Proceedings of the Twenty-First Annual Biochemical Engineering Symposium

Kenneth F. Reardon
Colorado State University

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PROCEEDINGS OF THE
TWENTY-FIRST ANNUAL
BIOCHEMICAL
ENGINEERING
SYMPOSIUM

20 April 1991

Kenneth F. Reardon
Editor

Department of Agricultural and Chemical Engineering

Colorado State University
PROCEEDINGS OF THE TWENTY-FIRST ANNUAL BIOCHEMICAL ENGINEERING SYMPOSIUM

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Department of Agricultural and Chemical Engineering
Colorado State University
Fort Collins, CO 80523

SPONSORED BY:
• Colorado Institute for Research in Biotechnology
• Colorado Bioprocessing Center
• Department of Agricultural and Chemical Engineering, Colorado State University
PREFACE

The 21st Annual Biochemical Engineering Symposium was held at Colorado State University on April 20, 1991. The primary goals of this symposium series are to provide an opportunity for students to present and publish their research work and to promote informal discussions on biochemical engineering research.

This is the third of these symposia to be sponsored by Colorado State University (others were the 11th and 15th). Previous meetings in this series have been hosted by Kansas State University (1st, 3rd, 5th, 9th, 12th, 16th, and 20th), University of Nebraska-Lincoln (2nd, 4th), Iowa State University (6th, 7th, 10th, 13th, and 17th), University of Missouri-Columbia (8th, 14th, and 19th), and University of Colorado (18th).

I would like to thank the authors of the papers presented at the symposium, especially those who submitted manuscripts for inclusion in these proceedings; the symposium attendees; the symposium organizing committee (Dr. Eric Dunlop, Linda Henk, Dr. Allen Rakow, Dr. Naz Karim, and Elaine Dunlop); the graduate students from the Department of Agricultural and Chemical Engineering (CSU) who helped set up and put away poster boards; and the Department of Agricultural and Chemical Engineering (CSU), the Colorado Institute for Research in Biotechnology, and the Colorado Bioprocessing Center for financial support.

Kenneth F. Reardon  
Editor
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Colorado State University  
Fort Collins, CO  
20 April 1991

Session 1: Oral Presentations (130 Engineering South)
8:30 Welcome (E.H. Dunlop)
8:55 "Influences of Medium Composition and Cultivation Conditions on Recombinant Protein Production by Bacillus subtilis," K. Park and K.F. Reardon, Colorado State University
9:15 "Characterization of a Novel Recombinant Bacterial Expression System Induced by Bacteriophage Infection," F. Miao and D.S. Kompala, University of Colorado-Boulder

9:55 BREAK

Session 2: Oral Presentations (130 Engineering South)
10:40 "Evaluation of a New Electrophoretic Device for Protein Purification," M.-J. Juang and R.G. Harrison, University of Oklahoma
11:00 "Crossflow Microfiltration and Membrane Fouling for Yeast Cell Suspensions," S. Redkar and R. Davis, University of Colorado-Boulder
11:20 "Interaction of β-Galactosidase-Maltose Binding Fusion Protein with Starch," L. Taladriz and Z. Nikolov, Iowa State University
11:40 "Predicting the Solubility of Recombinant Proteins in Escherichia coli," D. L. Wilkinson and R.G. Harrison, University of Oklahoma

noon LUNCH

Session 3: Oral Presentations (130 Engineering South)
2:30 "Practical Considerations for Implementation of a Field Scale in Situ Bioremediation Project," J.P. McDonald and L.E. Erickson, Kansas State University
2:50 Closing Comments (E.H. Dunlop)
Session 4: (3:00 - 4:30) Poster Presentations (228 Lory Student Center)
"Micromixing as an Engineering Parameter in the Design of Bioreactors," K.S. Wenger and E.H. Dunlop, Colorado State University
"Laser Doppler Velocimeter-Measured Feed Zone Turbulence and Its Effect on Baker’s Yeast Metabolism in a Chemostat," R. Beyerinck and E.H. Dunlop, Colorado State University
"Effect of Environmental Parameters on Solid Substrate Fermentation," J. Sargantanis, M.N. Karim, and V.G. Murphy, Colorado State University
"Production of Acetyl-Xylan Esterase from Aspergillus niger," M.R. Samara and J.C. Linden, Colorado State University
"Cultivation of Artemisia annua for the Production of the Antimalarial Artemisinin," J.R. Haigh and J.C. Linden, Colorado State University
"Structured Kinetic Model for the Intracellular Composition of Serratia marcescens at Different Culture Conditions," T. Diken and D.K. Kompala, University of Colorado-Boulder
"Effects of Fluid and Thermodynamic Factors on Biological and Latex Particle Partitioning," D. Hawker, R. Davis, P. Todd, and R. Lawson, University of Colorado-Boulder
"Novel Bioreactor/Separator for Microbial Desulfurization of Coal," H. Gecol, R.H. Davis, and J.R. Mattoon, University of Colorado-Boulder/Colorado Spring
"Effect of Plants and Trees on the Fate, Transport and Biodegradation of Contaminants in the Soil and Ground Water," W. Huang, E. Lee, J.F. Shimp, L.C. Davis, L.E. Erickson, and J.C. Tracy, Kansas State University
"Acoustic Studies of Interfacial Effects in Airlift Reactors," L.A. Glasgow, J. Hua, T. Yiin, and L.E. Erickson, Kansas State University
"Soy Yogurt Fermentation of Rapid Hydration Hydrothermal Cooked Soy Milk," P. Tuitemwong, L.E. Erickson, and D.Y.C. Fung, Kansas State University
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University of Nebraska
Mike Meagher, Daryl Graefman, John Sweeney, Shane Gold

University of Oklahoma
Roger Harrison, Ming-Jiing Juang, Chris Haught, Kostas Zgasas, Sophie Bertaux, David Wilkinson
High-density Fed-batch Cultivation and Energy Metabolism of *Bacillus thuringiensis*

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Abstract

Fed-batch cultivations of *B. thuringiensis* were conducted in carbon-limited media with the aim of increasing the amount of cell mass during vegetative growth. With proper feeding strategy, cell concentration as high as 54 g dry weight/L was obtained. Potency of the δ-endotoxin produced during sporulation increased correspondingly but not proportionally with the amount of cell mass. The content of poly-β-hydroxybutyric acid (PHB), an energy reserve for sporulation, in the cells at the beginning of sporulation could be important in determining the completion of sporulation.

Introduction

*Bacillus thuringiensis* (*B.t.*) is a Gram-positive, spore-forming microorganism. The life span of it constitutes in sequence of vegetative growth, sporulation and free-spore liberation. Sporulation is induced when the environment becomes unfavorable for vegetative growth, such as in the case of starvation of nutrients. An important feature about *B. thuringiensis* is that, during sporulation, it also produces a proteinacious protoxin. The protoxin in a sporulating cell aggregates to form a diamond-shaped particle commonly known as crystal or parasporal crystal. The crystals possess insecticidal activity specifically against many kinds of insects in the families of *Lepidoptera* and *Diptera*. Yet, they are not harmful to humans or useful insects such as honey bees. Nor do they cause pollution problems, as do many synthetic chemical insecticides. Consequently, preparations of *B. thuringiensis* crystals are now widely applied in agricultural pest control[1-4].

Many investigators have studied the cultivation of *B. thuringiensis*. Sakharova et al.[5] studied crystal formation under various nutrient limitations and reported that sporulation and crystal formation could be induced by the limitations of glucose, yeast extract, magnesium or potassium. However, the type of limitation affected the nature of the kinetics of growth and sporulation, as well as the quality and quantity of crystal formed. The growth kinetics of *B. thuringiensis* was investigated by Sakharova et al.[6] in synthetic and complex media. These authors reported that
B. thuringiensis cells preferred amino acids as carbon and nitrogen source over glucose, indicating complex nutritional requirements of the organism. On the other hand, Khovrychev et al. [7] reported that the properties of spores and crystal toxicity were similar, irrespective of whether these were produced in a batch bioreactor or in a two-stage continuous bioreactor. Scherrer et al. [8] looked into the effect of glucose concentration on crystal production and found that high glucose concentrations resulted in big crystals only at the expense of including biologically inactive proteins along with the protoxin. Nickerson and Bulla [9] studied the minimal nutrient requirements of B. thuringiensis and noted that supplementing the minimal medium containing glucose and salts with aspartate, glutamate or citrate also improved crystal formation. Yousten and Rogoff [10] related metabolism of B. thuringiensis to spore and crystal formation and showed that low pH in broth inhibited synthesis of spore and crystal protein. Foda et al. [11] noticed that aeration and pH control were important during cultivation. Goldberg et al. [12] modified a medium by continuous cultivation for a high yield production of spore-crystal preparation. Arcas et al. [13] showed that yeast extract in the medium could be substituted with malt sprout extract for crystal production. Arcas et al. [14] recently attempted a fed-batch fermentation, but details were not given.

