Proceedings of the Twenty-Sixth Annual Biochemical Engineering Symposium

Larry E. Erickson
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Proceedings of the
Twenty-Sixth Annual Biochemical
Engineering Symposium
September 21, 1996

Larry E. Erickson
Editor

Department of Chemical Engineering
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Manhattan, Kansas 66506
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FOREWORD

This volume contains the Proceedings of the Twenty-Sixth Annual Biochemical Engineering Symposium held at Kansas State University on September 21, 1996. The program included 10 oral presentations and 14 posters. Some of the papers describe the progress of ongoing projects, and others contain the results of completed projects. Only brief summaries are given of some of the papers; many of the papers will be published in full elsewhere. A listing of those who attended is given below.

LIST OF PARTICIPANTS

Colorado State University - Dr. Allen Rakow, Adeyma Arroyo, Zoila Flores-Bustamante, Mark Heinrich, Narendra Poflee, Carlos Puente, and Laurent Simon.

Iowa State University - Dr. Carole Heath, Dr. Zivko Nikolov, Dr. Tunde Oguntimein, Dr. Peter Reilly, Ryan Cooper, Weiyu Fan, Ruth Fink-Winter, Peng Jin, Kevin Liu, Mark Mowry, Kaz Ohmori, Greg Rutkowski, Dave Wendt, and Chenming Zhang.

University of Colorado - Dr. Rob Davis, Dr. Dhinakar S. Kompala, Dr. Paul Todd, Gautam Banik, Naomi Breckenridge, Piotr Czekaj, Jeff Heys, Vinod Kuberkar, Huimin Ma, Sanxiu Lu, Rick St. John, Arun Tholudar, Jon Webb, and Jing Xu.

University of Kansas - Dr. Soma Chakrabarti, Dr. Marylee Z. Southard, Sachiko Iwashita, Moi Huah Liew, Michael Rigney, and Sridhar Sunderam.

University of Missouri - Dr. Rakesh Bajpai, Dr. George Preckshot, Jun Gu, and Xi-Hui Zhang.

University of Oklahoma - Dr. Roger Harrison, Greg Davis, Brad Forlow, and Laura Worthen.

Kansas State University - Dr. Larry E. Erickson, Ryan Green, Jiang Hu, Melissa Miller, Muralidharan Narayanan, Krishnakumar Nedunuri, Xiaowei Wu, and Qizhi Zhang.
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Symposium Program
Foreign Protein Production From SV40 Early Promoter in Continuous Cultures of Recombinant CHO Cells

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Key Word: Cell cycle, CHO cells, Continuous culture, SV40 promoter, β-interferon production, β-galactosidase, Growth-associated production.

ABSTRACT
Foreign protein expression from the commonly used SV40 promoter has been found to be primarily in the S-phase of the cell cycle. Simple mathematical models with this cell cycle phase dependent expression of foreign protein suggest that the specific production rate will be proportional to the specific growth rate, which is particularly disadvantageous in high cell density perfusion or fed-batch bioreactors. In this study we investigate this predicted relationship between the production rate and the growth rate by culturing recombinant CHO cells in a continuous suspension bioreactor. One recombinant CHO cell line, 2FS-21, has been stably transfected with the plasmid pSVifneo, which contains the β-interferon gene and the gene for resistance to neomycin under the control of the SV40 early promoter. The second recombinant CHO cell line, GS-26, has been stably transfected with the plasmid pSVgalneo, which contains the E. coli lac Z gene and the gene for the resistance to neomycin under the control of the SV40 early promoter. Both cell lines was cloned under a selection pressure of 400 µg/ml G418 and the two high producing cell lines were grown in suspension cultures over a range of specific growth rates in batch and continuous bioreactors. The secreted glycoprotein β-interferon was assayed using SDS-PAGE and densitometry. The intracellular β-galactosidase activity was assayed using a standard spectrophotometric method after breaking the cells open and releasing the enzyme.

INTRODUCTION
Two strikingly different patterns of the specific glycoprotein production rate versus mammalian cell growth rates are commonly found in the literature. The first pattern, observed primarily in monoclonal antibody production rate from murine hybridoma cells, shows an inverse relationship between specific antibody production rate per cell versus the cell growth rate. That is, the specific production rate increases significantly as the specific growth rate is decreased in continuous chemostat and perfusion cultures (Miller et al., 1988; Batt et al., 1990; Suzuki and Ollis, 1990). Repeated observations of higher specific monoclonal antibody production rate in many growth-inhibiting culture conditions, such as increased osmolality (Ozturk and Palsson, 1991a), high ammonia concentrations (Miller et
al. 1988), low pH (Miller et al. 1988; Oaturk and Palsson, 1991b), high pH (Miller et al. 1988) suggest the hypothesis that reduced cell growth rate under these different suboptimal growth conditions may be the common mechanistic factor in causing the higher antibody production rate. The second pattern of glycoprotein production rate versus cell growth rate is observed increasingly in recombinant mammalian cell cultures. In an early example of this pattern, the specific antibody production rate in transfected myeloma cell lines is found to be strongly proportional to growth rate (Robinson and Memmert, 1991). Further examples of this directly proportional relationship between specific production rate and cell growth rate were observed during the production of recombinant glycoproteins in Chinese hamster ovary (CHO) cells at elevated pCO$_2$ levels (Aumins and Hentsch, 1993; Kimura and Miller, 1996) and at varying serum concentrations (Leelavacharamas et al. 1994).

These two different patterns of protein synthesis have been reconciled by hypothesizing different cell cycle phase specific expression in a mathematical model for an intracellular reporter protein (Gu et al., 1994). The inverse relationship between the specific monoclonal antibody production rate and the cell growth rate has been generated by a previous cell cycle model (Suzuki and Ollis, 1989) assuming that the cells secrete the antibody only during the G1 phase of the mammalian cell cycle. Experimental confirmations of the G1 phase expression of antibody by hybridoma cells have now been obtained by several researchers (Suzuki and Ollis, 1990; Ramirez and Mutharasan, 1990; Kromenaker and Sieri, 1991). Prediction of the proportional relationship between the intracellular reporter protein content and the cell growth rate (Gu et al., 1994) is based on the assumption that the reporter protein is synthesized only during the S (DNA synthesis) phase of the cell cycle. Several experimental studies suggest that commonly used promoters and enhancer elements driving the expression of foreign proteins are active maximally in the S (DNA synthesis) phase of the cell cycle. Mariani et al. (1981) found S phase-specific synthesis of dihydrofolate reductase (DHFR) from SV40 early promoter in Chinese hamster ovary (CHO) cells. Kubbies and Stockinger (1990) found that the expression of tissue plasminogen activator (tPA) from SV40 early promoter in CHO cells was again S phase-specific. The expression of β-galactosidase from CMV promoter in CHO cells was also shown to be S phase-specific by Gu et al. (1993). In the work of Kubbies and Stockinger (1990) it appears however, that the expression of DHFR from adenovirus major late promoter (AMLP) in CHO cells was G1 phase-specific.

From the similar S phase synthesis of two different proteins (the intracellular enzyme DHFR and the secreted glycoprotein tPA) from the same SV40 early promoter in two different CHO cell lines, it may be argued that the cell cycle phase in which foreign genes are expressed remains unaffected by changing the structural gene. However, changing the promoter and enhancer elements driving the expression of the same structural gene dhfr (from the SV40 early promoter to the AMLP promoter) appears to alter drastically the cell cycle phase in which DHFR is expressed from S to G1 phase. Based on such observations we have hypothesized that the upstream promoter and enhancer elements uniquely determine the cell cycle phase in which the downstream structural genes are expressed. Particularly, we conclude that the SV40 promoter commonly used for driving the foreign gene expression in recombinant mammalian cells is S phase specific. In this study, we seek to determine the consequences of using this S-phase specific promoter on the production rate of a secreted glycoprotein (β-interferon) and an intracellular reporter protein (β-galactosidase) at different growth rates in continuous suspension cultures of recombinant CHO cells.

**MATERIALS AND METHODS**

**Cell Lines:** Host cell line CHO-202 was stably transfected with plasmid pSVifneo (Canaani and Berg, 1982), which contains the β-interferon gene and the gene for resistance to neomycin both under the influence of the SV40 early promoter. The same host cells were transfected separately with the plasmid pSVgalneo (obtained from Dr. Pratt, Cornell
University), which contains the Lac Z gene and the gene for neomycin resistance both under the influence of the SV40 promoter. The two transfected cells were maintained under selection pressure in the neomycin analog, G418 at 400 μg/ml to prevent the growth of plasmid free cells. The transfected cells were then cloned, and two high producing cell lines (2FS-21 for the β-interferon producing cells, and, GS-26 for the β-galactosidase producing cells) were isolated and used in all the experiments discussed below.

Medium and Supplements: Both 2FS-21 and GS-26 cell lines were cultivated in serum-free IS-CHO medium (Irvine Scientific Company, Irvine, CA). The medium contained 8 g/l glucose and 8 mM glutamine. Penicillin and streptomycin were added to the medium at 100 units/ml. Medium was supplemented with 400 μg/ml G418 sulphate (Sigma, St. Louis, MO) for selection pressure.

Cell Culture and Measurements: Both GS-26 and 2FS-21 cells were adapted for growth in suspension culture, and all data shown below were obtained during cell growth in suspension culture. Continuous suspension culture experiments were conducted in two 1.5 liter Celligen bioreactor vessels, which had a working volume of 1100 ml. The dissolved oxygen concentration was controlled at 20% of air saturation and the pH was controlled at 7.2 ± 0.1 by sparging a mixture of air, nitrogen, carbon dioxide and oxygen into the bioreactor culture medium.

The viable cell density and percentage of viable cells were determined using a haemacytometer to count the cells after staining with trypan blue. Before staining with trypan blue the cell samples were treated with 0.25% w/v trypsin for 15 minutes at 37°C since the cells had a small tendency to aggregate in suspension culture. Incubation in trypsin before counting did not decrease cell viability.

SDS-PAGE: For β-interferon analysis and quantification a discontinuous SDS-PAGE system was used. A ready made SDS-Polyacrylamide gel casting system (Life Technologies, Cat # 15574-015, Grand Island, NY) was used for preparation of all gels. 0.4 ml of 10% ammonium persulphate was added to 16.8 ml of distilled water. 10 ml of resolving buffer and 12.8 ml of 40% w/v acrylamide-bis solution to prepare 40 ml of the 12% resolving gel. The stacking gel was prepared by mixing 9 ml of the stacking gel buffer, 1 ml of 40% w/v acrylamide-bis solution and 0.05 ml of 10% ammonium persulphate. A vertical gel electrophoresis system (Gibco BRL Model V-16-2, Rockville, MD) was used for all electrophoresis experiments. 20 μl of sample, standard (human fibroblast β-interferon, Lee Biomolecular Inc., San Diego, CA, Cat # 10151 Lot # 88032), or prestained protein ladder was mixed with 20μl of 2X sample buffer (100 mM Tris-HCl pH 6.8, 100 mM DTT, 4% w/v SDS, 0.2% w/v bromophenol blue, 20% w/v glycerol), and the mixture was heated for 20 mins at 80°C in a water bath. 25 μl of the mixture was loaded onto the gel, and electrophoresis was carried out at 100 V for an hour until the bromophenol blue band moved into the resolving gel. The voltage was then stepped to 225 V. Electrophoresis was carried out for another 3 hours.

Densitometry: SDS-PAGE gels revealed a single band which corresponded with the band for β-interferon standard. Hence for facile quantification of β-interferon a densitometry technique was used. After running the samples and various concentrations of the standard by SDS-PAGE the gel was stained using Blueprint Fast-PAGE stain (Gibco cat # 15587-017). After destaining with 10% acetic acid for 20 min the gels were scanned using a densitometer (Molecular Devices, Inc., Sunnyvale, CA) and the peak intensity was measured using MD ImageQuant™ software. A calibration curve was prepared using the peak intensities of the β-interferon standard bands with known concentrations added to the
wells. For the range of concentrations of β-interferon in the culture samples the calibration curve was found to be linear with \( R^2 > 0.95 \). Known concentrations of standards were run on each gel, and the β-interferon concentrations in the culture samples were obtained from the standard curves determined for each individual gel.

**β-galactosidase Measurement:** The activity of intracellular reporter protein (β-galactosidase) was measured using an assay with ONPG (o-nitrophenyl-β-D-galactoside), which is hydrolyzed by the enzyme yielding a yellow colored product. 0.5 ml of cell suspension was sonicated (Heat Systems-Ultrasound, Inc., Model W-380) for 120 seconds at 50% duty cycle while immersed in an ice-water bath. 1.3 ml of fresh Z buffer (0.06 M Na₂HPO₄, 0.04 M NaH₂PO₄, 0.01M KCl, 0.01 M MgSO₄, 5.4 ml/L β-mercaptoethanol, pH 7.0) and the lysate solution were added to 0.2 ml of 0.4% ONPG solution. The absorbance of the reaction was measured as a function of time at 28°C using a programmable spectrophotometer at 420 nm. Because the reaction rate is zero order when ONPG is in excess, the activity of β-galactosidase is proportional to the slope of the product accumulation curve. To determine the concentration of β-galactosidase in the sample, a standard solution of β-galactosidase was also assayed using the same procedure. The concentration of β-galactosidase in the sample was calculated by comparing the slope of the sample assay with that of the standard β-galactosidase solution.

**RESULTS**

The 2FS-21 cell line was grown in a 1.5 liter Celligen bioreactor having a working volume of 1100 ml. The time courses of viable cell density and total cell density in the bioreactor are shown in Fig. 1a. The cells were grown initially in batch mode and when the cells were in the exponential phase of growth (at 50 h) medium feed and removal were initiated. Immediately after initiating the continuous mode the cells undergo a transient phase in which there is an initial washout of cells followed by rapid growth. Cells were grown in continuous suspension culture until a steady state was obtained for each of the seven dilution rates. The maximum specific growth rate of the 2FS-21 cells was determined from batch cultures to be about 0.8 day⁻¹. To obtain a wide range of steady state growth rates, very low dilution rates such as 0.12 day⁻¹ were used. These low dilution rates were obtained by connecting the feed pump to a timer device. The timer has an operation cycle of one minute and the pump operated at only a fraction of a minute to get the desired flow rate. Since the response time of the system is very large compared to the intermittent operation of the pump such an operation is justified in obtaining steady states. Even at very low dilution rate of 0.12 day⁻¹ the cell viability was high (>67%). The dilution rates were changed in a random order and not in a monotonically decreasing or increasing order to prevent any bias in obtaining our results.

Figure 1b shows both the time course of β-interferon concentration in the bioreactor and the specific β-interferon production rate on a viable cell basis. Immediately following the start of continuous mode there is a high variation in both the β-interferon concentration and the specific β-interferon production rate. These points are not necessarily outliers but may be related to the transients in cell growth rates after the transition from batch culture. The variation in specific β-interferon production rate throughout Fig. 1b is caused by material balance calculations using the measured value of β-interferon concentration and the viable cell density. Therefore errors in measurement of either of these quantities are reflected in the specific β-interferon production rate. From Fig. 1b however, it is possible to observe the trend that as the dilution rate is increased, there is an increase in the steady state specific β-interferon production rate. These trends are even more convincing since the dilution rates were varied in a random order.
The GS-26 cell line was grown in two 1.5 liter Celligen bioreactors having a working volume of 1000 ml each. The total and viable cell concentrations in one of the bioreactors are plotted versus time in Figure 2a. Cells were grown initially in the batch mode until maximum cell densities were obtained and then medium feed and removal were initiated. As is usually seen, immediately after initiating the continuous mode, the cells undergo a transient phase in which there is an initial washout of cells followed by rapid cell growth. Cells were grown until steady states were well established at a total of 4 different dilution rates. Dilution rate of 1.2 day\(^{-1}\) was repeated in both the reactors to check the reproducibility of the steady states in the two different bioreactors. Dilution rate of 1.3 day\(^{-1}\) exceeds the maximum specific growth rate slightly and at this dilution rate there is a washout of cells from the reactor, as expected.

Figure 2b shows both the volumetric \(\beta\)-galactosidase concentrations and the intracellular \(\beta\)-galactosidase content in the cells from the same bioreactor plotted versus time. The volumetric \(\beta\)-galactosidase concentration is a product of the viable cell density and the intracellular \(\beta\)-galactosidase content, and therefore reaches a steady state at a time which corresponds to a steady viable cell density in the reactor. When the dilution rate is switched from \(D = 0.65\) day\(^{-1}\) to \(D = 1.2\) day\(^{-1}\) both the volumetric \(\beta\)-galactosidase concentrations and the intracellular \(\beta\)-galactosidase content reach steady states faster since the cell doubling times are almost halved. At washout or at \(D = 1.3\) day\(^{-1}\) both the cell density and the volumetric \(\beta\)-galactosidase concentrations in the reactor decrease monotonically. However, the intracellular \(\beta\)-galactosidase content in the reactor reaches a steady state in about 40 hrs following the switch to \(D = 1.3\) day\(^{-1}\) which corresponds to a time interval of less than three times the doubling time of cells at maximum specific growth rate.

Figure 3a also shows the standard deviations for the specific \(\beta\)-interferon production rate values for each steady state. The results clearly show a linear increase in the specific \(\beta\)-interferon production rate with specific growth rate. The steady state values of the intracellular \(\beta\)-galactosidase content in the reactor is shown as a function of the dilution rates investigated in both the reactors in Figure 3b. The two data points at \(D = 1.2\) day\(^{-1}\) correspond to the two steady states - one in each reactor. The results clearly show a dramatic increase of steady state intracellular \(\beta\)-galactosidase content with the dilution rate (or the specific growth rate) of the GS-26 cells in continuous cultures.

**DISCUSSION**

Several investigators (Gu et al., 1994; Suzuki and Ollis, 1989) have suggested that foreign protein production can be maximized by utilizing different bioreactor operating strategies appropriate for the particular pattern in which the foreign protein production varies with the cell growth rate. For example, the inverse relationship between the production rate and growth rates, may be exploited by growing cells at reduced growth rates in high density fed-batch or perfusion cultures to maximize the production of foreign proteins, whereas the direct relationship between the two will require maintaining the cells at high growth rates in repeated batch cultures or cause severely reduced production during the low growth fed-batch and perfusion cultures. For hybridoma cultures, which primarily exhibit the inverse relationship between the specific antibody production rate and cell growth rate (Miller et al., 1988; Suzuki and Ollis, 1990), the volumetric monoclonal antibody production rate can be increased significantly by growing cells at low specific growth rates in continuous perfusion cultures (Batt et al., 1990). In contrast, glycoprotein production in recombinant mammalian cells is typically found to be strongly growth associated with respect to their specific productivity (Aunins and Henzler, 1993; Leelacharamas et al., 1994; Robinson and Memmert, 1991). This proportional relationship makes high cell density fed-batch and perfusion systems unattractive for chimeric antibody
production, as reduced growth rate in these systems also reduces the specific antibody production rate.

Our experimental results, discussed in more detail elsewhere (Banik et al., 1996; Banik et al., 1997) show the intracellular $\beta$-galactosidase content in the GS-26 cells and the specific $\beta$-interferon production rate in 2FS-21 cells are directly proportional to the specific cell growth rate. These results are in general agreement with the trend predicted for an S-phase specific expression (Gu et al., 1994) and indirectly suggests that the SV40 promoter commonly used to drive the expression of foreign protein in recombinant CHO cells is S phase specific. This suggestion correlates very well with previous experimental data where repeated S phase synthesis of two different proteins (the intracellular enzyme DHFR and the secreted glycoprotein tPA) occur from the same SV40 early promoter in two different CHO cell lines (Mariani et al., 1981; Kubbies and Stockinger, 1990).

The production rate of foreign protein has been predicted (Gu et al., 1994) to either increase (for S-phase synthesis) or decrease (for G1-phase synthesis) with increasing growth rate depending on the cell cycle specific expression characteristics of the promoter that is chosen to drive foreign gene expression. We have now demonstrated that the S-phase specific SV40 early promoter causes the growth-associated production of both intracellular as well as secreted proteins in CHO cells. This directly proportional pattern of specific production rate with growth rate requires constant high cell growth rates such as in repeated batch cultures for the maximization of production rate. However, as the CHO cells are merely the catalysts or mini-factories for the synthesis of glycoproteins, it is unfortunate to keep on producing these cells, when the real objective is to maximize the synthesis of glycoproteins. High cell density fed-batch or perfusion cultures, which represent the optimized production systems for hybridoma cells, cause severely reduced glycoprotein synthesis in these CHO cells with a S-phase specific promoter during their low growth phases, unless the popular SV40 promoter is replaced with a strong G1 phase specific promoter.

CONCLUSIONS

The results from this work have strong implications on foreign protein production from SV40 promoters in recombinant CHO cell cultures. We find that the SV40 early promoter and enhancer elements are S phase specific and lead to growth associated production of proteins from the downstream structural genes. Consequently, it will be necessary to maintain high growth rates in repeated batch or chemostat cultures for the maximization of glycoprotein synthesis. The commonly used SV40 promoter and enhancer elements will not be optimal for glycoprotein synthesis from recombinant CHO cells in high cell density fed-batch or perfusion cultures which typically have low cell growth rates.

ACKNOWLEDGEMENTS

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REFERENCES


Figure 1a. Time course of viable cell density (cells/ml) and total cell density (cells/ml) during continuous suspension culture.

Figure 1b. Time course of the β-interferon concentration (µg/ml) and specific β-interferon production rate (µg/10⁶ cells/day) during continuous suspension culture.
Figure 2a. Time course of viable cell density (cells/ml) and total cell density (cells/ml) in one of the celligen bioreactors during continuous suspension culture.

![Graph showing cell density over time](image)

**Figure 2b.** Time course of the volumetric $\beta$-galactosidase concentration (mg/ml) and specific intracellular $\beta$-galactosidase content (mg/10$^6$ cells) in one of the celligen bioreactors during continuous suspension culture.

![Graph showing enzyme concentration over time](image)
Figure 3a. The steady state specific β-interferon production rate (μg/10^6 cells/day) as a function of the specific growth rate (day⁻¹) during continuous suspension culture.

Figure 3b. The steady state specific β-galactosidase content (mg/10^6 cells) as a function of the dilution rate (day⁻¹) during continuous suspension culture.
ABSTRACT

The recruitment of white blood cells to sites of injury or tissue damage is an important role of the immune response system. Circulating white blood cells initially attach and roll on the blood vessel wall. This initial rolling attachment is mediated by the selectin family of adhesion molecules. However, once the selectin sites on the vessel wall are covered by cells, other receptors on the cells may aid in further recruitment of circulating cells. The rolling event was reproduced in a parallel plate flow chamber using HL-60 cells flowing over a surface consisting of a bilayer containing P-selectin. We show that at high P-selectin site densities (250-350 sites/μm²) and at moderate shear stresses of 0.7-1.4 dyne/cm² the cells bound to the bilayer become "sticky" enabling them to capture flowing cells which greatly increases the attachment rate of cells to a site. The mechanism of these cell-cell interactions is not yet known, but an additional receptor mediated event may play an important role in cell recruitment which amplifies the rate of cell accumulation at a site.

INTRODUCTION

One of the most important functions of the immune response system is the recruitment of white blood cells to areas of infection or tissue damage. White blood cells first leave the central stream of flowing blood and roll along activated endothelial cells lining the blood vessel wall. Figure 1 shows the inflammatory response process. Once white blood cells roll on the endothelial cell surface, they adhere tightly allowing them to emigrate between endothelial cells into areas of infection or injury. The rolling of white blood cells is a receptor-mediated process that occurs by receptor bonds forming at the leading edge of the cell and braking at the trailing edge. The receptor molecules responsible for white blood cells coming out of circulation and rolling along endothelial cells are P-selectin (1), expressed on activated endothelial cells, and its high affinity ligand, P-selectin glycoprotein ligand-1 (PSGL-1) (2), constitutively expressed on white blood cells. However, once cells are bound to the endothelial surface blocking P-selectin sites, additional receptors on the bound cells may further aid in the recruitment of cells to the site. One such mechanism is the L-selectin/PSGL-1 interaction on neutrophils that allows a bound neutrophil to "capture" flowing neutrophils bringing them to the surface (3). The process of white blood cell emigration is responsible for successful host response to
infection or tissue injury. This process can be potentially harmful and contributes to various diseases and inflammatory disorders. Understanding the molecular basis of this complex mechanism could lead to new therapeutic approaches for controlling the intensity and specificity of the immune response.

MATERIALS AND METHODS

The receptor-mediated rolling process was reproduced in vitro using a parallel plate flow chamber (shown in Figure 2) that mimics the flow of blood in the body. P-selectin was incorporated into a lipid bilayer composed of phosphatidylcholine and bound to a supercleaned glass slide. The supercleaned glass slide is attached to the flow chamber by an applied vacuum. The height of the flow channel is created by gasket material sealed between the chamber and glass slide. Experiments were done at P-selectin site densities ranging from <20 - 350 sites/µm². Cells were withdrawn through the flow chamber at a constant shear stress (0.1-2.7 dynes/cm²) using a syringe pump. The cells used were HL-60 cells, a human cell line having monocyte-like characteristics, expressing PSGL-1. Experiments were run using HL-60 cells resuspended in HBSS/1% FBS at a concentration of 500,000 cells/ml. The effect of P-selectin site density and shear stress was studied to determine what role they had on the attachment rate of cells to the surface and on any cell-cell interactions that occur with bound cells. Additional experiments were done with HL-60 cells fixed with 1% paraformaldehyde for 20 minutes at 4°C to determine if any type of cell activation was involved in the cell-cell interactions (4). To determine if ATP was required for the cell-cell interactions, experiments were run using HL-60 cells incubated with 50mM 2-deoxyglucose and 0.06% NaN₃ for 60 minutes at room temperature (4). Since L-selectin was shown to mediate cell-cell interactions on neutrophils, experiments were run with the addition of an anti-L-selectin antibody (Dreg200). The cells were incubated with the antibody at a concentration of 5 µg/ml for 10 minutes at room temperature. The flow experiments were viewed using phase contrast microscopy. An inverted phase contrast microscope was used with a video camera to record the experiments allowing them to be analyzed at a later time.

RESULTS AND DISCUSSION

Attachment Assay. Attachment assays were run at varying shear stresses and P-selectin site densities to determine what effect they had on how cells would initially attach to the bilayer and any cell-cell interactions the bound cells had on flowing cells. The number of cell events with the surface is defined as the sum of the number of transient events, cells binding directly to the surface, and cells binding to the surface after first interacting with a bound cell. A transient event is defined as a cell that stops (attaches) to the surface then releases into the
flowing stream without rolling on the surface more than 1 cell diameter. Figure 3 shows the attachment rate at varying P-selectin site densities and shear stresses. At <20 P-selectin sites/μm² there is a large increase in the attachment rate as the shear stress is decreased. This surface is not capable of promoting cell rolling; therefore, many transient events occur leading to the increase since one cell can interact transiently with the surface more than once in the field of view. At site densities capable of producing rolling (25-350 sites/μm²) the attachment rate was not affected greatly by changes in site density or shear stress. For most experiments, the attachment rate decreases slightly as shear is increased, going to 0 at 2.7 dynes/cm² for all site densities (data not shown). At a site density of 250-350 sites/μm² the cells that attach to the surface roll very slowly (creeping) or are firmly attached. It was under these conditions that the bound cells interacted with flowing cells by an unknown cell-cell interaction.

Cell-Cell Interactions. Figure 4 shows the attachment rate versus shear stress plot for experiments done at 250-350 sites/μm². Under these conditions, the cells on the surface appear “sticky” and are capable of capturing flowing cells through cell-cell interactions, bringing them to the surface. As the shear stress is increased, the cells are more efficient at capturing flowing cells until a shear stress is reached that does not allow bond formation. The cell-cell interactions or cell capturing leads to increased accumulation of cells on the surface. At lower stresses (0.1-0.4 dynes/cm²), cell-cell interactions still occur, but do not lead to the cell attaching to the surface. Therefore, at the lower shear stresses cell attachment to the surface occurs in random fashion. Figure 5 shows three frames of video from an experiment done at 0.4 dyne/cm². The maximum accumulation of cells occurs around 3-4 minutes and are spread out randomly throughout the field of view. In experiments run at higher shear stresses (0.7-1.4 dynes/cm²), the cells on the surface are much more efficient at capturing flowing cells and bringing them to the surface which leads to a greater accumulation of cells. The attachment of cells to the surface at shears of 0.7-1.4 dynes/cm² is mainly the result of cell capturing and not direct binding to the P-selectin surface. The cell-cell interactions leading to binding on the surface produce lines or strings of cells on the surface. Once enough cells accumulate on the surface, flowing cells are captured and roll along bound cells until they reach the end of the string allowing them to deposit on the P-selectin surface. Figure 6 shows three frames of video from an experiment run at 1.4 dynes/cm². Lines of cells form almost immediately after a couple cells initially bind to the P-selectin surface. The cell-cell interactions occurring at these shear stresses lead to a much greater accumulation of cells than in experiments run at lower shear stresses.

The effect of shear stress on the efficiency of cell capturing is shown in Figure 7. Lines of cells form in experiments run at 0.7-1.4 dynes/cm² while the attachment of cells directly to the surface is random in experiments done at lower shear stresses. The greater number of cells attaching to the surface via
bound cells at higher shear stresses might be a hydrodynamic effect or redistribution of receptors on the cell surface due to shear. Figure 8 shows how the percent of the total number of cells on the surface at the end of the experiment that bound by cell-cell interactions is affected by shear stress. In experiments done under conditions that promote cell-cell interactions (high P-selectin site density and moderate shear stresses) and enhanced recruitment of cells, the cells are getting to the surface mainly by cell capturing. This is different than experiments run at lower site densities and shear stresses where the majority of the cell binding is directly to the P-selectin surface. Figure 3 showed that the attachment rate was not much different for experiments done at different site densities or shear stresses. Figures 5-8 show that cells attach to the surface in different ways and at an increased rate when cell-cell interactions are involved in the recruitment process.

Characterization of cell-cell interactions. Cell-cell interactions predominately occur at high P-selectin site densities (250-350 sites/μm²) which make the bound cells firmly attached to the surface or creeping very slowly along the surface. Additional experiments were run at a P-selectin site density of 350 sites/μm² for 10-13 minutes to determine how the cell-cell interactions would be affected over time. Figure 9 shows the total cells on the surface at the end of each minute during the experiment at varying shear stresses. At the lower shear stresses, maximum accumulation occurred around 3-4 minutes and the cells bound randomly to the surface with little cell capturing. At higher shear stresses, enhanced cell accumulation occurs due to cell-cell interactions leading to capturing of flowing cells. The lines of cells develop almost immediately with many cell-cell interactions occurring in the first few minutes. After 4-5 minutes, the number of cell-cell interactions drops off drastically in most experiments. Figure 10 shows the number of cell-cell interactions normalized for the number of cells on the surface during that minute of the experiment. Even though the number of cells is increasing on the surface, the number of cell-cell interactions relative to the number of cells bound greatly decreases after 3-4 minutes in most experiments. A number of different explanations are reasonable for this decrease in the number of cell-cell interactions. The cells on the surface might be activated in some way by the shear or the receptor may be redistributed on the cell in response to the shear. Also, the receptor might be shed after some time or once used in capturing of cells. There is also a possibility that the cells are entering the flow chamber differently as the experiment progresses.

Determining the Mechanism of Cell-Cell Interactions. The same type of cell capturing occurs with neutrophils mediated by L-selectin and PSGL-1. HL-60 cells do not express L-selectin as shown by FACS analysis (data not shown). Experiments were run with HL-60 cells incubated with an anti-L-selectin antibody (DREG200) to verify that the cell-cell interactions were not L-selectin dependent. Figure 11 shows that the antibody did not inhibit cell capturing and enhanced cell recruitment. To determine if the cell-cell interactions were a result of cell
activation, experiments were run with HL-60 cells fixed with paraformaldehyde. This did not affect the initial attachment of cells to the surface or inhibit cell accumulation due to cell-cell interactions as shown in Figure 11. Figure 11 also shows that experiments run with cells treated with 2-deoxyglucose and sodium azide had no effect on cell-cell interactions suggesting that the process is not ATP dependent.

CONCLUSION

Cells that become firmly attached or roll very slowly on a high density P-selectin surface are capable of capturing flowing cells, bringing them to the surface. The mechanism of these cell-cell interactions is still undefined. It is not L-selectin dependent as with neutrophils, and does not appear to require cell activation. Some type of receptor redistribution in the bound cells may play a role in the cell-cell interactions. The capturing of flowing cells by these unknown cell-cell interactions leads to an enhanced recruitment of cells to the surface. Recruitment of cells by cell-cell interactions may play an important role in vivo when initially bound cells block or cover receptors on the blood vessel wall.

ACKNOWLEDGMENTS

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REFERENCES


Figure 1. Inflammatory Response Process

White blood cells come out of circulating blood by adhering and rolling on the blood vessel wall through the interactions of receptors. After white blood cells roll, they become firmly attached to the vessel wall. This allows them to then emigrate into the area of infection or injury.

Figure 2. Flow Chamber Setup Used for Flow Experiments

A lipid bilayer is bound to a glass slide and attached to the flow chamber by an applied vacuum. The height of the flow channel is provided by gasket material. The cells enter the flow chamber at a controlled shear stress using a syringe pump. The experiments done with the flow chamber are viewed by phase contrast microscopy with a video camera, allowing the experiments to be taped and analyzed at a later time.
Figure 3. Attachment Rate of Cells to a P-selectin Surface

The attachment rate (number of events per minute) was determined as a function of shear stress and P-selectin site density. The number of cell events is the sum of the cells binding directly to the surface, cells binding to the surface after contacting a bound cell, and transient events. A transient event is defined as a cell that attaches to the surface then releases back into the flowing stream without rolling on the surface. At P-selectin site densities that support rolling (25-350 sites/μm²), the attachment rate does not vary much with the site density or shear stress. P-selectin site densities less than 20 sites/μm² do not support rolling. Many transient events occur under these conditions giving the large increase in the attachment rate as shear is reduced.

Figure 4. Attachment Rate of Cells to Surface Containing 250-350 P-selectin sites/μm²

For most site densities, the attachment rate slightly decreases as shear is increased. However, for the highest site density the attachment rate increases as shear is increased until a shear stress is reached that does not allow bond formation. The high P-selectin site density produces a surface that makes bound cells firmly attached or roll very slowly. The bound cells are capable of capturing flowing cells bringing them to the surface. Cell-cell interactions occur at all shears, but more readily lead to the cell binding to the surface as the shear is increased. The cell-cell interactions leading to cell capturing greatly enhance the number of cells attaching to the surface.
Figure 6. Cells Accumulating on a P-selectin Surface by Cell Capturing.

In experiments run at 250-350 sites/μm², cells roll very slowly or become firmly attached to the surface. At higher shear stresses (0.7-1.4 dynes/cm²) cell-cell interactions lead to the cell being captured to the surface. This type of cell-cell interaction leads to cells binding to the surface in lines or strings. These lines of cells can support cells rolling on their surface before depositing to the P-selectin surface at the end of the line. The cell-cell interactions lead to an enhanced recruitment of cells to the surface with the majority of them being captured by bound cells.

