. 1998. Isolation, cultivation and characterization of iron- and manganese-reducing bacteria. *In* R.S. Burlage et al.(ed.), Techniques in microbial ecology:58-78.

Liu Y. 1996. Bioenergetic interpretation on the S_0/X_0 ratio in substrate-sufficient batch culture. Water Research 30(11):2766-2770.

Liu Y, Chen GH. 1997. Model of energy uncoupling for substrate-sufficient culture. Biotechnology & Bioengineering 55(3):571-576.

Liu Y, Chen GH, Paul E. 1998. Effect of the S_0/X_0 ratio on energy uncoupling in substrate-sufficient batch culture of activated sludge. Water Research 32(10):2883-2888.

Liu Y, Tay JH. 2001. Strategy for minimization of excess sludge production from the activated sludge process. Biotechnology Advances 19(2):97-107.

Mayhew M, Stephenson T. 1997. Low biomass yield activated sludge: A review. Environmental Technology 18(9):883-892.

Myers CR, Nealson KH. 1988. Bacterial Manganese Reduction and Growth with Manganese Oxide as the Sole Electron Acceptor. Science 240(4857):1319-1321.

Pirt SJ. 1965. The Maintenance Energy of Bacteria in Growing Cultures. Proceedings of the Royal Society of London. Series B, Biological sciences 163(991):224-231.

Pirt S. 1982. Maintenance energy: a general model for energy-limited and energy-sufficient growth. Archives of Microbiology 133(4):300-302.

Russell JB, Cook GM. 1995. Energetics of bacterial growth: balance of anabolic and catabolic reactions. Microbiological Reviews 59(1):48-62.

Senez JC. 1962. Some considerations on the energetics of bacterial growth. Bacteriological Revolutions 26(2 Pt 1-2):95-107.

Strand SE, Harem GN, Stensel HD. 1999. Activated-sludge yield reduction using chemical uncouplers. Water Environment Research 71(4):454-458.

Tsai SP, Lee YH. 1990. A model for Energy-sufficient culture growth. Biotechnology & Bioengineering 35:138-145.

Wei YS, Van Houten RT, Borger AR, Eikelboom DH, Fan YB. 2003. Minimization of excess sludge production for biological wastewater treatment. Water Research 37(18):4453-4467.

Zeng AP, Deckwer WD. 1995. A kinetic model for substrate and energy consumption of microbial growth under substrate-sufficient condition. Biotechnology Progress 11(1):71-79.

CHAPTER 3

Metabolic uncoupling of *Shewanella oneidensis* MR-1, under the presence of excess substrate and 3,3',4',5 Tetrachlorosalicylanilide

Saini, Gaurav and Brian D. Wood

ABSTRACT

The dissociation between catabolism and anabolism is generally termed as metabolic uncoupling. Experimentally, metabolic uncoupling is characterized by a reduction in observed cell yield. This condition can be brought about by: (a) excess-substrate conditions (as measured by S_0/X_0) and (b) addition of organic protonophores, like TCS. A comprehensive equation is proposed to model the effects of both excess-substrate and uncoupler addition on microbial cultures. The uncoupling action of 3, 3', 4', 5 tetrachlorosalicylanilide (TCS) on the metabolism of *S.oneidensis* MR-1 has been modeled using this equation. Degree of uncoupling was measured as fractional reduction in theoretical maximum yield. Conflicting cellular yield trends were obtained, when direct cell count and total protein content were used as measures of biomass. This could, in part, be attributed to possible increase in cellular protein content upon addition of TCS. Metabolic uncoupling by excess substrate and chemical uncoupler action may provide effective means of controlling biomass growth during microbial processes such as subsurface bioremediation and activated sludge treatment.

Keywords: Uncoupling; excess-substrate; TCS; yield; protein

INTRODUCTION

In aerobic cultures, under excess substrate growth conditions, it has been observed that a larger amount of substrate and oxygen are required to achieve the same levels of biomass growth as is observed for substrate-limited cultures (Liu 1996; Liu 1998; Liu and Chen 1997; Liu et al. 1998; Liu et al. 1999; Liu and Tay 2000; Zeng and Deckwer 1995; Wei et al. 2003). Substrate-limited cultures are characterized by a value of the relative substrate concentration (S_0/X_0), for which the theoretically maximum cellular yield is observed. However, in case of excess substrate cultures, the observed yield is lower than this theoretical maximum. In other words, excess substrate leads to wasting of energy and higher consumption of substrate.

This phenomenon of yield reduction of microbial cultures, while maintaining the substrate consumption relatively unchanged, is termed as *metabolic uncoupling*. Similar effects have also been observed when certain chemicals known as *uncouplers*, *decouplers*, or *protonophores* are added to microbial cultures. A number of uncouplers have been studied by researchers to induce metabolic uncoupling (Table 1). Of these chemicals, 3, 3', 4', 5-tetrachlorosalicylanilide (TCS) has been shown to be one of the more efficient chemical uncouplers for activated sludge cultures. It is capable of reducing the cellular yield effectively at low concentrations of the chemical compared to other uncouplers (Chen et al. 2002; Ye and Li 2005; Chen et al. 2000; Chen et al. 2004; Ye et al. 2003).

Table 1: Chemicals employed to induce metabolic uncoupling (Strand et al. 1999; Mayhew and Stephenson 1997; Wei et al. 2003; Liu 2003; Liu and Tay 2001; Mayhew and Stephenson 1998)

2,4- Dinitrophenol (DNP)	Phenylglyoxal
2,4,5-Trichlorophenol (TCP)	pyridoxal-5-phosphate (PLP)
o-nitro-p-chlorophenol	Quercetin
2,4,6-tribromophenol	Gramicidin D
2,6-dibromo-4-nitrophenol	8-Anilino-naphthalene sulfonic acid (ANS)
2,4- dichlorophenol (DCP)	Rotenone
4-chloro-2-nitrophenol (CNP)	Antimycin A
Cresol	Dicoumarol
Aminophenol	Pentachlorophenol (PCP)
3,3',4',5-tetrachlorosalicylanilide (TCS)	Oligomycin
Carbonyl cyanide p-(trifluoromethoxy) phenylhydrazone (FCCP)	
Carbonyl cyanide m-chlorophenylhydrazone (CCCP)	

Microbial processes find wide-spread use in environmental engineering applications. However, the efficiency and cost of some of these processes is adversely affected by the generation of excessive biomass resulting from lack of control over microbial growth. This can lead to changes in the composition of

subsurface microbial population and failure of the entire *in-situ* remediation scheme (Anderson et al. 2003). Similarly, activated sludge treatment process also suffers from production of an excessive amount of biomass, disposal of which is resource-intensive (Liu and Tay 2001; Mayhew and Stephenson 1997; Wei et al. 2003). Uncoupler addition in excess substrate growth conditions might prove to be an effective way of controlling cellular growth.

A large number of Department of Energy (DOE) sites are contaminated with metals and radionuclides. Among the various measures proposed for clean-up of these sites, the use of metal reducing bacteria like, *Shewanella oneidensis* MR-1 and *Geobacter metallireducens*, is a prominent one. These bacteria are capable of using the contaminants as terminal electron acceptors, which leads to a reduction in their oxidation state. This is usually accompanied by a decrease in their solubility, which renders them immobile. In this study, *S. oneidensis* MR-1 has been used as a model microbe for studying the effects of chemical uncouplers (primarily TCS) on the metabolism of pure cultures. Preliminary experiments were performed to select an effective uncoupler and an optimum time for uncoupler addition. Batch experiments were conducted to model the effect of uncoupler addition on cells grown under excess-substrate conditions.

Any microbial process is difficult to employ in the real world unless it is accompanied by a process model. Existing models for metabolic uncoupling describe either (1) the effect of excess substrate (Liu 1996), or (2) the effect of a chemical uncoupler (Liu 2000). However, no model is currently available to

explain the behavior of microbes under the influence of both excess substrate conditions and a chemical uncoupler. The current study aims to present and verify a comprehensive model to illustrate the uncoupling effect of TCS on the metabolism of *S. oneidensis* MR-1 culture, under excess substrate conditions.

Although the phenomenon of *metabolic uncoupling* is well known, little work has been done to define the mechanism of uncoupler action. Futile cycles, dissipation of membrane potential, ATP hydrolysis, overflow metabolism, and other processes have been discussed as the possible mechanisms of energy loss by cells (Liu and Tay 2001; Russell and Cook 1995). Perhaps the best understood mechanism is the loss of proton motive force (PMF) due to a reduction in cell membrane resistance caused by some uncoupling chemicals. A class of these chemicals, also known as the *protonophores* (e.g., TCS, DNP etc.), is capable of short-circuiting the proton flow through the cellular membrane. This loss of proton gradient across the cellular membrane would lead to a decrease in ATP generation and subsequent energy wastage (Cook and Russell 1994). The exact mechanisms by which metabolic uncoupling is induced in other cases (e.g., heat stress, S_0/X_0 value etc.) still remain to be explained comprehensively. The current study makes an attempt to explain the various possible mechanisms involved in metabolic uncoupling, induced by excess substrate and presence of organic protonophores.

MATERIALS AND METHODS

Cell Cultivation and Sampling

The facultative bacterium, *Shewanella oneidenisis* MR-1, was used in these studies. This proteobacterium was first isolated from the anaerobic sediments in Lake Oneida, NY in 1988 (Myers and Nealson 1988) and its genome has been fully mapped (Heidelberg et. al 2002). Frozen stock for the study was provided by Pacific Northwest National Laboratories (PNNL). The stock culture was prepared by growing cells in 10% Tryptic Soy Broth (TSB) solution (3 gm/L) for 24 hours. The cells were harvested and mixed with glycerin in 2:1 ratio and stored at -75 °C for future use. For experiments, the inoculum was grown in 10 ml volumes of appropriate media for 24 hours. Two different growth media were used (1) 10% TSB media, and (2) minimal media (a modification of the media used by Kostka and Nealson) with pyruvate as the sole carbon/energy source (Kostka and Nealson 1998).

After growth from frozen stock, the inoculum was diluted in 1:100 ratio in 250 ml of same media that was used for inoculum growth. The culture batches were constantly agitated using a shaker incubator (Barnstead Max^Q 4000) maintained at 30 °C. All the cultures contained 1 ppm Resazurin as an indicator of dissolved oxygen levels and aeration was continuously provided to maintain the aerobic conditions. At each sampling intervals, samples were taken for cell

enumeration, substrate measurement and protein content determination. Aseptic techniques were adopted for cultivation, sampling and analysis.

Preliminary uncoupling Tests

Three stages of experimentation were conducted to quantify the effect of chemical addition on the metabolism of *S. oneidensis* MR-1. In the first phase, 10 ppm each of 3, 3', 4', 5 tetrachlorosalicylanilide (TCS, Acros Organics) and 2, 4-dintrophenol (DNP, Alfa Aesar) were added to cultures grown in 10% tryptic soy broth media (3 gm/L) after 10 hours of growth, during the logarithmic growth phase. Samples were taken at every 2 hour intervals for first 10 hours and then at each hour for next 5 hours and then finally at 18 hours. Direct cell enumeration was carried out using a compound microscope and Petroff Hausser counting chamber (Hausser Scientific) (Wilson and Blitchington 1996). Absorbance was measured at 600 nm using UV-spectrophotometer (Jenway Genova). These results were used to determine which of the two uncouplers, TCS and DNP, would be used for further experimentation.

In the second stage, experiments were conducted to determine the optimum time for addition of the uncoupler to the cultures. For this purpose, 1 ppm of TCS was added at 0, 12, 24, and 36 hours to different bottles containing cells grown in 20 mM pyruvate minimal media. Biomass growth and substrate removal efficiency were determined for each treatment and compared with the value from a control culture.

In the third series of experimentation, varying doses of TCS were added to cells grown in 20 mM pyruvate minimal media after 24 hours of growth. Five different doses of TCS (0.2, 0.5, 1, 2 and 5 ppm) were used to study the effect of the uncoupler on bacterial metabolism. A control culture with no uncoupler addition was included for comparison purposes. Samples were collected every 6 hours after addition of the uncoupler for the total sampling duration of 24 hours. Biomass growth was determined by direct cell counts using epi-fluorescence microscopy.