The objective of this research is to grow Bacillus thuringiensis cells to a high concentration during vegetative growth phase and to study the effect of the growth conditions under high density cultivation upon the quality and the quantity of crystal protein produced. Fed-batch fermentation has been used to achieve the goal. A carbon-limited medium has previously [15] been developed for cultivation of Bacillus thuringiensis and is presented in Table 1. Use of this medium in shake flasks resulted in improved crystal protein production. Results of fed-batch cultivations have been presented in this paper. Amount of poly-β-hydroxybutyric acid (PHB) in the cells has been monitored during growth and sporulation, and its possible role in energetics of sporulation-events has been discussed.

Materials and Methods

Bacillus thuringiensis ssp. kurstaki HD-1 obtained from the Bacillus Genetic Stock Center, Ohio State University (stock no. 4D6) was used in this study and the procedures for maintaining/propagating the cells have been reported previously [15].

Inocula were prepared by cultivation of cells with 200 mL medium in each of two 1000 mL, baffled Erlenmeyer flasks in a reciprocal incubator operated at 30 °C and 200 strokes/min. The medium composition in shake flasks was the same as that of basal medium (Table 1). Each of the shake flasks was inoculated with a loopful of spores from slant culture. Before autoclaving, pH in shake flasks was adjusted to 7.5 by using concentrated KOH solution. There was no pH control during shake flask cultivations.

Batch and fed-batch cultivations were performed in a 14 L NBS Modular Microferm Bench Top Fermentor (Model MF-214). The fermentor was operated at 900 rpm, 6 L/min air and 30 °C with pH
controlled above 6.5 by using concentrated KOH solution. For very-high-density fed-batch cultivations, 1000 rpm and air supply mixed with pure oxygen were used. Yeast extract, corn steep liquor and minerals except MgSO₄·7H₂O were prepared in the fermentor and autoclaved. To avoid the caramelization of glucose when steam-sterilized along with nitrogen and the formation of magnesium precipitates at high temperature, glucose and magnesium were autoclaved separately from the other ingredients and added to the fermentor after cooling down. The fermentor was inoculated with 400 mL 9 hr old culture from the two shake flask cultures. Basal medium was employed in the fermentor for batch and the initial batch operation of fed-batch cultivations. Some other operating conditions for batch and fed-batch cultivations are listed in Table 2.

The feed composition for each fed-batch experiment was essentially a concentrate of the basal medium. The concentrations, in terms of multiples of glucose concentration compared to that in basal medium, are listed in Table 2. Feed was sterilized by passing through a 0.2 μm autoclaved filter paper. Constant feeding rates were used for the early low-cell-density experiments while stepwise elevated feeding rates for the later high-cell-density ones to fulfil the nutrient requirement of the massively growing culture.

The analyses of glucose, cell dry weight, potency of crystals and total protein contained in the crystals were reported earlier[15]. Acetic acid concentration in the fermentation broth was determined along with glucose by using an HPLC.

Glucose concentrations in broth were further confirmed by using a glucose oxidase/peroxidase enzymatic method, with the reagents obtained from Sigma Chemical Co. (procedure no. 510). The Sigma procedure is a modification of that reported by Raabo and Terkildsen[16]. 0.5 mL diluted, cell-free broth samples were mixed with aliquots of 5.0 mL solution of glucose oxidase, peroxidase and o-dianisidine (a chromogenic reagent), followed by incubation at 37 °C for 30 min. Absorbances of the brown color in the incubated samples were measured at 450 nm within 30 min and compared to those of standard and blank solutions treated in the same way as samples to calculate glucose concentration.

Spore counts were performed on fermentation broth by using a Petroff-Hausser hemocytometer under a Bausch-Lomb phase-contrast microscope.

To assay poly-β-hydroxybutyric acid[17], samples of cells were centrifuged from fermentation broth, washed and redissolved in sodium hypochlorite (commercial bleach) solution at 37 °C for 1 hour. PHB granules were isolated from the last solution by filtration, washed subsequently with water, acetone and alcohol, and finally extracted with boiling chloroform which was evaporated later. Concentrated sulfuric acid was added to the samples to convert PHB to crotonic acid. Absorbance of the organic acid was measured at 235 nm against a sulfuric acid blank. Concentration of PHB was calculated with reference to a standard PHB preparation obtained from Sigma Chemical Co. and treated in the same way as samples.
Results and Discussions

Fig. 1 demonstrates the profiles of a typical fed-batch experiment (Run #6). Feeding started when the culture was about to reach the stationary phase at 6.5 hr. The feed contained 165 g/L glucose and proportional yeast extract, corn steep liquor and minerals according to the composition of basal medium. The feed was designed to obtain 25 g/L cell dry weight. The actual maximum cell dry weight was 23.4 g/L. The feeding rate followed step increase with time from 1.5 to 3.5 mL/min. Immediate HPLC analysis of samples taken out of the fermentor aided in monitoring remaining glucose concentration level so as to control the feeding rate manually. Before feeding stopped at 16 hr, 1.5 L of feed had been added to the fermentor.

Fig. 2 represents the progression of cell growth in some of the successful high-density experiments. With adequate strategy of feeding, a level of 54 g/L cell dry weight could be reached.

Table 2 summarizes the operating conditions and performances in some successful high-density fermentations. Although 54 g/L cell dry weight could be obtained by using a proper feeding strategy in Run #8, the spore count did not show a proportional increase with cell mass. In reality, a very low degree of sporulation was observed in this run. Total crystal protein and potency of crystals did not show a proportional increase with cell mass, either. Noticeable is that cell-mass-average potency decreases with succeeding fed-batch runs meanwhile percentage PHB content also decreases with these runs. This fact implies a connection between PHB content and degree of sporulation. The rest of this report is devoted to postulating a reasonable connection.

During vegetative growth, *B. thuringiensis* cells use glucose to provide energy for vegetative protein synthesis. With carbon limited medium, as in the present study, sporulation is turned on when glucose is exhausted. During sporulation, vegetative protein is depolymerized into component amino acids for use in spore and crystal protein synthesis[18]. The process of new protein synthesis requires energy which is most likely provided by PHB[19]. In order to study this phenomenon in details, an understanding of the energetics of *B. thuringiensis* seems necessary.

Starzak and Bajpai[20] proposed a pathway for carbon source metabolism in *B. thuringiensis* system as shown in Fig. 3. During vegetative growth, glucose is converted by *B. thuringiensis* to acetyl-CoA which then enters a modified Krebs Cycle specific to *B. thuringiensis*. According to this pathway, when the production of pyruvate from glucose is fast, acetate is formed and accumulated, but soon it will be decomposed to acetyl-CoA for the synthesis of PHB. The accumulation and consumption of acetate were indeed observed in all the experiments. When glucose is exhausted, culture enters sporulation phase and PHB is degraded to acetyl-CoA to provide energy for spore and crystal protein synthesis. The degradation pathway is shown in Fig. 4. The governing chemical equations which follow describe the energy flow from a PHB monomer to a peptide bond[19, 20 and 20, respectively]:

\[ \text{PHB} + \text{ATP} \rightarrow \text{Protein} + \text{PEP} + \text{Pi} \]
in the modified Krebs Cycle

\[ \Delta \text{PHB(M=86.09)} + \text{ATP} \rightarrow 4\text{CO}_2 + 9\text{NADH}_2 \]

oxidative phosphorylation

\[ 0.5\text{O}_2 + \text{NADH}_2 \rightarrow \text{H}_2\text{O} + 1.824\text{ATP} \]

vegetative amino acid(M=113.19) + 4.12ATP \rightarrow \Delta \text{new protein} 

By combining the above equations with balanced stoichiometry, a schematic pathway can be constructed as shown in Fig. 5. Thus, one mole PHB monomer (mw=86.09) can provide at most the energy required for the synthesis of protein from 3.74 moles amino acid (av. mw=113.19). \textit{B. thuringiensis} sporangia were found to contain 15.2 wt% spore protein and 18.5 wt% crystal protein\[18, 21\]. The minimum amount of PHB required for complete synthesis of both spore and crystal protein can then be easily calculated as 6.85 wt%. If some other minor energy-requiring processes are taken into account, such as m-RNA turnover (about 10 % extra energy required) and cell envelope turnover (about 1% extra energy required), the amount of PHB required can be raised to 7-8 wt%. This result suggests that if the PHB content in the cells is below the minimum amount at the beginning of sporulation, insufficient energy supply will lead to incomplete sporulation. This rationale provides a reasonable answer to explain why in some of the very-high-density fed-batch experiments (Table 2) low degree of sporulation was observed when PHB content was low. The reason for low PHB content in those experiments is still under investigation.

Conclusions

1. With proper feeding strategy, high-density cultivation of \textit{Bacillus thuringiensis} can be achieved via fed-batch fermentation.

2. High level of \(\delta\)-endotoxin production may be obtained if the content of poly-\(\beta\)-hydroxybutyric acid (PHB) in the cells is high at the beginning of sporulation. For the purpose of reaching high PHB content, the metabolism of PHB in the cells must be studied further.