Figure 5. Cells Accumulating on a P-selectin Surface without Cell Capturing.

These are three frames of video from an experimenter run at 250-350 sites/μm² and at a shear stress of 0.4 dynes/cm². At the low shear stress, cell-cell interactions do not readily lead to the cell binding to the surface. The majority of cells directly binding to the P-selectin surface. The binding is random at the maximum accumulation of cells occurs after 3-4 minutes.
Figure 7. Percent of the Cell-Cell Interactions in which the Cell is Captured to the Surface

As the shear stress is increased, the efficiency of the cell-cell interactions to capture flowing cells to the surface is greatly increased. Cell-cell interactions occur over the entire range of shear stresses, but at the lower shears (0.1-0.4 dynes/cm²) they do not lead to the cell then binding to the surface.

Figure 8. Percent of Total Cells on Surface Bound by Cell Capturing

Cell-cell interactions occur at high P-selectin site densities (250-350 sites/µm²) where the bound cells are basically stuck to the surface. Under these conditions, there is an enhanced recruitment of cells to the surface and the majority of the cells bind to the surface by being captured by bound cells. This is in contrast to experiments done where cell-cell interactions and cell capturing is not readily seen. The cells in those cases bind directly to the P-selectin surface in random fashion.
Figure 9. Accumulation of Cells on a High Density (350 sites/\mu m^2) P-selectin Surface

The total number of cells on the surface was counted after each minute during the experiment. At low shear stresses (0.1-0.4 dynes/cm^2), cells bind directly to the P-selectin surface in a random fashion with little cell-cell interactions. The maximum accumulation of cells occurs after 3-4 minutes. In experiments run at higher shear stresses, cell capturing is much more efficient leading to enhanced cell recruitment at the site.

Figure 10. Number of Cell-Cell Interactions Normalized for Number of Cells on Surface.

The number of cell-cell interactions was counted during each minute and divided by the number of cells on the surface during that minute. This normalizes the number of cell-cell interactions for the number of cells on the surface. The number of cell-cell interactions is much greater in the first few minutes than in the later stages of the experiment. The reason for the drop off in cell-cell interactions is not known.

Figure 11. Cell-Cell Interactions are not a Function of L-selectin or Cell Activation.

Control experiments (A) were run with regular HL-60 cells at 1.4 dynes/cm^2. Experiments run using cells treated with 50mM 2-deoxyglucose and 0.06% sodium azide (B) or with cells fixed with 1% paraformaldehyde (C) did not inhibit attachment of cells to the surface or cell capturing by bound cells. Also, experiments run using cells incubated with an anti-L-selectin antibody did not inhibit cell-cell interactions or enhanced cell recruitment.
The Recirculation of Hybridoma Suspension Cultures: Effects on Cell Death, Metabolism and MAb productivity

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Abstract

A hybridoma cell line, H166, was recirculated from a stirred flask through an external loop with a peristaltic pump. The effects of different shear stresses and residence times per pass in the recirculation loop on cell death, metabolism, and MAb productivity were studied. While high shear stress renders an immediate disruption of cells, low level stresses induce cell death in a different pattern: cell damage remains slow and undetectable for a few days but the specific death rate increased thereafter. This acceleration of cell death following prolonged shearing time at the same low stress level can possibly be explained by the assumption that some unknown biochemical factors in addition to physical forces are also responsible for the cell death. Since cell death in recirculation is independent of overall exposure time to tubing shear per pass, there may exist certain critical points in the recirculation loop which provide an overwhelmingly higher stress than elsewhere and are responsible for the cell death during recirculation, as is confirmed by the following observations: the number of passes in recirculation rather than the residence time (in the tubing) per pass is correlated with the cell death rate; modification of the tubing section by adding the membrane cartridge does not alter the growth dynamics of the cell culture. Neither glucose depletion nor lactate or ammonia accumulation became a limiting factor leading to cell death during the recirculation of cell culture. Cell metabolism and MAb productivity were not significantly modified by shear stresses as long as cells remained viable except that at high stress most cell functions were nearly terminated.

Introduction

Various responses by cells to mechanical stresses have been reported. Investigation of the shear sensitivity of hybridoma cells in different modes of growth and culture age have shown that cells are more sensitive to well defined viscometric shear during the lag and stationary phases than during the exponential phase of batch cultures (Peterson et al., 1988, 1990). Several studies have been conducted on the cellular metabolism of mammalian cells subjected to hydrodynamic stresses (Frangos et al., 1988; Al-Rubeai et al., 1990; Papadaki et al., 1996; Ranjan et al., 1996). Frangos et al. (1988) demonstrated that shear stresses in certain ranges did not hinder but instead stimulated mammalian cell metabolism in human umbilical vein endothelial cells. Al-Rubeai et al. (1990) observed that for a hybridoma cell line DNA synthesis was inhibited under conditions of intense hydrodynamic stress while cellular metabolic activity was increased to assist repair mechanisms.
Cell death caused by shear stress is usually accompanied by the release of a large amount of lactate dehydrogenase (LDH) to the medium following cell membrane leakage. In the evaluation of cell death and lysis rate, LDH measurement has proven to be a very reliable approach and has been widely adopted (Shi et al., 1993).

Due to the complicated relationships among culture medium, cell metabolism, and bioreactor configuration, shear sensitivity of cultured hybridoma cells has shown large variability with respect to differences in cell line, mode of growth, and device hydrodynamics. Therefore, systems at the experimental scale which mimic the specific operational characteristics of pilot scale reactors are very important in reactor scale up. While most studies have been directed towards the investigation of fluid-mechanical effects in agitation, bubble interaction, bubble breakup or rheological properties of interfaces, research on recirculation culture has not been widely conducted.

Some studies have been conducted on recirculation through capillary tubing (McQueen et al., 1989; Kieran et al., 1995; Zhang et al., 1993). These capillary recirculation studies, where the shear stress levels were very high (on the order of $10^3$ dyn/cm$^2$) and the shearing times were fairly short (hardly exceeding 1 minute), were useful in establishing defined shear stress models (modeling was the main objective for most of these studies) but lack value in most recirculation systems where the shear stress level has to be kept much lower (in terms of dyn/cm$^2$).

The studies of recirculation used in perfusion culture are few in number. Cell cultures were circulated through filter modules with (Zhang et al., 1993) or without (Maiorella et al., 1991) withdrawal; average shear stresses up to 10.6 dyn/cm$^2$ (Zhang et al., 1993) or 25 dyn/cm$^2$ (Maiorella et al., 1991) did not damage hybridomas. While the above works pioneer the study of recirculation shear effects in perfusion, the actual factors responsible for cell death by recirculation were not identified. The following aspects of perfusion still need investigation: specific death rates in recirculation; the effects of residence time in recirculation tubing; cell metabolism under shear; and more importantly, the discrimination of the possible deleterious effects from the pump, from the flow through tubing, and from the flow through a hollow fiber membrane.

With the intention of enhancing our understanding of the mechanisms for viability loss in recirculation, this study was conducted to investigate the effects of shear stress level and residence time in the recirculation loop on specific death rate, MAb productivity, substrate consumption, and metabolite production. The experiments were designed to separate the effects of the pump, the tubing, and the membrane from each other in order to identify their individual contributions to cell death in recirculation. While most previous work has been directed towards short term, high shear stresses, this study included low shear stresses, whose effects may not be detectable in a few hours but may be profound in the long run. This investigation of shear effects covered an extended period of continuous cultivation of cells, making application of the results to long-term, and possibly large scale processes more reliable.
Materials and Methods

A murine hybridoma cell line, H166, producing an IgM antibody, was used in this study. Cells were cultivated in 10 ml volumes in 25 cm² TC flasks (Corning, Corning, NY) and maintained at 37°C in a humidified incubator with 5% CO₂. The culturing medium, hybridoma serum free medium (HSFM, Gibco BRL, Grand Island, NY) containing a very low level of defined protein (20 μg/ml insulin and transferrin) and adjusted to a pH of 7.2 by sodium bicarbonate, was supplemented with 10² units/ml penicillin-streptomycin and 0.05% pluronic F68. The initial glucose and glutamine concentrations in the medium were 24 mM and 4 mM, respectively.

Four Kontes spinner flasks with working volumes of 500 cm³ were adopted for each recirculation experiment. The reactors were then run in CSTR mode with a dilution rate of 0.0104 hr⁻¹ for 4-7 days until the viable cell density, viability, and other associated parameters such as substrate, waste, and MAAb levels stabilized at similar levels in all of the four reactors; recirculation was then started. The dilution rate of 0.0104 hr⁻¹ was chosen because it proved to be more suitable in maintaining high cell density and preventing cell wash-out than other dilution rates. It was thus adopted in all of the following experiments.

Reactor 1 was run in CSTR mode (without recirculation of cell culture) as a control system while Reactors 2, 3, and 4 were run in CSTR mode (with recirculation of the cell culture). Hold up volumes in the recirculation lines were kept at 20, 30, and 40 ml for Reactors 2, 3, and 4, respectively, by using different tubing lengths. By changing the hold up volume of the tubing, the exposure time to tube shear in a single pass was changed accordingly.

Three sets of experiments were run. In the first set, Reactors 2-4 were run (with 20, 30, and 40 ml hold up volumes, respectively) at constant recirculation flow rates of 40, 60, and 80 ml/min, respectively, using Reactor 1 as a control. In the second set, since flow rates as low as 40 ml/min were found to be deleterious to cell growth, recirculation rates were changed to 20, 30, and 40 ml/min in order to find out the critical rate beyond which cell growth was hindered. In the third set, a hollow fiber membrane cartridge (A/G Technology Corporation, Needham, MA) (50 polysulfone hollow fibers with 0.45 μm pore size, 0.032 m² membrane area, 1 mm lumen ID, 6 ml total hold up volume) was added to the 20 ml recirculation loop (without permeate flow) to simulate the perfusion system. Another reactor with a 20 ml hold up volume in the recirculation loop (without a membrane cartridge) was used as a control.

Viable cell density, percentage viability, glucose, lactate, ammonia, lactate dehydrogenase (LDH), and IgM antibody concentrations were measured at regular intervals. During the second set of experiments, cell samples from Reactor 3 (40 ml/min recirculation rate) at 0, 2, and 4 days following the onset of recirculation were run on a flow cytometer.
RESULTS AND DISCUSSION

Cell Death

Figures 1-3 show the changes of viability following recirculation at different flow rates (40-80 ml/min; first set) with three different tubing hold up volumes. The average specific cell death rates for different recirculation flow rates and exposure times to tubing shear (first set) are shown in Figure 4. Under wall shear stresses of 1.73, 2.59, and 3.45 dyn/cm², within 8, 7, and 2 days, respectively, the viabilities, which started at over 70%, dropped to around 50%. While cell death under low and medium shear stresses (1.73 and 2.59 dyn/cm²) proceeded slowly (with average specific death rates of 0.0160 ± 0.0017 and 0.0198 ± 0.0020 /h, respectively), the highest level of shear stress (3.45 dyn/cm²) caused significant cell disruption (with an average specific death rate of 0.0616 ± 0.0045 /h). At the lowest shear stress level (1.73 dyn/cm²), it took 2 to 3 days before cell death proceeded to a detectable level. As a matter of fact, the specific cell death rates under low stresses were higher in the later days than in the earlier days (Figures 5). In this study, if recirculation had not been extended over two days, shear stresses as high as 2.59 dyn/cm² could have been perceived as not harmful.

There was no significant difference of using tubing with various lengths for all recirculation rates on cell death, implying that increasing the overall exposure time to tubing shear in the recirculation loop for each pass did not influence the cell death rate. Thus it appears that another point in the recirculation loop, such as the peristaltic pump, may render a more brutal shearing environment than the tubing and maybe responsible for shear-induced cell death. If such a critical point does exist, the number of passes through the recirculation loop is more likely to be a dictating factor in the resulting cell death than the overall shearing time for each pass. Therefore, increasing the exposure time to the tubing would not cause an increased cell death.

From this point on, the operating mechanism of the peristaltic pump itself deserves further discussion. When the rollers of a peristaltic pump move across the tubing, a "pillow" of fluid is formed between the rollers. The flow rate is determined by multiplying the rotor speed by the size of the pillow. The pillow size stays fairly constant since it is specific to the tubing ID and the rotor geometry. The fluid flow is pulsatile, as is the shear. Increasing the flow rate actually has two effects: it increases the rotor speed, or in other words, the frequency of pillow transportation; meanwhile, it also increases the instantaneous acceleration of flow, because the pump needs to transport a fixed volume of fluid in less time during each cycle. Because of the pulsatility of flow, the average wall shear stresses derived from Poiseuille Equation, which applies to uniform laminar flow, may not best reflect the actual shear environment in the recirculation loop. There was still no definite answer from this study for how frequency affected the cell death from pumping. Therefore, unless experiments and
appropriate models which better describe the flow pulsation are developed, knowledge of the cell death mechanism across a peristaltic pump will remain incomplete. The present study did not finish this mission but pointed out its importance. The necessity of controlled pump experiments were also addressed by Kieran, who pointed out that it was essential to determine the damage due exclusively to the pumping mechanism (Kieran et al., 1995).

In order to determine the operating conditions which provide a damage-free recirculation operation for the cells, the recirculation rate was decreased to 20 ml/min. The plan was to increase the circulation rate by 10 ml/min every few days until the maximum allowable recirculation rate was found. A critical recirculation rate of 30 ml/min, corresponding to a tube shear stress of 1.29 dyn/cm², was found.

The addition of a hollow fiber membrane to the recirculation loop (without permeate flow) did not introduce any detrimental effects to the cell culture at a shear stress of 1.29 dyn/cm². The cell growth dynamics for the three conditions, shear free without recirculation, shearing at 1.29 dyn/cm² without the membrane cartridge, and shearing at 1.29 dyn/cm² with the membrane cartridge, were very similar. Because the average flow velocity in the hollow fiber section was only one fifth of that in the tubing section (due to the difference of total cross sectional areas), it is reasonable to expect that shearing in the hollow fiber section is less vigorous than in the tubing section.

Because the modification of transportation section (tubing, with the addition of the hollow fiber cartridge) did not affect cell death while the number of passes through the recirculation loop appears to have more influence than shearing time per pass (changed with the tubing length in the recirculation loop), the hypothesis is supported that certain critical points (very likely to be the peristaltic pump) exist in the recirculation loop whose detrimental effect is overwhelming to that of other sections. This was shown by the results of the first and second sets of experiments. By contrast, if the pumping is not mainly responsible for cell death but the flow through the tubing is, by increasing tubing shearing time per pass, we would expect to see an intensified cell death.

Substrate, Metabolite, LDH, and Monoclonal Antibody

Investigations of substrate and metabolite kinetics in cell culture are essential for providing a better knowledge of cell death. They offer information such as whether nutrient depletion or metabolite build up is a limiting factor rather than physical disruption in cell death, and more importantly, whether the cells that remain alive are still active metabolically under shear stress. This information is lacking in most shear studies, which has left a lot of uncertainties in the explanation of cell death.

The concentration of glucose remained above 4 mM for most conditions in this study, suggesting that glucose depletion was not responsible for cell death at either high or low shear stresses. At the high shear stress level (3.45 dyn/cm²), not only were the cells vigorously disrupted, but the cells which remained viable for more than two days ceased consumption of
glucose. The minor modification of glucose metabolism at low and medium shear stresses, however, was insufficient to cause cell death because it did not result in depletion of glucose.

Lactate metabolism was even less affected by shear stress than glucose. There was hardly any difference of lactate concentration and specific lactate production rate between shear-free culture and sheared culture. Moreover, lactate concentration did not exceed 16 mM in all cases, which was much lower than the typically inhibitory level of 24 mM (Michaels et al., 1991).

Ammonia metabolism was also not very responsive to shear stresses except that at high shear stress (3.45 dyn/cm²) production was almost totally blocked. Because the concentration remained below 3 mM, a toxic level of ammonia was not reached under any of the tested conditions.

In this study, as cell lysis became prevalent under damaging shear stresses, the LDH release level turned into a more accurate gauge for cell death than cell counting with a hemocytometer (cells fragmented directly from viable are not counted with a hemocytometer). With LDH measurement, dead cells whose skeleton remained intact and lysed cells that became fragmented were both included in the calculation of dead or nonviable cells. The buildup of LDH in the culture was dramatic following exposure of the cells to shear stresses over 1.73 dyn/cm². Above the critical shear stress of 1.73 dyn/cm², the specific release rate of LDH, q_{LDH}, increased with each shear stress increment, reaching a much higher level under the shear stress of 3.45 dyn/cm² than under the shear-free environment.

Antibody production appeared to be unaffected by shear stresses up to 2.59 dyn/cm² while there was a noticeable decrease in the specific MAb production rate at a shear stress of 3.45 dyn/cm². There is neither shear-stimulated nor shear-hampered MAb productivity in this cell line except under a shear stress of 3.45 dyn/cm² where other cell functions are greatly reduced. Therefore, efforts should still be devoted to sustain viable cells under favorable conditions in order to reach the ultimate goal of antibody productivity enhancement.

The similarity of substrate and metabolite levels as well as their consumption and production rates between shear-stressed and shear-free cultures indicated that neither glucose depletion nor the buildup of lactate or ammonia occurred in this study and suggested that none of them was responsible for cell death under shear stresses. On the other hand, continued metabolism showed that those cells remaining viable were not "disabled" by the shear stresses (except at 3.45 dyn/cm²) and were still growing. The dramatic release of LDH was a reliable marker for the release of cytoplasmic contents to the extracellular fluid. Cytoplasmic release generates successive reactions causing damage to neighboring cells (Tomei, 1991). Dying cells can release proteases or other intracellular enzymes into the medium which will inactivate proteins or have a negative influence on cell proliferation (Maiorella et al., 1993).

The exposure time to tube shear per pass did not play an important role in cell metabolism, antibody production, and LDH release. Because cell growth dynamics was not
affected by tube shearing time, we would expect to see biosynthetic functions unaffected as well.

The acceleration of cell death following prolonged shearing time (or increase in the number of passes) suggested that the response of cells to shear stress may not be merely physical, and that, besides physical disruption (instantaneous), cellular content release could be responsible for cell death (slowly increasing) under low level shear stresses. The chain response of cells to the released cellular contents provides one possible explanation for the fact that detectable cell death elevation at low stress occurred only after two days. The hypothesis that cellular content release is a dominant factor in cell death (low stress) rather than physical forces, however, needs proof from further experiments, which will be done in our future studies.

Flow Cytometry

Flow cytometric analysis was carried out with a Coulter EPICS Elite analyzer to obtain more information on cell characteristics. Forward angle light scatter (FS) and 90° light scatter (SS) were used to identify cell size and granularity. Propidium iodide (PI) dye was used to examine cell viability. There was a 25% increase in cell size after shearing under 1.73 dyn/cm² for 4 days (Figures 6). There could be several explanations for the increase of cell size with extended shearing. First, it was assumed that younger cells during the exponential growing phase, usually at a comparably larger size, are less vulnerable under unfavorable environments such as shear stress than older cells during the plateau phase, usually at a smaller size (Petersen et al., 1988, 1990). The vulnerability of cells in the plateau phase, however, might be explained by the nutrient limitations linked to this phase which result in the decreased production of certain cellular components responsible for the mechanical strength of the membrane rather than by the dependence of shear sensitivity on growth rate (Petersen et al., 1990). Because no nutrient limitation was found in this study, however, the above assumption is not appropriate. It was then postulated that the larger size population might consist of necrotic cells, which became swollen with increased granularity. This supposition appeared unreasonable at first sight: larger size cells are more likely to burst physiologically than smaller size cells under hydrodynamic forces because they require more surface energy to remain intact in a turbulent environment (Zhang et al., 1993). This apparent contradiction can be explained by a hypothesis that physical bursting play a less important role in long term low stress shearing than other unknown factors, such as toxicity from released cellular contents. If physical bursting was the dominant source of cell death, surviving cells should have been smaller in size. In short, cell death could be initially triggered by physical disruption of cells and exacerbated by other unknown parameters. This proposition is commensurate with the observation that cell death augmentation under low stress became noticeable only after two days and that an acceleration of cell death occurred thereafter, in coincidence with the accumulation of cell debris. Although the above hypothesis provides an explanation for the increase of cell size after extended time of shear stress, it needs further testing for verification.
REFERENCES


Figure 1. Time course of viability at the tube wall shear stress of 1.73\textsuperscript{a}, 2.59\textsuperscript{b}, and 3.45\textsuperscript{c} dyn/cm\textsuperscript{2} (first set).
Figure 2. Average value of specific cell death rate during 8 (1.73 dyn/cm^2), 6 (2.59 dyn/cm^2), and 2 (3.45 dyn/cm^2) days, as a function of wall shear stress and tubing hold up volume.

Hold up volume (ml)

Specific death rate (/hr)

Shear stress (dyn/cm^2)

Figure 3. Time course of specific cell death rate at the tube wall shear stress of 1.73 dyn/cm^2 (first set).

Figure 4. Time course of relative cell size at the tube wall shear stress of 1.73 dyn/cm^2 (second set).
The Importance of Enzyme Inactivation and Self-Recovery in Cometabolic Biodegradation of Chlorinated Solvents

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Abstract

The toxicity of chlorinated solvents or their metabolic intermediates has long been studied. However the self-recovery of microorganisms as a response to toxicity has been addressed only recently. Usually, the toxic inactivation rate of key enzymes within microbes is much greater than the rate of their natural decay. Inactivation is the major factor that results in the loss of microbial activity especially where high concentrations of chlorinated solvents exist. In this situation, the self-recovery of the microbes plays a crucial role in keeping them active in biodegradation of chlorinated solvents. The toxic inactivation and self-recovery of key enzymes are reviewed in this paper. Kinetic models are presented.

Keywords: Bioremediation, enzyme kinetics, inactivation, recovery, cometabolism

Introduction

Biodegradation of chlorinated solvents has often been addressed excessively during the past decade because of their environmental significance. Complete mineralization has been demonstrated under aerobic and anaerobic conditions. In most of the cases, it is believed that the biodegradation takes place cometabolically with primary substrates. Oxygenases have been identified as the key enzymes in aerobic microbial systems that mediate biodegradation of chlorinated solvents. It has also been demonstrated that the cometabolism of chlorinated solvents is a net energy consuming process; hence, the chlorinated solvents can not be used as a source of carbon or energy for microbial growth. In-depth mechanisms of biodegradation are being gradually revealed. These include microbial growth and bioenergetics, competitive inhibition, key enzyme induction and degradation, toxic inactivation and self-recovery action, etc. The enzymatic reactions and enzyme regulation can be described as in Figure 1 based on key enzyme pool in microbial system. Most of previous studies focused on the utilization of growth substrate (C), biodegradation of nongrowth substrate (C), and their competitive inhibition. However, any systematic study on the regulatory steps of key enzymes including enzyme induction

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and degradation, inactivation and self-recovery has not been reported. Usually, it is these regulatory steps that dominate the reaction rate in microbial system.

![Diagram showing reactions and regulations around the key enzymes in microbial system.](image)

**Figure 1.** Reactions and Regulations around the Key Enzymes in Microbial System.

The enzyme production and its degradation have been studied at genetic level (Bailey and Ollis, 1986). The presence of growth substrate may result in the production of key enzymes. The induced enzymes are naturally degraded also. However, the inactivation and recovery of enzymes have not yet been studied. Natural degradation and toxic inactivation of key enzymes result in the decline of the amount or activity of enzymes. The rate of toxic inactivation rate is much greater than the natural degradation rate, e.g., about 50-5000 times in the case of TCE degradation by methanotrophic bacteria (Chang and Alvarez-Cohen, 1995). The recovery of inactivated enzymes in microbial system is important for the cells involved in biodegradation of chlorinated solvents. This paper reviews the mechanisms of toxic inactivation and self-recovery of key enzymes in microbial system during methanotrophic biodegradation of trichloroethylene. Kinetic expressions of inactivation and self-recovery are also explored based on analysis of literature data.

**Toxic inactivation mechanisms of key enzymes in microbial system**

During biodegradation of toxic contaminants, inactivation of key enzymes usually results from either contaminants themselves or its biotransformation products. In the case of methanotrophic monooxygenase (MMO), several authors (Green and Dalton, 1989; Fox *et al.*, 1989, 1990; Alvarez-Cohen and McCarty, 1991A, 1991B) demonstrated that only the reactive intermediates (such as transient radicals or carbonation compounds) contribute to the toxic inactivation. Furthermore, inactivation is irreversible and nonselective. All the key enzyme components can be inactivated at the same time with the loss of metal ion from coenzyme where usually the enzymatic reaction sites locate (Fox *et al.*, 1990; Green and Dalton, 1989). Toxic inactivation of key enzymes involved in the cometabolic chain render the whole chain inoperative and have a profound or even fatal effect upon the organisms.

A similar situation exists with cometabolic transformation of chlorinated solvents by toluene (Wackett and Householder, 1989; Zylstra *et al.*, 1989), ammonia (Hyman *et al.*, 1988; Rasche *et al.*, 1991), alkene (Hartmans and Bont, 1992), or isoprene utilizers
In a $^{14}$C radiotraced TCE degradation experiment with *P. putida* F1 which uses toluene as growth substrate (Wackett and Householder, 1989), about 16.6% of the radioactivity added to the culture was accounted for in the total cell fractions; most of the $^{14}$C incorporated was found in the protein fraction. The authors suggested that the inactivation effects may stem from cometabolic activation of TCE by toluene dioxygenase to form reactive intermediates that modify intracellular molecules. The results with *P. cepacia* G4 presented by Heald and Jenkins (Heald and Jenkins, 1994) are consistent with this phenomenon. Rasche et al. (Rasche et al., 1990; Rasche et al., 1991) have also observed a turnover-dependent inactivation of ammonia monoxygenases (AMO) in *N. europaea* cells inoculated with TCE and several other halogenated hydrocarbons. The results showed that inactivation during TCE biotransformation was accompanied by covalent modification of cellular proteins and loss of O$_2$ uptake activity associated with both ammonia and hydrazine oxidation.

**Kinetics of key enzyme inactivation in microbial system**

The kinetics of inactivation of degradation process is complicated by yet unclear direct and indirect pathways. According to the inactivation mechanism discussed above, the inactivation rate is correlated with formation of reactive intermediates. The inactivation process can be expressed as:

$$E + (P_2)_{\text{active}} \xrightarrow{k_i} E^*$$

(1)

where $E$ is active enzymes, $E^*$ is inactivated enzymes, $P_2$ is the reactive intermediates resulting from biotransformation of chlorinated solvents, and $k_i$ is inactivation constant. Assuming a first-order, inactivation equation can be written as:

$$\frac{dE^*}{dt} = k_i \cdot [E] \cdot [(P_2)_{\text{active}}]$$

(2)

According to discussion of mechanism, inactivation rate of enzyme in methanotrophic cells depends on the formation of reactive intermediates, which is closely associated with TCE transformation. Relatively, the enzyme level in cells is less important and can be assumed as a constant. Then the inactivation equation can be modified as:

$$-\left[\frac{d}{dt} \left( \frac{E}{E_{\text{max}}} \right) \right]_{\text{inactivation}} = k'_i \cdot \left[ - \frac{dC_2}{dt} \right]$$

(3)

where $E_{\text{max}}$ is the maximum concentration of active enzymes, $X$ is biomass concentration, $C_2$ is the concentration of chlorinated solvents, and $k'_i$ is the modified inactivation constant. In this way, the relative level of active enzymes in microbial system can represent active biomass which is easy to measure in practice. Equation 3 is also
consistent with the currently used concept as transformation capacity defined by Alvarez-Cohen and McCarty (1991A).

Obviously, the inactivation equation derived above is different from that of purified enzymes. In the later case, usually the amount of enzymes available is the limiting factor (Sadana et al., 1980, 1986, 1987; Bailey and Ollis, 1986). Here

$$\frac{dE}{dt} = -k_r E$$ (4)

where $k_r$ is the macroscopic inactivation coefficient. Different from the purified enzyme, the inactivation of enzymes undertaking within cells may be influenced by some cell-level functions. In this situation, it seems that the inactivation constant ($k_r$) depends also on the transformation rate of substrate (Sad’ina et al., 1987; Gray, 1989).

The experimental data published in literature can support the above analysis. In 1990, Fox et al. evaluated the inactivation of purified key enzymes during TCE degradation. The experiment was conducted in batch way by use of soluble methanotrophic monooxygenase (sMMO) extracted from Methylosinus trichosporium OB3b. The specific inactivation rate of enzymes can be obtained by modifying the experimental data. The relationships of specific inactivation rate with either enzyme level or TCE degradation rate are presented in Figure 2 and 3. It can be found that there is a linear relationship between inactivation rate and enzyme level or TCE degradation rate. The regression coefficients are 0.958 and 0.948, respectively. This relationship is consistent with the classic inactivation model of purified enzyme (Sadana et al., 1980; Bailey and Ollis, 1986).

![Figure 2. Inactivation of Pure Enzyme as a function of Enzyme Level (Data from Fox et al., 1990).](image-url)
For the enzymes existed in microbial cells, the correlation between inactivation rate and TCE degradation can be obtained by modifying the data from the related literature. For example, Tschantz et al. (1995) conducted experiments in methanotrophic multi-stage bioreactor to explore sustainable biodegradation of TCE by keeping suitable maintenance conditions. In their experiments, the TCE degradation and key enzyme activity were evaluated. During the initial reaction period, it is reasonable to assume that the enzyme and biomass kept constant, then plotting the inactivation rate of key enzymes against TCE transformation rate (Figure 4). It can be seen that there exists a very good linear relationship with regression coefficient of 0.998. These results demonstrate that Equation 3 can properly describe the kinetics of enzyme inactivation during biodegradation of TCE. A comparison of Figure 3 with Figure 4 also reveal that the in \textit{vitro} ratio of TCE degradation or enzyme inactivation are several orders of magnitude

\begin{figure}[h!]
\centering
\includegraphics[width=0.5\textwidth]{figure3.png}
\caption{Inactivation Rate of Pure Enzyme as a function of TCE Degradation Rate (Data from Fox et al., 1990).}
\end{figure}

\begin{figure}[h!]
\centering
\includegraphics[width=0.5\textwidth]{figure4.png}
\caption{Enzyme Inactivation of Intact Cells in Bioreactor (Data from Tschantz et al., 1995).}
\end{figure}
higher than those \textit{in vivo}. Therefore, the influence of cell-level factors cannot be ignored for inactivation of enzyme in cells.

**Recovery mechanisms of key enzymes in microbial system**

Self-recovery is the ability of cells to recover from the damage caused by the toxic inactivation during contaminant transformation (Ely et al., 1995A, 1995B). It determines the ability of the microbial system to fight the toxicity effects and to sustain itself. The toxic intermediates may be degraded, the inactivated enzymes may be repaired, or new enzymes may be produced to compensate for inactivation. The recovery action has been observed in both aerobic and anaerobic processes. Kanazawa and Filip (1986) have conducted enzyme assays for $\beta$-glucosidase, $\beta$-acetylglucosaminidase, phosphatase, phosphodiesterase, and proteinase in soil samples collected two months after contamination with tetrachloroethylene (PCE), dichloroethane (DCA), and TCE. The results indicated that TCE, PCE and DCA inhibited activities of all enzymes tested at a concentration of 1000 $\mu$g per 100 g soil initially. However, after two months the enzymatic activities, especially in soil samples contaminated with PCE and DCA, were found to be at the same or higher level than in the control samples.

Similar phenomena have also been observed in other cases during biodegradation of chlorinated solvents (Henry et al., 1988; Folsom and Chapman, 1991; Hyman et al., 1995; Ely et al., 1995B). The characteristics of self-recovery processes can be summarized as follows: (1) Recovery action is associated with the extent of inactivation; (2) There exists a lag time for recovery action in response to inactivation, especially in the case of some very toxic chlorinated compounds; (3) The enzyme level after recovery action may be at near-maximum or even higher than that before inactivation; (4) Recovery action consumes energy. However, the recovery action has not been addressed at a mechanistic level in research dealing with biodegradation to chlorinated solvents. Study of such mechanisms is urgently needed in order to provide insight into the biodegradation process, especially for the refractory and toxic chlorinated solvents.

**Kinetics of recovery of key enzyme in microbial system**

Recently, Hyman et al. (1995) and Ely et al. (1995A) have addressed recovery kinetics by proposing the concept of inhibition/inactivation/recovery in microbial systems. According to this concept, the maximal sustainable degradation rate of chlorinated solvents is likely to be achieved as a result of a balance between the ability of the microbial cells to oxidize contaminants and their ability to repair and recover from the concurrent cellular damage caused by the contaminants and/or their oxidation products. Ely et al. (1995B) suggested that this concept provides a reasonable basis for understanding the toxic effects of biodegradation of chlorinated solvents on the enzyme and bacterial response. Hence, a comprehensive model involving inhibition/inactivation/recovery was developed for pseudo-steady-state conditions. The model
simulation gave better fit to the experimental data than those obtained without incorporating recovery effect. The recovery function is presented here.

\[ E_{\text{new}} = k_{\text{rec}} P_1 \]  

Where: \( E_{\text{new}} \) is newly synthesized enzyme, \( k_{\text{rec}} \) is specific recovery constant, and \( P_1 \) represents the concentration of oxidatoin products of primary substrate.

Unfortunately, the most significant departure of model prediction from the experimental data occurred in their most innovative part, i.e., recovery action. More than 76.3% deviation for the specific recovery constant was observed, even after discarding the lowest and highest estimates. The failure may result from lack of recovery mechanisms. No explicit relationship between recovery action and toxic inactivation was brought into their model. After comparing the model prediction with experimental results, the authors themselves also recognized that the toxic effect should itself be one of the driving forces for recovery action.

Similar research in other fields may be helpful to address the recovery kinetics of cometabolic biodegradation. In anaerobic treatment of wastewater, for example, an empirical expression describing the recovery action has been developed by Parkin and Speece (1982) as the following.

\[ G_R = B e^{k_I} \]  

where \( G_R \) is recovery rate; \( B \) is empirical constant, \( k_I \) here is the recovery rate constant. It is reported that this model was successful in describing the recovery pattern for a wide variety of toxicants such as cyanide, chloroform, formaldehyde, and copper (Parkin and Speece, 1982). This empirical model is very similar with that of recovery kinetics in radiobiology.

In radiobiology, recovery of radiation-induced damage (e.g. DNA) usually undertakes directly by impair enzyme (Kiefer, 1988). Incomplete-repair model has been developed to describe the repair kinetics of cells against the radiation-induced damage (Curtis, 1986; Ostasheveky, 1992; Joiner et al., 1993). The recovery level is related with the inactivated extent of cells in the first-order. Model equation is as follows.

\[ \frac{E - E_0}{E_0 - E_\infty} = e^{-k_I t} \]  

where \( E_0 \) is initial enzyme concentration, \( E_\infty \) is the enzyme concentration after complete recovery.

Based on the repair kinetics in radiobiology, following kinetic equation for recovery action in response to inactivation in the biodegradation of chlorinated solvents could be expressed as follows.
where \( k \) and \( K \) denote the kinetic coefficients of enzymatic recovery action in which \( K \) may be associated with recovery capacity of microbial system.