Uncoupling Experiment

Three different TCS doses were employed to model the uncoupling effect of this organic protonophore on cellular metabolism. Specifically 0.5, 1 and 2 ppm of TCS was added to cells grown in a minimal media containing 20 mM pyruvate. The experiments were conducted in duplicate. Control cultures were also included to act as indicators of uncoupling due to excess-substrate alone. The metabolic uncoupling induced by the excess pyruvate growth conditions in *S. oneidensis* MR-1 has already been demonstrated (Ch. 2). TCS was added to the cultures after 24 hours of growth. Samples were taken at 0, 24, 36, and 48 hours after inoculation of the growth media. High Performance Liquid Chromatography (HPLC) was used to determine the amount of substrate utilized during cell growth. Two different measures of biomass growth: (1) direct cell count (in terms of cells/ml), and (2) total protein content (as ppm protein) were adopted. Epi-fluorescence microscopy (Olympus, US) was used for direct enumeration of DAPI

stained cells. Micro BCA^{TM} Protein Assay Kit (Pierce Chemicals, IL) was used for measurement of total cell protein content. Protein samples were pre-treated with Compat-Able^{TM} Protein Assay Preparation Reagent Set (Pierce Chemicals, IL), to remove any interfering substances. Cell yield was measured as the ratio of cell concentration (in cells/ml) or total protein content (as ppm protein) at 48 hours to the corresponding amount of pyruvate utilized (in mM pyruvate).

MODEL DEVELOPMENT

A number of models have been proposed to describe the metabolic kinetics of cells. The earliest model, proposed by Pirt, assumes a constant maintenance energy requirement to account for all the energy not used for biomass synthesis (Pirt 1965). However, it was later observed that yield (Y) and specific growth rate (μ) are not directly proportional to one another under commonly used growth conditions because of partial dissociation between catabolism and anabolism. This phenomenon can be brought about by: (1) the presence of excess substrate, (2) presence of organic protonophores, metals or toxins, and (3) some physico-chemical conditions (Senez 1962; Chen et al. 2004; Cook and Russell 1994; Liu 1998; Liu 2000; Liu and Tay 2001; Mayhew and Stephenson 1997; Strand et al. 1999; Wei et al. 2003; Ye et al. 2003). Under uncoupled conditions, the rate of catabolism as measured by substrate utilization remains unaltered. However, the rate of anabolism is reduced causing a reduction in cellular yield. Several models have been developed to explain this phenomenon (Pirt 1982; Tsai and Lee 1990; Zeng and Deckwer 1995).

Liu (Liu 1996) proposed a simple model to explain the effect of presence of excess substrate on the cellular yield as a function of relative substrate concentration (S_0/X_0), given as

$$\frac{1}{Y_{obs}} = \frac{1}{Y_{obs}'} + \frac{1}{(Y_w)_{min}} \times \frac{(S_0/X_0)}{(S_0/X_0) + K_{s/x}} \qquad (1)$$

In this model, Y_{obs} is the observed growth yield, Y_{obs}' is the theoretical maximum growth yield (or observed growth yield under substrate-limited conditions), $(Y_w)_{min}$ is the minimal energy spilling related growth yield, and $K_{s/x}$ is a saturation constant related to S_0/X_0 ratio. The authors have successfully applied Eq. (1) to describe the metabolic uncoupling of *S. oneidensis* MR-1 culture, under excess-substrate growth conditions (Ch. 2).

A similar model has been proposed to describe the effect of uncoupler addition on the cellular yield as shown in Eq. (2), where $(Y_{obs})_{max}$ is same as Y_{obs}', C_u is the initial uncoupler concentration, and $K_{u/x}$ is a saturation constant related to C_u/X_0 (Liu 2000).

$$\frac{1}{Y_{obs}} = \frac{1}{(Y_{obs})_{max}} + \frac{1}{(Y_w)_{min}} \times \frac{C_u/X_0}{C_u/X_0 + K_{u/x}} \qquad (2)$$

None of the existing models describe the effect of simultaneous presence of excess substrate and chemical uncouplers. By definition, only excess substrate cultures exhibit metabolic uncoupling. Substrate limited cultures on the other hand, are characterized by maximum theoretical yield (Y_{obs}' or $(Y_{obs})_{max}$) and are

devoid of any uncoupling. Consequently, an uncoupler would be effective only in an excess substrate culture. Thus, Eq. (2) relating biomass yield to relative uncoupler concentration (C_u/X_0) is incomplete. The authors propose the following empirical expression to model the effect of both excess-substrate and chemical addition simultaneously

$$\frac{1}{Y_{obs}} = \frac{1}{(Y_{obs})_{max}} + \frac{1}{(Y_w)_{min}} \times \frac{S_0/X_0}{S_0/X_0 + K_{S/x}} + \frac{1}{(Y_{wu})_{min}} \times \frac{C_u/X}{C_u/X + K_{u/x}} \qquad (3)$$

In this equation, $(Y_{wu})_{min}$ is the minimal energy spilling related growth yield under uncoupler addition conditions and X is the biomass concentration of the culture at the instant when the uncoupler is added. All other parameters are as already defined for Eqs. (1) and (2). When no uncoupler is added, Eq. (3) reduces to Eq. (1). Under substrate-limited conditions, the observed yield (Y_{obs}) approaches the theoretical maximum yield, $(Y_{obs})_{max.}$

To determine the constants in Eq. (3), cells are grown in media having different values of (S_0/X_0) without addition of uncoupler. This data is used to determine the values of $(Y_{obs})_{max}$, $(Y_w)_{min}$ and $K_{s/x}$. Using these parameters, the first two parts of Eq. 3 are constant for a given value of (S_0/X_0) and the remaining parameters could be determined by growing cells in media loaded with different uncoupler doses (C_u/X).

The degree of uncoupling is quantified by a parameter termed as uncoupling coefficient (E_u) and is described by the following mathematical relation (Liu and Chen 1997; Liu et al. 1998)

$$E_u = \frac{(Y_{obs})_{max} - Y_{obs}}{(Y_{obs})_{max}} \qquad (4)$$

It can be effectively utilized to distinguish between the amount of uncoupling induced by excess substrate and chemical uncoupler addition.

RESULTS

Preliminary Uncoupling Tests

The effectiveness of two uncoupling chemicals, TCS and DNP, were tested by examining the response of cell growth to uncoupler addition. The experimental
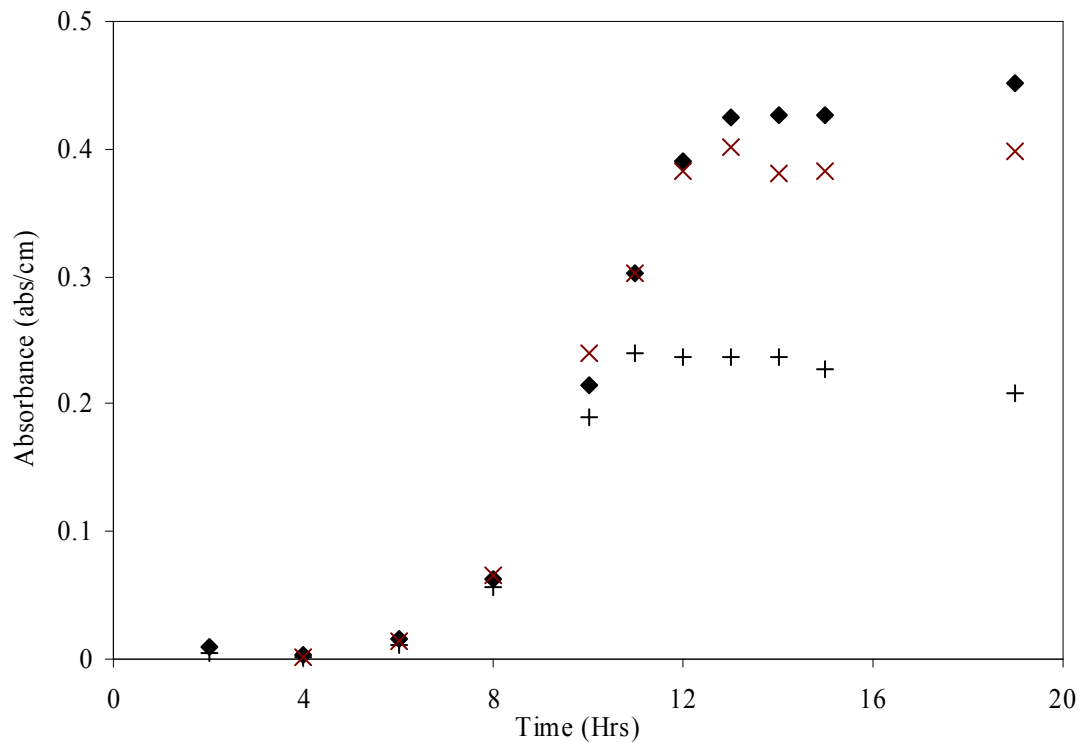
Figure 1: Effect of uncoupler addition on cell concentration, measured in terms of absorbance. ◆ represents control culture, x represents culture with 2, 4-DNP addition and + represents culture with TCS addition.

results are illustrated in Figs. (1) and (2). Experiments were conducted to determine the optimum time for addition of TCS based on cell growth and substrate removal efficiency (data not shown). TCS addition at 24 hours resulted in good cell growth (> 5 x 10^8 cells/ml) and a high total substrate removal efficiency (~97%).

At other dosing times, either the cell concentration or the substrate removal efficiency were too low to be practical (0 and 12 hour dosing times); or the uncoupler had negligible effect on biomass growth (36 hour dosing time) (data not shown).
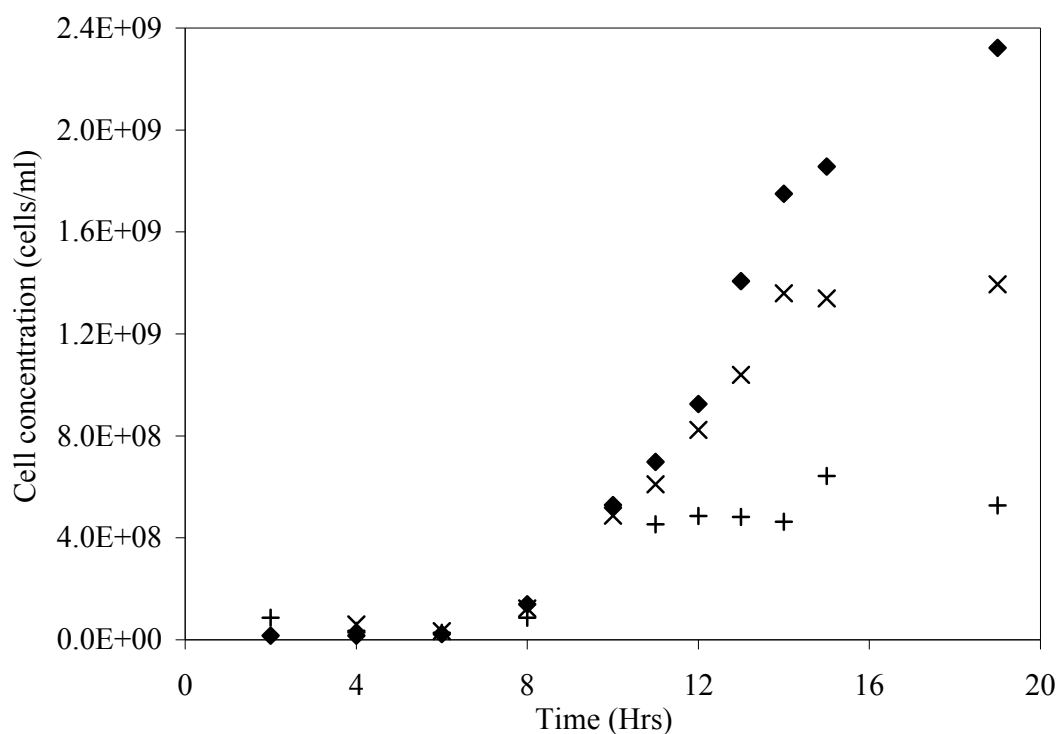
Figure 2: Effect of uncoupler addition on cells grown in 10% TSB media.   The symbol (◆) represents control culture, x represents culture with 2, 4-DNP addition and + represents culture with TCS addition.
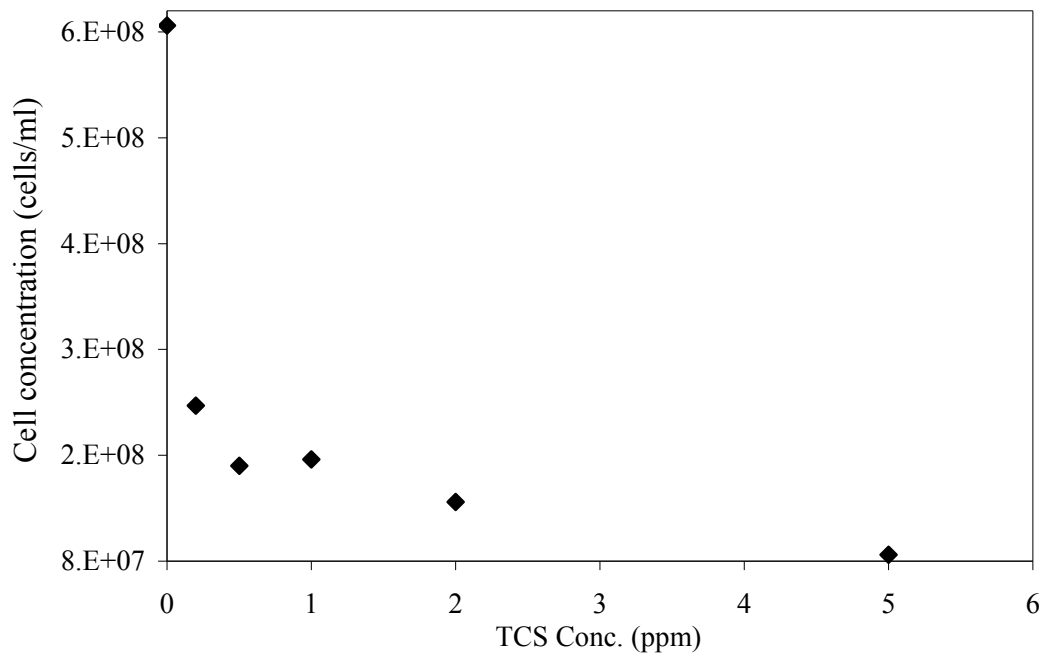
Figure 3. Effect of TCS on cell concentration, recorded at 48 hours after media inoculation. The cells were grown on 10% TSB. ◆ represents the observed cell concentration values.