References

5. Sakharova, Z. V., Yu. N. Ignatenko, M. P. Khovrychev, V. P. Lykov, I. L. Rabotnova, and V. V.

### Table 1: Basal Medium Composition

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose</td>
<td>8 g/L</td>
</tr>
<tr>
<td>(NH₄)₂SO₄</td>
<td>1 g/L</td>
</tr>
<tr>
<td>Yeast Extract</td>
<td>4 g/L</td>
</tr>
<tr>
<td>KH₂PO₄</td>
<td>3 g/L</td>
</tr>
<tr>
<td>K₂HPO₄</td>
<td>3 g/L</td>
</tr>
<tr>
<td>MgSO₄·7H₂O</td>
<td>4 g/L</td>
</tr>
<tr>
<td>CaCl₂·2H₂O</td>
<td>41 mg/L</td>
</tr>
<tr>
<td>MnSO₄·H₂O</td>
<td>30 mg/L</td>
</tr>
</tbody>
</table>

| Corn Steep Liquor | 4 g/L |
Table 2: Operating Conditions and Performances of Some Successful High-density Fed-batch Experiments.

<table>
<thead>
<tr>
<th></th>
<th>#1, batch</th>
<th>#6, fed-batch</th>
<th>#7, fed-batch</th>
<th>#8, fed-batch</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Initial working</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>volume, L</td>
<td>8</td>
<td>7</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td><strong>Feed conc., multiples</strong> of basal medium conc.</td>
<td>--</td>
<td>20.62</td>
<td>24.78</td>
<td>39.67</td>
</tr>
<tr>
<td><strong>Feeding rate, g glucose/min</strong></td>
<td>--</td>
<td>0.25-0.57</td>
<td>0.24-1.24</td>
<td>0.38-1.08</td>
</tr>
<tr>
<td><strong>Duration of feeding, hr-hr</strong></td>
<td>--</td>
<td>6.5-15.9</td>
<td>4.0-16.7</td>
<td>4.0-25.5</td>
</tr>
<tr>
<td><strong>Total glucose fed, g</strong></td>
<td>--</td>
<td>247</td>
<td>595</td>
<td>952</td>
</tr>
<tr>
<td><strong>Maximum cell dry weight, g/L</strong></td>
<td>5.9</td>
<td>23.8</td>
<td>36.4</td>
<td>53.7</td>
</tr>
<tr>
<td><strong>Maximum spore count, x10⁹/mL broth</strong></td>
<td>1.49</td>
<td>30.01</td>
<td>39.10</td>
<td>14.50</td>
</tr>
<tr>
<td><strong>Total crystal protein, µg/mL broth</strong></td>
<td>356.74</td>
<td>5329.76</td>
<td>3248.11</td>
<td>6089.33</td>
</tr>
<tr>
<td><strong>Potency of crystals, KIU/mL broth</strong></td>
<td>271.68</td>
<td>1210.48</td>
<td>1726.78</td>
<td>1680.20</td>
</tr>
<tr>
<td><strong>Specific potency, KIU/µg crystal protein</strong></td>
<td>0.762</td>
<td>0.231</td>
<td>0.532</td>
<td>0.276</td>
</tr>
<tr>
<td><strong>Cell-mass-average potency, MIU/g cell dry weight</strong></td>
<td>46.05</td>
<td>50.86</td>
<td>47.44</td>
<td>31.29</td>
</tr>
<tr>
<td><strong>% PHB content in cell dry wt at the beginning of sporulation</strong></td>
<td>20</td>
<td>12</td>
<td>4</td>
<td>2</td>
</tr>
</tbody>
</table>
Vegetative cells

Sporangiophores

Fig. 1. Concentration Profiles in A Typical Fed-batch Experiment (Run #6).

Fig. 2. Cell Concentration Profiles in Some Successful High-density Fed-batch Experiments.
Glucose $\rightarrow$ ATP $\rightarrow$ 2H $\rightarrow$ 2H $\rightarrow$ P-Glycerate $\rightarrow$ PEP $\rightarrow$ ATP $\rightarrow$ Pyruvate $\rightarrow$ Acetate $\rightarrow$ 2H $\rightarrow$ CO$_2$ $\rightarrow$ Acetyl-CoA $\rightarrow$ PHB $\rightarrow$ TSA $\rightarrow$ CO$_2$ $\rightarrow$ Malate $\rightarrow$ Oxaloacetate $\rightarrow$ Citrate $\rightarrow$ Isocitrate $\rightarrow$ 2H $\rightarrow$ Oxalosuccinate $\rightarrow$ Succinate $\rightarrow$ $\alpha$-Ketoglutarate $\rightarrow$ GABA $\rightarrow$ Glutamate $\rightarrow$ SSA $\rightarrow$ CO$_2$ $\rightarrow$ Glyoxylate $\rightarrow$ Malate $\rightarrow$ TSA $\rightarrow$ CO$_2$ $\rightarrow$ Fumarate $\rightarrow$ Oxaloacetate

Overall stoichiometry equation (without Acetate and PHB syntheses):
Glucose $+$ 12NAD $+$ 2ADP $+$ 2H$_3$PO$_4$ $+$ 4H$_2$O $\rightarrow$ 6CO$_2$ $+$ 12NADH$_2$ $+$ 2ATP

Fig. 3. Proposed Pathway for Glucose Metabolism in *Bacillus thuringiensis*. TSA: Tartronate semialdehyde, SSA: Succinate semialdehyde, GABA: $\gamma$-Aminobutyric acid.
Overall stoichiometry equation (combined with the modified Krebs Cycle):

\[ \text{PHB monomer} + \text{ATP} + 9\text{NAD} + 8\text{H}_2\text{O} \rightarrow 4\text{CO}_2 + \text{ADP} + \text{H}_3\text{PO}_4 + 9\text{NADH}_2 \]

Fig. 4. Degradation Pathway of PHB.

Fig. 5. Flow of Energy and Reducing Power during Sporulation.
INFLUENCES OF MEDIUM COMPOSITION AND CULTIVATION CONDITIONS ON RECOMBINANT PROTEIN PRODUCTION BY *BACILLUS SUBTILIS*

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The influences of medium composition and cultivation conditions on a genetically modified, low-protease mutant strain of *Bacillus subtilis* carrying a plasmid coding for β-lactamase are presented. The effects of temperature, pH, carbon source, nitrogen source, glucose and phosphate concentration, and medium quality on the growth and net β-lactamase production of plasmid containing cells were examined. The extracellular protease activity, which can influence heterologous protein production, was also measured.

INTRODUCTION

*Bacillus* species may be very useful microorganisms for the expression of heterologous proteins for two significant reasons. The first advantage is their ability to secrete several native proteins such as amylase, protease, and penicillinase. Each of these proteins has a signal peptide, which helps the cell membrane to recognize the protein to be transferred and is removed during the secretion process. When the structural gene of a foreign protein is fused to the signal peptide, the heterologous protein can be secreted into the medium. The other major advantage of *Bacillus* is their lack of endotoxin production.

Many laboratories have explored the fusion of the promoter and signal sequence from the gene of a secretory protein of *Bacillus* to the structural gene of a heterologous protein. The neutral protease prepro sequence, the alkaline protease prepro sequence, α-amylase signal sequence, and penicillinase signal sequence have been utilized to promote the secretion of heterologous proteins such as human interferon, human insulin, human growth hormone, mouse interferon, β-lactamase, nontoxic diphtheria toxin, and others. Promising results were reported.

A major problem facing extracellular heterologous protein production by *Bacillus* species is the production of extracellular proteases after the exponential growth phase. In order to reduce this protease activity, a genetically modified *Bacillus subtilis*, deficient in four
extracellular proteases, was used in our experiments.

The presence of plasmids in host cells affects the cellular metabolism and the cell growth due to the additional metabolic requirements for plasmid replication and protein expression. Thus, the cultivation conditions can be expected to have effects on the production of the cloned-gene product. The overall project goal is to understand the effects of the medium composition and the cultivation conditions on the cell metabolism and the plasmid-encoded heterologous protein production of a genetically modified, low protease \textit{B. subtilis}. In the first stage of this study, reported here, the objective was to identify the most important and influential cultivation parameters.

**MATERIALS AND METHODS**

**Organism, Plasmid, and Media**

\textit{Bacillus subtilis} DB428 was a gift from Dr. R.H. Doi of the University of California at Davis. This strain is deficient in four extracellular protease (\textit{apr}-, \textit{apr}-, \textit{epr}-, \textit{bps}-). Plasmid pVSL33 in \textit{B. subtilis} DB104 was a gift from Dr. S.L. Wong of the University of Calgary. This plasmid has the structural gene of \textit{\beta}-lactamase fused to the \textit{B. subtilis} subtilisin signal peptide to promote the secretion of \textit{\beta}-lactamase and also has the kanamycin resistance gene. The plasmid pVSL33 was transformed into DB428 in our laboratory. The strain was maintained on Tryptose Blood Agar Base plates with kanamycin (10 \(\mu\)g/mL).