Since most experiments reported in literature were conducted without identifying recovery from the other processes as inactivation, induction and degradation, specific data on recovery process is very limited. Hyman et al. (1995) have given a simple evaluation for the self-recovery of inactivated enzyme in microbial cells. In this experiment, the cells were first inactivated then were transferred to fresh culture to observe its self-recovery process. In this situation, the total enzymes including the inactivated enzymes at the beginning of recovery is a constant value as \( E_0 \) and the biomass also kept constant. The data is plotted in Figure 5 according to Equation 8. The regression coefficient is 0.962. It can be found that the recovery equation developed can roughly describe the recovery process kinetics within microbial system. Self-recovery rate is dependent on the inactivation extent of enzymes.

\[
\frac{\mathrm{d}}{\mathrm{d}t} \left( \frac{E}{E_{\text{max}}} X \right)_{\text{recovery}} = k_r \left( K_r - \frac{E}{E_{\text{max}}} \right) X
\]  

(8)

**Figure 5.** Self-Recovery Level of Enzyme in Cells as a Function Time  
(Data from Hyman et al., 1995).

In fact, the recovery action in microbial system during biodegradation of chlorinated solvents is not a simple or direct repair of inactivated enzymes (Hyman et al., 1995). Recovery action may cause the resynthesis of new enzymes. However, the detailed mechanisms are not clear. Thus, recovery models from radiobiology which based on the direct repair of damaged DNA may not be very suitable to the recovery action addressed here. Consequently, mechanistic model on recovery action is still need to be developed in the future.
Summary

Inactivation and recovery are very important to understand cometabolic biodegradation of chlorinated solvents and develop sustainable control strategies. The inactivation mechanisms have been studied in detail at enzymatic level. It is clear that reactive intermediates play significant role in enzyme inactivation. However, much uncertainty still exists in self-recovery process of cometabolic microbes. Modification and analysis of selected literature data indicated that inactivation rate of key enzymes is proportional to the biotransformation rate of chlorinated solvents. Self-recovery rate can be roughly described by incomplete-repair model in radiobiology. But mechanistic model is needed to explore.

Acknowledgment

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PHYTOREMEDIATION OF VOC CONTAMINATED GROUND WATER USING POPLAR TREES

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Abstract

Phytoremediation is a very new and cost effective way to treat contaminated ground water using vegetation. This paper presents a solution which uses poplar trees to treat volatile organic compound (VOC) contamination near the Riley County Landfill. The background includes the site history, a physical description of the land, a summary of contamination levels and locations, and an explanation of how phytoremediation will be used. The analysis section presents the methods used to determine water flow rates, the optimal tree planting scheme, and the predicted change in ground water movement after planting. The possible biodegradation of the contaminants in the root zone is discussed and supported using data from laboratory studies. The economics section summarizes the basic costs of the phytoremediation solution and compares these costs to those of a traditional mechanical pump and treat method.

Background

The Riley County Landfill is an 80-acre site about 2 miles south of Manhattan, located in the Kansas River Valley. The area surrounding the landfill is mostly farmland with some rural residences and a small trailer park. The landfill began operating as a municipal solid waste dump site in 1963. In 1985, the Kansas Department of Health and Environment sampled residential wells east of the landfill and found low levels of VOCs. In December 1990, the court ordered Riley County to evaluate the ground water in the area surrounding the landfill and propose remedial action. Riley County contracted CH2MHILL Inc. to install more sampling wells and further evaluate ground water quality. Figure 1 shows the location of the testing wells. CH2MHILL found VOC concentrations that exceeded the Kansas Action Level (KAL) (see glossary) for benzene and many chlorinated organic compounds (CH2MHILL, 1993; CH2MHILL, 1994; CH2MHILL, 1995). Although VOC contamination was above the KAL for some samples, most samples showed concentrations well below the KAL. Figure 2 shows the sum of all organic contaminants for every sampling for all wells. The average summed concentration of organic compounds in the ground water was 2 ppb. The concentrations fluctuated enormously and did not show any time related or position related trends.
Riley County recently closed the landfill, and CH2MILL installed an EPA approved cap to prevent vertical downward movement of water through the landfill. However, there is still horizontal movement of ground water through the entire area, including the landfill when the water table rises. As a result, the ground water comes in contact with further contamination whenever there are heavy rains or a rise in the river level. Figure 3 is a map of typical water table levels throughout the landfill area. Water flows perpendicularly to the lines of constant water level, resulting in ground water flow to the east. A 130 acre site directly east of the landfill is owned by Riley County and will be used for the phytoremediation project. Figure 4 shows a cross-sectional view of the site and its approximate dimensions.
Phreatic Surface Map

1007 1006 1005 1004 (mean feet above sea level)

landfill

Kansas River

North

Figure 3. Water Table Levels in April 1995

Figure 4. Cross-sectional View of the Site

Phytoremediation cleans up contaminated ground water in two ways. First, vegetation "pumps" the contaminated water from the aquifer into the root zone where VOCs can be biodegraded using microorganisms sustained by root exudates. Second, vegetation located near the landfill prevents the contamination from spreading throughout the aquifer to residential wells. The water and any remaining VOCs are brought to the atmosphere through evapotranspiration and released in unharmfully small quantities. Poplar trees and alfalfa have the unique ability to send their roots very deep and use water directly from the water table, which makes them very suitable for phytoremediation. Riley County preferred poplar trees because they require less maintenance than alfalfa.
The traditional approach to VOC removal is to mechanically pump the contaminated water out of the soil and treat it or spray it into the air, allowing the VOCs to volatilize into the air. Using phytoremediation, some of the VOCs are pumped through the roots to the atmosphere and some of the VOCs are biodegraded in the root zone. The cost of phytoremediation is often only a small percentage of the cost of a traditional pump and treat system.

**Analysis**

To determine what factors affected the water flow through the site, a simplified water balance was made. Figure 5 depicts a standard water balance.

![Figure 5. Standard Water Balance](image)

The water balance for the Riley County site can be reduced to precipitation, ground water inflow and outflow, and evapotranspiration. The runoff and runon terms in the standard water balance are eliminated for two reasons. First, the soil of the site and the surrounding fields is very sandy, and precipitation immediately penetrates the surface. Second, the land is essentially flat, so there is no slope for water to run off. The precipitation term was approximated, and the value we used was 2.5 (cubic feet
water)/(yr)(square foot land).

Next, the amount of ground water flowing through the 130 acre site was calculated. Darcy’s law uses the hydraulic conductivity, the aquifer’s cross-sectional area, and the hydraulic gradient to predict the amount of ground water flowing through the site. A value of hydraulic conductivity for the site was not available, so a modified version of Darcy’s law using transmissivity was used instead. The equations for Darcy’s law and the modified version we used can be found in the glossary. The transmissivity value for the site was 4560 square feet/day (Brummer, 1993). This value is on the high side of other literature values which range from 70-7000 square feet/day.

A width of about 1400 feet, which is a bit wider than the landfill itself, was chosen as the width of the phytoremediation site. Then, with the chosen width, the volumetric flow rate of water through the site was calculated. Because the rainfall and hydraulic gradient could fluctuate considerably for this part of Kansas, the volumetric flow rate of water was doubled to 9.8 million cubic feet/year. The length of the site was then determined based on the evapotranspiration rate and the optimal tree planting density for poplars.

There were two main considerations in designing the optimum tree planting plan: the number of trees needed and the best location to plant these trees. Two boundary conditions were set for the tree planted site. The northern border was assumed to be a no flux border, and the water table level at the eastern border was set to that of the river to eliminate the hydraulic gradient. We calculated the number of trees required to completely evaportranspire 9.8 million cubic feet of water per year. To do this, the poplar tree water removal rate was multiplied by the square feet of land to be planted with trees. Poplar trees remove water at a rate of approximately 6 (ft$^3$water)/(yr)(ft$^2$land) with a closed canopy (Davis et. al., 1996). A closed canopy exists when foliage covers the land entirely, and this results in the largest possible water removal rate. A tree density of 1500 trees/acre is optimum for phytoremediation sites using poplar trees (personal communication, Ecolotree, 1996). This density results in an 85% survival rate and a closed canopy the fifth year after planting. In approximately two years, the roots of the poplars extend 15 feet below the surface to the water table. With the chosen width of 1400 ft, the required length (east-west) of the planted site was determined to be approximately 2000 feet. During the heavy rains, the water level in the river and aquifer rise. As the water level drops, water flows through the aquifer in a very different manner. In some cases the water flows in the opposite direction from that observed in normal conditions. For this reason, the landfill should be surrounded with trees. It is estimated that approximately 2 acres are available on Riley County’s land between the landfill and the property line. It is suggested that as much of this land as possible be covered with trees as a buffer area in order to help in the removal of water during a change in the water level. The total number of trees proposed is 96,500 trees in the main planting area and 2,900 trees in the buffer area. The area east of the landfill to be planted with trees contains 64 acres. Figure 6 shows a layout of the optimum tree planting plan.
After the trees begin pumping the water from the area, the water table level will fall, creating a water sink on the site. Rough estimations with new hydraulic gradients showed that the site would still be able to evapotranspire all the water entering the area before it left the area. Figure 7 shows the new water table levels predicted for the area. Linear interpolation between two river sample sites was used to estimate the water levels throughout the aquifer. It should be noted that in the process of drawing more water into the site, the concentration of contaminants is predicted to fall well below the KAL for all contaminants.

Figure 6. Optimal Tree Planting Plan

Figure 7. Predicted Water Table Levels
In the calculations performed above, biodegradation of contaminants in the root zone was not considered. There has been much research done showing that alfalfa and poplar trees are capable of causing biodegradation of contaminants in the root zone (Davis et al., 1996; Narayanan et al., 1995). Figure 8 shows a proposed mechanism for the biodegradation of TCE and TCA, two common contaminants, in the root zone of the site.

Figure 8. Mechanism for Biodegradation of Contaminants in Root Zone

**Economics**

Compared to the alternatives, phytoremediation is extremely economical. CH2MILL estimated the mechanical pump and treat method to cost approximately $4,000,000 initially and $8,000,000 to operate for twenty years. Since no one knows the exact amount of contamination present at the landfill site, the pumping may go on much longer than 20 years, causing the cost of the pump and treat to increase. We estimated the phytoremediation solution to cost $217,000 initially, with a small annual maintenance cost. A summary of these costs can be seen Table 1.
<table>
<thead>
<tr>
<th>Item</th>
<th>Quantity</th>
<th>Cost</th>
</tr>
</thead>
<tbody>
<tr>
<td>Main Planting Area</td>
<td>64 acres (97,000 trees)</td>
<td>$169,000</td>
</tr>
<tr>
<td>Buffer Area</td>
<td>2 acres (3,000 trees)</td>
<td>$5,000</td>
</tr>
<tr>
<td>Transportation</td>
<td>4 trucks</td>
<td>$16,000</td>
</tr>
<tr>
<td>Irrigation (if needed)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Total Cost</td>
<td>$180,000</td>
</tr>
<tr>
<td></td>
<td>+20% Contingency</td>
<td>$217,000</td>
</tr>
</tbody>
</table>

Table 1. Summarized Costs of the Phytoremediation Project

The costs of the phytoremediation project may be decreased by planting in a stage wise fashion. Originally, a small section of the total remediation site may be planted. Then, offshoots of these trees may be taken and planted until the full area is covered.

There are several types of maintenance that might be needed for this site. Irrigation, fertilization, application of pesticides, and further well monitoring are the main areas of concern. If the site requires irrigation, it will cost approximately $8,000 per year for the first two years. After this time, the tree roots will have reached the water table and will no longer need irrigation.

In terms of actual cost to the average taxpayer, the phytoremediation project will cost about $5 per individual in a five year time span, whereas the mechanical pumping will cost $240 per individual in a twenty year time span.

Summary

The phytoremediation of the Riley County Landfill by use of poplar trees is a very economical and effective solution to treating ground water contamination. Poplar trees will be used to remove the contaminated water and biodegrade the contaminants. It was found that an area 1400 feet wide by 2000 feet long is required to prevent contaminant flow from the county's property. The removal rate of water from the land directly east of the landfill was calculated to be 9.8 million cubic feet per year. In addition, a buffer area of trees surrounding the landfill is recommended to compensate for changing water profiles during heavy rainfall. In addition to stopping the contaminated ground water flow, the trees will bring the contaminants from the aquifer into the root zone where they are biodegraded.
Glossary of terms and equations used

terms:
hydraulic conductivity - a property of an aquifer which measures its ability to transmit water under a hydraulic gradient.
hydraulic gradient - the drop in water level between two points divided by the distance between the two points
Kansas Action Level - (KAL) the maximum contaminant concentration allowed in ground water used for drinking
transmissivity - the product of hydraulic conductivity and aquifer thickness, this property measures an aquifer's ability to transmit water through its void space

equations:
Darcy's law

\[ Q = -k \cdot A \cdot \frac{dh}{dl} \]

where

\[ A = \text{cross-sectional area (square feet)} \]
\[ \frac{dh}{dl} = \text{hydraulic gradient (feet/feet)} \]
\[ k = \text{hydraulic conductivity (feet/yr)} \]
\[ Q = \text{water flow rate (cubic feet/yr)} \]

definition of transmissivity

\[ T = k \cdot b \]

where

\[ T = \text{transmissivity} \]
\[ b = \text{aquifer depth} \]

modified Darcy's law

\[ Q = -T \cdot w \cdot \frac{dh}{dl} \]

where

\[ w = \text{the width of the aquifer section (feet)} \]

rate of evapotranspiration

\[ R = L \cdot w \cdot C \]

where

\[ w = \text{the planting width of trees (feet)} \]
\[ C = \text{water removal rate (cubic feet/square foot of land \( \times \) year)} \]
\[ L = \text{the length from the landfill required for planting (feet)} \]
\[ R = \text{water evapotranspiration rate (cubic feet/yr)} \]
Acknowledgements

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BIOLOGICAL TREATMENT OF OFF-GASES FROM ALUMINUM CAN PRODUCTION: EXPERIMENTAL RESULTS AND MATHEMATICAL MODELING

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Increasing regulation of air quality has led companies to look for more cost effective technologies to reduce their emission of volatile pollutants. Bioreactors are a promising option.

The objectives of this research project were to construct and characterize a bench-scale biofilter and to develop a mathematical model of the reactor. The biofilter was designed for the treatment of gas containing 1-butanol, 2-butoxyethanol, and N,N-dimethylethanolamine, which are produced during the manufacture of aluminum cans. The experimental results were used to verify the mathematical model developed for the system. This presentation will outline the steps that have been performed to achieve these objectives.

A six-member consortium, capable of degrading the mixture of compounds, was isolated from activated sludge. Monod biodegradation kinetics were observed in suspended culture studies using single substrates. Inhibition was observed in studies with multiple substrates. A biofilter consisting of three stages of packed bed and air re-humidification sections has been characterized. High removal efficiencies were observed at 20, 30, and 45 seconds of residence time. Adsorption-desorption phenomena were observed in experiments when loading rates were changed. Mass balances were performed to obtain a set of two-dimensional equations that described steady state behavior. The modified Monod kinetics expressions obtained from experiments were incorporated into the model, which yielded predictions that were in close agreement with experimental data.
Inertial migration based separation of Chlorella microalgae in branched tubes

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Abstract

When a dilute suspension flows in the laminar regime through a tube, under certain conditions the suspended particles migrate laterally to an equilibrium radial position. Efficient concentration of the suspension in branched tubes may be achieved by adjusting the flow fraction through the branch such that the dividing stream surface is located within the dilute region of the flow field. Suspensions of microalgae, Chlorella vulgaris, were pumped through various diameter tubes for tube Reynolds number ranging from 47 to 1839 and photographed. Upstream particle concentration profiles were obtained by image analysis of the photographs. As the Reynolds number increased the concentrated particle region shifted towards the tube wall. The dividing stream surfaces in branched tubes were obtained from the three-dimensional numerical solutions of the Navier-Stokes equations for steady, laminar, and homogeneous flow through tubes having one and two orthogonal branches. Concentration factors for Chlorella suspensions in branched tubes, predicted by a general method, fall in the range 1.0 to 1.3. Experimental results for a single branch tube fall in this range.

1 INTRODUCTION

In 1962, Segre & Silberberg showed that when a dilute suspension of neutrally buoyant rigid spheres is transported along in Poiseuille flow, the particles are subjected to lateral forces that force them to migrate to an equilibrium radial region located approximately sixty percent of the radial distance from the tube axis to the tube wall. This phenomenon is referred to as inertial migration because Bretherton (1962) demonstrated that, if the inertia terms in the equations of motion of fluid are neglected, then no lateral force can exist on a body of revolution in a non-uniform, unidirectional flow. The mechanisms causing such a force distribution have been identified as inertial lift due to the presence of shear, wall repulsion due to the lubrication effect, lift due to particle rotation and, in the case of Poiseuille flow, a lift due to the added presence of velocity profile curvature (Feng et al., 1994).

The phenomenon of inertial migration has also been observed in non-spherical particles. Rakow and Chappell (1987) found that a suspension of helical shaped microalgae, Spirulina platensis, flowing through a 0.65 millimeter diameter tube migrated radially to a relatively narrow annular region. In a branched tube device, the nonuniform distribution of suspended particles across the parent (main) tube is utilized to achieve a separation by directing the outer flow field of the main tube through a branch. For Spirulina suspensions, by directing the outer dilute flow field through the branches it is possible to significantly concentrate the suspension leaving the main tube (Rakow and Fernald, 1991).

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Efficient skimming of the flow field in branched tubes may be obtained by adjusting the flow fraction through the branch such that the dividing stream surface is located within the dilute region of the flow field as the suspension arrives at the branch junction. The dividing stream surface is defined as the collection of streamlines acting as the boundary between the portions of the flow which enter each downstream branch. Knowledge of the dividing stream surface location and the concentration profile at an upstream location is required to evaluate the fraction of total particles going into the branch.

The present study will examine the inertial migration based concentration of suspensions of Chlorella vulgaris in branched tubes. Chlorella vulgaris is a microalgae, and is roughly spherical in shape with a size of approximately 5 μm. The study is divided into two major parts. The first part is concerned with the numerical determination of the location and shape of the dividing stream surfaces in branched tubes and the effect of parameters, upstream tube Reynolds number, $Re$, the diameter ratio of the branch to the main, $γ$, and the fraction of inlet flow leaving the branch, $Q_f$, on the dividing stream surface.

The second part consists of determining the inertial migration characteristics of Chlorella vulgaris in tube flow and the resultant radial particle concentration profiles in tubes. The particle concentration profiles in conjunction with the flow field in branched tubes are then used to determine the separation that may be achieved in branched tubes for various flow conditions. Also, experiments were performed to determine the concentration of Chlorella suspensions in single-branch systems.

2 Flow field in branched tubes

2.1 Governing equations and Numerical Scheme

The flow field in branched tubes has been determined by numerically solving the three-dimensional Navier-Stokes equations for the flow of a homogeneous fluid in branched tubes. The obtained flow field is then analyzed to determine the location of the dividing stream surfaces and the secondary flows in branched tubes. It is assumed that the fluid is incompressible and Newtonian. The equations are non-dimensionalized using the diameter of the main tube, $D_m$, as the characteristic length, the flow average velocity at the inlet of the main tube, $\langle V_{m,i} \rangle$, as the characteristic velocity, and $\rho \langle V_{m,i} \rangle^2$ as the characteristic pressure scale. The dimensionless forms of the governing equations, written in integral form, are then

$$\int \int_s u \cdot dA = 0, \quad (1)$$

and

$$\frac{\partial}{\partial t} \int \int_v u \, dV + \int \int_v u \cdot \nabla u \, dV = - \int \int_s p \, dA + \frac{1}{Re} \int \int_s (\nabla u + (\nabla u)^T) \cdot dA, \quad (2)$$

where the velocity vector is $u = (u_x, u_y, u_z)$, corresponding to the cartesian coordinates $(x, y, z)$, $p$ is the dynamic pressure, and the Reynolds number is defined as $D_m \langle V_{m,i} \rangle \rho / \mu$. $A$ represents the area vector of the surface of the control volume. A cell-centered finite-volume formulation has been used to discretize the governing Eqsns. (1) and (2). All variables are defined at the cell center and cartesian components are used for tensor quantities. The details of the finite-volume formulation used are described by Dandy & Dwyer (1990). Linear interpolation was used.
to evaluate the variables, operators, and geometric properties at each face. The solution algorithm and the pressure correction scheme is described in detail by Dwyer (1989). The three cartesian velocity components are computed iteratively using a predictor/corrector scheme and the pressure correction algorithm consists of solving a Poisson equation derived from the continuity equation.

Composite grid generation (CGM) method was used for mapping the domain. The primary motivation behind using CGM is the simplification in meshing a complex domain through the use of geometrically simpler overlapping subdomains, whose union covers the entire domain of interest. Each subdomain is then fitted with an independently generated component grid, ensuring that any physical boundary present in that region is accurately represented by the coordinate system chosen for the subdomain. The component grids overlap each other without any requirement that they exactly match up at the edges. A general approach for creating and using composite overlapping grids for the solution of elliptic and time-dependent partial differential equations has been adopted by Chesshire and Henshaw (1990). Two intersecting, mutually perpendicular cylinders are used as the geometric model for a orthogonal bifurcation. Cylindrical coordinates were used to generate the component grids, and the component grids overlapped each other at the junction, as illustrated in Figure 1. Details about composite grid are provided in Poflee, 1996.

Figure 1: Computational domain and component grids for a branched tube.

Each computation is carried out for specified values of the three parameters: $Re$, $\gamma$, and $Qf$. These quantities are prescribed at the beginning of each run. The fraction of the flow leaving the branch exit is used as a forcing condition by imposing a corresponding fully developed velocity profile at the exit of the branch. All computations started with a dimensionless time step of 0.001 and the time step is gradually increased in a geometric progression to $O(10^{-2})$ by the end of a simulation in order to attain relatively faster convergence towards the end of the computation. All computations were done on Hewlett Packard workstations (Series 700). For $Re = 250$, each global time step took approximately 49 cpu seconds on these workstations.

2.2 Results on flow field in two-branch bifurcations

The flow field obtained by the numerical solution of the Navier-Stokes equations in branched tubes is analyzed to obtain the dividing stream surface (branch influence zone) and secondary flows. At the outlet cross-section of the main tube and the branch tube, point particles are seeded and marched backwards in time to find their original positions at the inlet of the main tube. This procedure identifies the influence zone of the branch. The locus of the dividing stream surface at the inlet of the main tube can then be determined by fitting a smooth curve through the boundary of the influence zone. For tubes with one orthogonal branch, three dimensional numerical results for upstream $Re$
up to 2.5 are available (Enden & Popel, 1992). For tubes with two orthogonal branches, currently no numerical or experimental results are available in the literature regarding dividing stream surfaces or secondary flows. This type of system provides symmetrical flow skimming due to the presence of two diametrically opposed branches, which is advantageous from the point of view of separation. Since flow is symmetric with respect to the perpendicular plane in two-branch systems, only the right half of the domain is necessary.

Figure 2: The effect of $Q_f$ on the dividing stream surface in two-branch tubes for $\gamma = 0.5$ and $Re = 10$.

Figure 2 illustrates the effect of flow fraction on dividing stream surface location for $\gamma = 0.5$. The dividing stream surface moves away from the branch mouth with increasing flow fraction through the branch. Figure 3 shows the effect of the diameter ratio of the branch to main on the dividing stream surface. For larger diameter branches, the dividing stream surface is closer to the tube wall.

Figure 3: The effect of diameter ratio on the dividing stream surface in two-branch tubes for $Re = 47$ and $Q_f = 0.1$. The diameter ratios are 0.5 and 1.0.

Figure 4 illustrates the effect of Reynolds number for the smaller diameter ratio branched tubes. The shape of the dividing stream surface changes from concave to convex with increasing $Re$. Also, the curvature of the dividing stream surfaces increases with increasing Reynolds number. Moreover,
Figure 4: The effect of $Re$ on the dividing stream surface in two-branch systems for $\gamma = 0.5$ and $Q_f = 0.3$.

for $Re = 150$ and $250$, the dividing stream surface does not intersect the tube wall, but rather forms a closed dividing stream surface. In Poflee et al. (1994), regarding separation of Spirulina suspensions in branched tubes at high Reynolds number ($1200 \leq Re \leq 1800$), closed, circular dividing stream surfaces were assumed. Moreover, the radius of this circular dividing stream surface was determined based on mass balance, the Poiseuille velocity profile at the junction, and the assumption that the fluid outside the circular surface will enter the branches. Based on these assumptions, the radius of the ideal circular dividing stream surface for a given flow fraction, $Q_f$ through the branches is given by,

$$k = \sqrt{1 - \sqrt{Q_f}},$$

where $k = r/R$, $R$ is the radius of the main tube and $r$ is the radius of the circular dividing stream surface. In order to compare the ideal circular dividing stream surface with the closed dividing stream surface for $Re = 250$, $Q_f = 0.3$, $\gamma = 0.5$; the two dividing stream surfaces are plotted in Figure 5. From this result, it is arguable that a circular dividing stream surface is a reasonably good approximation of the actual dividing stream surface for $Re > 250$.

The secondary flows in branched tubes were observed as spiral flows in the branch mouth. No closed streamline wakes were found for the parametric range studied and the secondary flows did not interfere with the formation of a smooth dividing stream surface. This behavior is consistent with experimental observations of secondary flow by Rong & Carr (1990) and Karino et al. (1979) in tubes with an orthogonal branch.

3 **Inertial migration of Microalgae suspensions in tube flow**

The inertial migration characteristics of microalgae suspensions of Chlorella vulgaris is examined in this section. A suspension of Chlorella was pumped through various diameter tubes and photographed, and subsequently the photographs were digitized and analyzed. The image analysis is based on the assumption that the amount of incident light absorbed by a suspension is linearly
Figure 5: Comparison of the numerically computed dividing stream surface with the ideal circular dividing stream surface for $Re = 250$, $Q_j = 0.3$, and $\gamma = 0.5$, in a two-branch system.

The concentration is proportional to the concentration of the suspension. The image analysis procedure is described in Paflee et al., 1994.

Figure 6 shows the particle concentration profiles of Chlorella suspension obtained from image analysis of the photographs of suspension flowing through a tube of diameter 203 $\mu$m at $Re = 47$. The concentration is expressed in terms of grey scale obtained from the digitized images.

Figure 6: Upstream concentration profile of Chlorella vulgaris suspension obtained by image analysis; $Re = 47$ and tube diameter is 203 $\mu$m.

Figure 7 shows the upstream particle concentration profile of Chlorella vulgaris suspensions for the higher Reynolds numbers. The suspension was pumped through a 405 $\mu$m diameter tube. Note the cell depleted region around the tube axis as compared to that for the lower Reynolds number case shown earlier in Figure 6.

The concentration profiles are used in conjunction with the dividing stream surfaces in branched tubes to obtain the concentration factors produced in branched tubes. Concentration factor is defined as the ratio of the exit concentration to the inlet concentration, and it provides a measure of the separation. The procedure to compute concentration factor is as follows. It is assumed that the suspension is dilute, the particles are neutrally buoyant, and they have the same velocity as
Figure 7: Upstream concentration profile of Chlorella vulgaris suspension obtained by image analysis; Reynolds numbers are 490, 1176, and 1839, and the tube diameter is 405 μm.

A fluid particle would have at the position at which the particle is located. Then, by integrating the velocity field in conjunction with the concentration profile over the region demarcated by the dividing stream surface near the junction, it is possible to determine the amount of material going into the branch.

The dimensionless rate at which particles enter the inlet of the main tube is computed by:

$$S_{m,i} = \int_0^1 \rho c u 2\pi r \, dr,$$

where \( r \) is the radial position, \( c \) is the particle concentration, and \( u \) is the axial velocity. The fraction of particles, \( f_\psi \), going through the influence zone, \( \psi \), demarcated by the separating surface at the same upstream location (see Figure 8) is then given by:

$$f_\psi = \frac{\int_\psi \rho c u \, d\psi}{S_i}.$$

If \( Q_f \) is the fraction of the total inlet flow rate leaving through the branches, the concentration factor produced at the exit of the branch is given by:

$$\langle c_b \rangle = \frac{f_\psi}{Q_f}.$$

Similarly, the average concentration of particles leaving the exit of the main tube \( \langle c_{m,e} \rangle \) can be obtained by:

$$\langle c_{m,e} \rangle = \frac{(1 - f_\psi)}{(1 - Q_f)}.$$

Using the location of the dividing stream surfaces and the concentration profiles shown in Figure 7 the concentration factors for Chlorella suspensions at the exits of branch and the main tube are calculated and shown in Figure 9 for two-branch systems. Branch concentration factors as high as 1.3 are obtained for \( Re = 1176 \) for Chlorella suspensions.
Figure 8: Schematic for the evaluation of the flow through the influence zone at an upstream location in the main tube.

Experiments were conducted to determine the suspension concentration of Chlorella suspensions in branched tubes. These experiments were conducted in a glass branched tube having a single branch. The bifurcation angle of the branch was 45°. The diameter of the main tube and the branch was 405 μm, i.e., it was an equidiamic system. The upstream length of the main tube was 50 cm and the downstream length of the main tube was 10 cm. The branch length was also 10 cm. The experiments were conducted for Re = 1400 and for a flow fraction value of 0.43 through the branch. The experiments were conducted in a manner similar to that described in Rakow and Fernald, 1991. The concentrations of the suspensions leaving the main tube and branch were measured as percent dry mass (%DM). In these experiments, it was found that the branch concentration factors were close to 1. There is an error of 5% in the dry mass measurements. Concentration factors is a ratio of two concentrations and therefore the error in concentration factors will be 10%. Taking into account this error, the experimental results for concentration factors is close to what has been predicted for Chlorella suspensions in Figure 9. For single-branch systems, closed dividing stream surfaces were observed for Reynolds number 200 in our numerical computations and also in the experimental observations of Kotha (1993). At still higher Reynolds number, it is expected that these closed dividing stream surfaces are concentric with the main tube cross-section because of the large momentum of the core region of the flow. This observation implies that the numerical computation of the concentration factors based on the circular dividing stream surfaces for two-branch system is also applicable to the case of single-branch tube flows.

4 Discussion

In order to have a potential for separation by branched tubes, the concentration factors should be significantly greater than 1.0. As seen in the earlier section, the maximum concentration factor for Chlorella suspensions is 1.3 and that is not significantly high for a practical application. For Spirulina platensis suspensions concentration factors as high as 3.0 were obtained in branched tube experiments by Rakow and Fernald (1991). In the experimental observations of Rakow and Fernald (1991), Spirulina suspensions concentrated in the main tube and the dilute part of the flow field exited the branch. Spirulina particles occupy radial equilibrium positions closer to the tube axis in contrast to Chlorella which tend to concentrate closer to the tube wall. Chlorella concentrates in the branch, and the secondary flows are located at the branch mouth. At high Reynolds numbers,
Figure 9: Predicted concentration factors in two-branch systems for Chlorella vulgaris suspension as a function of $Q_f$; Reynolds number are 490, 1176, and 1839.

these secondary flows may negate the particle distribution created by inertial migration, thereby further reduce the concentration factors.

5 Conclusions and Future Work

A useful contribution of this work is the development of a three-dimensional composite overlapping grid scheme and its incorporation into a finite-volume based Navier-Stokes equation algorithm, which can be readily employed to study flows in complex geometries, for example, to study the flow of suspended particles in tubes.

The concentration factors for Chlorella suspensions in branched tubes is not significantly high enough for practical application. In our current investigation on concentrating Spirulina suspensions in branched tubes, very encouraging results have obtained in our laboratory experiments. It is planned to compute concentration factors for Spirulina suspensions in branched tubes for various flow conditions and geometrical configurations so as to determine the optimum conditions for its separation in branched tubes.

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References


INTRODUCTION

Since first used for bioseparations by Albertsson (Albertsson, 1986), aqueous two-phase systems have drawn interest because they offer mild conditions, short processing times, and ease of scale-up (Albertsson, 1986, Walter et al., 1985, Zaslavsky, 1994).

Considerable advances in the theory of aqueous two-phase systems and partitioning phenomena have been made in the last ten years (Albertsson, 1986, Walter et al., 1991, Abbott et al., 1990, Walter et al., 1985). Interactions determining protein partitioning in phase systems have been characterized (Albertsson, 1986). Among them, electrostatic interaction plays an important role in the protein partitioning in that protein usually carries net charge and it has been found that an interfacial electrostatic potential difference exists between two phases in a phase system (Johansson, 1985).

Albertsson (1986) proposed an expression for the partition coefficient when electrochemical force dominates the partitioning process:

\[
\ln K_p = \ln K_o + \frac{z_P F}{RT} \Delta \varphi
\]

where \( K_o \) is the partition coefficient in the absence of interfacial potential difference; \( z_P \) is the protein net surface charge; \( \Delta \varphi \) is the electrostatic potential difference; \( F \) is Faraday’s constant and \( T \) is the temperature.

The partition coefficient for a given protein in a phase system with the known \( K_o \) can be predicted from this equation provided the known potential difference and protein net charge. Conversely, the potential difference can be calculated from the slope of a plot of \( \ln K_p \) versus \( z_P \). Using the latter method, while varying \( z_P \) by changing pH, potential differences in the range of 0-20 mV were found (Johansson, 1974a, 1985, Hartounian et al., 1994, Haynes et al., 1989, Forciniti et al., 1992, 1991, Luther and Glatz, 1994, Schluck et al., 1995a, 1995b). However, a linear relationship between \( z_P \) and \( \ln K_p \) was not always obtained. One reason may be that \( \Delta \varphi \) was affected by pH (Albertsson et al., 1986).

Previous studies (Luther and Glatz, 1994) have employed genetic engineering techniques to alter a protein’s net charge so that the role of charge on partitioning can be investigated without modifying the phase system. A series of \( \beta \)-galactosidases (Zhao et al., 1990) with different lengths of polyasparate fused to the C-termini and T4 lysozymes (Dao-Pin, 1991) with charge-change point mutations were obtained and their partition coefficients in aqueous two-phase systems were measured. It was found that the partitioning behavior of T4 lysozyme mutants agreed only qualitatively with Equation 1, while for the \( \beta \)-galactosidase fusions, a linear relationship between \( \ln K_p \) and \( z_P \) was not observed. The different results for point mutants and fusions indicate non-electrostatic factors, lumped into the \( K_o \) term in Equation 1, also influence the protein partitioning in the phase systems. Particularly when adding long fusion tails to the protein, the size,
conformation, hydration and dipole moment could be changed as well leading to different molecular interactions in the phase solution. Alternatively, Equation 1 does not properly account for non-electrostatic effects.

However, because of the very different properties of T4 lysozyme and β-galactosidase, e.g. size, conformation, surface charge, hydrophobicity, it is difficult to compare and examine the influence of the addition of a charged fusion tail to the protein to its partitioning in an aqueous two-phase system. In this research, polyarginine tails were fused to the C-terminus of the T4 lysozyme triple point mutant to provide sets of point mutants and fusions of matching net charge. Comparing the partition coefficients of the two versions of lysozymes allows comparison of “charge” and “tail” effects.