Figure 3 demonstrates the effect of uncoupler concentration on the cell growth. Cell concentration measured by direct cell count (as cells/ml) has been plotted as a function of TCS dose.

Chemical Uncoupling Experiment

Figures 4 and 5 illustrate the observed cell yields upon addition of different TCS doses. Yields have been plotted as a function of relative uncoupler concentration (C_u/X). The model predictions generated by fitting Eq. (3) to the observed data, are also shown. The values of constants, $(Y_{obs})_{max}$, $(Y_w)_{min}$, and $K_{s/x}$ as obtained in the previous study with excess-substrate induced uncoupling (Ch. 2), were used in the present case. The current data set was used to compute the

values of $(Y_{wu})_{min}$ and $K_{u/x}$. Direct cell count and total protein have been used as measures of biomass in Figs. 4 and 5, respectively.

Figure 6 illustrates the average protein content of cells (as ppm protein/cell). Data is shown for only 36 and 48 hours to amplify the effect of chemical uncoupler on the protein content. The uncoupling coefficient (E_u) values are shown in Figs. 7 and 8. Model-predicted values of observed yield were used to calculate the values of E_u and these have been compared to the observed values.
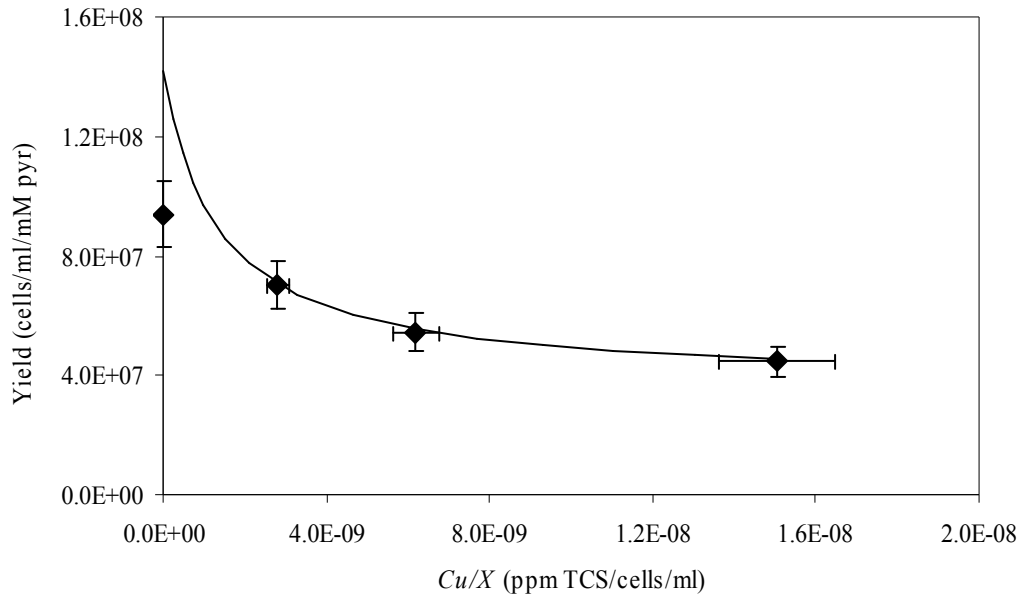
Figure 4. Effect of TCS on cellular yield. Biomass is measured by direct cell count as cells/ml. ◆ represents the observed cell yield and the model predictions are shown by line. The model parameters are $(Y_{obs})_{max}$ = 2.5x10^8 cells/ml/mM pyruvate, $(Y_w)_{min}$ = 3x10^7 cells/ml/mM pyruvate, $K_{s/x}$ = 4.06x10^{-5} mM pyruvate (cells/ml)$^{-1}$, $(Y_{wu})_{min}$ = 5x10^7 cells/ml/mM pyruvate and $K_{u/x}$ = 5.09x10^{-9} mM pyruvate (cells/ml)$^{-1}$. Error bars show 95% confidence intervals.
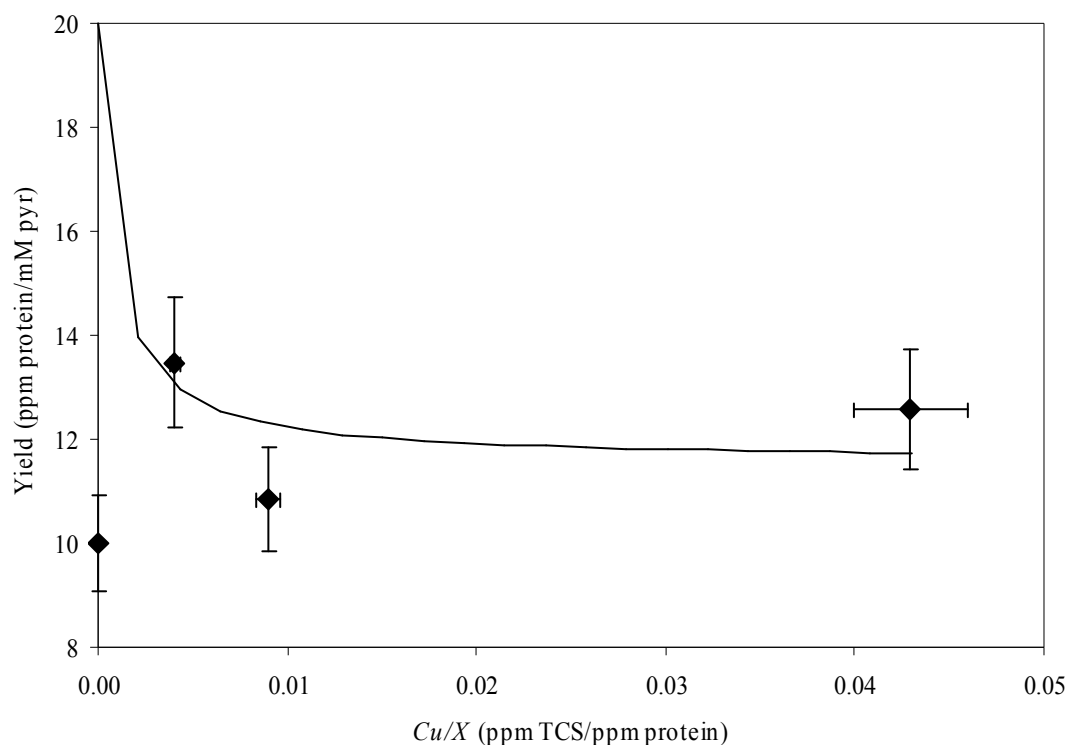
Figure 5. Effect of TCS on cellular yield. Biomass is measured by total protein content as ppm protein. ◆ represents the observed cell yield and the model predictions are shown by line. The model parameters are $(Y_{obs})_{max}$ = 30.21 ppm protein/mM pyruvate, $(Y_w)_{min}$ = -3.27 ppm protein/mM pyruvate, $K_{s/x}$ = -316.74 mM pyruvate/ppm protein, $(Y_{wu})_{min}$ = 27.46 ppm protein/mM pyruvate, and $K_{u/x}$ = 0.0015 mM pyruvate/ppm protein. Error bars show 95% confidence intervals.

DISCUSSION

Figures (1) and (2) show a similar pattern, and they illustrate that, for *S. oneidensis* MR-1, TCS was more effective than DNP as a metabolic uncoupler. At the uncoupler concentrations that were used in these experiments, it takes about three hours before a significant uncoupling effect is noticeable from DNP addition. In contrast, the effects of TCS addition are apparent as soon as it is introduced to
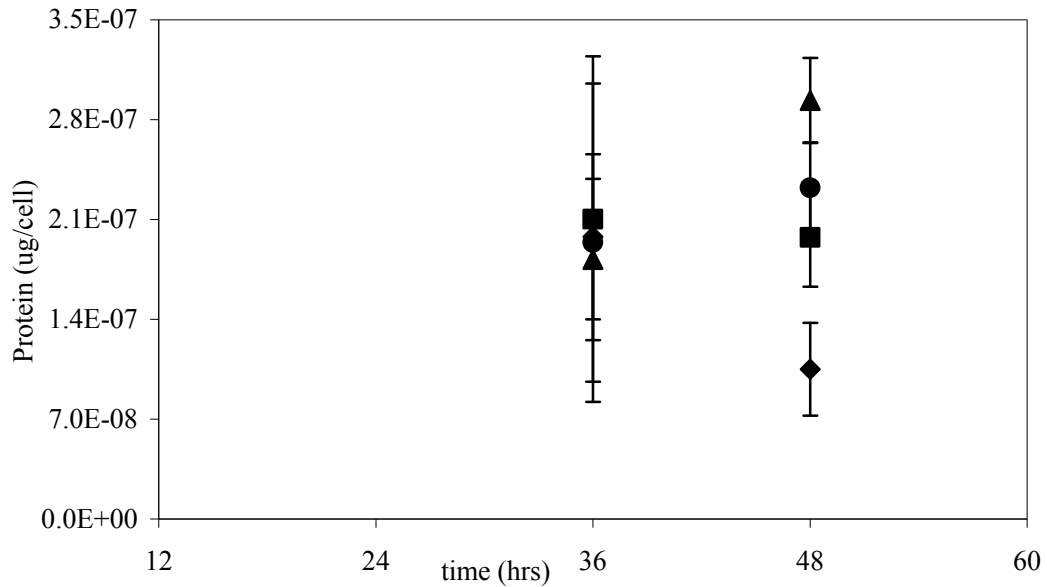
Figure 6. Average protein content of cells under TCS addition. ♦ represents control culture, ■ represents cultures with 0.5 ppm TCS, ● represents cultures with 1 ppm TCS and ▲ represents cultures with 2 ppm TCS.
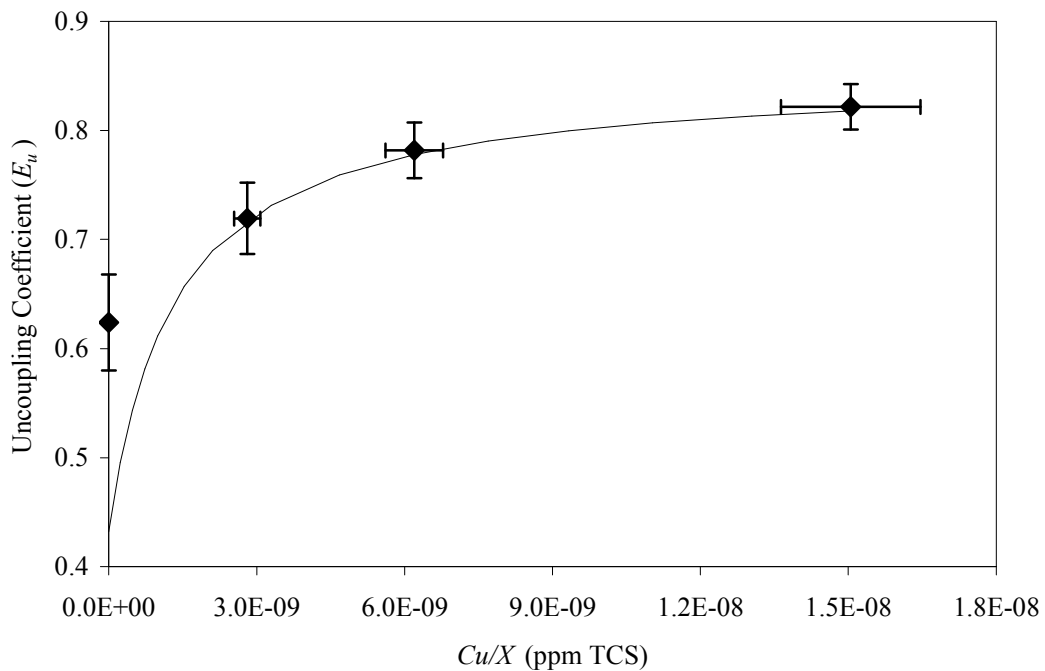
Figure 7. Uncoupling coefficient for TCS induced metabolic uncoupling in *S. oneidensis* MR-1. ♦ represents the observed E_u and the model predictions are shown by the solid line. 95% confidence intervals are shown by error lines. $(Y_{obs})_{max} = 2.5 \times 10^8$ cells/ml/mM pyruvate was used for E_u determination.