Precultures were grown on LB medium with kanamycin (10 \(\mu\)g/mL). The growth medium composition was\(^3\), per liter: glucose 2 g; yeast extract 1 g; nutrient broth 1 g; \((\text{NH}_4)_2\text{SO}_4\) 2 g; \(\text{MgSO}_4\cdot7\text{H}_2\text{O}\) 0.3 g; \(\text{KH}_2\text{PO}_4\) 3 g; \(\text{K}_2\text{HPO}_4\) 8.25 g; tryptophan 0.1 g; \(\text{FeCl}_3\) 2.251 mg; \(\text{MnSO}_4\) 1.41 mg; \(\text{ZnSO}_4\cdot7\text{H}_2\text{O}\) 2.2 mg; \(\text{CuSO}_4\) 0.25 mg; \(\text{CoCl}_2\cdot6\text{H}_2\text{O}\) 0.4 mg; \(\text{Na}_2\text{MoO}_4\cdot2\text{H}_2\text{O}\) 0.26 mg; \(\text{H}_3\text{BO}_3\) 0.4 mg; \(\text{KI}\) 0.06 mg; kanamycin 10 mg. The pH of the medium was adjusted to 7.0 before autoclaving, and shake flask cultures were conducted at 37 °C and 200 rpm.

**Assay Methods**

The activity of \textit{\beta}-lactamase was measured spectrophotometrically with cephalothin as the substrate\(^{14}\). One unit of \textit{\beta}-lactamase activity was defined as the amount of the enzyme which could hydrolyze 1 \(\mu\)mol of cephalothin at 25 °C and pH 7.0 in one minute.

Protease activity was measured with casein as the substrate\(^{4,17}\). Sample (0.2 mL) was incubated with 2.5 mL of substrate at 37 °C and pH 7.5 for 10 minutes. After the casein hydrolysis reaction was stopped, the precipitate (undigested casein) was removed by the centrifugation and the absorbance of the supernatant was measured at 280 nm.

Cell density was measured in a spectrophotometer at 600 nm and correlated with a dry weight analysis. Glucose concentrations were determined in a YSI glucose analyzer.
RESULTS AND DISCUSSION

Carbon Source

The effects of the type of carbon source on the cell growth and β-lactamase production were examined. Figure 1 presents the results of some flask experiments. The use of glucose increased cell density rapidly compared to the other two carbon sources, maltose and starch. Growth on maltose was faster than on starch. However, the maximum cell densities were in the reverse order: starch > maltose > glucose. Also, cell densities for the media with slowly metabolized carbon sources decreased slowly following the maxima in comparison with more rapidly metabolized carbon sources. Although the maximum β-lactamase activities for all three carbon sources were about 320 units/L, the β-lactamase activities in the maltose and starch cultures decreased to zero after 27 hours. This might be due to protease production after the cell growth phase. The β-lactamase activity with glucose decreased slowly and was more than 200 units/L even after 27 hours.

To test the effects of initial carbon level on cell growth and product formation, two initial glucose concentrations, 2 g/L and 5 g/L, were used (Table 1). The maximum cell density attained in the 5 g/L glucose medium was about 150 percent higher than that in the 2 g/L medium. However, the maximum β-lactamase activity in the 5 g/L medium was only slightly higher than in the other medium, indicating that little of the additional glucose was used for additional plasmid protein production.

Nitrogen Source

Three different nitrogen sources, (NH₄)₂SO₄, urea, and casamino acids, were tested (Figure 2). The cell density of the culture with casamino acids was always higher than that of the other two cultures. After growth stopped, the cell densities for the complex nitrogen source, casamino acids, decreased slowly compared with the cases of the simpler nitrogen sources. The maximum β-lactamase activities were the same in the casamino acids and urea media, and lower in the ammonium medium. The β-lactamase activities in the casamino acids and urea media decreased to zero after 27 hours, but the plasmid product level in the medium with the simplest nitrogen source decreased to about 200 units/L after 27 hours. These results are similar to the effects of carbon source.

Medium Quality

The effects of medium quality (yeast extract and nutrient broth levels) were examined by removing or reducing yeast extract and nutrient broth concentrations in the basic medium (Figure 3). Three different media were prepared: (1) with yeast extract (1 g/L) and without nutrient broth, (2) without yeast extract and with nutrient broth (1 g/L), and (3) 0.5 g/L yeast extract and 0.5 g/L nutrient broth. The culture with yeast extract only (medium 1) had the
highest cell growth rate and maximum cell density and the culture with nutrient broth only (medium 2) had the lowest cell growth rate. The cell growth rate, the maximum cell density and the β-lactamase production rate of the culture with 0.5 g/L each of yeast extract and nutrient broth were between those of the other two cultures. Yeast extract appeared to be the more important component for cell growth while nutrient broth was the more important component for β-lactamase production. The maximum β-lactamase activities for three media were about 325 units/L, which was slightly lower than that with 1 g/L each components. Either yeast extract or nutrient broth can be removed without significant reduction of β-lactamase activity, but with nutrient broth only the maximum cell density will be low.

**Phosphate Concentration**

The effects of phosphate concentration in the range from 65 to 205 mM were not significant for cell growth and β-lactamase production (Figure 4). The maximum cell density was only slightly lower in the media with higher phosphate concentrations.

**Temperature**

The influences on temperature on this cultivation were tested at 30 and 37 °C (Figure 5). The cell growth rate and the β-lactamase production rate were higher at 37 °C. The β-lactamase activity decreased during the later hours of the 37 °C culture but not at 30 °C. Protease activities were measured for both cultures. As expected, protease activity was detected in the samples from the 37 °C culture taken after the cell growth phase. However, no proteolytic activity was detected in the samples from the 30 °C culture.

**Culture pH**

Three pH values (6.0, 6.8, and 7.5) were examined for their effects on growth and product formation (Figure 6). The maximum cell density and the maximum β-lactamase were the highest at pH 6.8. At pH 6.0, degradation of β-lactamase started at the end of the exponential phase and β-lactamase activity decreased to 75 units/L after 26 hours. For the cultures at pH 6.8 and 7.5, degradation of β-lactamase started after the cell density decrease. The best pH level for cell growth and β-lactamase production in this system appears to be near 6.8.

**CONCLUSIONS**

Even though a strain deficient in four proteases was used, protease activity was detected. Protease production was found to depend on the medium composition and cultivation conditions. Longer stationary phases were associated with more rapid degradation of β-lactamase, probably due to greater protease production. There were
differences in cell growth and heterologous protein production under different cultivation conditions. The most important parameters for $\beta$-lactamase production were carbon source, temperature, and culture pH, while for cell growth the important factors were carbon source and concentration, nitrogen source, medium quality, temperature, and culture pH. The effects of phosphate concentration were not significant. The type of carbon and nitrogen source, temperature, and culture pH also affected the degradation of product.

ACKNOWLEDGEMENTS

The authors thank Dr. R.H. Doi (University of California, Davis) and Dr. S.L. Wong (University of Calgary) for supplying the host strain (DB428) and the plasmid (pVSL33). This research was supported by Biomedical Research Support Grant No. 7249.

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**Table 1. Effects of glucose concentration**

<table>
<thead>
<tr>
<th>GLUCOSE CONCENTRATION (g/L)</th>
<th>MAXIMUM CELL DENSITY (g/L)</th>
<th>MAXIMUM β-LACTAMASE ACTIVITY (UNITS/L)</th>
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<td>368.3</td>
</tr>
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Figure 1. Effects of carbon source. CD: cell density; BL: β-lactamase activity.

Figure 2. Effects of nitrogen source. CD: cell density; BL: β-lactamase activity.
Figure 3. Effects of medium quality. CD: cell density; BL: β-lactamase activity; Y.E.: yeast extract; N.B.: nutrient broth.

Figure 4. Effects of phosphate concentration.
Figure 5. Effects of temperature (A): 37 °C and (B): 30 °C. CD: cell density; BL: β-lactamase activity; Gl: glucose concentration.
Figure 6. Effects of pH at 37 °C.
Characterization of Foreign Gene Expression in a Recombinant T7 Expression System Infected with λ Phages

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Abstract

The recombinant T7 E. coli cells were infected with bacteriophages to over-produce a foreign protein. The gene for the protein is encoded by a plasmid and is regulated by a T7 promoter, which requires the utilization of T7 RNA polymerase. It is provided by infecting the cells with the phages carrying the gene for T7 RNA polymerase. The phage absorption, foreign protein formation and cell growth rates are studied at the various infection conditions, such as MOI and cell concentration at infection and type of the carbon source. It is found that infecting phages could be quickly absorbed by the cells and the target protein is significantly increased. High-level protein can be achieved when the cells are infected at a mid-exponential phase with MOI ranging from 7.5 to 20.