Light scattering can be used to investigate the “charge” and “tail” effects in polymer-protein interactions in phase systems. A semi-empirical model is proposed which accounts for the partitioning behaviors of these proteins in a series of phase systems. It is desired that the model can eventually be applied to guide the genetic alteration of protein charge toward enhanced selectivity in protein purification using aqueous two-phase systems.

THERMODYNAMIC FRAMEWORK

Many models have been proposed to predict the phase diagram and protein partitioning coefficient. Among them, Equation 1 remains one of the simplest and has been favored when electrostatic interaction dominates the partitioning process (Albertsson, 1986). The application of the model has been limited because it includes all the other interactions into the empirical $K_0$ such that it can only used into a specific system with a specific protein. Furthermore, it has to be pointed out that in the equation, electrostatic potential $\phi$ is the pure electrical potential and it doesn’t affect the activity coefficient of ionic species in the two phases. The chemical potential of an ionic species, however, is influenced by the electrostatic potential in a relatively simple way:

$$\mu_i = \mu_i^0 + RT \ln \gamma_i C_i + Fz_i \phi$$  \hspace{1cm} (2)

where $\mu_i$ is the chemical potential of species $i$, $\mu_i^0$ is the chemical potential at the standard conditions; $\gamma_i$ is the activity coefficient; $C_i$ is the concentration and $z_i$ is the charge of the ionic species.

Therefore, even though there is the argument that (Zaslavsky1995) an interfacial potential difference won’t affect the partitioning of ionic solutes because of the electroneutrality, we believe the potential difference term in Equation 1 does indicate the contribution of this pure electrical potential to the chemical potential of ionic species in phase solutions. Previous research has also indicated the usefulness of Equation 1’s treatment of electrostatic effects (Johansson, 1985, 1994, King et. al., 1988, Luther and Glatz, 1994). It has been used with success to determine isoelectric point of proteins (Johansson, 1974a). Here, we retain Albertsson’s treatment of the electrochemical effect to test its applicability using the two series of charge-mutants.

Nevertheless, it is important to notice that the potential difference measured with Ag/AgCl electrodes is different from the pure electrical potential difference in Equation 1. Guggenhein(1967) noted that the pure electrical potential difference was not measurable, while the measured potential difference is actually the difference of
electrochemical potential of the reference ion $r$ (in our case, is $K^+$), according to the quasi-electrostatic-potential theory developed by Newman, and later applied to phase systems by Haynes, et al. (1991). The electrochemical potential of reference ion $r$, $\Phi$, is defined as:

$$\mu_r = \mu_r^0 + RT \ln(m_r \gamma_r) = RT \ln(m_r) + z_r F \Phi$$

where $\mu_r$ is the chemical potential of the reference ion $r$, $\mu_r^0$ is the chemical potential at the standard state; $\gamma_r$ is the activity coefficient and $m_r$ is the molality of the reference ion. Equation 3 indicates that this electrochemical potential has actually included the nonidealities of the reference ion in the phase solutions.

Two other classes of model have been developed to account for non-electrostatic interactions, i.e. those included in $K_0$ of Equation 1. One employs the Flory-Huggins theory (a lattice model) (Flory, 1953, Huggins, 1942) and the other uses the osmotic virial expansion of Edmond and Ogston (Ogston et al., 1968).

Brooks et al. (1985) first applied the Flory-Huggins model to protein partitioning in aqueous two-phase systems by treating the protein as a third polymer component. For a system containing four components: 1-solvent, i.e. salt-water; 2-polymer 2, i.e. PEG; 3-polymer 3, i.e. dextran; 4-protein. Diamond et al. (1991) compared the magnitude of the five terms in the Flory-Huggins model for a PEG-Dextran phase system with low salt concentration, and were able to simplify the expression to two terms plus an electrostatic effect term with the potential difference $\Delta \varphi$.

$$\ln K_p = k_1(w_2^b - w_2^t) + k_2(w_2^b - w_2^t)^2 + \frac{z_p F}{RT} \Delta \varphi$$

where $k_1$ and $k_2$ are related to the Flory-Huggins interaction parameters, the molar volume ratios, and the protein molecular weight; they can be considered constant for a specific protein in a specific phase system. $w_2^b$ and $w_2^t$ are weight fractions of polymer 2, i.e. PEG, in the bottom and top phases, respectively. The third term was added to the model to take into account the electrostatic effect in the same manner as Equation 1.

The virial expansion model is based on a mathematical expansion of the thermodynamic properties of the polymer solution in terms of the concentrations of the polymer (Hill, 1962). King et al. (1988) derived the expression for the protein partition coefficient including both electrostatic and non-electrostatic effects:

$$\ln K_p = a_{2p}(m_2^b - m_2^t) + a_{3p}(m_3^b - m_3^t) + \frac{z_p F}{RT} \Delta \varphi$$

where $m_2^b$, $m_2^b$ are the molar concentration of polymer 2 in the top phase and bottom phase; $m_3^b$, $m_3^b$ are the molar concentration of polymer 3 in the top phase and bottom phase. $a_{2p}$ and $a_{3p}$ are the parameters directly related to the second virial coefficient $A_{2p}$ and $A_{3p}$, in units of $mL/mol/g^2$:

$$2A_{2p} = 1000 \frac{a_{2p}}{M_2 M_p}$$

$$2A_{3p} = 1000 \frac{a_{3p}}{M_3 M_p}$$
\( M_2, M_3, M_p \) are the molecular weight of polymer 2, polymer 3 and the protein respectively. Substituting Equation 6 into 5, we have:

\[
\ln K_p = 2A_{2p}M_p(w_2^B - w_2^T) + 2A_{3p}M_p(w_3^B - w_3^T) + \frac{z_p F}{RT} \Delta \phi
\]

(7)

where \( w_2 \) and \( w_3 \) are the weight fraction concentrations of polymer 2 and 3; subscripts \( T \) and \( B \) denote top phase and bottom phase respectively.

Clearly, Equation 4 and 7 are more alike than they first appear (Walter, et al., 1991). Equation 7 has neglected the higher order terms in the virial expansion. For parallel tie-lines, often a reasonable approximation, the polymer 3 concentration difference can be considered proportional to the polymer 2 concentration in Equation 7. Therefore, if we neglect the second order term in Equation 4, which is often reasonable when the concentration difference is small, Equation 7 and 4 both approximate to:

\[
\ln K_p = a(w_2^B - w_2^T) + \frac{z_p F}{RT} \Delta \phi
\]

(8)

where \( a \), a combination of the second virial coefficients \( A_{2p} \) and \( A_{3p} \), indicates the magnitude of the second virial coefficient between the protein and the two polymers in the phase system:

\[
a = 2M_p[A_{2p} + A_{3p} \left( \frac{w_3^B - w_3^T}{w_3^B - w_3^T} \right)]
\]

(9)

Moreover, it has been reported that the potential difference, \( \Delta \phi \), has a linear relationship with the tie-line length \( TLL \) (King, et al., 1988), which for parallel tie-lines, is in turn proportional to \( (w_2^B - w_2^T) \). Thus,

\[
\Delta \phi \propto (w_2^B - w_2^T)
\]

(10)

Substituting Equation 10 into Equation 8, the partition coefficient can be expressed as:

\[
\ln K_p = (w_2^B - w_2^T)(a + bz_p)
\]

(11)

where \( b = \frac{(F/RT)\Delta \phi}{w_2^B - w_2^T} \)

(12)

This linear relationship between partition coefficient and protein net charge can be rearranged to:

\[
\ln \frac{K_p}{w_2^B - w_2^T} = a + bz_p
\]

(13)

such that \( a \) and \( b \) can be determined from the intercept and slope of a plot of \( \frac{\ln K_p}{w_2^B - w_2^T} \) vs. \( z_p \).

**EXPERIMENTAL**

**Materials**

**Polymers.** Poly(ethylene glycol) (PEG-3350, \( M_w=3350 \)) and dextran (Dextran-40, \( M_w=39100 \)) were purchased from Sigma.
Proteins. Two series of genetically engineered T4 lysozymes were produced. One is the mutant series (strains provided by B. W. Matthews, University of Oregon) that has been modified through site-directed mutagenesis to replace lysine residues with glutamic acid. Each mutation results in an expected reduction of 2 units of charge at neutral pH. Using the notation of Dao-Pin, mutants containing one, two and three mutations are denoted as K16E, 16/135E, and 16/135/147E, respectively; WT denotes wild-type. The numbers 16, 135 and 147 represent the modification locations in the primary amino acid sequence. The second “fusion” series consists of 2 fusions of polyarginine tails containing 2 and 4 arginines (designated U1 and U2, respectively) to the carboxyl terminus of mutant T4 lysozyme 16/135/147E. The genes for all the T4 lysozyme are carried on the expression vector pHN1403 which has been transformed into E. coli strain RR1.

Full enzymatic activity is observed for all the mutants and fusions. The estimation of charge of T4 lysozyme wild type and mutants has been reported previously (Luther, 1994). U1 has the same charge as that of double mutant 16/135E while U2 has the same charge as that of the single mutant 16E. Figure 1 shows the result of the native gel electrophoresis indicating that U1 and U2 have the equivalent net charges as that of 16-135E and 16E respectively. Table 1 lists the values of net charge of all 6 lysozymes at pH 7.25.

Salts. All the salts were ACS Certified and obtained from Fisher Scientific. (Fair Lawn, NJ)

Methods

Protein production and activity assay

T4 lysozyme production was induced by the addition of isopropyl β-D-thiogalactopyranoside (IPTG) and purified as described by Luther and Glatz (1994). Lysozyme activity was measured by following the clearing of a Micrococcus lysodeiktkus cell suspension versus time. Protein concentration for the lysozyme mutants and fusions were determined by measuring the absorbance at 280 nm.

Aqueous two-phase systems

Bulk phase systems were prepared as described by Luther and Glatz (1994).

Protein partition coefficient measurements

Protein samples (ca. 1 mg/mL) were prepared by dialyzing against 0.01 M potassium phosphate at pH 7.25. Bulk PEG and dextran solutions were aliquoted into polystyrene tubes and 1 g protein solution was added. Water was added to the final weight 8 g. A blank phase system was also prepared by adding 1 g of 0.01 M potassium phosphate bulk solution. The systems were mixed by vortexing for 10 seconds and then were centrifuged at 5000 rpm for 5 minutes. Top phase samples were collected using Pasteur pipette while those from bottom phase were obtained by piercing the bottom of the tube and allowing to drain. The protein concentrations in each phase were determined. All partitioning experiments were performed in triplicate and at room temperature, 23°C.

Potential measurements

Electrodes made of a 1.5 mm glass capillary filled with 2% agar in 3 M KCl were used to measure Δφ as described by Luther and Glatz (1994). At least three measurements of each phase system were made. All potential differences reported are expressed as bottom phase minus top phase.
Multi-angle laser-light scattering

Light scattering measurement were made using the DAWN-B light scattering instrument (Wyatt Technologies Corporation, Santa Barbara, CA). All the measurements were performed at room temperature (23°C).

A graphical technique, the Zimm plot, was employed so that the second virial coefficients, \( A_v \), and molecular weight were obtained directly from the slope and intercept of the plot at zero angle.

RESULTS AND DISCUSSION

Figure 2 shows the phase compositions of four PEG 3350 and Dextran-40 phase systems. It also shows that “parallel tie-lines” is a good approximation. System A with the lowest polymer concentration has the smallest concentration difference between top and bottom phase. Tie-line length of the four systems are also listed in Figure 2.

The measured interfacial potential difference shows the same trend with tie-line length (Figure 3) as reported by others (King, et al., 1988, Haynes, et al., 1989, 1991, Luther, et al., 1994). A linear relationship between the potential difference and the PEG concentration difference between the top phase and bottom phase is observed. The junction potential between the electrode and each phase of the phase system contributes less than 10% of the measured electrostatic potential difference, estimated as:

\[
\Delta \phi_{\text{junction}} = -\frac{RT}{F} A \ln\left(\frac{B^\ast}{\bar{B}}\right)
\]

where

\[
A = \sum_i z_i u_i (c_i^\ast - c_i^p), \quad B^\ast = \sum_i z_i^2 u_i c_i^\ast, \quad \bar{B} = \sum_i z_i^2 u_i c_i^p
\]

where \( i \) represents any ionic species; \( e \) and \( p \) represents the solution in the electrode and the phase solution respectively; \( u_i \) is the mobility of species \( i \), which were determined from Nernst-Einstein equation. Similar results were reported by Haynes, et al. (1991).

Because of the low salt concentration of the phase systems, it is assumed here that the sum of the junction potential between one electrode and one phase and the junction potential between the second electrode and the second phase cancel out.

However, \( \Delta \phi \), the potential difference measured with Ag/AgCl electrodes, is the electrochemical potential difference and should be different from pure electrical potential difference \( \Delta \phi \) in Equation 1, 5 and 8. The partitioning results of T4 lysozyme point mutations verify this difference. Figure 4 shows the partition coefficients of the series of T4 lysozyme point mutants in the four aqueous two-phase systems. As the interfacial potential difference increases or the protein net charge increases, the partition coefficient decreases which is consistent with Equation 1. But using Equation 1 to calculate the interfacial potential difference from the slopes in Figure 5 gives values of interfacial potential difference different from those measured (Table 2).

One can not apply Equation 1 to different phase systems because Figure 5 clearly indicates that the four different phase systems have four different \( K_0 \) values. Equation 3 (or 11), on the other hand, takes into account the non-electrostatic effect on the protein partition behavior caused by the different proteins and different polymer concentrations.
in the phase systems. Figure 6 shows the linear relationship obtained for all four T4 lysozyme mutants in the four phase systems when plotted according to Equation 13.

Furthermore, for the same protein, the value of \( \frac{\ln K_p}{(w_z^p - w_z^T)} \) falls in the same range for all four phase systems, which indicates the effect of polymer concentration has been well included in the model. Linear regression of all four proteins in four different systems gives the expression for partition coefficient of T4 lysozyme mutants in PEG3350/dextran-40 phase systems:

\[
\ln K_p = (w_z^p - w_z^T)(5.9 + 1.4z_p)
\]  \( (16) \)

where the slope \( b \) can be used to calculate the relationship between the interfacial phase potential difference and the polymer concentration difference \( \Delta w \) from Equation 12.

Again, the calculated results yield a potential difference value different from the value measured from the experiment (Table 2). Equivalently, the prediction of protein partition coefficient from Equation 8 using \( a = 5.9 \) and the measured potential difference is not very satisfactory (Figure 7).

Equation 16 cannot be used to predict the partitioning behavior of the T4 lysozyme fusion series in the four phase systems. Figure 8 shows that the fusion tail lysozymes, U1 and U2, have different partition coefficients than the corresponding double and single point mutants even though U1 has the same net charge as that of double mutant and U2 has the same net charge as that of single mutant. Although a linear relationship in the form of Equation 13 can still be observed, Figure 9 shows the values of intercept and slope obtained from fusions in four different phase systems are different from those obtained from point mutants. For T4 lysozyme fusions, we have \( a = 9.4 \) and \( b = 0.37 \). The two different slopes and intercepts each have different implications.

First, the phase potential difference calculated from the slope of Figure 9 (\( b \) value of the fusions) is very different from the potential difference calculated from Figure 6 (\( b \) value of the point mutations) (Table 2), which indicates the electrostatic term \( \frac{z_p f}{RT} \Delta \varphi \) in the equation cannot be used to describe the electrostatic effect brought by the charged fusion tails. It has been pointed out (Luther and Glatz, 1994, Albertsson, 1986) that in order to use this electrostatic term, the protein is assumed to carry a point charge, which is a good approximation when the charge is distributed evenly over the surface. While all the point mutants have charged residuals distributed over the protein surface, T4 lysozyme fusions with their charged tails fused to the C-terminus of the protein perhaps can not be viewed in this way.

Second, the difference in the intercepts, \( a \), implies that the interactions between fusions and polymers in the solution are different from those between mutants and polymers since \( a \) reflects the magnitude of the second virial coefficients between the two polymers and the protein, depicting the interaction forces in the phase solution (Equation 9). Fusing a charged tail to the protein molecule obviously will change the protein dipole moment as well which brings the need to consider the dipole-dipole and dipole-charge interactions between the polymer and protein molecules in the phase solution. A high charge density tail at the end of the protein molecule will affect the hydration which
could in turn affect its interaction with the polymer molecule through competition for water molecules. Therefore, even though different genetic alterations, such as point mutation and charged tail fusion can bring the protein to the same charge, we can conclude that they can actually bring very different protein-protein and protein-polymer molecular interactions in phase systems such that different partitioning behavior is observed.

The second virial coefficients of PEG, dextran, and six T4 lysozymes in 0.01 M K-PO4 at pH=7.25 obtained from multi-angle laser light scattering are reported in Table 3. The differences in charge and charge distribution among the T4 lysozymes do change their molecular interactions with themselves and with the polymers. Positive values of the second virial coefficient indicate that the average force between the two molecules is repulsive, while negative values indicate an attractive mean force between the two molecules (Haynes, et al., 1989, 1991).

CONCLUSIONS

We have found T4 lysozyme mutants and fusions display different partitioning behavior in the aqueous two-phase systems. A model based on a thermodynamic framework and experimental regression is derived to include both non-electrostatic effects caused by the polymer concentrations and electrostatic effects caused by the interfacial potential difference. The model accounts for partitioning behavior of T4 lysozyme mutants but not for fusions where the addition of a charged tail has not only changed the net charge of the protein but also dipole moment, electrostatic potential distribution, hydration effect and so on which are not accounted for by the electrostatic effect term in the model. The potential difference regressed from protein partition coefficient data is different from the measured potential difference because of the two different thermodynamic meanings behind the two potential difference. The results from light scattering provided evidence that T4 lysozyme fusions have different protein-polymer interactions in the phase solution from those of mutants. However, applying the measured second virial coefficient from light scattering to the model did not give a good prediction of the protein partition coefficient.

REFERENCES


Table 1. Net Charge of T4 Lysozyme Mutants and Fusions at pH=7.25

<table>
<thead>
<tr>
<th>T4 Lysozyme</th>
<th>Wild Type</th>
<th>Single Mutant</th>
<th>Double Mutant</th>
<th>Triple Mutant</th>
<th>U1 Fusion</th>
<th>U2 Fusion</th>
</tr>
</thead>
<tbody>
<tr>
<td>Net charge</td>
<td>9.84</td>
<td>7.84</td>
<td>5.84</td>
<td>3.84</td>
<td>5.84</td>
<td>7.84</td>
</tr>
</tbody>
</table>

Table 2. Comparing the Phase Potential Differences Measured from Experiments to Those Regressed from Two Different Models

<table>
<thead>
<tr>
<th>System</th>
<th>System A</th>
<th>System B</th>
<th>System C</th>
<th>System D</th>
</tr>
</thead>
<tbody>
<tr>
<td>ΔΦ (measured, mV)</td>
<td>1.8</td>
<td>2.6</td>
<td>2.3</td>
<td>0.61</td>
</tr>
<tr>
<td>ΔΦ (from Equation 1 and Figure 5, using partitioning results of point mutations, mV)</td>
<td>2.5</td>
<td>3.7</td>
<td>3.3</td>
<td>0.87</td>
</tr>
<tr>
<td>ΔΦ (from Equation 12 and Figure 6, using partitioning results of point mutations, mV)</td>
<td>4.4</td>
<td>4.9</td>
<td>4.0</td>
<td>1.1</td>
</tr>
<tr>
<td>ΔΦ (from Equation 12 and Figure 9, using partitioning results of fusions, mV)</td>
<td>3.8</td>
<td>4.6</td>
<td>3.9</td>
<td>1.3</td>
</tr>
</tbody>
</table>

Table 3. Molecular Weight and Second Virial Coefficient of Single Salute All, from Multi-Angle Laser Light Scattering

<table>
<thead>
<tr>
<th>Published Molecular Weight</th>
<th>Molecular Weight Measured from Light Scattering</th>
<th>Second Virial Coefficient All mol-mL/g2</th>
</tr>
</thead>
<tbody>
<tr>
<td>PEG3350</td>
<td>3350</td>
<td>0.0044</td>
</tr>
<tr>
<td>Dextran-40</td>
<td>39100</td>
<td>0.00069</td>
</tr>
<tr>
<td>Wild Type</td>
<td>18600</td>
<td>0.958</td>
</tr>
<tr>
<td>Single Mutant</td>
<td>18600</td>
<td>0.949</td>
</tr>
<tr>
<td>Double Mutant</td>
<td>18600</td>
<td>0.017</td>
</tr>
<tr>
<td>Triple Mutant</td>
<td>18600</td>
<td>0.015</td>
</tr>
<tr>
<td>U1 Fusion</td>
<td>18800</td>
<td>0.045</td>
</tr>
<tr>
<td>U2 Fusion</td>
<td>19000</td>
<td>0.040</td>
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</table>

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Figure 1. Native gel of T4 lysozyme point mutants and fusion, pH 4.8.

Figure 2. Binodal and tie-lines for PEG 3350/dextran-40 phase systems. Points indicate the phase compositions for the four systems used in this work; tie-line length (TLL) for the A, B, C, D four systems are 18.5, 24.8, 29.3 and 32.7 WMT/WT%.

Figure 3. Potential difference vs. PEG concentration difference between two phases.

Figure 4. Partition coefficients of T4 lysozyme mutants vs. tie-line length in the four different phase systems.

Figure 5. Partition coefficient logK of T4 lysozyme mutants vs. protein net charge.

Figure 6. \( \ln K_p = \frac{1}{(w_2^p - w_1^p)} \) charge for all the T4 lysozyme mutants in A, B, C, D four phase systems.

Figure 7. Comparison of measured (data points) and predicted (lines) point mutant partition coefficients by using Equation 6, n=59, and measured potential differences for A, B, C, D four systems.

Figure 8. Comparing of partitioning coefficients of T4 lysozyme mutants to those of mutants in A, B, C, D four phase systems.

Figure 9. \( \ln K_p = \frac{1}{(w_2^p - w_1^p)} \) charge for T4 lysozyme fusions in A, B, C, D four phase systems.
Biodegradation of Some Commercial Surfactants Used in Bioremediation

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ABSTRACT

Biodegradation of four commercial surfactants (Triton X-100, Triton X-165, Tween 80, Dowfax 8390D) and methanol by two mixed cultures was investigated in this study. The cells growing on an acetic acid - minimal salt medium, were able to degrade only Tween 80 over a period up to 35 days. Even at an initial concentration of 30 g/L, activated sludge reduced residual Tween 80 concentration to below 1000 mg/L within 35 days. Starting with the same concentration, enriched culture degraded Tween 80 at a higher specific rate during the first two days, but the degradation stopped after two days. Triton X-100, Triton X-165 and Dowfax 8390D were not degraded at all. Methanol was also degraded by the cells, but not as rapidly as Tween 80.
INTRODUCTION

Several hydrophobic chemicals absorb very strongly on soil particles (Fu et al., 1994) and degrade very slowly in presence of soils. Surfactants and solvents have often been suggested for enhancement of their biodegradation in contaminated soils in recent years. The solubility enhancers assist biodegradation by reducing interfacial tension and mobilization of the hydrophobic compounds to the aqueous phase from where microbial uptake occurs (Bury and Miller, 1993; Fu et al., 1994; Stucki and Alexander, 1987). The surfactants commonly used are nonionic and anionic in nature. The anionic surfactants are more resistant to their own sorption in sandy and clay-containing soils and have been used in soil-washing. An example is a twin-head anionic surfactant, diphenyl oxide disulfonate (DPDS), with high effectiveness in soil washing due to its solubility and steric constraints (Rouse et al., 1996; Rouse and Sabatini, 1993).

Biodegradation of the solubility enhancers has practical implications for bioremediation processes. In soil washing process, whether the washing solution can be recycled or not, depends on the biodegradability of the surfactant. Surfactant biodegradability is also critical to bioslurry operation where their rapid biodegradation would cause economic concerns. On the other hand, in-situ operations may have difficulty if the additives are not biodegraded at all. Hence, it is important to know the biodegradation potential of the solubility enhancers under consideration.

Most of the published work has focused on ethoxylated nonionic and alkylbenzenesulfonate anionic surfactants. Alcohol ethoxylates are usually degraded by microorganisms within several days. Alkylphenol ethoxylates are more resistant to biodegradation. Three reasons have been attributed to the recalcitrancy of surfactants: (i) inhibition of growth of microorganisms. For example, cationic surfactants can only be degraded at very low ratio of the surfactant to microbes due to their toxic effect on cell growth, (ii) steric hindrance of the surfactant molecule to enzymatic activity responsible
for biodegradation, (iii) surfactant causing inactivating the enzyme responsible for its biodegradation, thus interfering with the normal cell metabolism (Swisher, 1970). In a previous work (Gu et al., 1996), we have investigated the effect of three nonionic surfactants Tween 80, Triton X-100 and Triton X-165 on microbial growth. Tween 80 enhanced microbial growth and the other two surfactants had only small effects on growth of two mixed cultures (Gu et al., 1996). In this manuscript, the biodegradability issues have been addressed.

Most of the published studies involving biodegradation of surfactants have dealt with low concentrations, not exceeding 100 mg/L (Birch, 1984; Huddleston and Allred, 1965; Kravetz, 1981; Kravetz et al., 1991; Larson et al., 1993; Lashen et al. 1965; Mann and Reid, 1971; Patterson et al., 1968; Scharer et al., 1979, Sundaram et al., 1994; Urano and Saito, 1985). On the other hand, concentrations >1% (w/v) have been suggested for use in bioremediation (Zappi et al., 1995). Hence, there is a need to conduct studies with high concentrations of surfactants. The focus of research presented in this article was concentrations in the range of 3-30 g/L.

MATERIALS AND METHODS

The anionic surfactant Dow8390D (DPDS) is a mixture of hexadecyl (sulfophenoxy) benzene sulfonic acid and its disodium salts; it was purchased from Dow Chemicals (Midland, MI). The nonionic surfactants (Triton X-100, Triton X-165 and Tween 80) were procured from Union Carbide Co. (Danbury, CT). The azo-dye, dimethylaminoazobenzene, was obtained from Merck Co. (Rahway, NJ). All other chemicals were from Sigma Chemical Co. (St. Louis, MO) and were of analytical grade. The activated sludge was obtained from Columbia Waste Water Treatment plant and the enriched culture was isolated from an aged soil contaminated with wood treatment chemicals. The isolation procedure and maintenance of these cultures was described in a previous paper (Gu et al., 1996).
All the experiments were conducted in 1000 ml baffled shake flasks. The shake flasks containing 196 ml mineral salt - acetic acid medium (Gu et al., 1996) were autoclaved at 121 °C for 20 minutes; pH of the sterile media were adjusted to 7.0 by addition of 1.0 N NaOH before addition of the appropriate amount of surfactant. Overnight-grown mixed culture cells were used to provide a 2% v/v inoculum. The flasks were incubated in a rotary shaker at the speed of 150 rpm at room temperature (~25 °C). Flasks in which no surfactant was added, were used as control. All the studies were conducted in triplicate.

5 ml samples were collected from each flask every six hours for the first two days and every twelve hours every day after that. Cell density in the samples was measured as absorbance at 540 nm in a spectrometer (SPECTRONIC 601, Milton Roy Company). The uninoculated medium was used as reference in the spectrometer.

For the measurement of surfactant concentrations, the samples were centrifuged at 10,000 rpm for 10 minutes in a refrigerated centrifuge. Supernatant fluid were kept for analysis and the pellets were discarded. The concentration of nonionic surfactants was measured by an azo-dye method. The method consisted of adding a small amount of the powdered azo-dye into the supernate and mixing it in a shaker for three hours. The solution was then centrifuged again for 10 minutes at 10,000 rpm; the absorbance of supernate was measured at 400 nm and converted into surfactant concentration with the help of a calibration curve. This method could be used only for concentrations of the nonionic surfactants greater than their critical micelle concentrations (CMC). The CMC values for Triton X-100, Triton X-165, and Tween 80 were 185 mg/L, 400 mg/L and 14 mg/L, respectively.

The concentration of anionic surfactant, Dowfax 8390D, was measured by measurement of absorbance in the sample-supernate at a wave length of 260 nm. At this wave length, the surfactant absorption spectrum shows a characteristic peak.
Methanol was analyzed by gas chromatography. After centrifuging the sample, the supernatant was removed and filtered in a 0.2 µm filter (Whatman, Kent, England). Gas chromatography was performed in a Perkin Elmer gas chromatograph (Norfolk, CT) equipped with a Super Q packed column (Alltech, Deerfield, IL) and an FID detector. The operating conditions were as follows: injector temperature 190 °C; detector temperature 220 °C; oven temperature varying from 150 °C to 170 °C at 4 °C/min; carrier gas nitrogen, carrier gas flow rate 15 ml/min.

RESULTS AND DISCUSSION

The results of biodegradation studies in shake flasks have been presented in Figures 1-4. The control represents inoculated minimal salt medium (with acetic acid) without any surfactant/methanol. Methanol control reflects the measurements in a flask that contained only the medium and methanol, but no cells. These figures show variations in the concentration of cells as well as those of surfactant/solvent in the flask. As reported earlier (Gu et al., 1996), there was only slight toxicity of the different chemicals to cell growth. However, there were considerable differences between the biodegradation patterns of the chemicals.

The concentration of Triton X-100 and Triton X-165 underwent almost no change from the initial concentrations of 3 and 10 g/L during the ten days of observations in presence of both the cultures. In a follow-up study, there was no change in the concentrations even after 35 days (data not shown). The mechanism of biodegradation of Triton has been suggested to be microbial attack on the ethoxylate chain (Swisher, 1970). However, the experimental reports on biodegradation of Triton X-100 are not consistent (Birch, 1984; Huddleston and Allred, 1965; Kravetz, 1981; Larson, et al., 1993; Lashen, et al., 1965; Mann and Reid, 1971; Patterson, et al., 1968; Scharer, et al., 1979). Generally, Triton X-100 has been found to be resistant to biodegradation. Since the
biodegradation rate depends upon the number of ethoxylate-groups in the surfactant molecule, Triton X-165 should be even less biodegradable. In our studies, biodegradation of both of these tritons was not detected, even though there was no significant toxicity of the tritons to cell growth (Gu, et al. 1996). The lack of any biodegradability of these chemicals may be attributed to a lack of acclimatization of the cells to these chemicals.

The anionic surfactant, Dowfax 8390D (DPDS) was also not biodegraded by the cells. Cell growth behavior in presence of Dowfax 8390D exhibited initial growth inhibition and lower total cell densities (figures 1-4). The structure of DPDS is different from the common anionic surfactant alkylbenzenesulphonate (ABS) whose biodegradability has been widely studied. Difficulties in biodegradation of DPDS may have been due to (i) poor transport across the cell membrane, (ii) steric hindrance of the chemical structure which inhibits the binding of enzyme to the target, and (iii) negative influence of DPDS on the enzyme activity of cell membrane. Swisher (1970) has shown that anionic surfactants interact with enzymes in the cell membrane and reduce cell growth which is confirmed by our observations also.

Tween 80 could be consumed by the cells relatively easily. At a concentration of 3 g/L, 95 % of Tween 80 disappeared within four days. Time required for complete degradation was related to the culture and to concentration of the surfactant. The enriched culture showed a higher biodegradation rate and took less time to degrade Tween 80 to levels below detection at initial Tween 80 concentrations of 3 g/L and 10 g/L, than the activated sludge culture. At the initial concentration level of 30 g/L (presented in Figures 5 and 6), however a completely different result was obtained. Starting from this concentration, the activated sludge completely degraded Tween 80 in fifteen days; the enriched culture, although it showed higher degradation rate in first two days, couldn’t consume Tween 80 any more during the rest of thirty days. In the meantime, the growth curve didn’t exhibit any loss of cell density.
Tween 80, a monoleate, has been reported to be utilized as carbon source for many species of microorganisms (Swisher, 1970). Intracellular bacterial lipases apparently degrade Tween 80 liberating free fatty acid which is then assimilated by the microorganisms. As shown in Figures 1-6, biomass growth patterns confirm this use of Tween 80 by the cells to produce additional cellular mass.

Methanol too was rapidly degraded by the cells. Methanol controls suggested that some (~25%) methanol may have been lost due to volatilization, but still a significant part of reduction in methanol concentration can not be attributed to volatilization. Methanol has commonly been used as energy and carbon source in wastewater treatment. In our studies, methanol was not found to be toxic to activated sludge and enriched culture cells at concentration as high as 10 g/L.

The specific rates of disappearance of Tween 80 and methanol were calculated from these data. These rates have been presented in Table 1. For both the cultures, the biodegradation rate of Tween 80 was around 1000 mg/OD/day and it did not change with the concentration of Tween 80 between 3-30 g/L. The enriched culture showed a very high biodegradation rate (2500 mg/OD/day) during the first two days but did not consume any more surfactant afterwards. This phenomenon needs to be investigated in future studies. The specific rate of disappearance of methanol varied between 500 - 1750 mg/OD/day. Considering 25% methanol losses due to volatilization, a biodegradation rate of 400 - 1400 mg/OD/day can be calculated.

CONCLUSION

Several commercially used solubility enhancers were investigated. Tween 80 was biodegraded at the concentration as high as 30 g/L by activated sludge, and 10 g/L by enriched culture. Triton X-100, X-165 and Dowfax 8390D were resistant to biodegradation at the concentration above 1 g/L. Methanol was degraded very easily.
ACKNOWLEDGMENTS

This project was supported by a research grant (#94-08) from the EPA Great Plains/Rocky Mountain Hazardous Substance Research Center in Kansas State University, Manhattan, Kansas.

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   "Bioslurry Treatment of a Soil Contaminated with Low Concentrations of Total
Figure 1 Biodegradation of Solubility Enhancers at Concentration of 3 g/L by Activated Sludge
Figure 2 Biodgradation of Solubility Enhancers at Concentration of 10 g/L by Activated Sludge
Figure 3 Biodegradation of Solubility Enhancers at Concentration of 3 g/L by Enriched Culture
Figure 4 Biodegradation of Solubility Enhancers at Concentration of 10 g/L by Enriched Culture
Figure 5 Biodegradation of Tween 80 at Concentration of 30 g/L by Activated Sludge
Figure 6 Biodegradation of Tween 80 at Concentration of 30 g/L by Enriched Culture
Table 1 Specific Biodegradation Rate of Solubility Enhancers

<table>
<thead>
<tr>
<th></th>
<th>Con. (g/L)</th>
<th>Tween 80</th>
<th>Methanol</th>
<th>Specific Biodegradation Rate (mg/day/OD)</th>
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<tr>
<td>Activated Sludge</td>
<td>3</td>
<td>989</td>
<td>671</td>
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<tr>
<td></td>
<td>10</td>
<td>1007</td>
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<td></td>
<td>30</td>
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<td>Enriched Culture</td>
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<td>30</td>
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</table>

a: This result is based on first two days.
MODELING THE ROLE OF BIOMASS IN HEAVY METAL TRANSPORT IN VADOSE ZONE

K.V. Nedunuri, L.E. Erickson, and R.S. Govindaraju, Departments of Civil and Chemical Engineering, Kansas State University, Manhattan, KS 66506.