the culture. Within five hours of its addition, TCS reduced the cell production by 66%, as compared to the unaltered control culture. For a similar time period, DNP reduced the cell production by only 28%. Based on these results, TCS was selected as the uncoupler for use in all the future experiments. Further, these results confirm to the previous findings on relative effectiveness of these two chemicals (Ye et al. 2003).

Experiment conducted to determine the effect of TCS dosing time showed that 24 hours could be considered to be 'optimal' from the perspectives of cell growth and total substrate removal. Therefore, in all the additional experiments, the uncoupler was added 24 hours after the inoculation of minimal media.
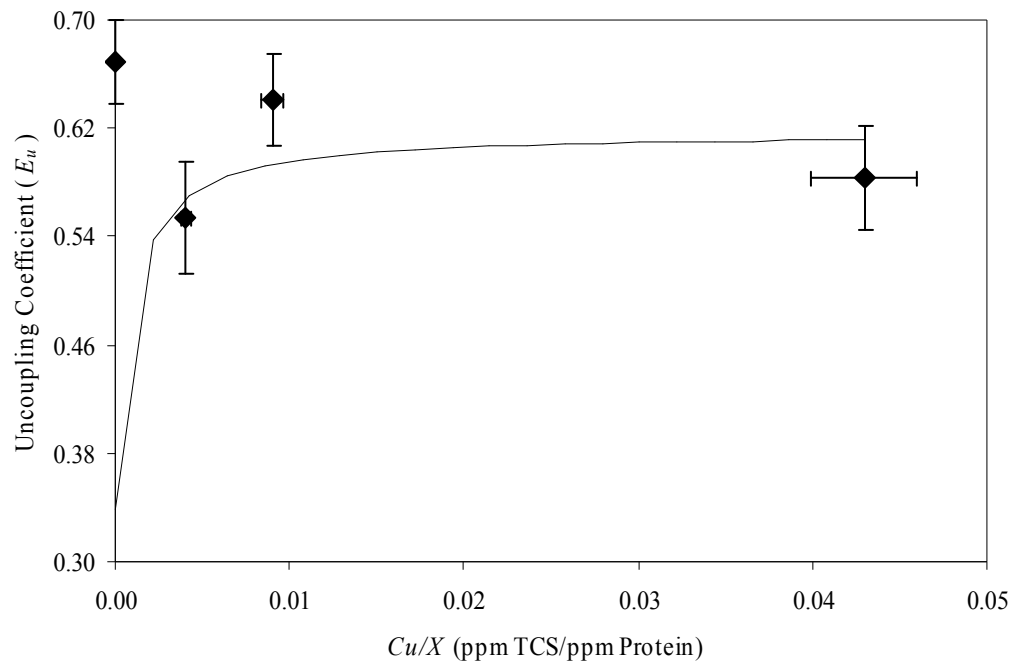
Figure 8. Uncoupling coefficient for TCS induced metabolic uncoupling in *S. oneidensis* MR-1 culture. The biomass is measured as the total protein content of the cells. ◆ represents the observed E_u and the model predictions are shown by the solid line. 95% confidence intervals are shown by error lines. $(Y_{obs})_{max} = 30.21$ ppm protein/mM pyruvate was used for E_u determination.

An increasing dose of TCS results in successive reduction in biomass, measured by epi-fluorescence microscopy (Fig. 3). The efficiency of TCS as an uncoupler is further verified by the reduction in cell concentration at even the very low dose of 0.2 ppm, where a 61% reduction in cell concentration (relative to control culture) was seen. At a dose of 5 ppm, an 85% decrease in cell concentration (compared to control culture) was observed. These results suggest that a reduction in cellular yield of *S. oneidensis* MR-1 can be brought about by the addition of small quantities of TCS, optimally at 24 hours.

It can be seen from Fig. 4 that a TCS dose of 2 ppm leads to yield reduction by about 53%, compared to control culture. Even a low dose of 0.5 ppm reduces the biomass yield by about 25%, demonstrating the efficacy of TCS as an uncoupler. The cellular yield follows a decreasing trend with increasing dose of the uncoupler (Fig. 4). However, when protein is used as a measure of biomass (Fig. 5), the yield remains more or less constant. A comparison between the two cases (Figs. 4 and 5) suggests that, in the presence of chemical uncoupler, the cells are accumulating protein. This could also justify the inability of the proposed model (Eq. 3) to explain the observed protein behavior (Fig. 5). The average protein content of cells verifies this hypothesis (Fig. 6). It can be seen that at 48 hours, when samples are taken for yield measurement, the average protein of cells increases with an increasing dose of TCS.

A similar observation was made when cells were grown under excess substrate conditions without any uncoupler addition (refer to Fig. 5, Chapter-2). It

appears that the average protein content of the cells increases in response to metabolic uncoupling, induced by excess-substrate and/or chemical uncoupler addition. Thus, changes in protein levels of cells could be a possible uncoupling pathway.

Plots of uncoupling coefficients (Figs. 7 and 8) are similar to those of cell yield (Figs. 4 and 5). The model provides very good fit at higher TCS concentrations, but does relatively poorly for the control culture (Fig. 7). The values of E_u represent the extent of uncoupling under given conditions and so it can be concluded from Fig. 7 that addition of 2 ppm TCS leads to 20% extra wastage of substrate, as compared to a control culture with no uncoupler addition. As already seen in Fig. 5, protein yields remain more or less constant in presence of the uncoupler, a similar behavior is observed in Fig. 8. The uncoupling coefficient does not dramatically change with increased TCS addition. From the uncoupling coefficient data, it can be concluded that substrate-excess conditions are more effective in uncoupling the metabolism of cells, as compared to TCS. Consequently, chemical uncoupler can be used in conjunction with excess substrate conditions to augment uncoupling.

In the current study, it was observed that acetate is accumulated in the culture. It is known that under oxygen-limited and/or lactate excess anaerobic conditions; anaerobic chemostat cultures of *S. oneidensis* MR-1 convert lactate to acetate and formate. When formate is the sole carbon source, acetate is accumulated (Fredrickson et al., 2004). Similar behavior is observed in *Shewanella*

putrefaciens, a closely related member of the same genus. For *S. putrefaciens* grown anaerobically on pyruvate, pyruvate is converted to acetate and formate and the later acts as the sole carbon source (Scott and Nealson 1994). Pyruvate can be respired either aerobically through the TCA cycle or anaerobically by formate dehydrogenation and fermentation (Liu et al. 2005). Figure 9 shows the possible pathway for metabolic conversion of pyruvate to formate and acetate.
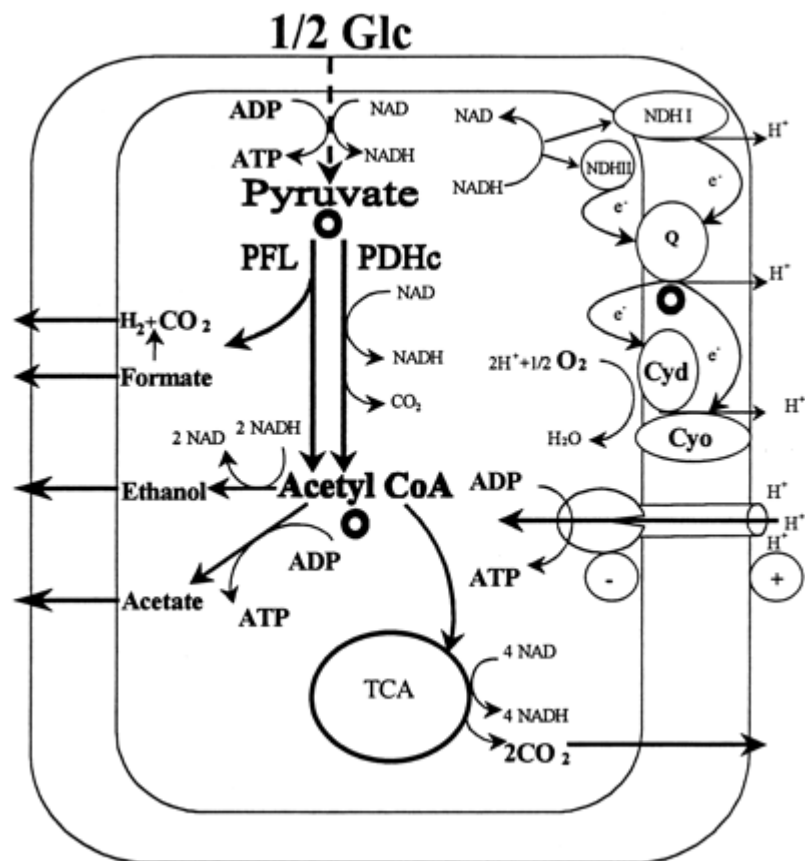
Figure 9. Pathways for catabolism of glucose and pyruvate (figure adopted from Alexeeva et. al 2000).

A similar phenomenon appears to be taking place for our culture of *S. oneidensis* MR-1 grown under aerobic conditions with pyruvate as the substrate. Very small quantities of formate were detected at some sampling intervals, suggesting that it is used up rapidly after it is produced. It appears that formate is being used for metabolic activities, while acetate is being accumulated in the system. Since, acetate is being accumulated it appears that *S. oneidensis* MR-1 is unable to use acetate as readily as pyruvate.

Among the available theories for mechanism of uncoupler action on microbial cultures, loss of cell membrane resistance to proton flow is most widely accepted (Cook and Russell 1994). According to this hypothesis, protonopohres like TCS and DNP, which are mostly lipid-soluble weak acids, carry protons through the cellular membrane into the cells. This leads to a disruption of proton motive force and thereby reduces the generation of ATP through oxidative phosophorylation (Mathews and vanHolde 1990). Under these conditions, the rate of substrate utilization remains unaffected and respiration is effectively uncoupled from phosphorylation. Other uncouplers like oligomycin, blocks proton channels which results in non-dissipation of the proton gradient. Other antibiotics like valinomycin act by inducing potassium ion (K^+) cycles through the membrane. When valinomycin is present with nigericin, it effectively leads to proton motive force dissipation, as nigericin is an K^+/H^+ antiporter (Mathews and vanHolde 1990). Futile cycles have also been known to cause energy spilling (Russell and Cook 1995).