INTRODUCTION

Recombinant viruses have attracted our recent interests for production of cloned gene products. Bacteriophages have been employed as expression vectors to over-express a gene of interest during their propagation through the lytic pathway on
bacteria.\textsuperscript{1} Such an approach of producing proteins eliminates the plasmid instability often encountered in recombinant microorganism cultures. Studies have been also conducted on insect cells infected with viruses to express recombinant genes.\textsuperscript{2} In addition, phage promoters are very efficient to control gene expression because of their unique characteristics. For instance, promoter $\lambda P_L$ and $P_R$ are thermostensitive,\textsuperscript{3} the expression of a controlled gene can be turned on or off by changing culture temperature. Bacteriophage T7 promoters are so active that genes under their regulation can be transcribed several time faster than other promoters.\textsuperscript{4} With this property, T7 promoters have been utilized for high-level production of foreign proteins in recombinant bacteria.

T7 promoters are activated only in the presence of the bacteriophage T7 RNA polymerase, which is lacked in natural microorganisms. Therefore, this promoter can be regulated through controlling the T7 RNA polymerase. This has been accomplished by either fusing the gene for T7 RNA polymerase into the host chromosome or by infecting the cells with the phages carrying this gene. A great advantage of using T7 promoter is that some proteins lethal to the strains can be successfully expressed in the recombinant T7 expression system. The expression of the T7-promoter-regulated gene will be completely quenched in the absence of T7 RNA polymerase. If a target protein encoded by the plasmid is toxic to the host, a basal level of such a protein leakily (without induction) expressed from the controlling promoter will kill the cell. In this situation, the plasmid can not be established. But, using the T7 promoter, the plasmid can be stably maintained by minimizing the basal T7 RNA polymerase level. Many efforts have been made to decrease the basal level of the protein in chemically-inducible T7 expression systems.\textsuperscript{5} An efficient way is to clone a gene for T7 lysozyme into the plasmid so that the leakily expressed T7 RNA polymerase will be quickly deactivated.\textsuperscript{6} But the plasmid-carrying cells usually have low growth rates and the plasmid still cannot be established if the protein is very toxic to the host. The most efficient method is to infect the plasmid-bearing cells with the bacteriophages bearing the T7 RNA polymerase gene. No target protein will be produced before infection since the cells do not contain any T7 RNA polymerase at all. After induction, the plasmid-bearing cells will be dying with the synthesis of the toxic protein.

This paper will investigate the foreign protein synthesis rate at the different infection condition, thereby elucidate an optimal infection strategy. In this study,
\(\beta\)-galactosidase is used as the model protein because of its simple assay. Although this protein is not toxic to the host, the infection with the phages that can undergo the complete lytic pathway may be a good simulation since the cells are dying upon infection. One of the main factors influencing the target protein synthesis rate is MOI (multiplicity of infection), defined as the phage number per cell at infection. We will investigate the protein synthesis rate at the MOI ranging from 1.0 to 20. The infection characteristics will be examined by measuring the foreign protein concentrations, the phage and the viable cell numbers with time course. The effect of cell concentration at infection and type of the carbon source for cell culture on the protein production was also explored.

MATERIALS AND METHODS

Phages, Cells and Plasmid

The host harboring the plasmid is MC1061, which has lambda phage receptors induced by maltose. Two cell lines, MY595 and C600, were used for the propagation of \(\lambda\) phage CE6 and its derivative respectively. Phage CE6 cannot lyse the cell line MC1061 because there is a Sam7 mutation, which prevents lysis of the infected cells. But, this phage can be propagated on the cell line MY595, where the Sam7 mutation can be repaired by a transfer RNA. The other type of phage is similar to CE6 except the Sam7 gene has been remutated back to the normal so that infection will cause cell lysis. This phage was propagated on the cell line C600. For both types of the phages, the \textit{int} gene has been replaced by the gene for T7 RNA polymerase, so that they will not undergo the lysogenic pathway. The plasmid used in this study is pDIP18/19D with a size of about 7.45 kb. It contains the Chloramphenicol-resistant gene and \textit{lacZ} gene controlled by a T7 promoter(\(\phi 10\)). The phages, the plasmid and the cells were kindly provided by Dr. Larry Gold and his colleagues (MCDB, CU Boulder).

Medium and Cell Culture

The medium for cell growth is M9CA minimal medium containing 6.0 g/l Na\(_2\)HPO\(_4\), 3.0 g/l KH\(_2\)PO\(_4\), 1.0 g/l NH\(_4\)Cl, 0.5 g/l NaCl, 0.002 M MgSO\(_4\), 0.0001 M CaCl\(_2\),
2.0 g/l maltose or glucose and 2.0 g/l casamino acids. Cell cultures were conducted in shaking flasks in a water bath (New Brunswick Scientific) maintained at 37°C. The cells grown overnight on the same medium supplemented with 10 μg/ml chloramphenicol was used as the inoculum. When the growing cells attained the designed cell concentration (measured by OD at wavelength 600 nm), the infection was implemented with the designed MOI. The cell culture was sat for 15 minutes for phage absorption, and then resumed shaking thereafter.

**Preparation of Phages**

The phages for cell infection were made in a fermentor (QUEUE with working volume of 1.0 liter). The LB medium was used for phage propagation. 20 ml cells grown overnight in the same medium were mixed with 0.4 ml pre-prepared phages with a concentration up to 10⁸/ml. The mixture was incubated for 15 minutes at 37°C for better absorption. Then the mixed cells and phages were injected into the fermentor for phage propagation during cell growth. After 7 hour fermentation, 2.0 ml chloroform was added to release the phages remaining in the cells. The phage broth was centrifuged at 4700 rpm and 5°C for 10 minutes. For the Sam7 unmutated phages, their concentration had been propagated up to higher than 10¹¹/ml and was high enough for infection. However, a further enrichment was required for the phages growing on the MY395 cell line because these phages propagated slowly. The most possible reason is because the infected cells need to repair the Sam7 mutation into the normal, thereby slowing down the propagating rate. The phage concentration was performed by precipitation with 50 g/l PG8000 on ice for 2-3 hours. The phage pellets were obtained by centrifugation and then resuspended with the 30 ml LB medium containing 2 g/l maltose and 0.001 M CaCl₂. The final phage concentration would reach typically more than 10¹¹/ml. The phages were kept at -4°C until use, usually within two weeks and exhibited high efficiency in forming plaques.

**Measurements of Phage and Cell Numbers**

The concentrations of both phages and cells were measured by plating method. The cells were seriously diluted with a sterile salt solution (6.0 g/l Na₂HPO₄, 3.0 g/l KH₂PO₄, 0.5 g/l NaCl and 1.0 g/l NH₄Cl). A certain volume of the diluted cells
was mixed with 2.5 ml soft agar (with 7.5 g/l agar) at 42°C and the mixture was cast on the LB plate. The plate was incubated at 37°C overnight and the colonies were counted. For the phage measurement, 200 µl of the exponentially-growing cells (MY595 or C600 corresponding to the different phages) were mixed with the diluted phages and incubated for 15 minutes before mixing with the soft agar and plating. The plate was incubated overnight, a titer would be formed from the growing cells. Each phage would absorb the cells surrounding it to form a plaque. The total plaques on the plate were counted, then the phage concentration was calculated.

β-Galactosidase Assay

β-galactosidase levels were measured with a chromogenic substrate, o-nitrophennoyl-β-galactosidase(ONPG). ONPG can be hydrolyzed by β-galactosidase to produce a yellow product and absorption can be measured at 420 nm. 0.6 ml sample taken from the culture was sonicated for 90 seconds to release the protein from the cells. A certain volume of the sonicated solution was mixed with ONPG(0.2%) solution and 1.3 ml Z buffer to a final volume of 1.5 ml. The absorbance of the reaction solution was continuously monitored with a programmable spectrophotometer, while maintaining the reaction temperature at 28°C. A pure β-galactosidase(from Sigma Chemical Co.) solution was also assayed in order to know the protein concentration of the sample.

RESULTS AND DISCUSSIONS

Infected cell growth in the maltose-containing M9 medium is depicted in Fig.1. The phages used are CE6 where the Sam7 gene has been remutated back to the normal gene so that the infection will cause cell lysis. As shown in the figure, the ODs of infected cells drop down significantly to the different levels, after keeping increased for a period of time, depending on the MOI applied. This indicates that cell lysis does not suddenly occur right after infection. The phages should be absorbed by the cells, and propagated, and then repackaged with the coat proteins into the phages for lysis. The cells are able to grow during these processes. At low MOIs, a long time of OD increase is attributed by the large fraction of well-growing uninfected cells and the infected cells but with low phage copies. These infected cells will not stop growing.
until the amplification of phage copies to lyse the cells. Therefore, the OD decrease occurs later after infection at lower MOIs.