ABSTRACT

Contamination of groundwater by heavy metals has been a serious environmental problem. In this study, the influence of active biomass in immobilizing heavy metals in the unsaturated region of the soil is investigated through mechanistic models. The movement of water in the soil is modeled using Richards equation. An advection-dispersion equation, with a sink term for metal uptake by biomass, is used for modeling the metal transport. The sink term is evaluated based on the non-linear kinetics of metal adsorption to the biomass. Partitioning of biomass into mobile and stationary fractions is hypothesized. Transport of the mobile fraction is modeled with an advection-dispersion equation, having a source term, that is evaluated based on Monod growth kinetics, and having a sink term, that is evaluated based on linear endogenous decay. A soil column with flow vertically downward, and containing metal, biomass and carbon substrate is chosen as a hypothetical system. Growth rate of biomass is one of the important parameters influencing the metal uptake by biomass, and hence its transport in the vadose zone.

Keywords: Heavy metals, Vadose zone, Mathematical modeling, Kinetics, Biomass.

INTRODUCTION

Remediation of metal contaminated sites can be achieved by immobilizing heavy metals in soil, thus preventing them from reaching the water table. Pierzynski et al. (1994) discuss different ways by which metals can be immobilized in soils. They suggest soil amendments such as phosphates for this purpose. The root exudates released by plants can bind with metals and increase dissolution from their parent minerals in soils. The different processes, by which metals can be distributed among soil constituents are illustrated in Figure 1.

Figure 1. Processes affecting the fate of metals (Pierzynski et al., 1994)
The ability of algae and bacteria to sequester heavy metals from dilute aqueous solutions and accumulate them in their cell structure is a well recognized phenomenon (Ting et al., 1989). Extensive work has been conducted in this area of research due to its potential for heavy metal immobilization (Baath, 1989). Algae are the most tolerant among the various species that can immobilize metals, followed by fungi, bacteria and actinomycetes (Hiroki, 1992). The following table shows the adsorption of different metals by different classes of biomass.

<table>
<thead>
<tr>
<th>Biomass Class</th>
<th>Metal Adsorption</th>
</tr>
</thead>
<tbody>
<tr>
<td>Algae</td>
<td>Strongly adsorbed</td>
</tr>
<tr>
<td>Fungi</td>
<td>Weakly adsorbed</td>
</tr>
<tr>
<td>Bacteria</td>
<td>Moderately adsorbed</td>
</tr>
<tr>
<td>Actinomycetes</td>
<td>Weakly adsorbed</td>
</tr>
</tbody>
</table>

† indicates non-availability of data

Source: Volesky and Holan (1995)

Lead is strongly accumulated by certain algal species such as Chlorella vulgaris. The results as shown in Table 1, support use of selected microorganisms in the clean up of heavy metal contaminated sites.

Modeling the metal uptake by biomass is important to gain insight into the various physical and biochemical processes in the soil that influence metal movement. Though there is strong experimental evidence of heavy metal adsorption to biomass in soils, attempts at modeling metal transport in soils under the influence of biomass have not been reported. In our study, the movement of heavy metal in a soil column, maintained under unsaturated conditions, is modeled by considering the metal uptake by biomass. The biomass is partitioned into mobile and immobile fractions. Mobile fraction of the biomass undergoes transport with the aqueous phase. Biomass grows in the presence of substrate whose concentration is assumed to be maintained at a fixed value. Monod kinetics is assumed for growth of the biomass. A metal uptake kinetic model proposed by Ting et al. (1989) for dilute aqueous systems is used to quantify the biosorption (sink term) of metal.

**BIOCHEMICAL MECHANISMS OF METAL UPTAKE AND BINDING**

Certain polysaccharides, amino and phosphoryl groups, and acetamido groups present on the surface of microbial cells have strong tendency to adsorb metals. This passive uptake takes place very rapidly and hence is modeled as an equilibrium process. Metal attached to the surface is assumed to be always in equilibrium with the metal in the soil solution. Some of the metal on the surface subsequently moves into the cell interior by a transport process which can only be performed by live cells. The process, known as active uptake, is modeled as a kinetic process.
MATHEMATICAL MODELING

The entire modeling activity consists of three steps. In the first step, Richards equation was solved to simulate the movement of water in the unsaturated zone of the column. In the second step, advection-dispersion equation was solved to obtain the physical concentrations of biomass and metal throughout the column. In the last step, the sink/source terms in the transport equations, such as growth/decay of biomass and uptake of metal by active biomass were modeled using the microbial kinetic theory. The physical concentrations of biomass and metal were modified to account for growth/decay of biomass and metal adsorption by biomass.

FLOW MODEL

The flow model is formulated using Richards equation for unsaturated movement of water in one dimension (Govindaraju and Kavvas, 1993). The equations governing vertical water movement are

\[ \psi(z) = -K(\frac{\partial \psi}{\partial z} - 1) \]  
\[ \frac{\partial \theta}{\partial t} = -\frac{\partial v(z)}{\partial z} \]

where \( \theta \) is the water content by volume (m\(^3\)/m\(^3\)), \( v(z) \) is the water flux (m/hr), \( K \) is the hydraulic conductivity (m/hr), \( \psi(<0) \) is the soil matrix potential (m), \( z \) is the vertical coordinate direction taken positive downward, and \( t \) is time (hr). The two equations can be combined to yield Richards equation in terms of \( \theta \) as

\[ \frac{\partial \theta}{\partial t} = \frac{\partial}{\partial z}[K(\theta)(\frac{\partial \psi}{\partial z} - 1)] \]

where the \( K(\theta) \) and \( \psi(\theta) \) are non-linear functions, and Brooks and Corey (1964) functional forms are chosen here. The Brooks and Corey equations are given by

\[ \frac{\theta_s - \theta}{\theta - \theta_o} = \left(\frac{\psi}{\psi_o}\right)^\lambda \]

\[ \frac{K(\psi)}{K_o} = \left(\frac{\psi}{\psi_o}\right)^\eta \]
where \( \theta_s \) is the saturated water content, \( \theta_r \) is the residual water content, \( \Psi \) is the air entry pressure head, \( K_s \) is the saturated hydraulic conductivity, and \( \lambda \) and \( \eta \) are the Brooks-Corey parameters whose values depend on soil type.

The partial differential equations are discretized in time and space using finite differences. At each time step, a set of non-linear algebraic equations are formulated. These equations are solved using Newton’s method to obtain the spatial distribution of the water content along the length of the column. Some of the input parameters to the model are saturated conductivity, water content at saturation and root water uptake parameters. The initial moisture content distribution and the water content or flux at the top of the column (inlet), have to be provided at all times as a boundary condition. The model computes the transient water content distribution and flow field along the length of the column.

**MODELING THE TRANSPORT OF BIOMASS**

In our work, biomass is partitioned into mobile and immobile fractions. The immobile fraction gets adsorbed to the soil according to a linear relationship

\[
C_{\text{soil biomass}} = K_{db} C_b
\]

where \( C_{\text{soil biomass}} \) (mg cell dry weight/kg soil) is adsorbed biomass and \( C \) (mg cell dry weight/l of aqueous phase of soil) is the biomass in soil solution.

Movement of the mobile fraction of the biomass is modeled using an advection-dispersion equation, which is given as

\[
\frac{\partial \left(C_b (\theta + \rho K_{db})\right)}{\partial t} = \frac{\partial}{\partial z} \left[ \theta D_b \frac{\partial C_b}{\partial z} \right] - \frac{\partial}{\partial z} \left[ \nu(z) C_b \right] + R_b \tag{7}
\]

where \( \rho \) is the bulk density of soil (kg/l of soil), \( D_b \) is the dispersion coefficient for the biomass (m^2/hr), \( K_{db} \) is the partition coefficient for the biomass (l/kg), and

\[
R_b = \frac{\mu_m C_t}{K_m + C_s} - K_d x
\]

is the source/sink term for the biomass. The total concentration of biomass (sum of the mobile and immobile fractions) is

\[
x = C_{db} [\theta + \rho K_{db}]
\]
In equation (8) $\mu_m$ is the specific growth rate (hr$^{-1}$), $Q$ is the dissolved organic substrate concentration (mg/l), and $K_m$ is the half-saturation constant (mg/l), and $K_d$ is the endogenous decay constant (hr$^{-1}$).

**MODELING METAL UPTAKE KINETICS BY MICROBIAL BIOMASS**

Kinetic models are based on the observation that metal uptake is a dual process; there is an initial rapid uptake (passive uptake) followed by a slower process (active uptake). During passive uptake, the metal ions adsorb onto the surface of the cells such that

$$C = K_p C_p$$  \hspace{1cm} (10)

where $K_p$ is the surface adsorption constant, $C$ (mol/L) is the metal in soil solution and $C_p$ (mol/mg cell dry weight) is the metal adsorbed on the cells. The metal ion attached to the cell surface subsequently traverses the cell membrane through the agency of carrier molecules. A detailed description of the carrier mediated mechanism is given elsewhere (Ting et al., 1989). The process is kinetically controlled (active uptake) and is given by the following rate expression,

$$R_a = \frac{d(xC_a)}{dt} = xR_1[C_p - R_2C_a]$$  \hspace{1cm} (11)

Here, $R_1$ (hr$^{-1}$) and $R_2$ (dimensionless) are carrier rate constants, $x$ is the concentration of the cell (mg cell dry weight/l of soil), and $C_a$ is the intracellular metal concentration (mol of metal/mg cell dry weight).

A certain fraction of total metal in the soil will be attached to the mobile fraction of the biomass. Hence, the intracellular concentration of the metal in the biomass needs to be considered, and it is given as

$$\frac{\partial[xC_a]}{\partial t} = \frac{\partial}{\partial z}[\theta D_b \frac{\partial(C_bC_a)}{\partial z}] - \frac{\partial}{\partial z}[\psi(z)C_bC_a] + R_{ma}$$  \hspace{1cm} (12)

where $R_{ma}$ is the source term given in equation (11).

**MODELING METAL TRANSPORT IN THE PRESENCE OF ACTIVE BIOMASS**

The movement of metal is governed by advection and dispersion. In our study, the metal moves in solution and with the biomass. The equation for the movement of metal is given as
\[
\frac{\partial[C(\theta + \frac{z}{K_p}) + x C_a]}{\partial t} = \frac{\partial}{\partial z}\left[\theta D \frac{\partial C}{\partial z}\right] - \frac{\partial}{\partial z}[v(z)C] + \frac{\partial}{\partial z}\left[\theta D_b \frac{\partial C_b}{\partial z}\right][\frac{C}{K_p} + C_a] - \frac{\partial}{\partial z}[v(z)C_b][\frac{C}{K_p} + C_a]
\]

(13)

where D is the dispersion coefficient for the metal (\text{m}^2/\text{hr}).

Total metal in the soil column at any time and at any location, C_{m}, is given as

\[
C_m = \theta C + x(C_a + C_a)
\]

(14)

SOLUTION STRATEGY FOR PHYSICAL TRANSPORT

The equations described in the preceding section are used to compute concentrations of the metal and biomass as these undergo vertically downward movement in the unsaturated soil. Finite difference method was used to discretize the equations. In order to circumvent the problem of numerical dispersion, an Eulerian-Lagrangian approach was adopted (Thomson et al., 1984). The solution procedure for the transport component was split into two steps. During the first step, only pure convection was considered. Then, the dispersion effects were included using Eulerian approach. The system of algebraic equations resulting from discretization at each time step, was solved by formulating a tridiagonal system and solving by the LU decomposition method.

IMPLEMENTATION OF THE DEVELOPED MODELS

The solution space for the transport model described above consists of three domains: spatial, biochemical and temporal (Walter et al., 1994). The advection-dispersion terms describing aqueous phase transport are spanning over spatial and temporal domains only, and the biochemical equations describing the translocation of the heavy metal are spanning over the biochemical and temporal domain only. Advection-dispersion equations and biochemical equations are decoupled and solved separately. The highly non-linear behavior of biokinetic equations is confined to the models describing the microbial reactions of the metal adsorption. Thus, the overall solution system during any time step consists of two steps: a physical step in which the advective-dispersive terms of the transport equation are solved keeping the reaction by biomass (source/sink) terms constant, and a biochemical step in which the metal uptake kinetics and biomass growth kinetics are solved at the same time step for biomass concentrations, and aqueous and bio-sorbed concentrations of the metal, for each nodal point in the spatial domain.

At each time step, the flow model is solved first yielding the pore water velocity at each spatial node. The other important process variable computed by the flow model is
spatial distribution of water content. The master program then calls the transport model. This model furnishes the concentrations at each node for the metal and biomass for the given velocity distribution provided by the flow model. The bio-kinetic model is then used to determine the active metal uptake in the biomass and the biomass growth. The new concentrations are used to modify the transport model. An iterative process is adopted to determine the correct concentrations of the metal and biomass at each time step. The flowchart depicting this methodology is shown in Figure 2.

![Flowchart](image)

Figure 2: Overall Solution Strategy

**SIMULATION RESULTS FOR KINETIC STUDY**

Figure 3 shows the microbial uptake of lead. The initial concentration of lead in soil solution is 1 mg/l. Initial concentration of the biomass (in this case Chlorella vulgaris) is 800 mg/l. A rapid uptake of metal takes place during the first 20 hours followed by a gradual increase at a slower rate. The biomass concentration increases during this process due to growth. Figure 4 shows the residual metal concentration in the aqueous phase. The concentration decreases by about 50 percent within 40 hours.

Figure 5 shows the uptake kinetics of cadmium. A comparison is made between the model predictions and experimental observations (Kurek et al., 1982) of cadmium uptake by bacterial cells (Serratia marcescens and paracoccus species). Initial concentration of the metal in soil solution is 10 µg/ml and the corresponding concentrations of the bacterial cells is 800 mg/l. The model results are in good agreement with the experimental data. Cadmium adsorption rate is large initially and reduces at later times of the simulation thus confirming the dual process.

**SIMULATION EXAMPLE FOR METAL TRANSPORT WITH BIOMASS**

The system chosen in this study is a 2 m long soil column with flow essentially in vertically downward direction. The soil has a bulk density of 1.3 gm/cc, saturated hydraulic conductivity of 0.05 m/hr and a dispersion coefficient of 0.1 m²/hr. The Brooks and Corey parameter (η) is taken as 5 and the air entry pressure head is taken as -15 cm of water. A constant initial moisture content distribution of 0.1 was chosen within the column. The boundary conditions at the top of the column were a constant water flux of 0.005 m/hr and
a constant inlet metal and biomass concentrations of 1 mg/l. At the bottom of the column, the concentration and pressure gradients are assumed to be zero.

Lead was chosen as the heavy metal of interest. *Chlorella vulgaris*, belonging to algae family, was chosen as the biomass species. The surface adsorption constant $K_p$ was taken as 3500 mg/l, and the kinetic constants $R_1$ and $R_2$, were taken as 0.1 hr$^{-1}$ and 0.22 respectively. Specific growth rate of the biomass is 0.1 hr$^{-1}$ and partitioning coefficient is 30 ml/g. The simulations were carried out for a 48 hour period.

Figure 6 shows the influence of growth rate on the soluble lead concentration after 48 hours of simulation. The wetting front in the column has moved to a distance of 1.4 m. It can be observed that lead concentrations in the aqueous phase with biomass growing in the column are lower than in the column without growth of the biomass. Adsorption of metal to the active biomass caused a reduction in the metal concentration in the aqueous phase. It can also be observed that there is a change in the slope of the concentration profiles at around 1.4 m from the top of the column. These trends in the concentration profiles may be attributed to the sharp change in the water content profile near the wetting front.

Figure 7 shows the influence of biomass on the downward movement of lead at different times of simulation. All the other parameters were the same as those described above. It can be observed that concentrations of the biomass after 48 hours are significantly greater than that after 24 hours. Concentrations of the biomass are increasing in the top portion of the column and then decreasing in the remaining part of the column due to the transient conditions and the retardation associated with biomass adsorption to soil. Difference in concentrations between total lead and lead in aqueous phase is due to the adsorption of lead to the biomass. It can be observed that the difference in the concentrations is much greater after 48 hours of simulation than that after 24 hours. This is due to increase in the lead adsorption with the growth of the biomass. Sharp changes in the slope of the concentration profiles at 0.65 m after 24 hours, and at 1.4 m after 48 hours, are due to the sudden changes in the water content profiles (near the wetting front) at these distances in the column, thus showing the influence of the water content on the movement of lead in unsaturated soils.

**CONCLUSIONS**

Certain microbial species such as algae have strong metal accumulating capabilities. Metal uptake by microbial species is generally a dual process as confirmed by experimental observations for cadmium. Kinetic modeling of the metal uptake provides understanding of the adsorption and diffusive processes that occur during metal immobilization. The kinetic model is used to estimate the amount of metal retained by the biomass in the vadose zone of soils. The model incorporates the transport of biomass as well, to evaluate the overall metal movement. Columns showed greater accumulation of the metal by the biomass with increased concentrations of the biomass. Water content has a significant influence on the fate of the metal in unsaturated soils.
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Multivariate Statistical Methods for monitoring process quality: Application to bioinsecticide production by *Bacillus thuringiensis*.

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Abstract

Data sets from nine batches of *Bacillus thuringiensis* fermentations were analyzed using principal component analysis (PCA) and cluster analysis. Each batch had different input conditions (X), and six output variables (Y) were measured every thirty minutes. An array of i=9 number of batches, j=6 variables and k=40 time steps, was obtained. Using this values, nine matrices were created for each time. PCA was then used to reduce the dimensionality of these matrices and obtain the scores of the more significant principal components (PC). Scores were compared to a group of four quality variables (Q) that were also determined for each batch. In this comparisons PC regression was done to relate the Q variables to the PC. Also, by plotting the first two PC, clusters that are related to good values for the Q variables were found. It is shown, that the PCA method can be applied to monitor the current stage and quality of *B. thuringiensis* fermentation. Fermentation conditions which result in a poor product quality can be separated from conditions generating desirable products. Using the PC models, on-line measurements can be used to understand the quality of the process instead of spending time and money analyzing the Q variables.
1 JUSTIFICATION

- The quality of many biochemical processes is measured by the values of certain variables which describe the characteristics of the process itself or the final product. Most of the time, the measurement of these variables involves a good amount of work and time. Monitoring the values of these variables results in a determination of the quality at the end of the process. Unfortunately this is not a preventive strategy of quality control. The product quality is only defined by the correct simultaneous values of all measured properties; that is, it is a multivariate property and it must be monitored at frequent time steps.

- In the biological industry where many specialties are produced by microorganisms in natural and genetic altered strains, the metabolic stage of the micromanufacturer plays a key role. Having the opportunity to observe those stages in a frequent time basis could help to take action if a problem is observed.

- Multivariate Statistical Methods are a good approach to monitor the quality of a process acting as a Multivariable Statistical Process Control (SPC) tool. They can describe data sets and associate the variability to certain behavior, quality or metabolic stage. Also they can help in finding the best conditions to run a process.

2 OBJECTIVES

- Develop an historical multivariable data set for B.t. fermentation using experimental design. Analyze the behavior of the variables to find the most significant ones in the B.t. process.

- Create representative PCA models using on-line measurements that describe the quality for the bioinsecticide production by B.t.

- Analyze the effects of process variables such as agitation rate (Kla) and percentage of inocula on the quality variables of the B.t. process using PCA multivariable statistical analysis.

- Suggest a Statistical Process Control System that allows us to monitor on-line, any output measurement or process variable change that could affect our expected quality.
3 BIOINSECTICIDE PRODUCTION

The bioinsecticide production by *Bacillus thuringiensis* (B.t.) is a good example to be analyzed. To produce the bioinsecticide, B.t. has to grow by consuming the substrate in the media. After glucose depletion the microorganisms start a sporulation process in which a delta-endotoxin protein (the bioinsecticide) in crystal form is also created. Different types of delta-endotoxins can be developed depending on the strain. Metabolic pathways are followed to determine this. The toxicity of each type of protein is specific for a group of insects. Many of them are important pests in agriculture. Therefore, the quality variables for this process would be:

- The biomass formed, related to the growth.
- The spore production.
- The bioinsecticide formation and the specificity of it.
- The rates at which all the above occurs.

All these variables have to be measured at the end of the process or require either complex or time consuming analytical techniques. We need to find other on-line measurement to apply our techniques and monitor the fermentation. The on-line measurements to be analyzed are:

- Pyruvic acid formation
- Glucose consumption
- Acetic acid formation
- Lactic acid formation
- Dissolved oxygen present
- Absorbance
- Phosphoric acid consumption for pH control
- Sodium hydroxide consumption for pH control
- pH
- redox

The interest on identifying the metabolic stages of B.t. is important when we need to determine the status of the process, the productivity or if the metabolic behavior observed will conduct to produce the bioinsecticide. The metabolic stages are:

- Lag or recess phase
- Log or exponential phase.
- Transition phase.
- Sporulation phase.
- Crystal production.
4. MULTIVARIABLE STATISTICAL PROCESS ANALYSIS AND CONTROL

Principal Component Analysis (PCA) and Cluster Analysis (CA) are part of the multivariable tools used. The objective of PCA is to reduce the dimensionality of a data set with a large number of interrelated variables while retaining as much as possible the variation present in the data set. The method is to transform the original variables to a new set of variables called Principal Components (PC) which are uncorrelated and ordered so that the first few retain most of the variation.

The nature of the batch data is shown in figure (1).

The approach of PCA is to classify batches as good or bad ones, based on their similarity to a group of batches that produced an acceptable product. The approach is based on the basic concepts of SPC whereby the future behavior of a process is monitored by comparing it against that observed in the past when the process was in a state of statistical control. In the PCA method we calculate the principal components at each time step. The first two or three describe most of the variance of the system and can be used to monitor the process. Using cluster analysis, clusters of good quality can be defined. Calculation of the distance of each new predicted point to the appropriate cluster is used to determine the quality status of each new batch.

![Diagram of batch process data](image)

Fig. 1. Nature of Batch data. The process is described by Y; Quality data by Q and Input Conditions by X.
Fig. 2. *Bacillus thuringiensis* system. The process is described by Y; Quality data by Q and Input Conditions by X. The PCA method is performed on the on-line measurements.
5 METHODOLOGY

To find PC we start with the data set

\[
\begin{bmatrix}
y_{11} & y_{12} & \cdots & y_{1n} \\
y_{21} & y_{22} & \cdots & y_{2n} \\
\vdots & \vdots & \ddots & \vdots \\
y_{p1} & y_{p2} & \cdots & y_{pn}
\end{bmatrix}
\]

We calculate the covariance matrix

\[
S = \frac{1}{n-1} \sum_{i=1}^{n} \left( x_{ij} - \bar{x}_i \right) \left( x_{ij} - \bar{x}_j \right)
\]

where

\[
s_{ii} = \sum_{j=1}^{n} (x_{ij} - \bar{x}_i)^2 \frac{1}{n-1}
\]

\[
s_{ij} = \sum_{i=1}^{n} (x_{ij} - \bar{x}_i)(x_{ij} - \bar{x}_j)
\]

Using Spectral Decomposition

\[
\sum = ADA^T
\]

where A is the matrix containing the eigenvectors and D a diagonal matrix containing the roots or eigenvalues associated to each eigenvector. The eigenvalues give an estimate of the variance explained by each principal component. That way we can select a few PC to explain the variability of the system instead of all the variables measured.

The PC selected are obtained by using the following formula

\[
Z = \alpha_1 x_1 + \alpha_2 x_2 + \cdots + \alpha_7 x_7
\]

where alpha are the weights of the eigenvector selected.

Once a cluster of good quality is found the calculation of the Euclidean distance of a new batch to that cluster can be performed

\[
D = \sqrt{(x_1 - y_1)^2 + (x_2 - y_2)^2}
\]
6 MONITORING THE QUALITY OF B.t. BATCHES

Using previous information and data sets from the literature, an analysis of the feasibility of using PCA to monitor the quality of B.t. fermentation was performed. Nine batches were analyzed using seven on-line measurements and were classified according to the result of the quality variables. In fig (3) and (4) it can be observed that using the PCA method, batches 1 and 3 which resulted in a lack of bioinsecticide production and a poor cell and spore production were classified as bad quality batches.
7 AGITATION AND PERCENTAGE OF INOCULA EFFECTS ON B.t. BIOINSECTICIDE PRODUCTION

Two input process variables were studied and varied to create a broad historical data of the B.t. system. The experiments helped us to understand better the B.t. system. It was observed that the agitation rate and the relation it has to the Kla influenced the production of the bioinsecticide. Oxygen is a key element in the metabolism of delta-endotoxin production. Batches which produced a good amount of bioinsecticide were never limited by oxygen. See fig (7). The performance of the organic acids acts as a response of the process conditions. It can be observed that a strong production of acetic and lactic acid and a small production of pyruvic can be associated to no production of bioinsecticide. On the other hand, a small production of acetic and lactic and a strong activity of pyruvic acid can represent a metabolism focused on the future production of the bioinsecticide. See fig (5), (6) and (8).
Fig. 5. Glucose consumption and acid production for B. t fermentation using a 2.5 percentage v/v of inocula. * 500 rpm, + 250 rpm, o 750 rpm

Fig. 6. Glucose consumption and acid production for B. t fermentation using a 3.75 percentage v/v of inocula. x 375 rpm, - 825 rpm.
Fig. 7. Dissolved oxygen and absorbance measurements for B.t fermentation. Using 2.50 percentage v/v of inocula. * 500 rpm, + 250 rpm, o 750 rpm. Using 3.75 percentage v/v of inocula. x 375 rpm, · 625 rpm.

Fig. 8. Delta-endotoxin production for B.t. fermentations using 2.50 percentage v/v of inocula. * 500 rpm, + 250 rpm, o 750. CrylA have a molecular weight of 133kDa. Cryll have a molecular weight of 71kDa.
The Use of Polymeric Flocculants in Bacterial Lysate Streams

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*Presenting

Flocculation of bacterial lysate can greatly increase downstream solid-liquid separation efficiencies and provides great potential cost benefits and capital savings to the biotechnological industry. Polymeric flocculants with varied chemistries, charge densities and molecular weights were evaluated for performance using a rapid centrifugal screening test on samples of E. coli lysate. Capillary suction time apparatus was also used to evaluate the increased ease of de-watering. Optimal polymer types and doses were further evaluated for floc size and strength, and the enhanced performance of the selected process aid was evaluated at the pilot plant scale in a flow centrifuge and in an ultrafiltration system.
Effect of Water Content on Transport of Trichloroethylene in a Chamber with Alfalfa Plants

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Abstract
Trichloroethylene (TCE), a carcinogenic and mutagenic pollutant, is prevalent in contaminated soils and groundwater. The effect of alfalfa plants on transporting contaminants from the saturated zone to vadose zone was investigated in a laboratory chamber contaminated with TCE because vegetation enhances the transport and biodegradation of organic compounds contaminating soils and groundwater. TCE was fed at a concentration of 100 µL/L in the entering groundwater. Concentrations of TCE in the aqueous phase were measured using gas chromatographic headspace analysis. FT-IR instrumentation (GASMET) was used to monitor gas phase concentrations and evapotranspirational fluxes of TCE from the soil to the headspace of the chamber. Numerical modeling of the fate of TCE was done by a Galerkin finite element approach with linear shape functions for one dimension. The model was developed based on equilibrium partitioning between gas, water, and solid phases. The boundary condition at the surface was written to account for free volatilization of TCE across a thin atmospheric boundary layer.

Key words: Volatile organics, trichloroethylene, gas phase diffusion, phytoremediation.

Introduction
Trichloroethylene (TCE), a frequently detected pollutant at Superfund sites, is a dense non-aqueous phase liquid (DNAPL) and is also relatively volatile in nature. Trichloroethylene contamination beyond the maximum contamination limits (1-5 µg/L) in groundwater used for drinking purposes is of major concern because of its potential carcinogenic nature. Industrial wastes, spills, and landfills leachates are sources for TCE in contaminated soils and groundwater [1]. The attenuation of TCE in soils may be due to transport related processes, adsorption onto soil particles, and microbial transformations involved in the subsurface. The loss of TCE through the transport pathway is significant but has not been fully investigated in soil environments [1-5].

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Mass Transport Phenomena

In contaminated soils and groundwater, TCE is often present in the soil gas due to significant partitioning of TCE from the polluted groundwater. To investigate the migration of TCE, subsurface related transport phenomena in both the liquid and gas phases need to be considered. Soils are usually comprised of water saturated and unsaturated zones sandwiching the tension-saturated zone. In the saturated and tension-saturated zones, the transport mechanisms are due to aqueous phase convection and dispersion. However, in the unsaturated zone mass transport is governed by gas diffusion as well, because of air fractions. Moreover, the water content distribution in soils changes significantly with plant growth and seasonal fluctuation and so, in turn, does the air distribution in the soils. Such dynamically changing water and air distributions could significantly affect the subsurface transport of TCE. The partitioning of TCE between phases in the subsurface could be modeled as a rate-limited nonequilibrium or an equilibrium process [2]. However, simple rate-independent phase equilibrium is assumed in this study.

Plant assisted phenomena

Plants in general help in the attenuation of organic compounds contaminating soils and groundwater. Several review papers and articles in books published recently highlight the increased attention that plant-based bioremediation is receiving [3]. In plant-soil environments, vegetation acts as a solar driven pump-and-treat system which pumps contaminated water economically during the regular photosynthetic process. Plants such as cottonwood, poplar, and alfalfa that are drought resistant and viable in contaminated soils, penetrate their roots to reach groundwater and draw up contaminated water to the vadose zone and rhizosphere (root zone of plants) [2, 4]. In other words, they play a significant role in the determination of water-content profiles in plant-soil systems. Plant roots, in particular, may be responsible for rapid depletion of water from soil and create unsaturated zones. Hence, the air-content profiles in vegetated soils are closely related to the pumping and photosynthetic processes of established vegetation, and suction pressures in roots as well. The objectives of this research are to investigate and model the transport processes involved for TCE in the vertical direction from the groundwater table to the surface of soil in an experimental chamber.

Experimental study

A chamber with two identical U-shaped channels each 10 cm wide, approximately 1.8 m in axial flow length, and 35 cm in depth was employed for the study [1]. The design, construction, and feeding operation of TCE to this chamber are already described in Narayanan et al. [1, 4]. The various analytical techniques involved for determining the groundwater concentration of TCE, headspace concentration of TCE, and aqueous phase concentration of TCE in the various zones of soil are described in Narayanan et al. [1].
Mathematical model

The development of the mathematical model and assumptions employed to investigate vertical transport of TCE in the experimental chamber is fully explained by Narayanan et al. [5]. The governing differential equation used for one dimensional vertical TCE transport is shown below:

\[
(\theta_w + \theta_d H + \rho K_d) \frac{\partial}{\partial z} \left( C_w \right) = D_w \frac{\partial}{\partial z} \left( \theta_w \frac{\partial}{\partial z} \left( C_w \right) \right) - V_w \frac{\partial}{\partial z} \left( C_w \right)
+ HD_a \frac{\partial}{\partial z} \left( \xi \frac{\partial}{\partial z} \left( C_w \right) \right) - \theta_w k C_w
\]

where,

\[
\xi = \frac{\theta_a 10/3}{\eta^2}
\]

Boundary and initial conditions

Model Eq. (1) is a partial differential equation with respect to \( C_w \). For the boundary condition at the bottom boundary of the chamber \( (z = 0) \) the concentration of TCE is assumed as the groundwater concentration \( C_{w0} \).

\[
C_w \big|_0 = \text{Concentration in groundwater}
\]

However, for the boundary condition at soil surface \( (z = L) \) free volatilization of TCE is considered. To account for free volatilization of TCE at the soil surface a stagnant boundary layer of thickness \( d \) is assumed to exist at the ground surface due to vegetation and surface roughness. Then, the flux of the contaminant from the soil surface to the atmosphere across this boundary layer is essentially the total flux of contaminant occurring from the subsurface to the soil surface of the chamber through the gas and liquid phases. The upper boundary condition may then be represented as a Robin’s kind of boundary condition:

\[
\left[ -\theta_w D_w \frac{\partial}{\partial z} \left( C_w \right) + V_w C_w - \xi HD_a \frac{\partial}{\partial z} \left( C_w \right) \right] \big|_L = \frac{D_a}{d} \left( HC_w \big|_L - C_{air} \right)
\]

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where $C_{\text{air}}$, concentration in headspace atmosphere, is usually assumed to be zero as the atmosphere acts as an infinite sink for the contaminant. For the initial conditions, the steady state simulation results were employed as initial conditions for Eq. (1); that is, Eq. (1) was solved with the left-hand-side set equal to zero to obtain initial conditions for the simulation.

**Computational Method**

The governing Eq. (1) was solved for a one-dimensional vertical soil column using a weighted residuals Galerkin finite element technique. Linear shape functions were assumed over the element for the weak form of Galerkin formulation of Eq. (1). For representing the time derivative, Crank-Nicholson's method was used with a weighting parameter equal to 0.5.

**Discussion of results**

To simulate the transport of TCE in aqueous and gas phases, the parametric values listed in Table 1 were used in conjunction with collected data [5]. The distribution of porosity assumed in the system is shown in Fig. 1. The porosity was assumed to be 0.35 in the bottom 25 cm of soil and assumed to increase in the top 10 cm of soil due to the generation and degeneration of plant roots. A typical distribution of water content and corresponding air content distribution is also shown in Fig. 1. The air content was calculated from the difference between the porosity and the water content. Figure 2 shows the distributions of water content assumed to investigate the concentration profile and flux of TCE for different cases. The air content distributions (not shown) were calculated for all cases as the difference between the water content and porosity distributions. The porosity distribution shown in Fig. 1 was used for all cases. For cases 1, 3, 4, 6, and 7 the water content values were assumed to vary from saturated water content values of 0.35 cm$^3$/cm$^3$ to different constant surface water content values of 0.3, 0.25, 0.2, 0.15, and 0.1 cm$^3$/cm$^3$, respectively. A steep transition in water saturation was assumed at distances 5-10 cm, 10-15 cm, 15-20 cm, 20-25 cm, and 25-30 cm from the surface for cases 1, 3, 4, 6, and 7, respectively. These distributions were assumed to be due to significant water extraction by roots of actively growing alfalfa plants. Cases 1, 3, 4, 6, and 7 are more realistic in a soil where TCE is dissolved in the aqueous phase and is convectively and dispersively transported through soil-water prior to partitioning into soil gas and volatilizing from the surface of the soil. In cases 2, 5, and 8 water content values were assumed to be constant and equal to 0.3, 0.2, and 0.1 cm$^3$/cm$^3$, respectively. Cases 2, 5, and 8 are more close to situations in soils when large spills of TCE occur in the vadose zone. In these situations, TCE rapidly partitions from an infinite source capacity to the gas phase and volatilizes through the soil surface.