Eucaryotes are known to contain uncoupling proteins (UCP) which act as a regulator of biomass and lead to energy over-expenditure under certain conditions (Klaus et al. 2005; Ricquier 2005). Similar proteins are also known to occur in prokaryotes (Diehl and Hoek 1999). Although *S. oneidensis* MR-1 is not yet known to contain similar proteins, their presence or production under certain specific conditions (like, presence of uncouplers) could not be ruled out. Thus, detailed analysis for changes in protein content and composition would be useful in determining the effect of uncoupling, both under excess substrate and uncoupler addition conditions, on the proteome.

Although the addition of TCS has been well documented to cause metabolic uncoupling, there are still reservations about its applications in the real world systems. Long term effectiveness of this substance in pure culture systems is still not understood and it is possible that organisms may adapt to the long term presence of chemical uncouplers. Further research in this direction, with pure cultures, is likely to yield measures for better control over microbial growth. TCS is known to inhibit the DNA replication under both aerobic as well as anaerobic conditions in *Escherichia coli*. It also inhibits the activity of several enzymes, with DNA polymerase III being the most sensitive (Weigel and Englund 1977). Similar inhibitory effect might be expected for enzymes involved in cellular metabolism. Since, genus *Shewanella* is closely related to genus *Escherichia*, TCS could have comparable effects on *Shewanella*. An extensive study of changes in the gene

expression of *S. oneidensis* MR-1, under the application of uncouplers, would provide a better insight into uncoupling mechanisms.

CONCLUSIONS

The effectiveness of TCS for reducing the yield of *S. oneidensis* MR-1 has been verified through experimentation. A decrease in cell number based yield was observed (Fig. 4) starting at a very small dose of 0.5 ppm TCS, while the substrate consumption remained unaltered. An expression was proposed for describing the observed behavior under excess substrate and/or presence of chemical uncoupler. The model was able to explain the experimental observations reasonably well, when direct cell counts were used as the biomass measure. The uncoupling coefficient was used to distinguish between the uncoupling effects of excess substrate and TCS addition. The protein yield remained relatively unaffected by the presence of TCS and proposed expression was unable to fit the observed protein data.

The average protein content of the cells was shown to increase with an increase in TCS dose. Apart from the increase in protein levels, acetate accumulation was also observed. We believe that these are two possible pathways for non-growth associated metabolic activities that lead to uncoupling. More research is required to determine the exact mechanisms of metabolic uncoupling.

However, it is clear that TCS can effectively, in conjunction with excess-substrate conditions, uncouple cellular metabolism.

Notation

S_0 :   initial substrate concentration (mM pyruvate)

X_0 :   initial biomass concentration (cells/ml or ppm protein)

Y :   cell yield (cells/ml/mM pyruvate or ppm protein/mM pyruvate)

m :   maintenance coefficient (

μ :   specific growth rate

Y_G :   true or theoretical maximum yield

Y_{obs} :   observed growth yield (cells/ml/mM pyruvate or ppm protein/mM pyruvate)

Y_{obs}' :   observed growth yield under substrate-limited conditions (cells/ml/mM pyruvate or ppm protein/mM pyruvate)

$(Y_w)_{min}$:minimal energy-spilling related growth yield (cells/ml/mM pyruvate or ppm protein/mM pyruvate)

$K_{s/x}$:   saturation constant related to S_0/X_0 (mM pyruvate/(cells/ml) or mM pyruvate/ppm protein)

E_u :   uncoupling coefficient

TCS :   3, 3', 4', 5 Tetrachlorosalicylanilide

C_u :   initial uncoupler concentration (ppm)

$K_{u/x}$:   saturation constant related to C_u/X_0 (mM pyruvate/(cells/ml) or mM pyruvate/ppm protein)

X :   biomass concentration at the time of uncoupler addition

$(Y_{obs})_{max}$: (same as $Y_{obs}'s$)

DNP: 2, 4-dinitrophenol

REFERENCES

Alexeeva S, DeKort B, Sawers G, Hellingwerf KJ, deMattos MJT. 2000. Effects of limited aeration and of the ArcAB system on intermediary pyruvate catabolism in *Escherichia coli*. Journal of Bacteriology 182 (17):4934-4940.

Anderson RT, Vrionis HA, Ortiz-Bernad I, Resch CT, Long PE, Dayvault R, Karp K, Marutzky S, Metzler DR, Peacock A and others. 2003. Stimulating the in situ activity of Geobacter species to remove uranium from the groundwater of a uranium-contaminated aquifer. Applied And Environmental Microbiology 69(10):5884-5891.

Chen GH, Mo HK, Liu Y. 2002. Utilization of a metabolic uncoupler, 3, 3', 4', 5-tetrachlorosalicylanilide (TCS) to reduce sludge growth in activated sludge culture. Water Research 36(8):2077-2083.

Chen GH, Mo HK, Saby S, Yip WK, Liu Y. 2000. Minimization of activated sludge production by chemicallly stimulated energy spilling. Water Science & Technology 42(12):189-200.

Chen YX, Ye FX, Feng XS. 2004. The use of 3, 3', 4', 5-tetrachlorosalicylanilide as a chemical uncoupler to reduce activated sludge yield. Journal of Chemical Technology & Biotechnology 79(2):111-116.

Cook GM, Russell JB. 1994. Energy-spilling reactions of Streptococcus bovis and resistance of its membrane to proton conductance. Appl. Environ. Microbiol. 60(6):1942-1948.

Diehl AM, Hoek JB. 1999. Mitochondrial Uncoupling: Role of Uncoupling Protein Anion Carriers and Relationship to Thermogenesis and Weight Control The Benefits of Losing Control. J Bioenerg Biomembr 31(5):493-506.

Fredrickson, J, Beliaev A, Cannon B, Gorby Y, Lipton M, Liu P, Romine M, Smith R, Trease H. 2004. Global and physiological responses to substrate shifts in continuous and controlled batch cultures of *Shewanella oneidensis* MR-1. Genomics:GTL Program Projects.

Heidelberg J, Paulsen I, Nelson K, Gaidos E, Nelson W, Read T, Eisen J, Seshadri R, Ward N, Methe B and others. 2002. Genome sequence of the dissimilatory metal ion-reducing bacterium Shewanella oneidensis. Nature Biotechnology 20(11):1118-23.

Klaus S, Rudolph B, Dohrmann C, Wehr R. 2005. Expression of uncoupling protein 1 in skeletal muscle decreases muscle energy efficiency and affects thermoregulation and substrate oxidation. Physiological Genomics 21(2).

Kostka JE, Nealson KH. 1998. Isolation, cultivation and characterization of iron- and manganese-reducing bacteria. *In* R.S. Burlage et al.(ed.), Techniques in microbial ecology:58-78.

Liu Y. 1996. Bioenergetic interpretation on the S_0/X_0 ratio in substrate-sufficient batch culture. Water Research 30(11):2766-2770.

Liu Y. 1998. Energy uncoupling in microbial growth under substrate-sufficient conditions. Applied Microbiology & Biotechnology 49(5):500-505.

Liu Y. 2000. Effect of chemical uncoupler on the observed growth yield in batch culture of activated sludge. Water Research 34(7):2025-2030.

Liu Y. 2003. Chemically reduced excess sludge production in the activated sludge process. Chemosphere 50(1):1-7.

Liu Y, Chen GH. 1997. Model of energy uncoupling for substrate-sufficient culture. Biotechnology & Bioengineering 55(3):571-576.

Liu Y, Chen GH, Paul E. 1998. Effect of the S_0/X_0 ratio on energy uncoupling in substrate-sufficient batch culture of activated sludge. Water Research 32(10):2883-2888.

Liu Y, Gao W, Wang Y, Wu L, Liu X, Yan T, Alm E, Arkin A, Thompson DK, Fields MW, Zhou J. 2005. Transcriptome analysis of *Shewanella oneidensis* MR-1 in response to elevated salt conditions. Journal of Bacteriology 187(7):2501-2507.

Liu Y, Tay JH. 2000. A kinetic model for energy spilling-associated product formation in substrate-sufficient continuous culture. Journal of Applied Microbiology 88(4):663-668.

Liu Y, Tay JH. 2001. Strategy for minimization of excess sludge production from the activated sludge process. Biotechnology Advances 19(2):97-107.

Mathews CK, vanHolde KE. 1990. Biochemistry.525-528.

Mayhew M, Stephenson T. 1997. Low biomass yield activated sludge: A review. Environmental Technology 18(9):883-892.

Mayhew M, Stephenson T. 1998. Biomass yield reduction: Is biochemical manipulation possible without affecting activated sludge process efficiency? Water Science & Technology 38(8-9):137-144.

Myers CR, Nealson KH. 1988. Bacterial Manganese Reduction and Growth with Manganese Oxide as the Sole Electron Acceptor. Science 240(4857):1319-1321.

Pirt S. 1982. Maintenance energy: a general model for energy-limited and energy-sufficient growth. Archives of Microbiology 133(4):300-302.

Pirt SJ. 1965. The Maintenance Energy of Bacteria in Growing Cultures. Proceedings of the Royal Society of London. Series B, Biological sciences 163(991):224-231.

Ricquier D. 2005. Respiration uncoupling and metabolism in the control of energy expenditure. Proceedings of the Nutrition Society 64(1):47-52.

Russell JB, Cook GM. 1995. Energetics of bacterial growth: balance of anabolic and catabolic reactions. Microbiological Reviews 59(1):48-62.

Scott JH, Nealson KH. 1994. A biochemical study of the intermediary carbon metabolism of *Shewanella putrefaciens*. Journal of Bacteriology 176(11):3408-3411.

Senez JC. 1962. Some considerations on the energetics of bacterial growth. Bacteriological Reviews 26(2 Pt 1-2):95-107.

Strand SE, Harem GN, Stensel HD. 1999. Activated-sludge yield reduction using chemical uncouplers. Water Environment Research 71(4):454-458.

Tsai SP, Lee YH. 1990. A model for Energy-sufficient culture growth. Biotechnology & Bioengineering 35:138-145.

Wei YS, Van Houten RT, Borger AR, Eikelboom DH, Fan YB. 2003. Minimization of excess sludge production for biological wastewater treatment. Water Research 37(18):4453-4467.

Weigel P, Englund P. 1977. Inhibition of DNA replication in Escherichia coli by Dibromophenol and other uncouplers. The Journal of Biological Chemistry 252(4):1148-1155.

Ye FX, Li Y. 2005. Reduction of excess sludge production by 3, 3', 4',5-tetrachlorosalicylanilide in an activated sludge process. Applied Microbiology & Biotechnology 67(2):269-274.

Ye FX, Shen DS, Li Y. 2003. Reduction in excess sludge production by addition of chemical uncouplers in activated sludge batch cultures. Journal of Applied Microbiology 95(4):781-786.

Zeng AP, Deckwer WD. 1995. A kinetic model for substrate and energy consumption of microbial growth under substrate-sufficient condition. Biotechnology Progress 11(1):71-79.

CHAPTER 4

CONCLUSIONS

Microbial processes are very useful to environmental engineers and form the basis of some of the most important applications like waste water treatment and bioremediation. Despite its significance, the cellular metabolism is still not completely understood. And the inability to control these processes leads to the production of excess secondary biomass, which entails heavy costs in disposal and could cause failure of the entire treatment scheme.

The effect of excess substrate and uncoupler addition on the metabolism of *S. oneidensis* MR-1 was studied. The cellular yield was shown to decrease under excess-substrate and/or TCS addition. Further, the substrate removal efficiencies remained relatively constant for all the cultures. Two different measures of biomass, direct cell count and total protein content, were used for determination of cell yield. The experimental observations were modeled by relating the cellular yield to relative substrate (S_0/X_0) and relative uncoupler (C_u/X) concentrations. A mathematical expression is proposed to quantify the effect of both of these conditions simultaneously and it provided a good fit to the observed data. Uncoupling coefficient was used to determine and distinguish between the uncoupling effects of excess substrate and TCS addition conditions. Under the presence of TCS, the protein yields remained fairly unaffected and the model failed to capture this observed behavior.