The infection effect can be better illustrated by Fig.2, where the viable cells measured with plating method are plotted with post-infection time (the time after infection). The figure shows that at low MOIs, say MOI=1, the viable cell concentrations go up initially after infection and then drops down, indicating many cells are not infected. But, at MOIs higher than 5, the ODs of the cells have been decreased to the lowest within about two hour infection, and then they do not significantly vary with time. Nevertheless, we know from Fig.4 that almost all the phages are suddenly absorbed by the cells after infection. But at this time there are many viable cells as shown in Fig.2. This means that the infected cells will not immediately lose their ability of forming colonies on LB plates.

β-galactosidase formation rates at the various MOIs are investigated and the experimental data are presented in Fig.3. It shows that the foreign protein levels are very low within 0.5 hour after infection and then rapidly increased. At MOIs higher than 5, the protein synthesis rates maintain high within 2-3 hour infection then slow down. We also may observe that the protein concentration increases with an increase of MOI ranging from 1 to 7.5 and then does not significantly varies at higher MOIs. This is in consistent with the results obtained from Studiers et al.4 Higher MOIs will result in a more rapid synthesis of the T7 RNA polymerase which is constitutively expressed from the gene on the phages. The foreign protein synthesis rates will increase with an increase in T7 RNA polymerase level until saturation (T7 RNA polymerase in large excess). But, higher infection with higher MOIs will result in a more rapid lysis of the cells. For instance, the protein synthesis will be remarkably inhibited at MOIs higher than 50 because the cells will be quickly killed by the infecting phages.4

Besides MOI, another imoportnat factor affecting foreign protein production is cell concentration at infection. The study is conducted by maintaining MOI(10) and varying cell concentration of infection. The results for cell growth are presented in Fig.5, which shows that the lowest final OD is obtained at infection with the lowest OD. But, it is interesting to note that at the lowest cell concentration of infection the OD can reach a maximum and then fall down to a minimum, and then recover to the higher. This phenomenon is because some well-growing uninfected cells attribute the observed growth rate pattern. The OD recovery also indicates that the phages

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released from the infected cells poorly reinfect the other uninfected cells. This can be more clearly illustrated by Fig.6, where the viable cell concentration is plotted with post-infection time. It shows that the viable cell number recover significantly after 6 hour infection. These infected cells cannot produce the target protein but deplete the nutrients. Therefore, a high-level protein will not be obtained in this case. The foreign protein levels at the various infection cell concentrations are shown in Fig.7. The figure elucidates that there is an optimal OD for phage infection for maximizing the final protein concentration. This optimal OD is around 1 in this case, and it may vary with the sugar concentration. But, this OD is located at the mid-exponential phase, which is similar to a chemical induction.

The characteristics of the T7 expression system infected with the phages which cannot undergo the lysis process are described in Figs.8, 9, and 10. In contrast to the expression system infected with Sam7-unmutated phages, the present phages do not lyse the infected cells so that the ODs will not decline. As Fig.8 shows, the infected cells still keep growing and the growth rates do not decrease significantly even at high MOIs. But, we may observe that the final proteins levels in this infection system are not better than the phages causing cell lysis. However, unlike the former phage infection, the protein concentrations do not vary significantly at MOIs higher than 2.5. This is probably because the phages can keep propagating inside the infected cells without causing cell lysis. The total phage numbers are shown in Fig.10, demonstrating that the phage numbers increase with time, since the propagated phages are not released to reinfect other cells.

The effect of sugar type on infection is investigated and the results are presented in Figs.11 and 12. Where the circle symbol refers to the cells growing in the maltose medium but without stopping shaking after addition of phages, as we usually did in other shake flask experiments. The black pie refers the cells growing in the glucose medium and the cell culture was incubated for 15 minutes after infection. Obviously, glucose medium is poor for phage infection. The viable cell concentration is still increased after infection and the final protein level is much lower than that in the maltose medium. The poor infection in the presence of glucose is because glucose inhibits the λ phage receptors. From these figures, we also may observe that without incubation the infection is quite good and high-level protein is obtained. This indicates once again the high efficiency of the phage infection in maltose medium.
CONCLUSIONS

High levels of foreign protein can be obtained by infecting the T7 E. coli expression system with bacteriophage λ. The phage infection is so efficient in maltose medium that almost all infecting phages are quickly absorbed even without need for incubation after infection. But glucose is the poor carbon source for phage infection since the lambda receptors are repressed. The cell infection with the phages undergoing cell lysis is recommended to use since such phages are easily to be propagated. For this infection system, the optimal infection MOIs are around 7.5 to 20 and the optimal OD of infection is about 1.0, which is at the mid-exponential phase. With infection by the phages without causing cell lysis, the cells can keep growing upon infection. The foreign gene can be also overexpressed with infection at MOIs in the range of 2.5 to 20.

references

Figure 1. Cell growth at various MOIs, as marked in the figure. The phages used can lyse the infected cells since they do not have a stop codon at s gene.

Figure 2. Variations of viable cells with post-infection time.

Figure 3. Foreign protein formation rates at various MOIs.

Figure 4. Variations of total phages with post-infection time.
Figure 5. Cell growth with infection at various cell concentrations. The MOI used here is equal to 10.

Figure 6. Variations of viable cell numbers with post-infection time.

Figure 7. Variations of β-galactosidase levels with post-infection time.

Figure 8. Cell growth with infection at various MOIs, as marked in the figure. The phages used here cannot lyse the infected cells.
Figure 9. Variations of β-galactosidase levels with time course

Figure 10. Variations of phage numbers with post-infection time

Figure 11. Variations of viable cell numbers with post-infection time at two different culture conditions

Figure 12. Foreign protein formation at the different culture conditions
SIMULATION OF AN ENZYMATIC MEMBRANE SYSTEM WITH FORCED PERIODIC SUPPLY OF SUBSTRATE

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Chemical reactions in an enzymatic membrane are of both theoretical and practical interest. The present work has investigated numerically the nonlinear dynamic behavior of a diffusion-reaction system involved in the enzymatic membrane containing papain, which is exposed to a substrate whose concentration is periodically perturbed. The autonomous oscillations induced in the membrane system have been analyzed with the concentration of the external substrate as the parameter. To investigate in detail the periodic, quasi-periodic, or aperiodic behavior of the system, numerically generated time-series data have been analyzed by three diagnostic tests, i.e., the spectral analysis, the correlation integral analysis, and the estimation of Lyapunov exponent. A broad spectrum of frequencies has been observed in the power-spectrum diagram. The correlation integral analysis has yielded a saturated value of 1.68 for the correlation dimension, $\nu$. A positive value of 0.23 bits/s has resulted from the calculation of the largest Lyapunov exponent. These results suggest that the aperiodic oscillations may be chaotic.

INTRODUCTION

The formation of spatial patterns or temporal oscillatory behavior has been widely observed in chemically reacting systems under conditions far from equilibrium.¹ Such reacting systems may play an important role in living organisms for controlling the cell

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metabolism and the cell synthesis itself. The majority of biochemical reactions are catalyzed by enzymes, each of which is highly specific for one type of reactions. The rates of chemical reactions in living organisms depend on the rates of diffusion of participating chemical species since the enzymes are immobilized within structured systems, e.g., membranes.

Diffusion-reaction problems of enzymatic membranes were studied extensively from both the theoretical and practical points of view. Kinetically, a typical example is a membrane containing immobilized papain. The chemical reactions involved are substantially less complex than the well-known Belousov-Zhabotinskii reaction, which induces spontaneous chemical oscillations; the kinetic parameters in the former have been fully characterized.

The kinetics of the hydrolysis of α-N-benzoyl-L-arginine ethyl ester (BAEE) in solution in the presence of papain has been well established by Whitaker and Bender. Naparstek et al. observed oscillations in pH when BAEE diffuses through a synthetic membrane imbedded with immobilized papain. Zabusky and Hardin simulated the oscillations on computer with and without considering the existence of a boundary layer adjacent to the membrane and have proposed a linear theory for small amplitude oscillations without a boundary layer. Specifically, oscillatory or periodic operation of an immobilized enzyme reactor is of particular interest because of the nonlinearity induced by the coupling of the diffusion and complex reactions in the reactor.

The dynamic behavior of a papain enzymatic membrane with periodically forced operation has been investigated numerically in the present work. Under certain circumstances, such a membrane exhibits chaotic behavior.

KINETIC MODEL AND THE REACTION-DIFFUSION EQUATIONS

The following reaction scheme of papain kinetics has been chosen for analysis in this study.

\[
E + S \xrightleftharpoons{K_S} ES \xrightarrow{k_2} ES' + P_1 \xrightarrow{k_3} E + P_1 + P_2
\]

where E is the enzyme papain; S, the substrate (BAEE); ES, the enzyme-substrate complex; ES', the acyl-enzyme complex; and P1 and P2, alcohol and acid produced by the splitting of the ester substrate, respectively. The dissociation constant, K_S, is considered to be fixed at 54.5, i.e.,
and the dependence of the reaction rates, $k_2$ and $k_3$, on the hydrogen-ion concentration, $H$, can be described, respectively, by

$$k_2 = \frac{64.5}{1 + 10^{1.28} H + 10^{-5.49} / H}$$

$$k_3 = \frac{20.2}{1 + 10^{0.97} H}$$

The reaction rate as a function of both substrate and hydrogen ion concentrations is given by the following equation.

$$R = \frac{k_2 k_3 S E_0}{k_2 S + k_3 S + k_3 K_S}$$

where $E_0$ is the concentration of the enzyme. For convenience, a new variable, $A$, is defined as

$$A = H - K_w / H$$

where $K_w$ is the dissociation constant of water. The rate of dissociation of water into hydrogen and hydroxyl ions is much faster than the rate of an enzymatic reaction; thus, the former reaction is assumed to be in equilibrium.