Steady state fluxes of TCE volatilization from the soil in cases 1, 3, 4, 6, and 7 were found to be 350 mg/(m$^2$.day), whereas for cases 2, 5, and 8 the corresponding fluxes were 350, 1929,
and 9203 mg/(m².day), respectively. In a variably saturated (saturated and unsaturated) soil situation the flux of TCE is entirely governed by the convective-dispersive fluxes in aqueous phase but in unsaturated soil the flux of TCE is dependent on the soil-gas fraction through which the gaseous diffusion of TCE occurs. Moreover, since gaseous diffusive flux of TCE is one-two orders of magnitude larger than the convective-dispersive fluxes of TCE through the aqueous phase, the values of fluxes for cases 2, 5, and 8 are larger than for cases 1, 3, 4, 6, and 7. Figure 3 shows the simulated profiles of TCE in the aqueous phase of the soil system relative to the groundwater concentration. The profiles of TCE were obtained for the various cases shown in Fig. 2. The gradient in concentration of TCE in the aqueous phase was significant for cases 1, 3, 4, 6, and 7 at depths 5-10 cm, 10-15 cm, 15-20 cm, 25 cm, and 30 cm, respectively. A steep gradient in TCE concentration in the aqueous phase was observed because of the decrease in water phase and appearance of the air phase at the corresponding depths of soil. Since TCE is highly volatile; rapid partitioning from the water phase into the air fraction of the subsurface soil occurs. Consequently, migration of TCE occurs in the gas phase in the upward direction through a gas diffusional transport mechanism. This results in a rapid drop of concentration of TCE in the aqueous phase.

In an interacting plant-soil field environment where water content profile may be continuously varying due to either seasonal changes or fluctuations in groundwater table, the concentration of TCE in the aqueous phase would significantly change in the unsaturated zone. It must however be emphasized that the TCE concentration in the aqueous phase in the top 5 cm of soil surface is always less than 20% of the groundwater TCE concentration in the chamber. Evidently, plant roots that grow actively in the top 5 cm of vegetated soil would encounter contaminant concentrations in water phase that are relatively low and less phytotoxic. Consequently, it may be possible to manage active vegetation as a solar driven pumping system on the top of a highly contaminated soil without detrimental effects to the plants.

Conclusions

Trichloroethylene is a highly volatile contaminant which partitions and migrates by gas phase diffusion in the vertical direction. Due to significant partitioning of TCE to soil gas of subsurface soils, the actual concentration of TCE in the aqueous phase experienced by plant roots in the vadose zone may be considerably lower than the groundwater concentrations. In vegetated systems, the soil porosity may be larger in the top soil due to the actively growing root masses and hence enhance the TCE migration in the air phase of the subsurface soil environment. A model developed based on local phase equilibrium concepts was used to simulate subsurface transport of TCE in the experimental chamber. Numerical studies indicate that fluxes of TCE from the surface soil to the headspace atmosphere increase with upward movement of water from the subsurface due to evapotranspiration associated with actively growing alfalfa plants. Earlier studies indicated
that the major loss of TCE is through volatilization from the soil. Vegetation mediated bioremediation is limited to relatively shallow unconfined aquifer systems in actual field environments. Modeling results obtained here are directly applicable to other compounds of comparable volatility and Henry's law constant.

Acknowledgments

This research was partially supported by the U.S. EPA under assistance agreements R-815709 and R-819653 to the Great Plains-Rocky Mountain Hazardous Substance Research Center for regions 7 and 8 under project 94-27, an EPA EPSCoR grant, and an EPA grant (CR81-7790-01-1) to Drs. Fateley and Hammaker. It has not been submitted to the EPA for peer review and, therefore, may not necessarily reflect views of the agency and no official endorsement should be inferred. The U.S. Department of Energy, Office of Environmental Restoration and Waste management, Office of Technology Development and the Center for Hazardous Substance Research also provided partial funding.

Nomenclature

- \( C_w \): concentration of contaminant in aqueous phase (g/cm\(^3\))
- \( d \): boundary layer thickness at the soil surface (cm)
- \( D_a \): gas phase diffusion coefficient of contaminant (cm\(^2\)/hr)
- \( D_w \): hydrodynamic dispersion coefficient in water phase (cm\(^2\)/hr)
- \( H \): Henry's law constant of contaminant (g/cm\(^3\)/g/cm\(^3\)) (dimensionless)
- \( k \): first order decay constant for TCE (1/hr)
- \( K_d \): adsorption coefficient of contaminant onto soil particles (cm\(^3\)/g)
- \( L \): distance of the soil surface from the bottom of the chamber (cm)
- \( t \): time (hr)
- \( V_w \): convective volumetric flux or superficial velocity in upward direction (cm/hr)
- \( z \): Cartesian coordinates for vertical direction (cm)

Greek

- \( \alpha_w \): dispersivity in the aqueous phase in the upward direction (cm)
- \( \eta \): soil porosity (cm\(^3\)/cm\(^3\)) (dimensionless)
- \( \theta_a \): volumetric gas porosity (cm\(^3\)/cm\(^3\)) (dimensionless)
- \( \theta_w \): volumetric soil-water content (cm\(^3\)/cm\(^3\)) (dimensionless)
- \( \rho \): bulk density of soil (g/cm\(^3\))
- \( \xi \): tortuosity factor for gas phase diffusion in the soil
References

Table 1. The various parameters and values employed for the simulation study of volatilization of TCE from channel soils (@ 26°C).

<table>
<thead>
<tr>
<th>Soil Parameters</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bulk density</td>
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</tr>
<tr>
<td>Porosity in the bottom 25 cm of soil</td>
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</tr>
<tr>
<td>Dispersivity</td>
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</tr>
<tr>
<td>Darcy’s flux</td>
<td>0.01 cm/hr</td>
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<tr>
<td>Organic carbon content</td>
<td>1%</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>TCE Parameters</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Organic carbon-water partition coefficient</td>
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</tr>
<tr>
<td>Dimensionless Henry’s law constant</td>
<td>0.38</td>
</tr>
<tr>
<td>Gas phase diffusivity</td>
<td>300 cm²/hr</td>
</tr>
<tr>
<td>Decay rate</td>
<td>0.0</td>
</tr>
<tr>
<td>Surface boundary layer thickness</td>
<td>1 cm</td>
</tr>
</tbody>
</table>
Figure 1. Profiles of porosity, water content, and air content assumed for the chamber.
Figure 2. Profiles of various cases of water content distribution assumed in the system.
Figure 3. Simulated concentration profiles of TCE in the experimental chamber for the different cases depicted in Fig. 2.
Detection of Specific Microorganisms Using the Arbitrarily Primed PCR in Bacterial Communities of Vegetated Soil

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Abstract

Plants are believed to be able to enhance the activity and growth of microorganisms in soil, and have considerable potential for enhancing the biodegradation of organic contaminants in soil. However, further investigations about the impact of plants on bacterial community diversity, population, and the fate of particular bacteria in the contaminated soil are needed for the purpose of manipulation of plants or microorganisms specifically for bioremediation. Conventional microbiological methods suffer some disadvantages, the key ones being the labor-intensive and time-consuming nature of the assays, and the lack of sufficient specificity to allow unambiguous identification. We developed a method using polymerase chain reaction with arbitrary primers to amplify the total genomic DNA from a Klebsiella pneumoniae strain. For this strain, the amplification profiles are reproducible and characteristic. The same approach has been applied with other bacterial strains. We will be able to detect a specific strain in the soil and study the bacterial community by using this method.

Introduction:

We are very interested in the role plants play in the biodegradation process. We are especially interested in the impact of plants on the indigenous bacterial community and the fate of an introduced microorganism in contaminated soil. For this type of investigation, we need some simple, rapid and reliable methods to identify soil isolates and to detect the presence of a specific strain. Polymerase Chain Reaction (PCR) with arbitrary oligonucleotide primers amplifying bacterial genomic DNA has been applied in the characterization and differentiation of clinical isolates and environmental strains (1, 2, 3, 4, 5, 6). It is a suitable method for our purpose because it requires little information on the phylogeny or molecular biology of the species being studied. It is rapid, easy to perform; and it requires only a small amount of unpurified DNA.
In this study, we have used this newly developed methodology to develop the fingerprint which is the characteristic amplification profile of a specific bacterial strain, *Klebsiella pneumonia* M5a1, by screening 20 randomly chosen arbitrary primers. Hopefully, we will be able to apply the same strategy to our future study.

**Materials and Methods**

*Bacteria and culture conditions*

Wild type *Klebsiella pneumoniae* M5a1 was from our stock culture. It was incubated at 37°C and cultured on LB solid medium.

*Preparation of DNA for PCR analysis*

Whole cells of bacteria after overnight growth on LB were suspended in deionized water and diluted to a certain turbidity. Otherwise single colonies were picked and suspended in 100 μl deionized water. Different amount of cell suspensions were used as templates.

*Oligonucleotide primers and PCR amplification*

A total number of 20 10-mer arbitrary primers were purchased from Operon Technologies Inc. (Alameda, CA). The nucleotide sequences of the twenty primers were as follows: OPA01: 5' CAGGCCCTTC-3'; OPA02: 5' TGCCGAGCTG-3'; OPA03: 5' AGTCAGCCAC-3'; OPA04: 5' AATCGGGGCTG-3'; OPA05: 5' AGGGGTCTTG-3'; OPA06: 5' GGTCCCTGAC-3'; OPA07: 5' GAAACGGGTG-3'; OPA08: 5' GTGACTGAGG-3'; OPA09: 5' GGGTAACGCG-3'; OPA10: 5' GTGATCGCAG-3'; OPA11: 5' CAATCGCCGT-3'; OPA12: 5' TCGGCATAG-3'; OPA13: 5' CAGCACCCAC-3'; OPA14: 5' TCTGTGCTGG-3'; OPA15: 5' TTCCGAACCC-3'; OPA16: 5' AGCCAGCGAA-3'; OPA17: 5' GACCCTTGT-3'; OPA18: 5' AGGTGACCGT-3'; OPA19: 5' CAAACGTCGG-3'; OPA20: 5' GTTGCGATCC-3'.

The conditions were recommended by the producer and have been tested to be the suitable conditions for our purpose. Briefly, each PCR reaction held a total volume of 50μl, which contained 25 picomoles primer, 5μl 10 times PCR buffer, 5μl MgCl2 (25mM), 1μl dNTP (10mM), and 2.5 u Taq polymerase. Unless mentioned, 1μl cell suspension was used as template. The cycling condition consisted of 45 cycles of 1 min at 94°C, 1 min at 36°C, and 1 min at 72°C. The amplified products were analyzed by electrophoresis in 1.5% agarose gel in 40
mM Tris acetate buffer. The gels were stained with ethidium bromide and photographed under ultraviolet transillumination.

Results and Discussion

Screening for suitable primers

A total of 20 different arbitrary oligonucleotide primers (10-mers) were used to amplify total genomic DNA of *Klebsiella pneumoniae* M5a. Under conditions of low stringency (i.e. annealing temperatures of 36°C for 40 cycles), amplification of DNA fragments were observed with majority of the primers except OPA05, OPA08, OPA12, OPA15 and OPA19 (figure 1.). We found the amplification with primer OPA01 and OPA03 were reproducible. Therefore, they were selected to develop a fingerprint for this strain.

Effect of amount of template on DNA amplification

A problem with the use of arbitrary primers and low stringency conditions is that there is no consistent amplification profile. What is obtained is a series of bands whose number, size and intensity are determined by many factors, such as the
Figure 2. DNA amplification with different amount of templates using primer OPA01. Lane 1, PCR product for undiluted template; lane 2, undiluted template; lane 3, PCR product for 5 fold dilution; lane 4, 5 fold diluted template; lane 5, PCR product for 25 fold dilution; lane 6, 25 fold diluted template; lane 7, PCR product for 100 fold dilution; lane 8, 100 fold diluted template.

annealing temperature, the amount of template, and the concentrations of the reaction components. What is required for PCR fingerprinting is a standard condition which gives the clearest and most reproducible amplification pattern. We found the amount of template used was crucial for our system. In figure 2, we suspended *Klebsiella pneumoniae* cells in deionized water and made 5, 25, and 100 fold serial dilutions. Then 1 μl of cell suspension from each dilution was used as template. As showing in figure 1, the amplification patterns varied with different template amount, and the most variable parts are the bands with less intensity. However, two bands with high intensity appeared consistently.

**Effect of colony age on DNA amplification**

It was discussed elsewhere (3) that colony age is a major factor affecting the quality and reproducibility of the fingerprints obtained. We found it is true for our system also. In figure 2, we did DNA amplification with fresh colonies (incubated overnight at 37°C) or with old colonies (incubated overnight at 37°C, stored at 4°C for 9 days). The amplification pattern for fresh colonies are more consistent than that of old colonies. It was suggested and proved by our experiment that variations in the quantity of DNA added to the reactions resulted in changes in the amplification patterns obtained. The efficiency with which DNA is released and the number of cells sampled might also change with colony age, resulting in varying amounts of DNA being added to the PCR reaction. We think that might be a reasonable explanation.

*Primers OPA01 and OPA03 can be used to generate reproducible DNA amplification patterns*

Figure 4 shows the DNA amplification pattern using templates from ten single
Figure 3. DNA amplification with fresh or old templates using OPA03. Panel A, DNA amplification with fresh colonies as templates. Lane 0, molecular weight marker; lane 1-8, PCR product for single colonies 1-8; lane 9, negative control, reaction without Taq polymerase; lane 10, negative control reaction without template. Panel B, DNA amplification with ten days old colonies as templates. Lane 0, molecular weight marker; lane 1-9, PCR product for single colonies 1-9.

Figure 4. DNA amplification with different colonies using primer OPA01. Lane 0, molecular weight marker; lane 1-9, PCR products for single colonies 1-9.

Figure 5. DNA amplification with primer OPA03. Lane 0, molecular weight marker; lane 1-5, single colonies 1-5, 32.5 l template; lane 5-10, single colonies 1-5, 10 l template; lane 11-15, single colonies 1-5, 5 l template.
colonies. Briefly, we picked up 9 single colonies randomly and plated them out on 9 petri dishes. After overnight growth on LB, whole cells were suspended in deionized water and diluted to certain turbidities, which were 27, 20, 30, 32, 28, 32, 35, 30, 24( 54 filter) klett units respectively. There are some faint bands missing, but the consistent appearance of two bands around molecular weight 1.8 kb suggested that OPA01 is able to amplify a reproducible DNA profile for Klebsiella pneumoniae.

For the purpose of identifying soil isolates, we tried to simplify the template preparation procedure. We simply picked up some single colonies, suspended them in 100μl deionized water, and used 32.5 μl, 10 μl or 5 μl cell suspensions as templates. As showing in figure 5, even 5μl template from a single colony suspension can produce characteristic DNA amplification profile for this strain. The diagnostic bands for this strain with primer OPA01 are two bands with molecular weight around 0.5 kb.

Conclusion:

From this study, we found that bacterial genomic DNA amplification with arbitrary primer is a feasible approach for the purpose of identifying a specific strain. We also simplified the procedure for the preparation of template. No prior molecular biology information of the strain is needed. The procedure is simple and the amplification profiles are characteristic.

The same approach has been applied to some Pseudomonas and Xanthobacter strains which are soil microorganisms and are believed to be able to degrade some organic contaminants such as toluene and TCE.

Reference:


Acknowledgments

This research was partially supported by the U.S. EPA under assistance agreement R-819653 to the Great Plains-Rocky Mountain Hazardous Substance Research Center for regions 7 and 8 under project 94-27. It has not been submitted to the EPA for peer review and, therefore, may not necessarily reflect views of the agency and no official endorsement should be inferred.
Flux Enhancement Using Backpulsing

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ABSTRACT

A promising method for reducing membrane fouling during crossflow microfiltration of biological suspensions is backpulsing. In this in situ method of cleaning the membrane, clear fluid is periodically forced through the membrane in the opposite direction to that of normal operation, by reversing the transmembrane pressure at high frequency. This results in partial removal of the fouling deposit formed during forward filtration. This work focuses on experiments done with bacterial suspensions. Very short backpulse intervals (0.1 sec - 1.0 sec) have been used to increase the net flux (calculated on the basis of permeate gained during forward filtration minus that lost during reverse filtration). As a result, the net flux for a 1% E. coli suspension under optimum conditions is approximately ten times higher than that obtained during normal crossflow microfiltration operation.

Keywords: Membranes, Microfiltration, Backpulsing
CHROMATOGRAPHIC PURIFICATION OF Oligonucleotides: COMPARISON WITH ELECTROPHORESIS

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University of Colorado, Boulder
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ABSTRACT

The purification of nucleic acids on the basis of molecular weight is usually accomplished by gel electrophoresis, which is not readily scaled to industrial production levels or to levels required for structural and clinical studies. Therefore, a direct comparison was made between chromatography and gel electrophoresis for the separation of closely related oligonucleotide species, in this case a 5'-hydroxylated and a 5'-phosphorylated RNA dodecamer synthesized by transcription and modified (or not) by 5' dephosphorylation using calf intestine alkaline phosphatase. The two methods were compared on the basis of resolution (calculated in the standard way), speed (time required to collect pure material from a starting reaction mixture), and capacity (mass that can be processed in a scaled unit operation). Under conditions studied to date, the two species can be separated with higher resolution and higher speed by chromatography. Under denaturing conditions (high column temperatures, e.g. 80°C), unphosphorylated and phosphorylated dodecamer can be separated from each other with good peak resolution. Under native conditions (low column temperatures, e.g. ≤ 30°C), mixtures of the two forms of dodecamer give chromatographic peaks which are not fully resolved and which may contain heterogeneity. In addition, a higher loading capacity is possible with chromatography than with gel electrophoresis.

INTRODUCTION

Gel electrophoresis is often the method of choice for laboratory-scale purifications of oligonucleotides. However, with the development of oligonucleotides for therapeutic use, the need for a robust, high-resolution, large-scale purification technique becomes obvious, since gel electrophoresis does not lend itself to scale-up. Chromatography has become established as the purification workhorse in therapeutic protein production and appears to also hold promise for oligonucleotide purification.

Much research has been conducted by Yamamoto on scaling-up and optimizing gradient and stepwise elution chromatography, the two most common operational modes in protein (and oligonucleotide) chromatographic purification. Yamamoto et al. (1987) derived an equation for the resolution of proteins in linear gradient elution ion-exchange and hydrophobic interaction chromatography, which is very useful for scaling-up from small columns. This resolution relationship was tested experimentally and found to be “valid except for very short columns with a shallow slope of the gradient and for low flow-rates” (Yamamoto et al., 1987, p. 101). Yamamoto et al. (1990) also reported a method for
determining stepwise elution conditions from data obtained from linear gradient elution experiments. A method for predicting the zone-sharpening effect in linear gradient and stepwise elution chromatography of proteins was also reported (Yamamoto et al., 1993).

The usefulness of chromatography for separating closely related oligonucleotide species that have very subtle sequence or conformational differences has not been extensively studied. Delort et al. (1984) used reversed-phase HPLC to separate oligonucleotides up to 19 nucleotides in length that differed only in the type of end-group at the 5' terminus, that is, they were 5'-hydroxylated or 5'-phosphorylated, but otherwise the same in nucleotide sequence. Reversed-phase HPLC has also recently been used to detect secondary structure differences (B- versus B'-conformation of double strands) in sequence isomeric self-complementary oligonucleotides (Potaman et al., 1993).

The focus of this research was to apply strong (an)ion-exchange chromatography to the separation of very closely related RNA oligonucleotide species. Several reasons can be cited for preferring ion-exchange chromatography to reversed-phase chromatography (RPC). RPC typically requires large quantities of hazardous solvents such as methanol and acetonitrile. Larger oligonucleotides often require chromatography to be carried out under strong denaturing conditions, such as using heated columns. High temperature chromatography is commonly used with ion-exchange columns, but not with reversed-phase columns where the solvents used are often highly volatile and the stationary phases unstable. In addition, traces of the organic solvents used in RPC can hamper the crystallization of the purified molecule (Giege et al., 1986) and approval for pharmaceutical application.

**MATERIALS AND METHODS**

**UU-dodecamer (12mer) RNA**

A 12mer RNA (UU-dodecamer) with the following primary sequence was used as the model oligonucleotide:

5'-GGCGCUUGCGUC-3'

It was synthesized by in vitro transcription using the immobilized DNA template method developed by Marble and Davis (1995) and purified by gel electrophoresis. Final UU-dodecamer purity was typically about 90%. The 5'-phosphorylated transcription product was converted, if desired, to the 5'-hydroxylated form by CIP (calf-intestine alkaline phosphatase) digestion prior to gel electrophoresis. Throughout this paper, the terms "CIP 12mer" and "unCIP 12mer" will be used, respectively, for UU-dodecamer transcription samples that were CIP digested, and therefore primarily 5'-hydroxylated, and those that were not, and therefore primarily 5'-phosphorylated.

Depending on the ionic strength of the surrounding solution, the UU-dodecamer, which is self-complementary, can occur either in a hairpin (low salt) or a duplex (high salt) structure (Jucker, 1995).

**FPLC of UU-dodecamer**

FPLC (Fast Protein Liquid Chromatography) experiments were conducted using a Pharmacia FPLC system, consisting of a GP-250 Plus Programmer module, a UV-M11 monitor and detector module, two P-500 pump modules, a solvent mixer, a V-7 valve, a Frac-200 fraction collector, and an XK 26/40 (26 mm x variable length up to 400 mm) column.
The column was packed with 50 mL of Pharmacia SOURCE™ 15Q, a strong anion-exchange media. The voltage signal output from the UV-MII monitor was collected using a Houston Instrument OmniScribe Series D5000 Recorder or a Nova computer with a data acquisition board. Column heating was achieved by flowing heated water from a recirculating water bath through the thermostatic jacket of the XK 26/40 column, which was insulated using glass fiber pipe insulation. The FPLC system, including the column but not the recirculating water bath or data collection units, was located in a sliding-glass door refrigerator. Specific operating conditions are given with the relevant results discussed below.

**HPLC of UU-dodecamer**

HPLC (High Performance Liquid Chromatography) experiments were conducted using a Waters Chromatography HPLC system. A NucleoPac™ PA-100 column (4 × 250 mm) was used with the Waters system, consisting of a 600S Controller, 486 Tunable Absorbance Detector, 626 Pump, and 717plus Autosampler, all controlled by Waters software using an NEC computer. Column heating was achieved using the column heater built into the 626 Pump unit. Specific operating conditions are given with the relevant results discussed below.

**Analytical gel electrophoresis of UU-dodecamer**

Analytical denaturing 20% polyacrylamide gel electrophoresis was conducted using Sigma (St. Louis, MO) Techware equipment and BIORAD power supplies (3000Xi or PowerPac 3000). RNA bands were visualized using Stains-All (Sigma), and permanent records of the gels were made using a Personal Densitometer™ and ImageQuant™ software (Molecular Dynamics, Inc., Sunnyvale, CA) to scan the gels and process the image data.

**RESULTS AND DISCUSSION**

**FPLC of CIP 12mer and unCIP 12mer mixtures**

Figures 1 and 2 show the results of low (-15°C) and high (70°C) temperature FPLC separations of equal-mass mixtures of gel-purified samples of CIP 12mer and unCIP 12mer using the operating conditions given in Table 1. At low temperature, it appears that the four labeled peaks correspond to the different UU-dodecamer species (5'-hydroxylated and 5'-phosphorylated) since at high temperature these collapse into a single, sharp peak. At the time, it was speculated that Peak 1 in Figure 1 corresponded to UU-dodecamer species in a hairpin structure and peaks 2 to 4, to homogeneous and heterogeneous dimers of the species.

Attempts were made to resolve the various peaks at low temperature, in order to collect and analyze them. This proved to be very challenging. Many separations were conducted at various gradient slopes, and, based on simple linear elution gradients, more complex schemes were attempted which combined intervals of isocratic and gradient elution. The best resolution achieved is represented in Figure 3. The corresponding operating conditions are given in Table 2. For purposes of peak collection and analysis, better resolution was desired. Due to column fouling and unrelated difficulties with the FPLC system, further studies were carried out using HPLC.
Table 1: FPLC operating conditions and gradient elution scheme for the separation of mixtures of CIP 12mer and unCIP 12mer (see figures 1 and 2). Elution conditions at specified timepoints change linearly between timepoints.

<table>
<thead>
<tr>
<th>System: Pharmacia FPLC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Column: Pharmacia XK 26/40 (26 × variable length up to 400 mm) packed with 50 mL of Pharmacia SOURCE™ 15Q media</td>
</tr>
<tr>
<td>Column Temperature: ~15°C and 70°C</td>
</tr>
<tr>
<td>Elution Buffers: A = 25 mM Tris, pH 8.0</td>
</tr>
<tr>
<td>B = A + 0.8 M ammonium chloride, pH 8.0</td>
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</table>

Gradient Elution Scheme

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<th>Time (min)</th>
<th>Flow Rate (mL/min)</th>
<th>% A</th>
<th>% B</th>
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</thead>
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<td>80.0</td>
<td>20.0</td>
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<td>20.0</td>
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<td>70.00</td>
<td>4.70</td>
<td>80.0</td>
<td>20.0</td>
</tr>
</tbody>
</table>

Figure 1: Low temperature (~15°C) FPLC chromatogram of an equal-mass mixture of CIP 12mer and unCIP 12mer. See Table 1 for the complete operating conditions and gradient elution scheme. (Experiment conducted by C. Y. Lee.)

Figure 2: High temperature (70°C) FPLC chromatogram of an equal-mass mixture of CIP 12mer and unCIP 12mer. See Table 1 for the complete operating conditions and gradient elution scheme. (Experiment conducted by C. Y. Lee.)
Figure 3: Improved low temperature (−27°C) FPLC chromatogram of an equal-mass mixture of CIP 12mer and unCIP 12mer. Tangent lines were drawn in for determination of peak widths (used in the resolution calculations). See Table 2 for the complete operating conditions and gradient elution scheme.

Table 2: Improved FPLC operating conditions and gradient elution scheme for the separation of a mixture of CIP 12mer and unCIP 12mer (see Figure 3). Elution conditions at specified timepoints change linearly between timepoints.

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Flow Rate (mL/min)</th>
<th>% A</th>
<th>% B</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.00</td>
<td>4.70</td>
<td>80.0</td>
<td>20.0</td>
</tr>
<tr>
<td>20.0</td>
<td>4.70</td>
<td>80.0</td>
<td>20.0</td>
</tr>
<tr>
<td>25.00</td>
<td>4.70</td>
<td>50.0</td>
<td>50.0</td>
</tr>
<tr>
<td>45.00</td>
<td>4.70</td>
<td>50.0</td>
<td>50.0</td>
</tr>
<tr>
<td>55.00</td>
<td>4.70</td>
<td>40.0</td>
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<tr>
<td>65.00</td>
<td>4.70</td>
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<td>100.0</td>
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<td>83.00</td>
<td>4.70</td>
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<td>20.0</td>
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<tr>
<td>100.0</td>
<td>4.70</td>
<td>80.0</td>
<td>20.0</td>
</tr>
</tbody>
</table>
HPLC of CIP 12mer and unCIP 12mer mixtures

The investigation into separating the closely related UU-dodecamer species was continued at NeXstar Pharmaceuticals using a Waters Chromatography HPLC system and a NucleoPac™ PA-100 (4 x 250 mm) column. A preliminary elution scheme suggested by Christina Hall (personal communication, 1996) at NeXstar had given very similar results to those seen in figures 1 and 2, namely, several poorly resolved peaks at low temperature and a single, sharp main peak at high temperature. It was expected that considerable effort would be required to achieve the desired separation. However, simply decreasing the gradient slope considerably resulted in very good separation at high temperature (80°C) and somewhat improved separation at room temperature. Table 3 below gives these optimized operating conditions.

Table 3: HPLC operating conditions and gradient elution scheme for the optimized separation of 5'-hydroxylated and 5'-phosphorylated UU-dodecamer (see Figure 4). Elution conditions at specified timepoints change linearly between timepoints.

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Flow Rate (mL/min)</th>
<th>% A</th>
<th>% B</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.00</td>
<td>1.50</td>
<td>90.0</td>
<td>10.0</td>
</tr>
<tr>
<td>2.00</td>
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<td>90.0</td>
<td>10.0</td>
</tr>
<tr>
<td>52.00</td>
<td>1.50</td>
<td>30.0</td>
<td>70.0</td>
</tr>
<tr>
<td>55.00</td>
<td>1.50</td>
<td>30.0</td>
<td>70.0</td>
</tr>
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<td>56.00</td>
<td>1.50</td>
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<td>100.0</td>
</tr>
<tr>
<td>60.00</td>
<td>1.50</td>
<td>0.0</td>
<td>100.0</td>
</tr>
<tr>
<td>61.00</td>
<td>1.50</td>
<td>90.0</td>
<td>10.0</td>
</tr>
<tr>
<td>70.00</td>
<td>1.50</td>
<td>90.0</td>
<td>10.0</td>
</tr>
</tbody>
</table>

Figure 4 summarizes the results at both temperatures. Chromatograms a) and c), respectively, show the high temperature separations of the individual CIP 12mer and unCIP 12mer samples. The CIP 12mer sample appears to have been quite pure, while the unCIP 12mer sample obviously contained at least three different species. In increasing order of retention times, the peaks apparently correspond to the 5'-hydroxylated and two 5'-phosphorylated UU-dodecamer species (probably mono- and di-phosphorylated based on previous HPLC results in which three closely eluting main peaks were observed). Chromatogram e) in Figure 4 shows the high temperature analysis of the equal mass CIP/unCIP 12mer mixture and confirms the 5'-hydroxylated species peak assignment. Chromatograms b) and d) show the low temperature results for the individual samples, in which it is seen that the 5'-hydroxylated species elutes later than the 5'-phosphorylated species. Since this elution order is the reverse of that at high temperature (in which charge and size should be the basis for separation), secondary structure differences apparently have a
Figure 4: HPLC chromatograms of individual samples and equal-mass mixtures of CIP 12mer and unCIP 12mer using a Dionex NucleoPac™ PA-100 (4 x 250 mm) column at both high (80°C) and room temperature (RT). Chromatograms: a) CIP 12mer, 80°C; b) CIP 12mer, RT; c) unCIP 12mer, 80°C; d) unCIP 12mer, RT; e) equal-mass mixture, 80°C; and f) equal-mass mixture, RT. See Table 3 for the complete operating conditions and gradient elution scheme.
significant effect at low temperature. In addition, Chromatogram f) shows that a third intermediate peak appears at low temperature when a mixture of the two samples is injected. Since the new peak cannot be explained away by simply numerically summing the absorbance data in chromatograms b) and d), it must represent a real component, probably a heterogeneous dimer. The flanking peaks probably represent the homogeneous dimers.

Comparison with gel electrophoresis

The best resolutions obtained using low-temperature FPLC and high-temperature HPLC were compared to that achieved using analytical gel electrophoresis. The chromatogram in Figure 3, a magnified version of chromatogram e) in Figure 4, and the line graph of the area integration of the boxed gel lane shown in the Figure 5 insert (see below) were used to calculate the resolutions. Since length measurements on the chromatograms (and line graph) are linearly proportional to the elution volume units (and migration distance units in the gel), these were used instead in the classical definition (in terms of peak elution volumes and peak widths) of the resolution between two adjacent peaks. For FPLC, the best resolutions were 0.56 and 0.76; for HPLC, 3.5 (between the peaks corresponding to the 5'-hydroxylated and 5'-mono-phosphorylated species), and for gel electrophoresis, 1.2.

![Figure 5: Line graph of the area integration of the boxed gel lane shown in the insert. The gel scan is of mixtures of 5'-hydroxylated UU-dodecamer and 5'-phosphorylated UU-dodecamer separated on an analytical denaturing 20% polyacrylamide gel. Tangent lines and vertical lines at the peak maxima were drawn in for determination of peak widths and distance between peak maxima (used in the resolution calculations). (Experiment conducted by C. Y. Lee.)](image-url)
Table 4 compares estimated production times for the UU-dodecamer if chromatography (proposed for the FPLC system) were used as the primary purification step rather than gel electrophoresis (as currently done). The decrease in production time from 49 hours to 32 hours if chromatography were employed strongly encourages the expenditure of effort in scaling up the high-resolution analytical chromatographic separation discussed above.

Table 4: Comparison of UU-dodecamer production times using chromatography versus gel electrophoresis as the purification step.

<table>
<thead>
<tr>
<th>Step</th>
<th>Time (hours)</th>
<th>Step</th>
<th>Time (hours)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chromatography</td>
<td></td>
<td>Gel Electrophoresis</td>
<td></td>
</tr>
<tr>
<td>Transcription</td>
<td>5.0</td>
<td>Transcription</td>
<td>5.0</td>
</tr>
<tr>
<td>EtOH Precipitation</td>
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<td>EtOH Precipitation</td>
<td>4.0</td>
</tr>
<tr>
<td>Desalt Sample</td>
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<td>Desalt Sample</td>
<td>8.0</td>
</tr>
<tr>
<td>CIP Rxn</td>
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<td>CIP Rxn</td>
<td>2.0</td>
</tr>
<tr>
<td>High Temp FPLC</td>
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<td>Gel Electrophoresis</td>
<td>15.0</td>
</tr>
<tr>
<td>Desalt Product Fraction</td>
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<td>Band Elution</td>
<td>5.0</td>
</tr>
<tr>
<td>Concentrate Sample</td>
<td>4.0</td>
<td>Desalt Sample</td>
<td>6.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Concentrate Sample</td>
<td>4.0</td>
</tr>
<tr>
<td>Total Time</td>
<td>32.0</td>
<td>Total Time</td>
<td>49.0</td>
</tr>
</tbody>
</table>

CONCLUSIONS

High temperature anion-exchange chromatography appears to be a promising technique for purifying UU-dodecamer RNA (and probably any oligonucleotide of up to at least 20 or 30 nucleotides) from very closely related contaminants. For example, analytical high temperature anion-exchange HPLC was able to separate 5'-hydroxylated UU-dodecamer from its 5'-phosphorylated analogs with a resolution that was much better than that of analytical gel electrophoresis (a resolution of 3.5 as compared to 1.2), which is encouraging since chromatography is much more scaleable than gel electrophoresis. The successful scale-up from analytical HPLC columns still must be accomplished, but it would be a very worthwhile endeavor, even for the UU-dodecamer production scale required in our laboratory, given the relative labor intensiveness of gel electrophoresis. Low temperature anion-exchange chromatography appears to hold promise as an analytical tool for investigating secondary structural differences of oligonucleotides, both of a single oligonucleotide and of very closely related ones.

ACKNOWLEDGMENTS

This research was supported by the Colorado RNA Center, Colorado Institute for Research in Biotechnology, National Institutes of Health (Integrated Training Grant for Leadership in Biotechnology), NeXstar Pharmaceuticals, Inc., and the National Aeronautics and Space Administration. Christina Hall and Stephanie Nieuwlandt at NeXstar deserve special recognition for their help with the HPLC part of this research.
REFERENCES


Determining Singular Arc Control Policies For Bioreactor Systems Using a Modified Iterative Dynamic Programming Algorithm

Arun Tholudur and W. Fred Ramirez
Department of Chemical Engineering
University of Colorado, Boulder

Abstract

Dynamic Programming is a very powerful technique for the optimization of dynamic systems. With the ready availability of high speed computers and the development of the Iterative Dynamic Programming (IDP) algorithm, a feasible alternative to the calculus of variations approach to the optimal control problem is now available. Inherent in the IDP algorithm is the application of piecewise constant discretized controls. This often leads to singular optimal control policies that are highly active. The aim of this research is to modify the IDP algorithm to reduce the control energy variations that are typical of IDP while at the same time developing policies that are very close to the true optimal control. This is achieved by including a median filter within the IDP procedure. Application of this modified algorithm to two bioreactor systems that yield singular optimal control profiles is presented and the usefulness of this scheme is demonstrated.