Increase in internal protein content of cells might be a possible explanation for this behavior. In addition, acetate accumulation was also observed in the cultures. It is hypothesized that pyruvate is being converted to acetate and formate; with latter acting as the carbon-source. The increase in average protein content of the cells and the accumulation of acetate has been hypothesized as possible pathways for metabolic uncoupling, under excess substrate and uncoupler addition conditions.

The current research illustrates that the metabolic uncoupling of a metal reducing bacteria can be satisfactorily modeled and the concentrations of substrate and uncoupler can be varied to have desired level of biomass growth. A comprehensive understanding of this phenomenon could provide better control over microbial growth. Further research is required for determining the exact mechanism of uncoupling and developing a model to explain the uncoupled growth comprehensively.

BIBLIOGRAPHY

Alexeeva S, DeKort B, Sawers G, Hellingwerf KJ, deMattos MJT. 2000. Effects of limited aeration and of the ArcAB system on intermediary pyruvate catabolism in *Escherichia coli*. Journal of Bacteriology 182 (17):4934-4940.

Anderson RT, Vrionis HA, Ortiz-Bernad I, Resch CT, Long PE, Dayvault R, Karp K, Marutzky S, Metzler DR, Peacock A and others. 2003. Stimulating the in situ activity of Geobacter species to remove uranium from the groundwater of a uranium-contaminated aquifer. Applied And Environmental Microbiology 69(10):5884-5891.

Chen GH, Mo HK, Liu Y. 2002. Utilization of a metabolic uncoupler, 3, 3', 4', 5-tetrachlorosalicylanilide (TCS) to reduce sludge growth in activated sludge culture. Water Research 36(8):2077-2083.

Chen GH, Mo HK, Saby S, Yip WK, Liu Y. 2000. Minimization of activated sludge production by chemicallly stimulated energy spilling. Water Science & Technology 42(12):189-200.

Chen YX, Ye FX, Feng XS. 2004. The use of 3, 3', 4',5-tetrachlorosalicylanilide as a chemical uncoupler to reduce activated sludge yield. Journal Of Chemical Technology And Biotechnology 79(2):111-116.

Cook GM, Russell JB. 1994. Energy-spilling reactions of Streptococcus bovis and resistance of its membrane to proton conductance. Appl. Environ. Microbiol. 60(6):1942-1948.

Diehl AM, Hoek JB. 1999. Mitochondrial Uncoupling: Role of Uncoupling Protein Anion Carriers and Relationship to Thermogenesis and Weight Control The Benefits of Losing Control. J Bioenerg Biomembr 31(5):493-506.

Fredrickson, J, Beliaev A, Cannon B, Gorby Y, Lipton M, Liu P, Romine M, Smith R, Trease H. 2004. Global and physiological responses to substrate shifts in continuous and controlled batch cultures of *Shewanella oneidensis* MR-1. Genomics:GTL Program Projects.

Heidelberg J, Paulsen I, Nelson K, Gaidos E, Nelson W, Read T, Eisen J, Seshadri R, Ward N, Methe B and others. 2002. Genome sequence of the dissimilatory metal ion-reducing bacterium *Shewanella oneidensis*. Nature Biotechnology 20(11):1118-23.

BIBLIOGRAPHY (Continued)

Klaus S, Rudolph B, Dohrmann C, Wehr R. 2005. Expression of uncoupling protein 1 in skeletal muscle decreases muscle energy efficiency and affects thermoregulation and substrate oxidation. Physiological Genomics 21(2).

Kostka JE, Nealson KH. 1998. Isolation, cultivation and characterization of iron- and manganese-reducing bacteria. *In* R.S. Burlage et al. (ed.), Techniques in microbial ecology: 58-78.

Kostka JE, Stucki JW, Nealson KH, Wu J. 1996. Reduction of structural Fe(III) in smectite by a pure culture of *Shewanella putrefaciens* strain MR-1. Clays And Clay Minerals 44(4):522-529.

Li, J. 2004. MS Thesis. Oregon State University.

Liu Y. 1996. Bioenergetic interpretation on the S_0/X_0 ratio in substrate-sufficient batch culture. Water Research 30(11):2766-2770.

Liu Y. 1998. Energy uncoupling in microbial growth under substrate-sufficient conditions. Applied Microbiology & Biotechnology 49(5):500-505.

Liu Y. 2000. Effect of chemical uncoupler on the observed growth yield in batch culture of activated sludge. Water Research 34(7):2025-2030.

Liu Y. 2003. Chemically reduced excess sludge production in the activated sludge process. Chemosphere 50(1):1-7.

Liu Y, Chen GH. 1997. Model of energy uncoupling for substrate-sufficient culture. Biotechnology And Bioengineering 55(3):571-576.

Liu Y, Chen GH, Paul E. 1998. Effect of the S_0/X_0 ratio on energy uncoupling in substrate-sufficient batch culture of activated sludge. Water Research 32(10):2883-2888.

Liu Y, Gao W, Wang Y, Wu L, Liu X, Yan T, Alm E, Arkin A, Thompson DK, Fields MW, Zhou J. 2005. Transcriptome analysis of *Shewanella oneidensis* MR-1 in response to elevated salt conditions. Journal of Bacteriology 187(7):2501-2507.

Liu Y, Tay JH. 2000. A kinetic model for energy spilling-associated product formation in substrate-sufficient continuous culture. Journal of Applied Microbiology 88(4):663-668.

BIBLIOGRAPHY (Continued)

Liu Y, Tay JH. 2001. Strategy for minimization of excess sludge production from the activated sludge process. Biotechnology Advances 19(2):97-107.

Mathews CK, vanHolde KE. 1990. Biochemistry.525-528.

Mayhew M, Stephenson T. 1997. Low biomass yield activated sludge: A review. Environmental Technology 18(9):883-892.

Mayhew M, Stephenson T. 1998. Biomass yield reduction: Is biochemical manipulation possible without affecting activated sludge process efficiency? Water Science & Technology 38(8-9):137-144.

Myers CR, Nealson KH. 1988. Bacterial Manganese Reduction and Growth with Manganese Oxide as the Sole Electron Acceptor. Science 240(4857):1319-1321.

Pirt S. 1982. Maintenance energy: a general model for energy-limited and energy-sufficient growth. Archives of Microbiology 133(4):300-302.

Pirt SJ. 1965. The Maintenance Energy of Bacteria in Growing Cultures. Proceedings of the Royal Society of London. Series B, Biological sciences 163(991):224-231.

Ricquier D. 2005. Respiration uncoupling and metabolism in the control of energy expenditure. Proceedings of the Nutrition Society 64(1):47-52.

Russell JB, Cook GM. 1995. Energetics of bacterial growth: balance of anabolic and catabolic reactions. Microbiological Reviews 59(1):48-62.

Scott JH, Nealson KH. 1994. A biochemical study of the intermediary carbon metabolism of *Shewanella putrefaciens*. Journal of Bacteriology 176(11):3408-3411.

Senez JC. 1962. Some considerations on the energetics of bacterial growth. Bacteriological Revolutions 26(2 Pt 1-2):95-107.

Strand SE, Harem GN, Stensel HD. 1999. Activated-sludge yield reduction using chemical uncouplers. Water Environment Research 71(4):454-458.

Tsai SP, Lee YH. 1990. A model for Energy-sufficient culture growth. Biotechnology & Bioengineering 35:138-145.

BIBLIOGRAPHY (Continued)

Wei YS, Van Houten RT, Borger AR, Eikelboom DH, Fan YB. 2003. Minimization of excess sludge production for biological wastewater treatment. Water Research 37(18):4453-4467.

Weigel P, Englund P. 1977. Inhibition of DNA replication in Escherichia coli by Dibromophenol and other uncouplers. The Journal of Biological Chemistry 252(4):1148-1155.

Ye FX, Li Y. 2005. Reduction of excess sludge production by 3, 3', 4', 5-tetrachlorosalicylanilide in an activated sludge process. Applied Microbiology & Biotechnology 67(2):269-274.

Ye FX, Shen DS, Li Y. 2003. Reduction in excess sludge production by addition of chemical uncouplers in activated sludge batch cultures. Journal of Applied Microbiology 95(4):781-786.

Zeng AP, Deckwer WD. 1995. A kinetic model for substrate and energy consumption of microbial growth under substrate-sufficient condition. Biotechnology Progress 11(1):71-79.

APPENDICES

APPENDIX A-1

STOCK MICROBIAL CULTURE PREPARATION

AIM

To prepare a stock culture of Shewanella oneidensis MR-1, for long-term experimentation.

MATERIALS REQUIRED

10% TSB culture media
Starter S.oneidensis MR-1 culture
Centrifuge
2ml sterile Cryo-Vials (Corning)
Glycerin
0.1 µm, sterile, syringe filter

PROCEDURE

1. Autoclave 250 ml of 10% TSB solution in a 500ml capacity Kimax$^{®}$ bottle.

2. Add 250 µl of starter stock culture to the media and incubate at 30ºC in a shaker table, to provide adequate agitation.

3. Harvest cells at ~20 hours and divide them (40 ml each) into 6, 50 ml sterile centrifuge tubes and centrifuge them at 6000rpm for 10 minutes.

4. Pour out supernatants slowly, so as not to loose the cell pellets.

5. Add 4 ml of 10% TSB media into each tube and vortex vigorously to dissolve the cells.

6. Divide this culture and add 0.667 ml of it to each cryo-vial.

7. Add 0.3 ml, 0.1µm filter sterilized Glycerin to each vial.

8. Mix well by shaking and store in refrigerator at -75 ºC.

NOTE

1. For gfp-tagged MR-1 cells, use 1.3ml of 5000ppm Kanamysin Sulfate for cell growth in addition to all the above steps.

2. Gfp-tagged cells usually show a slower growth rate and so harvest them at ~24 hours.

MR-1 MINIMAL MEDIA PREPARATION

AIM

To prepare minimal growth media for *Shewanella oneidensis* MR-1 cells.

MATERIALS REQUIRED

Sterile centrifuge 50 ml plastic tubes
Sterile 5ml Falcon tubes
Autoclavable glass bottles, 2 liters capacity
Eppendorf Reference® (Adjustable volume) pipette, 500-2500 µl capacity
Eppendorf Reference® (Adjustable volume) pipette, 100-1000 µl capacity
Eppendorf Reference® (Adjustable volume) pipette, 50-200 µl capacity
Eppendorf Reference® (fixed volume) pipette, 25 µl capacity
epT.I.P.S, 500-2500 µl capacity, autoclavable
epT.I.P.S, 100-1000 µl capacity, autoclavable
epT.I.P.S, 20-300 µl capacity, Biopur, autoclavable
Sterile aerosol pipette tips, 1-40µl capcity
Acrodisc Supor 25 mm 0.1µm sterile syringe filters
B-D Plastic Syringes, 10 ml and 30ml capacity
Sterile 18G1 ½ or 20G1 ½ syringe needles
Magnetic stir bars
Thermolyne Cimarec® magnetic stirrer
Wheaton 500 ml sterile glass bottles
Aluminum foil
Autoclavable tape
Mettler Toledo weighing scale
symphony SR 601C pH/ISE/conductivity meter
DI Water

CHEMICALS REQUIRED

Please refer to MR 1 Minimal Media sheet
Sodium Hydroxide (10N and 1N)
Hydrochloric Acid (1N)

PROCEDURE

1. AMINO ACID MIX

a) Weigh 100mg of each of L-glutamic acid, L-arginine and D-L-serine using Mettler Toledo weighing scale.

b)   Add the measured salts to DI in a 250ml glass bottle and keep on adding DI to bring the solution upto 50ml mark.

c) Using the magnetic stir bar and Thermolyne Cimarec® magnetic stirrer, stir the solution to obtain a pure solution, with no particles seen floating.

d)   Adjust the pH of the solution to 7.0 using sodium hydroxide and hydrochloric acid solution. The pH measurements are made by using VWR symphony SR 601C meter.

e) Using a B-D plastic syringe (30 ml capacity) and Acrodisc Supor 0.1µm syringe filter, filter-sterilize the above prepared solution to a 50 ml sterile centrifuge plastic tubes.

f)   Store the solution at 4ºC in the walk-in refrigerator in a secondary containment.