A membrane containing papain is formed on a wall and immersed in a bath of substrate. The membrane is permeable to the substrate, but not to the enzyme. For simplicity, it is assumed that diffusivities of hydrogen and hydroxyl ions are identical. This gives rise to the following pair of nonlinear partial differential equations governing the diffusion-reaction coupling system in the enzymatic membrane.

$$\frac{\partial S}{\partial t} = -R(H, S) + D_S \frac{\partial^2 S}{\partial x^2}$$

$$\frac{\partial A}{\partial t} = R(H, S) + D_H \frac{\partial^2 A}{\partial x^2}$$

By introducing the dimensionless variables defined by

$$U = S / S_m$$

$$V = A / A_0$$
\[ \eta = x/L \quad (11) \]

and

\[ \theta = D_s t/L^2 \quad (12) \]

Equations 7 and 8 are transformed into, respectively,

\[ \frac{\partial U}{\partial \theta} = -aR'(V,U) + \frac{\partial^2 U}{\partial \eta^2} \quad (13) \]
\[ \frac{\partial U}{\partial \theta} = bR'(V,U) + \frac{\partial^2 V}{\partial \eta^2} \quad (14) \]

where

\[ a = \frac{L^2}{S_m D_s} \quad (15) \]
\[ b = \frac{L^2}{A_0 D_s} \quad (16) \]

and

\[ c = \frac{D_R}{D_s} \quad (17) \]

This is a one-dimensional system; no spatial variations exist except in the direction perpendicular to the membrane surface. The initial and boundary conditions are given by

\[ U=0, V=1, \quad 8=0, 0<\eta<1 \quad (18) \]
\[ \frac{\partial U}{\partial \eta} = \frac{\partial V}{\partial \eta} = 0, \quad \eta=0, 0<\theta \quad (19) \]
\[ V=1, \quad \eta=1, 0<\theta \quad (20) \]

Forced periodic perturbation exerted on the concentration of the external substrate is represented by

\[ U = S_o / S_m = 1 + \epsilon \cos(\omega \xi \theta), \quad \eta=1, 0<\theta \quad (21) \]

where the mean value, \( S_m \), is specified in the present study. Based on its physical significance, \( \epsilon \) is restricted to be non-negative. This forced periodic supply of external substrate changes the system into a two-parameter, non-autonomous system.
NUMERICAL METHOD

For numerical simulation, a finite difference method, specifically the forward explicit method, has been adopted. Simulations were carried out with the values of $L = 1.0 \times 10^{-4}$ m, $E_0 = 1.0$ mol/m$^3$, $H_0 = 1.0 \times 10^{-7}$ mol/m$^3$, $D_H = 6.4 \times 10^{-10}$ m$^2$/s, and $D_S = 1.3 \times 10^{-10}$ m$^2$/s.

RESULTS AND DISCUSSION

The numerically generated time series of the concentration of substrate at $\eta = 1.0$ has been analyzed. The sampling interval has been held at 1 s. An m-dimensional phase space portrait can be reconstructed by multiple delays. Nevertheless, it is often difficult to distinguish a chaotic motion from a motion that has a long-period oscillatory behavior. Moreover, quasi-periodic oscillations occur where two or more incommensurate periodic motions are combined. Various methods have been proposed to determine whether the oscillations are periodic or aperiodic. The correlation dimension, Lyapunov exponent, and power spectra have been selected in the present study to identify chaotic oscillations.

Decomposition of a signal into a set of sinusoidal or harmonic signals is a common technique to analyze aperiodic behavior. Figure 1 depicts the spectral density function of the time series. A broad spectrum of frequencies appears in this diagram. There are a number of peaks containing the main frequencies produced from the forced oscillations, and other frequencies. Broad-band power spectra perhaps serve to confirm the occurrence of chaotic phenomena; however, this is not always certain because of the presence of noise.

The method for computing the correlation dimension has been given by Grassberger and Procaccia. The correlation integral, $C(r)$, can be calculated by using the number of points in the hypersphere of radius $r$ and center $z_i$ at some point on the orbit of time trajectory.

$$C(r) = \lim_{m \to \infty} \frac{1}{m^2} \sum_{i=1}^{m} \sum_{j=1, i \neq j}^{m} H(r-|z_i(t)-z_j(t)|)$$  \hspace{1cm} (22)

where $m$ is the number of data points, and $H$ is the Heavyside function, i.e.,
\[
H(r - |z_i(t) - z_j(t)|) = \begin{cases} 
1 & \text{if } r > |z_i(t) - z_j(t)| \\
0 & \text{otherwise}
\end{cases}
\] (23)

\[C(r) \text{ has been found to be a power function of } r \text{ for small } r's, \text{i.e.,}
C(r) \sim r^\nu
\] (24)

Thus the correlation dimension, \( \nu \), may be defined as
\[
\nu = \frac{\ln C(r)}{\ln r}
\] (25)

This expression indicates that the slope of the plot of \( \ln(C(r)) \) versus \( \ln(r) \) yields an estimate of \( \nu \) for a given embedded space dimension. If the slope reaches an asymptotic value when the embedded space dimension is increasing, the signal may be chaotic. The correlation dimension determined must be less than the minimum phase space dimension. Figure 2 portrays a correlation integral analysis of the time-series data from a numerical simulation. Note that the slope of \( \ln(C(r)) \) versus \( \ln(r) \) saturates at a value with increasing embedding dimension of the phase space. The correlation dimension asymptotically reaches a value of \( \nu = 1.68 \), when the embedding space dimension is greater than 4, as illustrated in Figure 3. This result implies that the attractor has a non-integer dimension; the minimum phase-space dimension in this case is 2.

A strange attractor is highly sensitive to the initial conditions. The Lyapunov exponent represents the average exponential rate of divergence of nearby trajectory in the phase space. The i-th one-dimensional Lyapunov exponent of a continuous dynamical system in a p-dimensional phase space defined by Wolf et al. is
\[
\lambda_i = \lim_{t \to \infty} \frac{1}{t} \log_2 \left[ \frac{d_i(t)}{d_i(0)} \right]
\] (26)

where \( d_i(t) \) is the length of the ellipsoidal principal axis arising from the long-term evolution of an infinitesimal p-sphere of initial conditions, and the \( \lambda_i \)'s are ordered from the largest to the smallest values.

The sign of the largest Lyapunov exponent provides the criterion for the dynamic behavior of the system; a positive value suggests chaotic behavior. Figure 4 shows the temporal convergence of \( \lambda_1 \) as a function of the number of data points. The calculations have been performed with the algorithm presented by Wolf et al. As indicated in Figure 4, the value of the largest Lyapunov exponent, \( \lambda_1 \), is 0.23 bits/s.
CONCLUDING REMARKS

The membrane impregnated by the enzyme, papain, gives rise to a nonlinear diffusion-reaction system when an externally supplied substrate is transported through the membrane and reacts enzymatically. When the external substrate concentration is periodically perturbed, this system responds periodically, quasi-periodically the relative forcing frequency and the amplitude or aperiodically depending on the magnitude of the parameters, the forcing frequency, $\omega_f$, and the amplitude, $\epsilon$. Moreover, a period-doubling cascade has been observed in the space defined by the two parameters. Poincaré maps have been constructed to facilitate visualization and comparison of the quasi-periodic and aperiodic behavior. A broad spectrum of frequencies in the power-spectrum diagram, the saturation value of the correlation dimension, $\nu$, at 1.68, and the positive value of the largest Lyapunov exponent, $\lambda_1$, (0.23 bits/s), have been obtained from the numerically generated time series of the aperiodic behavior. The results imply that the aperiodic oscillations generated are probably chaotic. Their practical significance is obvious since industrial chemical processes often involve nonlinear diffusion-reaction systems.