Keywords: iterative dynamic programming, optimization, median filtering, bioreactors, recombinant protein production.

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Pressure Effect on Subtilisins measured via FTIR, EPR and Activity Assays, and its impact on Crystallizations


Department of Chemical Engineering, University of Colorado, Boulder, Colorado, 80309-0424; *Department of Chemical Engineering, Iowa State University, Ames, Iowa 50011; **Department of Pharmaceutical Sciences, School of Pharmacy, University of Colorado Health Sciences Center, Denver, Colorado, 80262.

High pressure experiments have been conducted on two subtilisin strains, Subtilisin Carlsburg and a variant from Bacillus Lentis provided by Genencor. Fourier transform infrared (FTIR) and electron paramagnetic resonance (EPR) spectra have been collected for the high pressure treated and non-pressure treated subtilisins. The enzymes were exposed to pressures of 1000 bar and 2000 bar for varying times. FTIR spectra for the pressure treated samples were compared to those for the unpressurized samples per the method developed by Kendrick, et. al. (1996) and showed no significant secondary structural alterations after pressurization for the conditions tested. Further, EPR and activity assay data show no after-pressure effect on either variant. For the conditions tested, evidence to date suggests that no permanent changes to the proteins are occurring. These data, coupled with crystallization rate data from Iowa State, suggest that enhanced crystallization of the Bacillus Lentis variant is possible with brief pressure treatments.


Key Words: crystallization, protein, subtilisin
Intercellular Calcium Changes in Endothelial Cells Exposed to Flow

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School of Chemical Engineering and Material Science
University of Oklahoma, Norman, OK 73019

Endothelial cells line the blood vessel wall and are the source of vascoactive compounds that regulate many physiological processes, including those involved in regulating inflammation, vascular tone, and coagulation. In previous experiments, we have shown, that endothelial cells release the Willebrand Factor (vWF) in response to the initiation of physiological levels of fluid mechanical shear stress. This release is enhanced in the presence of histamine and thrombin, mediators of inflammation and coagulation. Other investigators have shown that changes in intercellular calcium levels may play a part in the signal transduction cascade leading to the release of vWF. We have examined the changes in intercellular calcium concentrations in human endothelial cells exposed to fluid flow in the presence of inflammatory mediators. This study will enhance our understanding of the mechanism by which the endothelial cells can regulate coagulation in the presence of shear stress.
APPLICATION OF LIQUID-LIQUID EXTRACTION IN PROPIONIC ACID FERMENTATION

Zhong Gu, Bonita A. Glatz and Charles E. Glatz
Iowa State University, Ames, IA 50011

A liquid-liquid extraction system in a hollow fiber supported liquid membrane module was incorporated with an immobilized-cell fed-batch fermentation system for concurrent propionic acid production and recovery. 40% (v/v) Alamine 304-1 in oleyl alcohol was the optimized solvent for acid extraction. With such an extractive fermentation process, 90% of the propionic acid produced by fermentation was extracted to the organic phase so that its inhibitory effect on cell growth was alleviated. The overall yield of the propionic acid was doubled from that of the non-extractive fermentation.
Purification of Recombinant T4 Lysozyme from E. coli: Ion-Exchange Chromatography

Weiyu Fan  Matt L. Thatcher Charles Glatz
Department of Chemical Engineering, Iowa State University, Ames, Iowa 50011

Summary
Two series of genetically engineered charge modifications of bacteriophage T4 lysozyme were produced from E. coli. The retention behavior of ion-exchange chromatography of these lysozymes was studied. Both charge modifications, one in the form of charged-fusion tails and the other in the form of charge-change point mutations, affected protein retention. However, the two series displayed different retention behavior in both weak and strong cation-exchange column.
Recovery of Recombinant β-Glucuronidase from Transgenic Corn

Ann R. Kusnadi¹, Roque Evangelista¹, and Zivko L. Nikolov¹,²
¹Department of Food Science and Human Nutrition.
²Department of Agricultural and Biosystem Engineering
Iowa State University, Ames, IA 50011
and
John Howard, ProdiGene Inc.
College Station, TX 77845

Transgenic plants producing recombinant proteins with different potential applications have been widely reported. To our knowledge no effort has been made in identifying possible downstream processing issues specific to transgenic crops. The objective of this study was to investigate those issues by using recombinant β-glucuronidase (rGUS) produced in transgenic corn. rGUS was stable during storage at -290°C and 10°C for up to three months. Moreover, large-scale milling conditions for producing corn flour did not affect the rGUS activity. The preferential accumulation of rGUS in the corn germ provides an opportunity to increase the enzyme concentration in the extract by separating the endosperm from the germ by a dry-milling process. Under the established extraction condition 70% of rGUS was extracted, and complete extraction was achieved when 2% β-mercaptoethanol (ME) and 1% sodium dodecylsulfate (SDS) were added to the extraction buffer. Using a three-step chromatography, between 40% and 50% of purified rGUS was recovered from flaked corn kernels, full-fat germ, and defatted germ. The extraction and purification of rGUS was not affected by the presence of starch and/or oil in the extract.

INTRODUCTION

Transgenic plants are potentially one of the most economical systems for a large-scale production of proteins and peptides for industrial, pharmaceutical, veterinary, and agricultural uses. Advantages of plant systems as protein "factories" include low cost of growing on a large scale, easy scale-up (increase of planted acreage), natural storage organs (tubers, seeds), and established practices for efficient harvesting, transporting, storing, and processing of the plant material (1). Several cereal and oilseed crops appear to be attractive as factories for heterologous protein production. The choice of crop will largely depend on the ability to efficiently recover the recombinant protein without interfering with the extraction of primary crop products (1). Our long-term goal is to develop an approach to recombinant protein recovery and purification from plant seeds that will be readily adaptable to a wide variety of protein products and at the same time allow the co-production of the traditional seed products.

We used rGUS as a model protein to investigate the recovery of recombinant protein from transgenic corn. This systems was selected because of adequate expression levels of rGUS, simplicity and sensitivity of the enzyme assay, and the availability of the transgenic corn seed in sufficient amounts to address various aspects of downstream processing. In this study we are reporting some of the factors in the downstream processing of transgenic corn that affect rGUS activity, purity, and the recovery yield.
MATERIALS AND METHODS

Production of transgenic corn

The transgenic corn kernels were provided by Pioneer Hi-Bred International (Johnston, IA). The transgenic corn producing rGUS was prepared by using transformation, selection, and plant regeneration methods reported by Hood et al. (2).

Degermination

The germ was separated from the endosperm by a dry-milling process (3). Five hundred grams of dry corn kernels (12% moisture) were placed in a polyethylene bag and moisture conditioned at 40°C to the preselected moisture content (MC) by adding water according to the schedule shown in Table 1. After conditioning the kernels were passed through a horizontal custom-made dehuller/degermer operated at 16,000 rpm with a feed rate of approximately 300 g/min. The dehulled and degermed kernels were air-dried at ambient temperature to around 15% moisture and fractionated through a series of sieves (No. 3.5, 5, 7, 12, 25, and 50 Standard testing Sieves, Fisher Scientific, Philadelphia, PA). The fractions retained by sieves No. 3.5 through 12 were collected and aspirated to remove the hulls using a Test Model Duo-aspirator (Carter Day International, Minneapolis, MN). The fraction retained by sieve No. 3.5 consisted mostly of whole or partially broken kernels. The majority of the germ was retained by sieves No. 5 and 7. The germ fractions from sieves No. 5 and 7 were aspirated at higher air velocity to obtain a germ-rich fraction. The combined germ-rich fractions contained 77% of the initial germ amount.

Table 1. Conditioning schedule for germ separation from corn kernels

<table>
<thead>
<tr>
<th>Moisture content (% wet basis)</th>
<th>Holding time (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IM to 16</td>
<td>16</td>
</tr>
<tr>
<td>16 to 21</td>
<td>1.5</td>
</tr>
<tr>
<td>21 to 24</td>
<td>0.25</td>
</tr>
<tr>
<td>IM - initial moisture (12-14%)</td>
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</tr>
</tbody>
</table>

Distribution of rGUS in the corn kernel tissue

Corn kernels (100 g) were soaked in deionized water for two days. The hulls, endosperm, and germ were manually separated. Each fraction was air-dried at ambient temperature and ground with a coffee grinder (Salton/Maxim Housewares Inc., Mt. Prospect, IL). rGUS activity and the moisture content of each ground sample were determined. rGUS activity was determined using the assay described by Jefferson and Wilson (4). One unit (U) of rGUS activity was defined as the amount of enzyme that converts one nmole of p-nitrophenyl β-D-glucuronide per minute at pH 7.0 and 37°C.

Storage and heat stability

Dry corn kernels (12% moisture, 500 g per batch) were cracked using a roller mill (Ferrel-Ross, Oklahoma City, OK), then flaked to 0.3 mm thickness using Roskamp flaking rolls (Model K, Roskamp Mfg., Inc., Waterloo, IA). Seven 500-gram batches of flaked corn kernels were placed in zip-lock bags and stored at each of four different temperatures for up to 95 days. The storage stability study was performed in triplicate; three 50-g samples per time and per
temperature. To determine the activity of GUS, a 20-g sample was taken from each of the three 50-g bags at the specified time and extracted with 200 ml of 20 mM Tris-HCl pH 7.9 containing 500 mM NaCl and 1 mM CaCl₂. Extraction was carried out using a paddle mixer at ambient temperature for 30 min.

The heat stability of rGUS at temperature above 50°C was studied by heating corn kernels in a convection oven IsoTemp 500 series (Fisher, Pittsburgh, PA). Ten-gram batches of corn kernels were placed in separate aluminum pans and placed in the oven at four different temperatures (50, 70, 90 and 125°C) for up to eight hours. The heat stability study was performed in duplicate; two 10-g samples per time and per temperature. Heat-treated corn kernels were ground using a coffee grinder prior to extraction. Three-gram ground samples were extracted with 30 ml of 50 mM NaPi pH 7.5 and GUS activity in the extract was determined.

rGUS extraction

A portion of the germ sample prepared by the degemination process was defatted by using a Soxhlet apparatus. Forty grams of full-fat germ were extracted with 300 ml of hexane for five hours, and then air-dried at ambient temperature to remove the hexane.

All samples (flaked kernels, full-fat germ and defatted germ) were ground using a coffee grinder before protein extraction. The samples were extracted at 1:4 or 1:10 solid-to-liquid ratio with 50 mM NaPi buffer of pH 7.5. The extraction was carried out for 15 min at ambient temperature by using a magnetic stirrer for mixing less than 50-ml and a paddle mixer for greater than 50-ml volumes. After stirring, the suspension was centrifuged at 26,000g for 25 min at 0°C, and then filtered through a 4-layer of cheese cloth. The extract was assayed for GUS activity (4) and protein (5).

The efficiency of rGUS extraction was qualitatively examined by staining the spent solids with 0.1% X-Gluc (5-bromo-4-chloro-3-indoly-β-D-glucopyranoside) solution. After the extraction, spent solids were separated by centrifugation at 26,000g, rinsed extensively with water to remove the remaining rGUS, and dried at ambient temperature. To a 100 mg of washed and dried solids, 2 ml of 0.1% X-Gluc solution were added and the suspension was incubated at 37°C for 30 min. The presence of a blue precipitate (stain) in the solids indicated incomplete extraction of rGUS.

The extraction yield was estimated by SDS-PAGE followed by a Western blot analysis. A 500-μl aliquot of the 2 x SDS-PAGE sample buffer was added to 100 mg of washed and dried solids. The mixture was boiled for 5 min, and 15-μl aliquots were loaded on the gel. SDS-PAGE was carried out on a 10% resolving gels at a constant voltage of 150 V (6). Following the SDS-PAGE, the gel was soaked for 10 min in Bjerrum and Schafé-Nielsen transfer buffer (7). Protein bands from the presoaked acrylamide gel were transferred onto a 0.2-μm nitrocellulose membrane at a constant voltage of 10 V for 30 min using a semidy-electroblotting apparatus (BioRad, Richmond, CA). The nitrocellulose membrane was incubated in a blocking solution containing 10% milk diluent solution (Kirkegaard and Perry, Gaithersburg, MD) in PBS-T at ambient temperature for 30 min followed by an overnight incubation with polyclonal anti-GUS in the blocking solution. The excess antibody was removed by washing the membrane with PBS-T. The membrane was then incubated with Protein-A gold solution (Bio-Rad, Hercules, CA). The immunoreactive protein bands were enhanced by silver staining (Bio-Rad, Hercules, CA) and quantified by densitometry.

Purification

rGUS was purified from ground kernels, full-fat germ and defatted germ using a three-step chromatography. Ground corn kernels (75 g), full-fat germ (7.5 g), and defatted germ (7.5 g)
samples were extracted with 50 mM NaPi buffer pH 7.5 (buffer A) at 1:4 or 1:10 solid-to-liquid ratio as described in the previous section. Fifteen ml of DEAE-Toyopearl (Supelco, Bellefonte, PA) resin in buffer A were added to the centrifuged and filtered extract to adsorb (capture) rGUS. The slurry was mixed with a magnetic stirrer for 15 min at ambient temperature, filtered through a funnel-supported No. 1 filter paper, and washed with 400 ml of buffer A. The washed DEAE resin was packed into a glass column with an inside diameter of 1.5 em. The adsorbed rGUS was eluted with 0.3 M NaCl in buffer A. The fractions containing rGUS activity were combined, ammoniumsulfate (AS) was added to a final concentration of 1 M, and the solution was filtered through a 0.45 μ filter. The filtered solution was applied to a 12 ml of octyl-Sepharose column (Pharmacia, Piscataway, NJ) pre-equilibrated with buffer A containing 0.3 M NaCl and 1 M ammonium sulfate (buffer B). The column was first washed with 15-20 column volumes (CV) of buffer B followed by buffer A (9-13 CV) containing 150 mM NaCl and 500 mM AS to elute the loosely bound protein. rGUS was eluted by applying 200 ml of NaCl/AS (105 mM /350 mM to 52 mM/175 mM) gradient in Buffer A. The flow rate during sample loading, washing, and elution was maintained at 2 ml/min. The fractions containing GUS activity were combined and dialyzed against distilled water using a Centriprep-30 concentration unit (Amicon, Beverly, MA). The dialyzed sample was applied to a second DEAE-Toyopearl column pre-equilibrated with Buffer A. After loading the sample, the column was washed with 3 CV of the same buffer, and rGUS was eluted with 200 ml of 0 to 0.3 M NaCl gradient in Buffer A. The purity of rGUS was estimated by densitometry of protein bands on the SDS-PAGE gel (6), which was stained with Coomassie blue.

RESULTS AND DISCUSSION

Storage and heat stability

The handling of harvested corn includes drying the kernels to a final moisture content below 15% and storing the corn at different temperatures depending on the required length of storage, relative humidity of air, the kernel moisture content, and the end application. Drying, storage, and processing (milling) temperatures of transgenic corn is of a particular importance because integrity and activity of the recombinant protein must be preserved. For example, during drying as well as in the commercial corn milling kernels could be exposed to temperatures as high as 100°C. At the end, the primary (upstream) processing of corn (milling, degemination, fractionation, oil extraction, etc.) will probably be performed at a site different than the bioprocessing (downstream processing) plant, and processed corn may require different temperature regime for transporting and storing than that established for commercial grain. For these reasons, we have investigated the storage and heat stability of rGUS in processed corn (flaked kernels) and whole kernels, respectively.

Storage stability study (Figure 1) showed that no significant loss of rGUS activity occurred when flaked kernels were stored at -29, 10, or 25°C for up to 2 weeks. When flaked kernels were stored at 37°C for one week, 23% of the initial rGUS activity was lost. Storing the flaked kernels at -29 and 10°C for up to 3 months resulted in 14% and 23% loss of rGUS activity, respectively. At ambient temperature (25°C), a sample spoilage was observed after three weeks and a loss of 13% of the initial GUS activity was measured. Therefore, flaked kernels can be transported and stored at ambient temperature (25°C) for a period of less than two weeks without affecting rGUS activity. If necessary, flaked kernels can be stored for up to two months at 10°C. A similar storage stability data were obtained for recombinant avidin in transgenic corn (3).
The heating of whole kernels with initial MC of 12.6% at 50°C for up to eight hours did not affect rGUS activity (Figure 2). The prolonged heat stability study indicated that rGUS activity in the kernels was fully retained for up to six days at 50°C (data not shown). Heating at 70°C for one hour also did not significantly affect rGUS activity, but after 8 hours 60% of its initial activity was lost. No residual rGUS activity was observed after heating the kernels for one hour at 90°C and for 10 min at 125°C.

The heat stability results obtained in the laboratory were validated on a large scale. During the processing of 1,000 kg of transgenic corn in a custom-milling plant located in Grinnell, IA, the corn was exposed for a short time (less than 1 min) to 90°C. The milled corn was haged at approximately 50°C, transported at ambient temperature (30°C) for two hours to Iowa State University, and then stored overnight at 10°C before the rGUS activity was determined. Sampling of milled transgenic corn revealed no loss of rGUS activity, which indicated that lab-

Figure 1. Storage stability of rGUS in flaked corn.
scale heat-stability data could be used to predict the effect of the storage and processing temperature on the recombinant protein.

Figure 2. Heat stability of rGUS in corn kernels.

**Extraction of rGUS**

To determine the effect of extraction parameters on rGUS activity, eight process variables were screened using a quarter factorial design experiment. Based on the analysis of screening experiments, the following extraction conditions were used in the subsequent studies: 1) 50 mM NaPi buffer of pH 7.5; 2) minimum of 1:4 solids-to-buffer ratio; and 3) mixing for at least 15 min at ambient temperature. The screening experiments revealed that particle size distribution of the ground corn (i.e. flaked vs. milled corn) and the mixing speed did not significantly affect the extraction yield. In addition, the presence of 0.5 M NaCl, 0.05% (v/v) of Tween-20 and a cocktail of protease inhibitors consisting of 10 mM ME, 5 mM ethylenediamine tetraacetic acid (EDTA), 1 mM phenylmethyl sulphonyl fluoride (PMSF), 0.2 M diazoacetyl D.L-norleucine methyl ester (diazo AcNleOMe) in the extraction buffer did not increase the recovery of rGUS activity.
To determine whether a complete extraction of rGUS from corn kernels, full-fat germ and defatted germ was achieved, the remaining solids were stained with X-Gluc. The visual examination of the spent solids revealed the presence of blue stained particles indicating an incomplete extraction of rGUS from ground corn kernels, full-fat germ, and defatted germ. The Western blot analysis of the spent solids confirmed the presence of unextracted rGUS. We estimated that about 70% of rGUS was extracted from the ground kernels with the phosphate or borate buffer alone (Figure 3, lanes 2 and 4). Changing the pH of the extraction buffer to pH 10 did not affect the extraction efficiency. When 1% (v/v) of SDS and 2% (v/v) of ME were included in the extraction buffer rGUS was completely extracted (Figure 3, lane 3), but the enzyme was inactive. The subsequent dialysis of SDS did not yield active protein. Interestingly, a complete extraction of rGUS could not be achieved when either SDS or ME was missing in the extraction buffer suggesting a synergistic action of these two components.

![Figure 3](image)

**Figure 3.** Western blot analysis of corn kernels before and after extraction. Lanes: 1) before extraction; 2) after extraction at pH 7.5 without ME and SDS; 3) after extraction at pH 7.5 with ME and SDS; 4) after extraction at pH 10.0 without ME and SDS; 5) after extraction at pH 10.0 with ME and SDS; 6, 7, 8, 9) 0.05, 0.01, 0.1 and 0.2 μg GUS.

Further attempts to increase the extraction yield by adding 1% (v/v) of either Triton X-100 or Tween-20 to the extraction buffer did not show any yield improvement. Because in general, the primary product recovery is critical for effective downstream processing, an additional research effort to maximize the extraction yield of rGUS will be needed.

**Distribution of rGUS in the kernel**

The fractionation of the transgenic kernels into hulls, endosperm, and germ tissue, showed that approximately 93% of the total rGUS activity was located in the germ and the remaining 7% in the endosperm (Table 2). The staining of the kernel cross-sectional area with X-Gluc solution confirmed that majority of rGUS activity was produced in the germ tissue. Neither the extraction nor the tissue staining showed any rGUS accumulation in the hulls. Because the germ usually accounts for 10-13% of the dry kernel weight and contains 93% of rGUS activity, the separation of the germ tissue before the protein extraction step could considerably reduce the cost of downstream processing by reducing the amount of total solids in the process and by increasing the concentration of rGUS in the extract. For example, the concentration of the rGUS (μg/mg of soluble protein) in the germ extract was twice as high as that in the whole kernel extract (Table 3).
Table 2. Distribution of rGUS in the corn kernel

<table>
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<tr>
<th>Amount of tissue (%)</th>
<th>Germ</th>
<th>Endosperm</th>
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<tr>
<td>rGUS activity (U/g tissue)</td>
<td>6800</td>
<td>80</td>
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<tr>
<td>rGUS activity (%)</td>
<td>93</td>
<td>7</td>
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Table 3. Extraction of rGUS from ground corn kernels, full-fat germ, and defatted germ

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<th>Corn kernels</th>
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<tr>
<td>Solid-to-liquid ratio</td>
<td>1:4</td>
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<tr>
<td>Initial oil content (% db)</td>
<td>3.3</td>
<td>20.0</td>
<td>2.0</td>
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<tr>
<td>rGUS (µg/ml)</td>
<td>5</td>
<td>35</td>
<td>60</td>
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<tr>
<td>rGUS (µg/g dry solids)</td>
<td>17</td>
<td>170</td>
<td>180</td>
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<tr>
<td>rGUS (µg/mg soluble protein)</td>
<td>1.9</td>
<td>3.4</td>
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<tr>
<td>Total protein (mg/g dry solids)</td>
<td>9.3</td>
<td>50</td>
<td>47</td>
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</table>

*All values are average of two replications with less than 10% deviation*

**Effect of the starting material on the extraction and purification of rGUS**

Because the distribution study showed that more than 90% of rGUS is located in the germ, we have separated the germ by a dry-milling process. The degermination of transgenic corn kernels performed in our laboratory resulted in a germ-rich fraction of 50% purity; the balance consisted of endosperm and hulls. After extracting the corn oil from part of the germ-rich fraction, the three different starting materials (ground kernels, full-fat germ, and defatted germ) containing rGUS activity were extracted and compared (Table 3). The ground kernel, full-fat germ, and defatted germ fractions contained 3.3, 20.0, and 2.0% (w/w) corn oil, respectively (Table 3). The rGUS concentration (µg/ml) in the germ extract was six-to ten times greater than that in kernel extract at either 1:4 or 1:10 solid-to-liquid. Because total corn protein concentration was also greater in the germ than in the kernel extract, the concentration of rGUS was approximately 0.4% and 0.2% of the total soluble protein in the germ and the kernel extract, respectively. For all three starting materials, 30% more rGUS and soluble protein per gram of dry solids were extracted at 1:10 than at 1:4 solid-to-liquid ratio. The amounts of rGUS extracted from the full-fat germ and defatted germ at either 1:4 or 1:10 solid-to-liquid ratio were
similar indicating that 1) the corn oil extraction with hexane (–60°C) did not affect the activity of rGUS, and 2) the high-oil content (20%) in the initial material did not interfere with the protein extraction. The stability of rGUS during hexane extraction is an important finding which shows that, prior to the protein extraction, the corn oil could be recovered and sold as a co-product. When full-fat germ is considered as a starting material for recovery of recombinant proteins, 1:10 solid-to-liquid ratio is recommended because at 1:4 ratio the extract was rather viscous. Although not observed on a lab-scale, the increased extract viscosity could be a problem in the scaled-up capture chromatography step.

The purification data summarized in Table 4 indicate that rGUS can be purified with a similar effectiveness from either extract. Apparently, neither starch in the kernel extract nor the oil presence in full-fat germ extract affected the purification yield and the final purity (specific activity) of rGUS, which was estimated at 50%. To determine whether and how much the extract impurities would foul the DEAE resin after repeated use, we have performed ten on-and-off capture chromatography cycles using the full-fat germ extract (1:10 ratio). No change in the resin capacity and rGUS yield was observed after ten cycles. The rGUS concentration factor achieved after the capture chromatography step was fifteen fold for the kernel extract and five fold for the germ extracts, whereas the purification factor for all three extracts was about five fold.

Table 4. Comparison of rGUS purification from different starting materials.

<table>
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<tr>
<th></th>
<th>Corn kernels</th>
<th>Full-fat germ</th>
<th>Defatted germ</th>
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<tr>
<td>Loaded GUS (U)</td>
<td>70,000</td>
<td>41,000</td>
<td>53,000</td>
</tr>
<tr>
<td>Yield (%)</td>
<td>36</td>
<td>44</td>
<td>47</td>
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<tr>
<td>Purification fold</td>
<td>300</td>
<td>350</td>
<td>330</td>
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<tr>
<td>Specific activity (U/mg)</td>
<td>36,000</td>
<td>45,000</td>
<td>36,000</td>
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<tr>
<td>Purified rGUS per g starting material</td>
<td>0.006</td>
<td>0.040</td>
<td>0.056</td>
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</table>

We believe that by further optimizing hydrophobic interaction chromatography and the last anion-exchange step one can obtain greater than 90% purity of rGUS.

In conclusion, the extraction and purification of rGUS starting with either ground corn kernels, full-fat germ, or defatted germ had similar efficiency. Thus, the choice of starting material will depend on process economics including fractionation of transgenic corn, downstream processing and the revenues from potential co-products.
ACKNOWLEDGEMENTS

We thank Dr. Elizabeth E. Hood of ProdiGene, Inc., College Station, TX for analyzing the rGUS distribution in transgenic kernels by X-gluc staining. Dr. L. Logan helped with the experimental design and statistical analysis of rGUS extraction. This research was supported by grants from Pioneer Hi-Bred International, Inc. and United States Department of Agriculture, Grant No.950310.

REFERENCES


Effects of auxins and cytokinins on formation of Catharanthus roseus G.Don multiple shoots

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Abstract

The effects of different combinations of plant growth regulators and light intensity on the formation of multiple shoots of Catharanthus roseus (L.) were studied. By composing three dimensional surfaces and their topographical views from experimental data, it was clear that Murashige-shoog (MS) medium supplemented with 7.0 mg/l BA and 1.0 mg/l NAA strongly stimulated the formation of shoots, whereas medium supplemented with 2,4-D suppressed the formation of shoots or presented shoot differentiation. Light intensities of 550-700 Lux were found to be beneficial to the formation of shoots when MS medium was supplemented with 2 mg/l 6-BA and 0-1.0 mg/l NAA.

Abbreviations: BA-6-benzyladenine,
NAA-α-naphthaleneacetic acid,
2,4-D-2,4-dichlorophenoxyacetic acid
Fate and Effect of Trichloroethylene As Nonaqueous Phase Liquid in Chambers with Alfalfa

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Abstract

A system was constructed consisting of six chambers (sixty six liters each) with five planted to alfalfa and one unplanted. Trichloroethylene (TCE) was introduced into the system as a nonaqueous phase liquid (NAPL) groundwater contaminant. The effect of vegetation on TCE fate was experimentally investigated. TCE concentrations in both the groundwater and the gas phase above the soil were measured. Comparison of the planted chambers to the unplanted one showed that the plants significantly increased the upward water flux and therefore enhanced the relative contaminant flux rates. The groundwater flow pattern and the corresponding flow model coefficients were determined by using the bromide tracer method. For the five months since the TCE was introduced, alfalfa plants have been growing well, without being markedly affected by the contaminant.

Key words: groundwater, vegetation, NAPL contaminant, flow pattern

Introduction

Nonaqueous phase liquids (NAPLs) in the subsurface are long-term sources of groundwater contamination, and may persist for centuries before dissolving completely in adjacent groundwater. The most frequently cited group of NAPL contaminants to date are the halogen-containing compounds. Of this group, trichloroethylene (TCE) is a chlorinated organic solvent used by various industries. TCE is also the most frequently found contaminant at hazardous waste sites on the EPA's National Priority List.

Natural communities of microorganisms in various contaminated habitats are able to metabolize and often mineralize an enormous number of organic molecules. The halogenated compounds would rapidly pollute the earth if there were no microbial dehalogenation pathways. This is one of the reasons why bioremediation techniques appear promising in remediating large scale soil
and groundwater contamination.

While searching for cost-effective bioremediation techniques, people have found that plants can play an important role. With growing plants, the rhizosphere is a zone of intense microbial activity. This activity is a consequence of the large number of bacteria that utilize the simple organic compounds continuously excreted by the roots of plants during their active stages of development. Because TCE is more readily destroyed in the rhizosphere than in nonrhizosphere soil, the use of growing plants to promote bioremediation of TCE-contaminated soil has been proposed.

Understanding the NAPL contaminant fate under vegetation and the effect of NAPLs on plant growth will provide significant insight in determining the potential effectiveness of vegetation. To detect and delineate the presence and fate of NAPL contaminants, a system consisting of six chambers was equipped, and TCE was introduced into the system as a NAPL groundwater contaminant.

Experimental Section

Experimental Setup

The chamber system used in this study is schematically shown in Figure 1. There are six independent chambers of the same size, five of them are planted with alfalfa and one is unplanted to allow studying the effect of vegetation. The key features include the following:

1. Soil depth is 60 cm;
2. Length of each chamber is 110 cm;
3. Width of each chamber is 10 cm;

During this study, the saturated zone was maintained at approximately 35 cm deep. Distilled water was fed from water jugs to maintain a stable water supply to the system. Pure TCE was introduced to the inlets of five chambers (1,2,4,5,6) of the system through a syringe pump at 20 ml per chamber. Alfalfa is harvested monthly before blooming.

Experimental Methods

To estimate the upflow water amount $V_{up}$ (L/day) due to plant transpiration and water evaporation, each day the jug level was restored to a fixed value and the amount needed was recorded. The volume of water exiting was measured. $V_{up}$ was calculated from the difference between the restored and the exiting amount. After one month, water was maintained by addition of
a constant volume per day ($L$) with head pressure allowed to vary somewhat.

Groundwater samples were regularly taken from the exit sampling ports with a syringe and transferred to small vials. The head space TCE concentration in the vial, $C_G (mM)$, was determined using a gas chromatograph equipped with a flame ionization detector and then converted to the liquid sample TCE concentration, $C_L (mM)$, as follows:

$$C_L = \frac{C_G}{H} + \frac{C_G (V_B - V_S + V_f)}{V_S}$$

where $H(=0.383)$ is the Henry's constant of TCE; $V_B, V_S$ and $V_f$ are volumes ($ml$) of the vial bottle, liquid sample and the chromatograph syringe, respectively.

On some occasions, a sealed metal hood was placed over the chamber so as to detect TCE coming from the soil and plants (shown by the dashed line in Figure 1.). The gas within the hood was extracted continuously at constant extraction rate and analyzed by a Gasmet FTIR analyzer for TCE and other compounds. Gas phase concentration was obtained as mole fraction $y$ and converted to $C (\mu M)$. Assuming complete mixing within the hood, the mass balance equation of this extraction process for a pulse input of trichloroethane (TCA) tracer gas can be written as:

$$V \frac{dC}{dt} = -qC$$

where $V$ is the hood volume (=38$L$), $t$ is time (min.), and $q$ is the extraction rate ($L/min$) which was estimated by injecting TCA as half-time marker. When TCA concentration attenuated to 50% of the injected value $C_0$, the time was recorded as half-time $t_{1/2}$ and was used to find $q$ by integrating from zero to $t_{1/2}$, i.e.,

$$q = \frac{V}{t_{1/2}} \ln\frac{C_0}{C} = \frac{V}{t_{1/2}} \ln2$$

Once the TCE concentration reached a steady state value, the contaminant flux rate into the hood was obtained as:

$$f = \frac{q \cdot C}{A}$$

in which $f$ is flux of TCE into the hood ($\mu$ mole/$m^2/min$), $A$ is the sectional area of each chamber exposed to the atmosphere (=0.11$m^2$).
The flow pattern of groundwater through soil is important for system characterization and development of contaminant transport models. Therefore, a bromide tracer experiment was performed. By switching the inlets from water jugs to KBr solution tanks, the tracer (KBr) solution was added to each chamber in the same way the water was supplied. Samples were taken from the exit sample ports immediately after the switching and every half day thereafter. The concentration of bromide was determined by the phenol red method with flow injection analysis techniques, which was set up in our laboratory. This method can quickly detect concentrations of bromide down to 0.1 mg/l.²

Results and Discussion

Comparison of Upflow Water

Table 1 lists the evapotranspiration rates \( V_{up} \) of the six chambers. The data varies on different dates and for different chambers because evapotranspiration rate depends on room humidity, the growth rate of the alfalfa, and the soil moisture. On the day when alfalfa was harvested, \( V_{up} \) was lowest (as marked with stars). However, it is obvious that the planted chambers 1, 2, 3, 5, and 6 have higher evapotranspiration rates than the unplanted chamber 4. The mean (\( \bar{V}_{up} \)) and standard deviation (s) are also listed. \( V_t (L) \) is the total water volume added to each chamber, \( \sum V_{up} \) is the summation of all evapotranspiration volumes (L). The differences between the \( \sum V_{up}/V_t \) ratios of chambers 1, 2, 3, 5, 6 and that of chamber 4 indicate that the upward water fluxes were significantly increased by the vegetation.

Breakthrough Curves

KBr tracer solution (1.1 liters) was added to each chamber at the concentration of 26.9 mg/l of \( Br^- \) within 12 hours. Water samples were taken at the exits every half day. Figures 2 and 3 are the breakthrough curves of bromide as a function of the cumulative volume of exiting solution from planted and unplanted chambers, respectively, which show that similar water flow patterns exist in these chambers.

After there was no \( Br^- \) detected at the exits, we obtained the \( Br^- \) amount coming out, \( m_{Br} \) (mg), by integrating over the volume numerically. For chamber 2 and chamber 4, \( m_{Br} \) equals to 9.54 mg and 13.2 mg, accounting for 32.2% and 47.3% of all \( Br^- \) injected, respectively. The difference of these two ratios (15.1%) is in agreement with that of the corresponding greater evapotranspiration fraction (18%) shown in Table 1. In other words, chamber 2 takes 15% to 18% more of entering water up due to the vegetation.
Groundwater TCE Concentration

The changes in groundwater TCE concentration as a function of the exit water volume are shown in Figures 4 and 5. Figure 4 represents the results of the planted chambers and Figure 5 signifies that of the unplanted chamber. We can see that the TCE concentration was lower in planted chambers than in the unplanted one. By numerically integrating the TCE concentration vs. effluent volume profiles, we found that, four months after the injection, about 75% of the TCE in the planted chambers was carried out by the groundwater, while 95% has been carried out from the unplanted chamber. Therefore, it may be concluded that plants either increase the upward TCE flux, as predicted by the modeling studies which have shown that plants enhance the upward transport of TCE \(^6,7\), or enhance the degradation and immobilization of TCE.