2. VITAMIN MIX

a) B-12 Solution: Measure 3.5mg B-12 and add it to 3.5ml of DI and mix well.   Filter sterilize using 0.1µm syringe filter into a 5ml sterile plastic Falcon tube.

b) Add 500µl of stock B-12 solution to DI and add the corresponding quantities of other chemicals (see media recipe sheet) and bring the solution upto 100ml mark in a 250ml glass bottle.

c)   Using magnetic stirrer and stir bar arrangement, mix the solution thoroughly.

d)   Adjust the pH of the solution to 7 using sodium hydroxide and/or hydrochloric acid, if required.

e)   Using 0.1µm syringe filter, filter-sterilize the solution into an autoclaved glass bottle. Store the solution at 4ºC in the walk-in refrigerator in a secondary containment.

3. MINERAL MIX

a)   Measure corresponding quantities of chemicals (see media recipe sheet) and add to DI in a glass bottle. Mix by vigorous shaking.

b)   Add 10N sodium hydroxide to bring the pH of solution between 8-8.5, to dissolve NTA.

c) Add more DI to bring the solution upto 100ml mark.

d) Autoclave the solution for 30-35 minutes and store at 4ºC in the walk-in refrigerator in a secondary containment, after cooling it down to room temperature.

4. MAIN MEDIA

a) Sodium Selenate: Weigh 19mg of sodium selenate and add it to DI to make 10ml of solution and mix vigorously. Store the solution in 20ml glass scintillation vial at 4ºC in the walk-in refrigerator in a secondary containment.

b) Ferric NTA: Add 410mg Sodium bicarbonate, 640mg trisodium NTA and 405mg ferric chloride to DI and bring the solution upto 25ml mark in a 50ml plastic tube and mix thoroughly. Filter sterilize using 0.1μm syringe filter into a 50 ml sterile plastic Falcon tube. Store the solution at 4ºC in the walk-in refrigerator in a secondary containment.

c) Measure corresponding quantities of all the chemicals (see media recipe sheet) except sodium selenate, ferric NTA, mineral mix, amino mix and vitamin mix solutions. Add the measured chemicals to DI, in a 2 liter glass bottle and bring the solution to 1 liter mark. Mix it well using magnetic stirrer and stir bar arrangement.

d) Add 1m of 1mM sodium selenate or 0.1ml of stock selenate solution prepared above to the solution. Also add 1ml of mineral mix to the solution.

e) Using sodium hydroxide and/or hydrochloric acid solutions, bring the pH of the solution to 7.0.

f) Autoclave the solution for 30-35 minutes.

g) After bringing down the solution to room temperature, add 0.2ml of vitamin mix and 0.5ml of ferric NTA in a sterile fashion. Mix well and measure the pH and bring it to 7 by adding sodium hydroxide and hydrochloric acid. Make up for any volume lost by adding filter sterilized (through 0.1 μm filter) DI.

NOTE

a) For long term storage, keep all solutions sterile either by autoclaving or by filter- sterilization and keep them at 4ºC in secondary containment.

b) Always make sure to adopt sterile transfer techniques when working with sterile solutions. Use sterile pipettes and sterile plastic tubes or autoclaved glass bottles.

c) Always label all the solutions with the name of chemical inside, date prepared, concentration, name of person who prepared it and any other relevant information.

d) On any autoclaved bottle make sure to attach autoclavable tape before putting it in the autoclave.

e) Always make sure to work with the sterile solutions under a laminar flow hood to avoid any external contamination.

INOCULUM GROWTH

AIM

To grow *Shewanella oneidensis* MR-1 cells from frozen stock, to be used as starting culture in experimentation.

MATERIALS REQUIRED

50 ml sterile plastic tubes
Frozen *S.oneidensis* MR-1 stock
Sterile 10ml plastic pipette
Pipette bulb
MR-1 minimal growth media, 100mM pyruvate
Tryptic Soy Broth (TSB) media, full strength (30 gm/liter)
Eppendorf Reference® (Adjustable volume) pipette, 50-200 µl capacity
epT.I.P.S, 20-300 µl capacity, Biopur, autoclavable
95% ethanol solution
DI

PROCEDURE

a) Add 1 ml of stock full strength TSB media to 9 ml filter sterilized DI in a 50 ml sterile plastic falcon tube and mix well. In case of MR-1 minimal media, just take 10ml of sterile MR-1 minimal media in a 50ml sterile plastic falcon tube using a sterile 10ml plastic pipette.

b) Take out a vial of *S.oneidensis* MR-1 stock culture from the freezer (-75ºC) and keep it in the laminar flow hood for some time to bring it to room temperature. Add 100µl of these stock cells in a sterile fashion, using an autopipette and sterile tips, to plastic tube containing 10ml of growth media and mix well.

c) Incubate the plastic tubes at 30 ºC in the shaker table at an rpm of about 300 to allow proper shaking.

d) Harvest these cells when they are in exponential growth phase and add them to proper media at such a dilution factor so as to have an initial cell concentration of 10^6-10^7 cells. Usually, the harvesting period would be 12-15 hours and 24 hours for TSB and minimal media, respectively.

METABOLIC UNCOUPLING BY EXCESS SUBSTRATE

AIM

To study the effect of varying the pyruvate concentrations on the yield of *Shewanella oneidensis* MR-1

MATERIALS REQUIRED

500 ml glass bottles with caps
Frozen Stock of S.oneidensis MR-1
Micropipettes and sterile tips
Autoclave
Incubator
Compressed air supply
100 mM pyruvate Minimal Media
Blank minimal media
Sterile 25ml pipettes
Sterile 10 ml pipettes
Sterile 10 syringes
Sterile 0.1 syringe filters
Sterile 50ml plastic tubes
400 ppm Resazurin stock (Sterile)

PROCEDURE

1. STERILIZATION

   a) Sterile all the glassware and aeration arrangement in the autoclave.

   b) Let the autoclaved products cool down to room temperature. This might take probably couple of hours.

2. CULTURING

   a) INOCULUM: Bring the frozen stock to room temperature and sterilize its cap using ethanol and flame. Transfer about 100µl of frozen stock to 10ml, 100mM pyruvate minimal media and mix it by shaking. In a similar fashion, grow inoculum in 2 other tubes, totaling 30ml inoculum. Use sterile pipettes for transferring the media to the tube. Incubate the culture for 24 hours.

b) BATCH BOTTLES: Pour the calculated amounts of blank and pyruvate media into bottles so as to have pyruvate concentration of 5 mM, 20mM, 50mM and 100mM in different bottles, in 250 ml total media. Flame the lip of the media bottle each time a transfer is made. The cultures are to be grown in <u>duplicates</u>. A total of 8 bottles would be prepared. Use the following volumes to obtain desired substrate concentrations

S0 desired (mM Pyr)	Volume of blank media (ml)	Volume of 100mM pyruvate media (ml)	Final Volume (ml)
5	237.5	12.5	250
20	200	50	250
50	125	125	250
100	0	250	250

c) Resazurin: Add 625 µl resazurin to each bottle and mix.

d) After 24 hours of growth, take the inoculum bottles out of incubator and mix them in the Laminar-flow hood. Add 2.5ml of inoculum to each bottle and mix by shaking.

e) Incubate the cultures at 30°C and appropriate rpm. (~ 150-200)

f) AERATION: As soon as the cultures turn purple (usually ~8-9 hours), start the aeration using compressed air cylinder.

g) SAMPLING: 1) Substrate utilization: Take roughly 2-3 ml of samples at *t=0, 24,36 and 48 hours* using sterile syringes and filter them using sterile syringe filters and freeze them.
2) Direct Cell Enumeration: Take samples from inoculum for initial cell count. Withdraw about 1ml of cultures from each bottle at t=24, 36 and 48 hours.

h) Run the experiment for 48 hours and then dismantle the arrangement and analyze the samples.

i) ANALYSIS: Use HPLC for measuring the amount of pyruvate utilized as well as acetate produced. Epifluorescence microscopy is to be used with DAPI as staining dye.

j) Use sterile 5ml plastic tubes for taking out samples.

k) Use the following model for fitting the data:

$$\frac{1}{Y_{obs}} = \frac{1}{Y_{obs}'} + \frac{1}{(Y_w)_{min}} \times \frac{(S_o/X_o)}{(S_o/X_o) + K_{s/x}}$$

Yield is calculated as the ratio of cell concentration to amount of pyruvate utilized, in the corresponding time interval.

METABOLIC UNCOUPLING BY TCS ADDITION

AIM

To study the effect of addition of different TCS concentrations on the yield of *Shewanella oneidensis* MR-1

MATERIALS REQUIRED

500 ml glass bottles with caps
Frozen Stock of S.oneidensis MR-1
Micropipettes and sterile tips
Autoclave
Incubator
Compressed air supply
100 mM pyruvate Minimal Media
Blank minimal media
Sterile 25ml pipettes
Sterile 10 ml pipettes
Sterile 10 syringes
Sterile 0.1 syringe filters
Sterile 50ml plastic tubes
400 ppm Resazurin stock (Sterile)
Stock TCS solution (500ppm)

PROCEDURE

1. STERILIZATION

   a) Sterile all the glassware and aeration arrangement in the autoclave.

   b) Let the autoclaved products cool down to room temperature. This might take probably couple of hours.

2. CULTURING

   l) INOCULUM: Bring the frozen stock to room temperature and sterilize its cap using ethanol and flame. Transfer about 100µl of frozen stock to 10ml, 100mM pyruvate minimal media and mix it by shaking. In a similar fashion, grow inoculum in 2 other tubes, totaling 30ml inoculum. Use sterile pipettes for transferring the media to the tube. Incubate the culture for 24 hours.

m) BATCH BOTTLES: Pour 50 ml, 100mM pyruvate media in a 500ml sterile Kimax bottle and add 200ml blank minimal media to it, making 20mM pyruvate minimal media. Flame the lip of the media bottle each time a transfer is made. The cultures are to be grown in <u>duplicates</u>. A total of 8 bottles would be prepared, a similar fashion.

n) RESAZURIN: Add 625 µl resazurin to each bottle and mix.

o) After 24 hours of growth, take the inoculum bottles out of incubator and mix them in the Laminar-flow hood. Add 2.5ml of inoculum to each bottle and mix by shaking.

p) Incubate the cultures at 30ºC and appropriate rpm. (~ 150-200)

q) AERATION: As soon as the cultures turn purple (usually ~8-9 hours), start the aeration using compressed air cylinder.

r) TCS ADDITION: At t=24hours, add 0.25 ml, 0.5ml and 1 ml of 500ppm sterile TCS stock solution to 2 bottles each. This would give a TCS concentration of 0.5, 1 and 2 ppm respectively to 2 bottles each (duplicates). Remaining 2 bottles would serve as the controls.

s) SAMPLING

    i. Substrate utilization: Take roughly 2-3 ml of samples at *t=0, 24,36 and 48 hours* using sterile syringes and filter them using sterile syringe filters and freeze them.

    ii. Direct Cell Enumeration: Take samples from inoculum for initial cell count. Withdraw about 1ml of cultures from each bottle at *t=24, 36 and 48 hours.*

t) Run the experiment for 48 hours and then dismantle the arrangement and analyze the samples.

u) ANALYSIS: Use HPLC for measuring the amount of pyruvate utilized as well as acetate produced. Epifluorescence microscopy is to be used with DAPI as staining dye.

v) Use sterile 5ml plastic tubes for taking out samples.

w) Use the following model for fitting the data:

$$\frac{1}{Y_{obs}} = \frac{1}{Y_{obs}'} + \frac{1}{(Y_w)_{min}} \times \frac{(S_o/X_o)}{(S_o/X_o) + K_{s/x}} + \frac{1}{(Y_{wu})_{min}} \times \frac{(C_u/X_o)}{(C_u/X_o) + K_{u/x}}$$

Yield is calculated as the ratio of cell concentration to amount of pyruvate utilized, in the corresponding time interval.