TCE Gas Phase Concentration and Upflow Flux

Table 2 displays five batches of TCE concentration measured in the gas phase above the soil and plants of the closed chamber and the corresponding TCE upward flux rates. The data indicate that small amounts of TCE are carried up by volatilization and transpiration processes, and that the planted chambers have higher TCE flux rates.

Mass balances on TCE

In order to evaluate the effect of vegetation on the fate of TCE, mass balances on TCE were carried out based on assumptions as follows:

- When there is no TCE detectable at the groundwater exits (169 days after the introduction of TCE), the remaining part of TCE within chambers was either biodegraded or immobilized,
- The groundwater TCE concentrations measured each time represent average concentrations of that day the samples were taken, and
- The amount of TCE carried up with upward water can be estimated using the average values of concentration data in Table 2 and averaged upward water flow rates in Table 1 over 169 days.

Mass balance results are listed in Table 3 in which the \textit{exiting in groundwater} (g) is the numerical integration result of TCE groundwater concentration over effluent water volume, \textit{exiting into gas phase} (g) is the amount of TCE carried up with upward water resulted from volatilization and evapotranspiration.
Conclusions

A 6-chamber system was constructed to study the effect of vegetation on contaminated groundwater. Alfalfa was planted and TCE was the contaminant examined. Comparison of experimental results of planted chambers with that of the unplanted one indicates that alfalfa obviously increased the upward flux rates of water and, consequently, of TCE; enhanced the biodegradation and/or immobilization of TCE; but did not greatly affect the lateral groundwater flow pattern within chambers. Alfalfa plants have been growing well since TCE was introduced.

Acknowledgments

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References

Table 1. Comparison of evapotranspiration rates of individual chambers

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</table>

| \( \bar{V}_{w} \) (L/day) | 0.56  | 0.54  | 0.50  | 0.22  | 0.43  | 0.50  |
| s (L/day) | 0.20  | 0.21  | 0.19  | 0.08  | 0.14  | 0.19  |
| \( \sum V_{w} \) (L) | 14.63 | 13.91 | 13.02 | 5.81  | 11.23 | 13.04 |
| \( \bar{V}_{w} \) (L) | 31.45 | 28.86 | 33.17 | 19.08 | 31.58 | 34.70 |
| \( \sum V_{a}/V_{b} \) (-) | 0.47  | 0.48  | 0.39  | 0.50  | 0.36  | 0.38  |

*Data on the harvesting day.

Table 2. Soil gas TCE concentration and upward flux rate

<table>
<thead>
<tr>
<th>chamber</th>
<th>1</th>
<th>2</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>1</th>
<th>2</th>
<th>4</th>
<th>5</th>
<th>6</th>
</tr>
</thead>
<tbody>
<tr>
<td>Date</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7/18</td>
<td>0.112</td>
<td>0.201</td>
<td>0.045</td>
<td>0.067</td>
<td>0.150</td>
<td>1.069</td>
<td>1.324</td>
<td>0.428</td>
<td>0.641</td>
<td>1.432</td>
</tr>
<tr>
<td>8/8</td>
<td>0.063</td>
<td>0.067</td>
<td>0.099</td>
<td>0.018</td>
<td>0.067</td>
<td>0.599</td>
<td>0.841</td>
<td>0.086</td>
<td>0.171</td>
<td>0.641</td>
</tr>
<tr>
<td>8/12</td>
<td>0.089</td>
<td>0.057</td>
<td>0.111</td>
<td>0.022</td>
<td>0.067</td>
<td>0.855</td>
<td>0.641</td>
<td>0.107</td>
<td>0.214</td>
<td>0.641</td>
</tr>
<tr>
<td>8/30</td>
<td>0.134</td>
<td>0.031</td>
<td>0.031</td>
<td>0.038</td>
<td>0.125</td>
<td>0.764</td>
<td>0.178</td>
<td>0.178</td>
<td>-</td>
<td>0.713</td>
</tr>
<tr>
<td>9/30</td>
<td>0.149</td>
<td>0.128</td>
<td>0.085</td>
<td>0.021</td>
<td>0.149</td>
<td>1.188</td>
<td>0.873</td>
<td>0.679</td>
<td>0.339</td>
<td>0.891</td>
</tr>
</tbody>
</table>
Table 3. Mass balance for TCE; 29.2g TCE injected into each chamber

<table>
<thead>
<tr>
<th>Chamber Number</th>
<th>Exiting in Groundwater (g)</th>
<th>Exiting into Gas Phase (g)</th>
<th>Total Mass Exiting Chamber (g)</th>
<th>Mass of TCE Lost/Remaining in Chamber (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>20.16</td>
<td>1.85</td>
<td>22.01</td>
<td>7.19</td>
</tr>
<tr>
<td>2</td>
<td>23.67</td>
<td>1.61</td>
<td>25.28</td>
<td>3.92</td>
</tr>
<tr>
<td>4</td>
<td>30.93</td>
<td>0.24</td>
<td>31.17</td>
<td>--</td>
</tr>
<tr>
<td>5</td>
<td>26.68</td>
<td>0.43</td>
<td>27.11</td>
<td>2.09</td>
</tr>
<tr>
<td>6</td>
<td>27.81</td>
<td>1.68</td>
<td>29.49</td>
<td>--</td>
</tr>
</tbody>
</table>

Figure 1. Schematic diagram of a planted chamber system
Figure 2. Bromide Breakthrough Curve (Chamber 2, planted)

Figure 3. Bromide Breakthrough Curve (Chamber 4, unplanted)
Figure 4. TCE Concentration in Aqueous Effluent vs. Effluent Volume (Chamber 2, planted)

Figure 5. TCE Concentration in Aqueous Effluent vs. Effluent Volume (Chamber 4, unplanted)
INTRODUCTION

An airlift reactor is a bubble column in which a buoyancy-driven circulatory flow is set up by sparging only part of the cross-section. It is simple in design with a minimum of internals (Fig. 1) and has good oxygen transport and gentle agitation for a given power input since it does not require an impeller. This makes it suitable for fragile animal and plant cells.

However, it is not suitable for highly viscous media due to the large frictional losses involved. Also, the interfacial stresses associated with bubble dynamic events are harmful to cellular entities. Transport characteristics vary widely over the range of airlift designs and geometries. The operating strategy in a specific case is thus a function of a combination of cell type, airlift geometry and fluid properties. In choosing an optimal strategy, oxygen depletion at low aeration rates and intense shear stress conditions at high sparge rates must be avoided (assuming that environmental conditions such as pH and working temperature have been predetermined for the system).

Figure 1. Schematic of an internal loop draught tube-sparged airlift reactor.

Working volume = 9 liters
Height/Diameter = 6.86
Riser area/Downcomer area = 0.225
Draft tube length/Liquid depth = 0.7

In this work, the properties of culture media are simulated using suitable fluids. The minimum permissible sparge rate that will provide adequate oxygen transport and mixing in the airlift are determined. Theories extant in the literature are used to estimate fluid shear in key regions and assess the possibility of cell damage.
THEORY

Properties of culture media

Culture media have variable physical properties and non-Newtonian rheology, affected further by the presence of cells or microcarriers in suspension. According to a theory developed by Einstein (see Hiemenz, 1977), for a dilute suspension of microspheres the viscosity is changed in a Newtonian fashion and can be calculated from the equation

$$\mu = \mu_0 (1 + 2.5 \phi + 10 \phi^2)$$  \hspace{1cm} (1)

Table 1. Properties of various media

<table>
<thead>
<tr>
<th>Medium</th>
<th>T, C</th>
<th>Density, g/cc</th>
<th>Apparent viscosity, cP</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pure water</td>
<td>25</td>
<td>1.008</td>
<td>0.87</td>
<td>This work</td>
</tr>
<tr>
<td>DMEM*</td>
<td>37</td>
<td>1.0156</td>
<td>0.91</td>
<td>This work</td>
</tr>
<tr>
<td>DMEM (+ 25 g/l beads)</td>
<td>37</td>
<td>1.0162</td>
<td>0.97</td>
<td>Equation (1)</td>
</tr>
<tr>
<td>5% glycerol</td>
<td>25</td>
<td>1.0239</td>
<td>1.17</td>
<td>This work</td>
</tr>
<tr>
<td>DMEM</td>
<td>37</td>
<td>1.003</td>
<td>0.71</td>
<td>Croughan et al, 1989</td>
</tr>
<tr>
<td>FCS**</td>
<td>37</td>
<td>1.015</td>
<td>0.88</td>
<td>-ditto-</td>
</tr>
<tr>
<td>DMEM, 5% v/v FCS</td>
<td>37</td>
<td>1.004</td>
<td>0.74</td>
<td>-ditto-</td>
</tr>
<tr>
<td>DMEM, 25% v/v FCS</td>
<td>37</td>
<td>1.006</td>
<td>0.78</td>
<td>-ditto-</td>
</tr>
</tbody>
</table>

* Dulbecco’s Modified Eagle Medium  ** Fetal calf Serum

Oxygen Transport

The oxygen transfer rate is given by the equation

$$OTR = \frac{dC_L}{dt} = Kp \left(C^* - C_L\right)$$  \hspace{1cm} (2)
If the oxygen requirement OUR is known for the chosen cell line then the minimum permissible sparge rate \( U_{s,\text{min}} \) corresponds to \( K_a \) determined from the safety condition

\[
OTR \geq OUR
\]

\( i.e. \quad K_a \geq \frac{OUR}{C_L^* - C_L} \) 

The reactor should always be operated above \( U_{s,\text{min}} \). \( K_{a,\text{min}} \) is tabulated for typical cell lines in Table 2 below.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>OUR, (( \mu \text{mol} , \text{O}_2/10^6 \text{ cells h} )) ( \times 10^3 )</th>
<th>( K_{a,\text{min}}, (\text{s}^{-1}) \times 10^5 )</th>
</tr>
</thead>
<tbody>
<tr>
<td>BHK 21c13s(^1)</td>
<td>110</td>
<td>35.8</td>
</tr>
<tr>
<td>Namalwa(^1)</td>
<td>75</td>
<td>24.4</td>
</tr>
<tr>
<td>Mouse L929(^1)</td>
<td>65</td>
<td>21.2</td>
</tr>
<tr>
<td>Human foreskin FS-4(^1)</td>
<td>85</td>
<td>16.3</td>
</tr>
<tr>
<td>Hybridoma(^2)</td>
<td>343.75</td>
<td>111.9</td>
</tr>
<tr>
<td>Bacteria/yeast(^3)</td>
<td>56250</td>
<td>1831.5</td>
</tr>
</tbody>
</table>

1- Bailey and Ollis, 1983 2- Birch et al., 1987 3- Chisti, 1989

Fluid Flow and Mixing

The response to a pulse input in a plug flow (with axial dispersion) reactor with total recycle is given by the equation

\[
\frac{C_t}{C_{\infty}} = \left( \frac{Pe}{4\Pi \lambda} \right)^{1/2} \sum_{p=1}^{\infty} \exp \left[ - (p-\lambda)^2 \frac{Pe}{4\lambda} \right]
\]

where \( Pe = \frac{(U_L \lambda)}{D_x} \), \( \lambda = \frac{\nu}{\epsilon} \) (Chisti, 1990). If we assume plug flow with axial dispersion throughout the circulation path of an airlift reactor we can fit
pulse tracer response data to equation (5) to determine the Peclet number and the liquid circulation time. Knowing the length of the circulation path \( L_c \), we can get the liquid circulation velocity \( U_{lc} = \frac{L_c}{t_c} \). A material balance on the liquid flow yields liquid velocities \( U_{lr} \) and \( U_{ld} \) in the riser and downcomer respectively. The flow regime in each of these sections can then be judged from the prevailing Reynolds number at that sparge rate.

**Shear Within the Airlift Reactor**

Shear stresses due to velocity gradients and eddy dissipation are prevalent throughout the column. Additionally, the bubble sparging and bubble disengagement areas impose stresses on the fluid. The main focus of this work is to model the shear in bubble wakes, stresses through viscous dissipation, and shear along the reactor walls. The limits for these have been found in the literature. McQueen reports that the threshold for damage in laminar flow is about 1800 dyne/cm². Croughan determined that the Kolmogorov eddy length must be smaller than 2/3 of the microcarrier diameter in order to damage the cells. The eddy lengths decrease with increasing energy dissipation (thus increasing stress). Models for the flow around the bubbles are dependent on the shape and size of the bubbles.

**Shear in Bubble Wakes**

The bubbles rising through the reactor impart momentum to the fluid in the form of vortices. These vortices eventually shed from behind the bubble, and dissipate their energy into the fluid. A typical bubble has a "cap" shape and is from .4 to .8 cm in diameter. The size and intensity of the vortices can be predicted using the model from Tsuchiya and Fan (1990), given by the equations

\[
k_{pw} = \frac{3}{2} \Delta k_{pw} - 2(\sqrt{2} - 1)\left(\frac{b}{h}\right)\left(\frac{d_{bl}}{b}\right)\frac{1}{\pi} \quad (6)
\]

\[
\tau = \mu \frac{dv}{dx} \quad (7)
\]
High Stress Region

The high stress region in the bulk flow of the reactor, noticed during dye tracer studies, is the region of fluid turn-around. This action sets up a vortex on the outer wall of the draft tube at the top of the column. The shear in this profile is highest very close to the reactor wall in the laminar flow sub-layer. The stress in this layer can be modelled by Newton's law of viscosity and Diezler's formula (Bird *et al.*, 1960), which combine to give

\[ \tau_0 = \frac{d\bar{v}}{ds} + \rho n \bar{v} \bar{v} s(1 - \exp[-n \bar{v} s/\bar{v}]) \frac{d\bar{v}}{ds} \]  

(Eq. 8)

Eddy Dissipation

The stress generated by the dissipation of eddies can be seen as a function of the average dissipation, or Kolmogorov, eddy length. As the turbulence increases, and the amount of mechanical energy that can be imparted on an object increases (in the form of stress), the average eddy length decreases. According to Croughan, the eddy length at which cell damage begins is approximately $2/3$ of the diameter of a microcarrier. This eddy length can be calculated by the equation $\eta = \nu^{3/4}$.

MATERIALS AND METHODS

Densities were measured gravimetrically and viscosities using a Brookfield viscometer for shear rates from 45 to 450 s$^{-1}$.

The local volumetric oxygen transfer coefficient $K_a$ was estimated using the well-known static gassing-out method (Scrugg, 1991). The reactor was purged with nitrogen until a low oxygen concentration was reached and then sparged with air at a known constant flow rate until saturation. The response was followed by an Ingold oxygen probe and transmitter hooked up to a PC. The values measured at three locations along the riser and downcomer were averaged to give an overall $K_a$ for the reactor.

Fluid flow and mixing were analyzed by injecting a pulse of 0.5M KCl solution into the top of the downcomer and following the response in conductivity with time at a point lower down using an Omega conductivity probe and meter which were also wired to a PC for on-line data acquisition.
RESULTS AND DISCUSSION

To simulate properties of DMEM with a microcarrier loading of up to 25 g/l, experiments were conducted using both pure water and 5% glycerol, which bracket the actual medium properties (see Table 1).

Oxygen Transport in the Airlift Reactor

Using the data shown on Fig. 3 below, $K_a$ was correlated with the superficial gas velocity $U_{sg}$ for pure water and 5% glycerol by

$$K_a = 1.324 \ U_{sg}^{0.345}, \ 0.06 < U_{sg} < 0.33 \ cm \ s^{-1} \ (9)$$

The constants in the correlation depend on reactor geometry and fluid properties.

![Figure 3](image.png)

Figure 3. Overall $K_a$ versus superficial gas velocity $U_{sg}$ for pure water and 5% glycerol.

$K_a$ is almost linear with sparge rate as would be expected at such low aeration. The spatial variation of $K_a$ was less than 10%, indicating that the reactor can be assumed to be well-mixed. An increase in viscosity promotes coalescence which reduces the gas-liquid interface and justifies the marginally lower $K_a$ for 5% glycerol. Actual culture media contain various components such as salts, proteins, organic acids, etc. which can have a prominent effect on the surface tension of the dispersion. This effect must be analyzed and studied to make this simulation valid for the real case.

The saturation solubility of oxygen in water $C_{L}^s$ is equal to 0.000284 mol/L (Haegert, 1993). Typically mammalian cells require $C_L$ at 25-40% satn. (Bailey and Ollis, 1986). Assuming a maximum average cell concentration of $2 \times 10^6$
cells/mL, from equation (9) we get the minimum permissible sparge rate $U_{sg,\text{min}}$ to be less than $6 \times 10^{-4}$ m/s for all the mammalian cell lines in Table 2. This is at the lowest end of the range of sparge rates investigated. Therefore, low aeration seems to be sufficient for mammalian cells and oxygen transport may not be the deciding factor for $U_{sg,\text{min}}$. It remains to be seen whether excessive frothing or settling occur at $U_{sg,\text{min}}$.

**Fluid Flow and Mixing in the Airlift Reactor**

The Peclet number $Pe$ and liquid circulation time $t_\text{c}$ were calculated from the tracer response curve (see Figs. 4, 5). Liquid velocities $U_{L\text{r}}(\text{riser})$ and $U_{L\text{d}}(\text{downcomer})$ were correlated with $U_{sg}$ as

$$U_{L\text{r}} = 339.09 \ U_{sg}^{1.15} , \quad U_{L\text{d}} = 76.29 \ U_{sg}^{1.15} \quad (10)$$

for pure water and

$$U_{L\text{r}} = 254.6 \ U_{sg}^{1.11} , \quad U_{L\text{d}} = 57.35 \ U_{sg}^{1.11} \quad (11)$$

for 5% glycerol. These regressions compare closely with values from the iterative procedure of Chisti et al. (1988). Low fluid velocities result in low Peclet numbers. This implies that convection is not very dominant. Moreover, the time for 95% mixing of tracer is far less than time required for 95% oxygen saturation. Therefore it is apparent that the reactor is well-mixed. From Fig. 6 it is evident that $Re$ is lower for 5% glycerol due to greater viscosity, indicating slower circulation. Predominantly turbulent flow occurs in the riser, with flow in the downcomer in the transition regime. The knowledge of fluid velocities determined in this study is useful in estimating fluid shear.

When compared with the results of McQueen and Bailey (1989) the shear behind bubbles, as well as the wall shear (Fig. 7), appear too low to promote damage. At higher superficial gas velocities, the dissipation eddy lengths approach the damage threshold of 2/3 the microcarrier diameter, or 100 microns (Fig. 8). Therefore, at higher sparge rates, some damage could occur.

**CONCLUSIONS**

Gentle aeration is sufficient to provide oxygen and good mixing for most mammalian cell lines in this airlift reactor. At the sparge rates investigated, noticeable cell damage is not expected from the agencies considered here.
Pulse response in conductivity

Figure 4.

Figure 5.

Figure 6. Reynolds number in the riser and downcomer versus $U_{sg}$ for water and 5% glycerol.
Figure 7. Wall and wake shear stress versus superficial gas velocity.

Figure 8. Eddy dissipation scale in the riser and downcomer versus superficial gas velocity.
Nomenclature

b  bubble cap diameter
C_L  dissolved oxygen concentration; "*" indicates saturation
d_v  diameter of primary vortex behind bubble cap
D_z  axial dispersion coefficient
h  height of bubble cap
K_a  volumetric oxygen transfer coefficient
k_w  factor that dictates how much larger the wake volume is than the bubble cap volume
L_c  length of liquid circulation loop
n  a resistance-to-flow constant. (0.124 for long smooth tubes)
s  dimensionless radius (R-r), R = outside radius
\tau  liquid circulation time
U_l  linear liquid velocity
U_\infty  superficial gas velocity
v_z  free stream velocity in the direction of flow, z
\mu  absolute viscosity
\nu  kinematic viscosity
\phi  volume fraction of microcarrier beads in suspension
\eta  Kolmogorov eddy length
\epsilon  energy dissipated into fluid by eddy action
\tau  shear stress

References

Effects of Cyclic Shear Stress on Mammalian Cells Under Laminar Flow Conditions: Apparatus and Methods

M.L. Rigney, M.H. Liew, and M.Z. Southard, Department of Chemical and Petroleum Engineering, University of Kansas, Lawrence, KS 66045

The work being presented today is part of our study of optimization of cell growth in airlift bioreactors. Some of this research is being conducted in cooperation with a group at Kansas State University, led by Dr. Larry Glasgow. My particular area of interest is the effect of cyclic shear stresses on mammalian cell viability. This presentation will focus on experimental apparatus and methods which we are developing to investigate these effects. To begin, I would like to discuss why we believe cyclic shear stress is important and how it is generated. Then I will describe how our experimental setup and techniques for measuring the effects of shear stress on mammalian cells. I will conclude with a few words about what we will do with this information.

Substantial research has already been done to investigate the effects of shear stress on a variety of cell lines. However, most of this work has focused on the effects of constant shear stress, and as I will show, this is not the best description of the physical reality which cells encounter in an airlift reactor.

Based on the varied hydrodynamics of an airlift reactor, the reactor can be split into four different flow regions. The first is the sparger outlet, at the base of the reactor. The important events here are bubble formation and energy
introduction into the reactor. The second region is the central riser area. Here cells and microcarriers interact with the wakes left behind the bubbles as they rise through the medium. The microcarriers and cells are also impacted directly by the bubbles. The third area is the top of the reactor, where bubbles disengage from the medium and rupture. The energy released by a bursting bubble is significant from a cellular perspective. The fourth region is the annular downcomer area. The number of bubbles in this area is minimal, but fluid flow is typically turbulent, and could be energetic enough to give rise to eddies small enough to impact cells on the surface of a microcarrier.

While there are distinct and different mechanisms of potential damage to cells in the four areas, there are some similarities. One overarching theme is the way in which the magnitude of the shear stresses to which the cells are exposed varies throughout their transit. Whether it is a case of a bubble impacting a microcarrier, or a microcarrier being nearby when a bubble bursts or being hit by a turbulent eddy small enough to impact cells on its surface, the stress felt by the cells will rise quickly from some mean or central value and then fall back towards that mean. Clearly, a constant shear stress does not model this type of encounter very well.

So the challenge is to develop a more realistic approximation of the physical situation within an airlift reactor. One step is to investigate the effects of a cyclic, rather than constant, shear stress on cellular viability.

We have chosen to use Chinese hamster ovary (CHO) cells as the cell line for our experiments. These cells are relatively well known, being widely used in industry. The particular strain we are using is a CHO-K1 line, supplied to
us by Dr. Paul Kitos of the KU Biology Department.

The cells are grown in an incubator at 37°C and 95-100% relative humidity. The pH is maintained with a HEPES buffer at 7.3. Rapidly growing cells are subcultured onto slides and allowed to attach. The slides are used within 18-24 hours after being seeded, insuring that cells are in the exponential area of the growth curve.

The cells are grown in medium which is a 1:1 blend of Dulbecco's Modified Eagle Medium (DME) and Ham's F-12 (F-12). The DME has a high glucose concentration, 4500 mg/L, and acts as the main energy source for the cells, but does not contain proline, which is necessary for CHO cell growth. The F-12 contains only 1000 mg/L of glucose, but supplies the required proline. The blend of media is supplemented with 10% (v/v) fetal bovine serum (FBS), which is a poorly defined mix of growth factors, trace elements, and other necessary nutrients for successful in vitro cell maintenance. As already noted, the final medium also contains HEPES buffer, which helps maintain the pH at optimal levels.

The fluid used in experiments is Hanks' Balanced Salt Solution (HBSS), an aqueous solution of glucose, NaCl, KCl and other salts. It is isotonic with respect to growth medium. It has a viscosity of 0.68 cP. HBSS is used instead of medium in experiments because it is significantly cheaper and completely defined.

Once the cells are attached to the slides, they are subjected to shear stress with a laminar flow device, shown in Figure 1 on the next page. The device consists of four pieces. The bottom is a polycarbonate block, with entrance and exit ports milled out. The entrance port also serves as a bubble trap when the chamber is inverted during the
experiment. A connection for syringe attachments to withdraw bubbles is not shown. A gasket is placed between the base and a plastic slide, covered with cells in the exponential growth phase. The gasket serves two purposes: it seals the chamber to prevent leakage and also defines the gap height for the chamber. The assembly is completed with a metal plate fastened with screws which thread into the base.

For laminar flow through a parallel plate geometry, the fluid flow is well characterized, and the hydrodynamic equations relating flow rate to shear stress are fairly easy to solve.

The pertinent equations are:

\begin{align*}
\text{(1)} & \quad \text{Re}_f = \frac{Q \rho}{\mu b} \\
\text{(2)} & \quad \tau = \frac{6Q \mu}{bh^2}
\end{align*}
\( L = 0.04(h)Re \)

Table 1 shows values of Reynolds number, shear stress, and entry length for various flow rates through the chamber.

<table>
<thead>
<tr>
<th>( Q ) (cm(^3)/min)</th>
<th>( \text{Re} )</th>
<th>( \tau ) (dyne·s/cm(^2))</th>
<th>( L ) (cm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.0</td>
<td>0.9</td>
<td>3.4</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>10.0</td>
<td>9.0</td>
<td>33.8</td>
<td>0.005</td>
</tr>
<tr>
<td>70.0</td>
<td>63.3</td>
<td>236.5</td>
<td>0.032</td>
</tr>
</tbody>
</table>

Clearly, from the Reynolds number and entry length, the flow is laminar at all flow rates through the chamber, so the shear stress equation is valid. For pulsatile flows, the flow may be taken to be laminar and described by the above equations while the Wormersley number is less than one. The Wormersley number can be calculated as

\( \alpha = \omega R^2/v \)

When \( \alpha \) is small compared to 1, the solution of the Navier-Stokes equation simplifies to a Poiseuille flow that fluctuates with time. If \( \alpha \) is larger, the solution to the Navier-Stokes equation yields a flow whose velocity profile varies periodically with time, and equations (1)-(3) are no longer valid. In our system, values of \( \omega \) less than 0.1 sec\(^{-1}\) will insure that the flow remains laminar and equations (1)-(3) remain valid.
Such a pulsatile flow will create a cyclic shear stress on the cells. This should be a better approximation of the shear phenomena in an airlift reactor. In order to produce such a flow, we have developed a flow circuit, as is shown on the next page, in Figure 2.

The heart of the circuit is a dual syringe pump (Isco 260D), which is configured so that one cylinder refills while the other is emptying. This allows us to program a continuous, constant flow of fluid. The fluid in the pump cylinders is maintained at 37°C with a water jacket, and the rest of the flow circuit is held at 37°C inside a constant temperature air enclosure.

The fluid flow is split into two legs as it exits the pump, a primary and secondary leg. Both legs are run through metering valves, which are calibrated and set before each run. The metering valves are not changed during a run. This allows precise control of the flow through each leg.

As shown, the primary leg will always be sent to the laminar shear chamber. The secondary leg is run through a computer-controlled three-way valve. When a voltage is applied by the computer to the valve, the flow is sent to the right, where it recombines with the primary leg before entering the laminar shear chamber. When a voltage is not applied, the secondary leg flow is shunted directly to the reservoir. The effect is produce a high-low, or pulsatile, flow across the shear chamber. The computer-controlled valve is much quicker and efficient than controlling the pump output directly.

After the HBSS exits the shear chamber it is collected in
a stirred reservoir where samples are pulled before the HBSS is recycled to the pump.

The cellular response to shear stresses are monitored with a microscope and camera connected to a VCR. The microscope and camera are on loan to us from Dr. Glasgow at KSU. The resulting images can be digitized and analyzed with a computer. These frames can be analyzed to give the number of cells remaining at a given time. This number of cells remaining is our basic measure of cellular viability.

At this point, we are working with members of the Electrical and Computer Engineering department at KU to adapt their image analysis program for our needs. We want the computer to count and record changes in cell shapes and orientations at specific times in the run. Previous researchers have shown that cells exposed for a length of time to constant shear stress will elongate and then change their orientation so that the cells' long axis is parallel to the direction of flow. We would like to see if the results are duplicated by cells exposed to pulsatile flows.

We can also monitor cell removal with an enzyme assay. We have chosen to work with a lactate dehydrogenase (LDH) assay), which was originally developed for diagnosis of muscle damage in humans, as might occur during a heart attack. LDH is an intracellular enzyme, released upon cell lysis. Its natural function is as a catalyst for the homolactic fermentation reaction:

\[
\text{LDH} \quad \text{Pyruvate} + \text{NADH} + \text{H}^+ \quad \longrightarrow \quad \text{Lactate} + \text{NAD}^+ 
\]
NADH strongly absorbs radiation at 340nm. Since NAD\(^+\) does not have significant absorbance at this wavelength, the LDH concentration can be measured by combining the enzyme, substrate and co-factor in a spectrophotometer cuvette. The rate of change in absorbance at 340nm is linearly related to the enzyme concentration.

This assay was originally developed for reactions involving 100-200 I.U. of enzyme, but our samples have LDH concentrations in the 0.01-0.1 I.U. range. In addition, the accuracy of the assay is affected by substrate and product inhibition, as well as secondary reactions in the sample fluid. However, by closely controlling test conditions we can get reproducible results. Differences between multiple tests run on a single sample are on the order of 1-2%.

To summarize, we have developed a laminar shear chamber, with which we can produce well-defined shear stresses on CHO cell monolayers grown on glass slides. We have also put together a flow circuit which allows us to subject our cells to pulsatile, rather than constant shear stresses. Currently, we are measuring cell lysis with an enzyme assay. We can also collect and digitize images of the cells and are also working to modify an algorithm for analyzing the number, shapes, positions, and orientations of cells as they are exposed to flow.

Experiments will begin when these methods have been tested. Information from the experiments will then be combined with knowledge obtained by the reactor design part of my research group, to begin large-scale culture of cells.
Nomenclature (typical values in parenthesis)

\begin{itemize}
  \item \textbf{Re} \quad \text{Reynolds number}
  \item \textbf{\tau} \quad \text{shear stress}
  \item \textbf{L} \quad \text{entry length for transition to laminar flow}
  \item \textbf{Q} \quad \text{volumetric flow rate} \quad \text{(}<1-70\text{ml/min.})
  \item \textbf{\rho} \quad \text{fluid density} \quad \text{(}0.995\text{g/cm}^3\text{ for HBSS at }37^\circ\text{C})
  \item \textbf{\mu} \quad \text{fluid viscosity} \quad \text{(}0.68\text{cp for HBSS at }37^\circ\text{C})
  \item \textbf{b} \quad \text{length along the plates} \quad \text{(}3.8\text{cm})
  \item \textbf{h} \quad \text{width between plates} \quad \text{(}0.013\text{cm})
  \item \textbf{v} \quad \text{kinematic viscosity}
  \item \textbf{\alpha} \quad \text{Wormersley number}
  \item \textbf{\omega} \quad \text{period of oscillation of a } \text{pressure gradient driving a flow} \quad \text{(}0.1\text{ sec}^{-1})
\end{itemize}
8:30 Welcome

8:35 Foreign Protein Production from SV40 Early Promoter in Continuous Cultures of Recombinant CHO Cells - Gautam Banik, Paul Todd, and Dhinakar Kompala, University of Colorado

9:00 Enhanced Cell Recruitment Due to Cell-Cell Interactions - Brad Forlow and Matthias Nollert, University of Oklahoma

9:25 The Recirculation of Hybridoma Suspension Cultures: Effects on Cell Death, Metabolism and Mab Productivity - Peng Jin and Carole A. Heath

9:50 Poster Session

10:30 The Importance of Enzyme Inactivation and Self-Recovery in the Biodegradation of Chlorinated Solvents - Xi-Hui Zhang, Shanka Banerji, and Rakesh Bajpai, University of Missouri-Columbia

10:55 Phytoremediation of VOC Contaminated Groundwater using Poplar Trees - Melissa Miller, Jason Dana, L.C. Davis, Murlidharan Narayanan, and L.E. Erickson, Kansas State University


11:45 Lunch at Pizza Hut, 1121 Moro in Aggieville

1:00 Poster Session

1:30 Inertial Migration Based Separation of Chlorella Microalgae in Branched Tubes - N.M. Poflee, A.L. Rakow, D.S. Dandy, M.L. Chappell, and M.N. Pons, Colorado State University

1:55 Contribution of Electrochemical Charge to Protein Partitioning in Aqueous Two-Phase Systems - Weiyu Fan and Charles C. Glatz, Iowa State University

2:20 Poster Session

3:00 Biodegradation of Surfactants Used in Enhancing PAH Solubility during Bioremediation - Jun Gu and Rakesh Bajpai, University of Missouri-Columbia

3:25 Modeling the Role of Biomass in Heavy Metal Transport in Vadose Zone - K.V. Nedunuri, L.E. Erickson, and R.S. Govindaraju, Kansas State University

3:50 Invitation to Attend 1997 Symposium
Poster Papers

Multivariable Statistical Methods for Monitoring Process Quality: Application to Bioinsecticide Production by Bacillus Thuringiensis - C. Puente and M.N. Karim, Colorado State University

The Use of Polymeric Flocculants in Bacterial Lysate Streams - H. Graham, A.S. Cibulskas and E.H. Dunlop, Colorado State University

Fate of Trichloroethylene in a Chamber in Presence of Alfalfa Plants: Experimental and Modeling Studies - Muralidharan Narayanan, Lawrence C. Davis, and Larry E. Erickson, Kansas State University

Detection of Specific Microorganisms using the Arbitrary Primed PCR in the Bacterial Community of Vegetated Soil - X. Wu and L.C. Davis, Kansas State University

Flux Enhancement Using Backpulsing - V.T. Kuberkar and R.H. Davis, University of Colorado

Chromatographic Purification of Oligonucleotides: Comparison with Electrophoresis - Stephen P. Cape, Ching-Yuan Lee, Kevin Petrini, Sean Foree, Micheal G. Sportiello and Paul Todd, University of Colorado

Determining Singular Arc Control Policies for Bioreactor Systems Using a Modified Iterative Dynamic Programming Algorithm - Arun Tholudur and W. Fred Ramirez, University of Colorado

Pressure Effect on Subtilisins Measured via FTIR, EPR and Activity Assays, and Its Impact on Crystallizations - J.N. Webb, R.Y. Waghmare, M.G. Bindewald, T.W. Randolph, J.F. Carpenter, C.E. Glatz, University of Colorado and Iowa State University

Intercellular Calcium Changes in Endothelial Cells Exposed to Flow - Laura Worthen and Matthias Nollert, University of Oklahoma

Application of Liquid-Liquid Extraction in Propionic Acid Fermentation - Zhong Gu, Bonita A. Glatz, and Charles E. Glatz, Iowa State University

Purification of Recombinant T4 Lysozyme from E. Coli: Ion-Exchange Chromatography - Weiyu Fan, Matt L. Thatcher, and Charles E. Glatz, Iowa State University


Effects of Auxins and Cytokinins on Formation of Catharanthus Roseus G. Don Multiple Shoots - Ying-Jin Yuan, Yu-Min Yang, Tsung-Ting Hu, and Jiang Hu, Tiangjin University and Kansas State University

Fate and Effect of Trichloroethylene as Nonaqueous Phase Liquid in Chambers with Alfalfa - Qizhi Zhang, Brent Goplen, Sara Vanderhoof, Lawrence C. Davis, and Larry E. Erickson, Kansas State University