MICROSCOPIC ENUMERATION OF MICROBIAL CULTURES
(using PETROFF-HAUSSER Counting Chamber)

AIM

> To obtain a direct count of cells in a given culture and estimate the cell concentration

MATERIALS REQUIRED

> Phase contrast microscope
> Petroff-Hausser counting chamber
> Eppendorf Reference® (fixed volume) pipette, 25 µl capacity
> Sterile aerosol pipette tips, 1-40µl capcity
> 95% ethanol solution
> Methylene blue dye
> Glass coverslips, No. 1 ½

PROCEDURE

1. Add 25µl of diluted culture on each side ditch of the counting chamber using micropipette and sterile pipette tips.

2. Hold the glass coverslip at an angle and gently place it over the central portion of the counting chamber. Avoid any air bubbles between the coverslip and chamber.

3. View the counting chamber through the microscope starting at the lowest resolution and progress to higher resolution (usually 40X eyepiece). Avoid the use of oil-immersion microscopy (100X eyepiece), unless required.

4. Count the cells in square groups at the ends and in the centre to get a representative enumeration of the culture.

5. Start with the cells at the bottom interface of a square group. Usually, the bottom interface is characterized by sharp image of the lines on the counting chamber. Count the clearly visible cells that are relatively immobile and leave out the cells that are moving through the squares. At the boundaries, count the cells lying on the upper & right corner and leave the cells on the left corner or the lower side. Note down the micrometer reading on the side of the microscope.

6. Rotate the fine focus slightly till the top interface is reached. The top interface is usually characterized by bare visibility of the cells. Generally, the top interface contains much less cells compared to the bottom interface. Note down the micrometer reading for the top interface and calculate the cell film thickness as the difference in the micrometer readings for the top and bottom interfaces.
7. Move through the top and bottom interfaces to account for any cells missed out in the counting. It is generally easy to count all the cells in each square group (consisting of 16 small squares) instead of counting cells in each small square.

8. Use the same procedure to count the cells in other square groups. Count as many cells as is possible within the given time frame, usually a count of 500-600 is sufficient. In other words, a count of 5-15 cells per small square is considered appropriate. The cell concentration in the original culture is calculated by using the following equation:

Cell concentration (as cells/ml) =

$$\frac{\text{Total cells counted} \times \text{dilution factor} \times 4 \times 10e8}{\text{Number of small squares counted} \times \text{Average cell film depth (in micrometers)}}$$

9. Clean the counting chamber with soap, water and alcohol and let it air dry.

Note: A tally sheet for enumeration is attached. It contains 25 square groups, each consisting of 16 small squares. Ideally, count all the cells in the four corners and one central square group i.e. count 80 small squares in all. Any other suitable and representative number of squares can also be counted.

SLIDE PREPERATION FOR EPI-FLUORESCENCE MICROSCOPY

AIM

To measure the cell concentration (in cells/ml) using epi-fluorescence microscopy.

MATERIALS REQUIRED

Epifluorescence Microscope (Nanonics Imaging Ltd., Israel)
UPLFL 100x objective
DAPI filter-cube
1 ppm 4'-6-Diamidino-2-phenylindole (DAPI) solution
Hybridization Buffer (150 mM NaCl, 20 mM Tris/HCl, 5mM EDTA and 0.01% sodium dodecyl sulfate)
Glass Slide and Cover Slip (No.1)
Prolong® Gold Antifade reagent (Molecular Probes, Eugene, OR)

PROCEDURE

1. Add 0.5 ml hybridization buffer to 0.1ml of appropriately diluted cells in a sterile falcon tube using auto-pipette and sterile pipette tips. Vortex the mixture and let it stand for 1 min.

2. Add 1 ml of 1ppm DAPI solution and vortex it thoroughly. Wrap the falcon tube in aluminum foil to avoid photo-exposure of the dye. Allow the solution to stand for about 1 min.

3. Filter the mixture on a 0.22 µm black, polycarbonate filter using filter assembly at a vacuum pressure of 5 psi.

4. Pour 2 ml of filter sterilized DI over the filter to rinse any remaining DAPI, wait for 2 minutes and let it dry under air pressure.

5. Using forceps, place the filter onto a glass slide and pour a drop of Prolong Gold antifade reagent over it. Seal the filter with a No. 1 cover slip. Store the slide in freezer.

PYRUVATE MEASUREMENT BY HPLC

AIM

To determine the amount of Pyruvate utilized by the cells, using High Performance Liquid Chromatography (HPLC).

MATERIALS REQUIRED

Dionex HPLC
Auto-sampler Cassette
Dionex Polyvials, 0.5 ml capacity
Autopipettes and sterile pipette tips
Gluteraldehyde
Acetonitrile
Phosphoric Acid (H_3PO_4)
Potassium Phosphate monobasic (KH_2PO_4)
Nano-pure DI
Blank Minimal Media

PROCEDURE

1. Prepare the eluent for HPLC by mixing 6804.5 mg KH_2PO_4, 40 ml H_3PO_4 and 75 ml Acetonitrile and making a solution volume of 2 liters in nano-pure DI.

2. Open the cap of eluent bottle a little bit and fill it with the eluent. Make sure that the piping remains always dipped in the eluent. Close the cap of eluent bottle.

3. Adequately dilute the samples and pour 600 µl of each into polyvials and close them with vial-filters. Prepare standards in a similar fashion. Always start the analysis with a 10% glutaraldehyde sample, to purge any microbial accumulation in the system.

4. Open the gas flow by turning the knob on left side of eluent bottle, gently in the anti-clockwise fashion twice.

5. Load the samples into autosamplers using the autosampler cassettes. Don't put more than 4 casettes at a time. Press the "Hold/Run" switch on the autosampler panel.

6. Go to the main menu button on the Dionex HPLC program and press it. Open up a new schedule and load the sample names, method to be used and data storage file name. Save the file. The last entry is always the stop program.

7. Go back to the main menu and load the schedule.

8. Wait for 10-15 minutes till the number on the AD_{20} panel is close to 0.

9. Start the HPLC by clicking on the appropriate symbol in the main screen. Each sample takes roughly 20 minutes.

10. Once all the samples are done and the HPLC reads the stop program, shut off the gas flow by turning the knob in clockwise fashion twice. Open the cap of eluent bottle to release the pressure and close it again.

PROTEIN MEASUREMENT

AIM

To determine the total protein content of a given microbial sample.

MATERIALS REQUIRED

2.0 ml VWR® Microcentrifuge Tubes
Micro BCA™ Protein Assay Kit (Pierce Biotechnology, IL)
Compat-able™ Protein Assay Preparation (Pierce Biotechnology, IL)
Autopipette and sterile pipette tips
Blank culture media
Water Bath
Centrifuge

PROCEDURE

1. Prepare diluted BSA standards as follows, using the Stock BSA standard (2 mg/ml):

Sample #	Stock BSA Vol (ul)	Blank vol (ul)	Final BSA conc (ppm)
A	100 of Std	900	200
B	75 of Std	925	150
C	50 of Std	950	100
D	375 of A	625	75
E	250 of A	750	50
F	167 of B	833	25
G	100 of C	900	10
H	100 of E	900	5
I	0	1000	0

2. Dispense 100 µl of adequately diluted sample into microcentrifuge tube.

3. Add 500 µl of Compat-Able reagent 1 to each tube. Mix each tube and allow to stand at room temperature for 5 minutes.

4. Add 500 µl of Compat-Able reagent 1 to each tube. Mix each tube and centrifuge at 14000 G for 10 minutes.

5. Invert each tube and discard the supernatant. Blot the open end of inverted tube on clean paper. The protein pellet would be seen at the sides of the tube.
6. Make working reagent (WR) solution by mixing 6.25 ml of Reagent MA, 6 ml of Reagent MB and 0.25 ml of Reagent MC.

7. Add 100 µl of WR to each tube to dissolve the protein pellet. Vortex vigorously to solubilize the pellet.

8. Add 1 ml of WR solution into each tube, mix and heat at 60º C for 60 minutes, using the water bath.

9. Centrifuge for 2 minutes and cool down at room temperature for 20 minutes.

10. Measure absorbance at 562 nm within 10 min, using a visible-spectrophotometer.

NOTE

1. The protein samples remain stable for upto 1 week when stored in refrigerator.

2. The protein standards should be prepared fresh each time the analysis is done, however the stock BSA solution remains stable upto 24 hours, once the vial is opened.

3. Always verify the OD results by running random samples again.

4. Measure the absorbance as soon as possible. With time, the samples tend to turn dark.

5. Always use blank media (media without substrates), for standard preparation and sample dilutions.

6. The samples should be diluted to reduce the protein content to less than 50 µg/ml.

APPENDIX B

MR 1 Minimal Media

per L	g	ml	FW	mM
PIPES	0.91		302.4	3.0
Sodium Pyruvate	11.004		110.04	134.1
NaOH	0.3		40	7.5
NH4Cl	1.5		53.5	28.0
KCl	0.1		74.55	1.3
NaH2PO4H2O	0.6		137.99	4.35
fumaric acid (if no O2)	4.8		160.1	30.0
NaCl (if O2)	1.753		58.44	30.0
Na2SeO4		1 ml of 1mM into 1 liter		1 um
Fe(III)NTA (100mM)		0.5 of 100 mM		0.05
mineral solution		1 of 10X		
amino acids solution		1of 1000X		
vitamin solution		0.2 of 50X		

Set pH to 7.0 after all the components have been added
using NaOH or HCl (After Autoclaving)
Fe(III) NTA, amino mix and vitamin mix: add after autoclaving rest of media
dissolved in 970 ml water, add NaOH pellets until pH 7.0

APPENDIX B (Continued)

Vitamin Mix (Lovely's)			Prepared 50X	
			Conc in media	Conc in media
	mg/L	g/L	(g/L)	(mg/L)
biotin	2	0.002	2.00E-05	0.02
folic acid	2	0.002	2.00E-05	0.02
pyridoxine HCl	10	0.01	1.00E-04	0.1
riboflavin	5	0.005	5.00E-05	0.05
thiamine HCl	5	0.005	5.00E-05	0.05
nicotinic acid	5	0.005	5.00E-05	0.05
d-pantothenic acid	5	0.005	5.00E-05	0.05
B-12	0.1	0.0001	1.00E-06	0.001
p-aminobenzoic acid	5	0.005	5.00E-05	0.05
thioctic acid	5	0.005	5.00E-05	0.05

Adjust pH to 7, filter sterilize

Amino Acid Mix			Prepared 1000X	
			Conc in media	Conc in media
	mg/L	g/l	(g/L)	(mg/L)
L-glutamic acid	200	0.2	2.00E-03	2
L-arginine	200	0.2	2.00E-03	2
D-L- Serine	200	0.2	2.00E-03	2

Adjust to pH 7, filter sterilize

Mineral Mix (Lovely's)			Prepared 10X	
			Conc in media	Conc in media
	mg/L	g/l	(g/L)	(mg/L)
NTA	1500	1.5	1.50E-02	15
MgSO4	3000	3	3.00E-02	30
MnSO4 * H2O	500	0.5	5.00E-03	5
NaCl	1000	1	1.00E-02	10
FeSO4 * 7 H2O	100	0.1	1.00E-03	1
CaCl2 * 2 H2O	100	0.1	1.00E-03	1
CoCl2 * 6 H2O	100	0.1	1.00E-03	1
ZnCl2	130	0.13	1.30E-03	1.3
CuSO4 * 5 H2O	10	0.01	1.00E-04	0.1

AlK(SO4)2 * 12 H2O	10	0.01	1.00E-04	0.1
H3BO3	10	0.01	1.00E-04	0.1
Na2MoO4	25	0.025	2.50E-04	0.25
NiCl2 * 6 H2O	25	0.025	2.50E-04	0.25
Na2WO4 * 2 H2O	25	0.025	2.50E-04	0.25

after adding NTA. pH to 8-8.5 with 10N NaOH to dissolve NTA.

Allow each ingredient to dissolve completely before adding
Autoclave or filter sterilize: Not necessary, but sterilize in media

APPENDIX C-1

Figure 1: Epifluorescence image of S.oneidensis MR-1, without uncoupler addition. (Image size: 40 µm x 40 µm)

Figure 2: Epifluorescence image of S.oneidensis MR-1 , with addition of 1

ppm TCS (Image size: 40 μm x 40 μm)