NOMENCLATURE

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>$A$</td>
<td>concentration difference between hydrogen and hydroxyl ions, mol/m$^3$</td>
</tr>
<tr>
<td>$C(r)$</td>
<td>correlation integral</td>
</tr>
<tr>
<td>$D_H$</td>
<td>diffusion coefficient of the hydrogen ion, m$^2$/s</td>
</tr>
<tr>
<td>$D_S$</td>
<td>diffusion coefficient of the substrate, m$^2$/s</td>
</tr>
<tr>
<td>$d$</td>
<td>length of the ellipsoidal principal axis</td>
</tr>
<tr>
<td>$E$</td>
<td>enzyme papain</td>
</tr>
<tr>
<td>$E_e$</td>
<td>concentration of the enzyme, mol/m$^3$</td>
</tr>
<tr>
<td>$E_S$</td>
<td>enzyme-substrate complex</td>
</tr>
<tr>
<td>$E'_S$</td>
<td>acyl-enzyme complex</td>
</tr>
<tr>
<td>$H$</td>
<td>concentration of the hydrogen ion, mol/m$^3$</td>
</tr>
<tr>
<td>$K_s$</td>
<td>dissociation constant, mol/m$^3$</td>
</tr>
<tr>
<td>$K_w$</td>
<td>dissociation constant of water, mol$^2$/m$^6$</td>
</tr>
<tr>
<td>$k_2$</td>
<td>reaction rate, s$^{-1}$</td>
</tr>
<tr>
<td>$k_3$</td>
<td>reaction rate, s$^{-1}$</td>
</tr>
<tr>
<td>$L$</td>
<td>thickness of the membrane, m</td>
</tr>
<tr>
<td>$P_1$</td>
<td>alcohol produced by the splitting of the substrate</td>
</tr>
<tr>
<td>$P_2$</td>
<td>acid produced by the splitting of the substrate</td>
</tr>
<tr>
<td>$p$</td>
<td>embedded phase-space dimension</td>
</tr>
<tr>
<td>$r$</td>
<td>radius of the hypersphere</td>
</tr>
<tr>
<td>$S$</td>
<td>substrate</td>
</tr>
<tr>
<td>$U$</td>
<td>dimensionless concentration of the substrate</td>
</tr>
<tr>
<td>$V$</td>
<td>dimensionless $A$</td>
</tr>
<tr>
<td>$x$</td>
<td>distance from the reservoir, m</td>
</tr>
<tr>
<td>$z$</td>
<td>time trajectory</td>
</tr>
</tbody>
</table>
Greek letters

\[ \varepsilon = \text{amplitude} \]
\[ \eta = \text{dimensionless distance} \]
\[ \lambda = \text{one-dimensional Lyapunov exponent} \]
\[ \nu = \text{correlation dimension} \]
\[ \theta = \text{dimensionless time} \]
\[ \omega_f = \text{forced frequency, rad/s} \]
\[ \omega_n = \text{natural frequency, rad/s} \]

LITERATURE CITED


Figure 1. Typical power spectra of the aperiodic oscillations:
$\epsilon=0.9$ and $\omega_f/\omega_n=2.5$.

Figure 2. Logarithmic plot of the correlation integral corresponding to the aperiodic oscillations:
$\epsilon=0.9$ and $\omega_f/\omega_n=2.5$.

Figure 3. Variation of the correlation dimension as a function the embedded dimension:
$\epsilon=0.9$ and $\omega_f/\omega_n=2.5$.

Figure 4. Dependence of the estimated largest Lyapunov exponent on the number of data points for the aperiodic oscillations:
$\epsilon=0.9$ and $\omega_f/\omega_n=2.5$. 
Batch Extraction of Dilute Acids in a Hollow Fiber Module

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SUMMARY

One of the major problems in fermentation is the recovery of dilute products some of which inhibit the fermentation. Extractive fermentation has been proposed to overcome inhibitory effects by extraction of the products as they are formed. The extraction must provide for product removal without being toxic to the cells. 20 weight percent tri-n-octylphosphine oxide in kerosene was found acceptable from the toxicity standpoint for the production of propionic and acetic acids using Propionibacterium acidipropionici. In preliminary mass transfer studies it has been used to extract acetic and propionic acids from a 0.25 M aqueous solution by contacting the two phases in a hollow fiber module. The hollow fibers (Celgard X20 polypropylene fibers with a 400 micron internal diameter and pore dimensions of 0.065x0.19 (wxl) microns) are wetted by this extractant. Liquid velocities were varied on both the shell and tube sides to determine mass transfer design parameters accounting for the individual resistances to mass transfer. The solvent shell side velocities were varied from 0.75 to 9.05 cm/s while the aqueous tube side velocities were varied from 13.3 to 82.9 cm/s. Over these velocity ranges the organic film phase resistance for acetic acid decreased from 28.1 to 3.2 percent of the total resistance. The percentage resistance for the propionic acid decreased from 16.6 to 1.7 percent of the total resistance. However, the majority of the resistance was due to the membrane itself. The aqueous phase velocity had no significant effect in reducing the resistances in the system. The design parameters will be used to optimize the extraction of the acids from a fermentation broth.

INTRODUCTION

Conventional road deicers such as calcium chloride and sodium chloride cause corrosion to roads, bridges, cars, etc. These salts cause detrimental effects to the environment, destroying vegetation and polluting water streams. A possible alternative to calcium and sodium chloride is calcium magnesium acetate, CMA. CMA does not cause corrosion and is environmentally safe. The major hindrance to using CMA at present is the cost of producing the acetate portion of CMA from acetic acid. The acetic acid is produced from petroleum and natural gas which makes the cost of CMA at least ten to twenty times the cost of sodium chloride. With so many biological waste streams from wheys, woody biomass residues, municipal solid wastes, and sewage sludges, methods of
using these waste streams as a fermentation substrate for bacterium to produce acetic acid have been studied as a possible less expensive source of acetic acid. The acetic acid can then be used to produce CMA.

One of the major problems with using a fermentation system to produce organic acids is that the acids produced inhibit the further production of acids when they reach a certain concentration. This inhibition concentration for most of the acetic acid producing bacterium is less than 3 weight percent. Therefore, researchers in the Iowa State Food Technology Department are working on increasing the fermentor productivity by increasing the resistance of the \( E. \text{acidipropionici} \) to acid concentration. There are many studies of potential bacterium to be used for the production of acetic acid. Also, if it is possible, a system of acid recovery whereby the acids can be removed from the fermentation system while the fermentation process is on going is desirable. In this manner the inhibition effects of the acetic acid and propionic acid are reduced by continuous removal. As has been shown by several experimenters, the cell density and net acid production of a fermentation system with continuous removal of acid are significantly greater than that of a fermentation system without removal of the acids.

The second major problem of fermentation systems is the recovery of the dilute (approximately 1-3 weight percent) acetic and propionic acids from the fermentation broth. The recovery system must be enclosed to prevent contamination of the bacteria. However, it must continuously remove the desired acid products while leaving the nutrients for further acid production.

This paper takes a look at the second problem, acid recovery from dilute systems. As noted, a fermentation system with continuous acid removal is desired. One method of continuous removal is that of supported liquid membrane extraction. There have been many studies that show the effectiveness of using membranes for extraction purposes. Membrane systems have many advantages over distillation and extraction towers. Membrane systems are typically of a compact design and have large surface area to volume ratios. They also avoid the problems of flooding, loading, and channeling which occur in extraction and distillation columns.

There are two major problems with the use of membrane systems. The first problem is to find a solvent which has a good partition coefficient. The second problem is to find a solvent that is non toxic to the fermentation system.

There have been quite a few studies of partition coefficients for acetic acid and propionic acid with different solvents. The best solvents tended to be secondary and tertiary amines. Their partition coefficients ranged from 1.27 to 33.4 respectively for 50 volume percent Amberlite LA-1 in Chevron 25 and Adogen 283-D. However, many of these tertiary amines are toxic to fermentation systems. Therefore, solvent systems such as tri-n-octylphosphine oxide (TOPO) in kerosene have been used. The organic solvents themselves usually have a partition coefficient much less than one. However, the addition of a
tertiary amine or TOPO increases the distribution coefficient to above one. The partition coefficients at an initial acetic acid concentration of 0.0893M for kerosene versus 0.209M TOPO dissolved in kerosene is respectively < 0.001 and 1.143.

Here we report on the first phase of this work: batch extraction of dilute acetic and propionic acid using a hollow fiber module. The hollow fiber module is composed of a glass shell surrounding hollow fiber membranes. The best design parameters for the extraction of the acetic and propionic acid are desired. In a succeeding study, the parameters determined from this model system will be used to design an extractor to couple to the fermentation.

MATERIALS AND METHODS

The hollow fiber module\textsuperscript{21,22} is shown in Figure 1. The hollow fibers are Celgard X20 400 Microporous Membranes provided by Hoechst Celanese Corporation, Charlotte, NC. The microporous membrane properties are: a porosity of 40%; pore dimensions of 0.65 x 0.19 microns (w/l); an internal diameter of 400 microns; a wall thickness of 30 microns; an outside diameter of 460 microns; composed of polypropylene which is hydrophobic. The shell was made of glass by the Iowa State Glass Blowing Shop with dimensions of: an internal diameter of 8 millimeters; an outside diameter of 10 millimeters; a length of 7.5 inches (0.75 inches on either side for epoxy). There are a total of 52 fibers in the hollow fiber module.

The dilute acid samples were analyzed on an HPLC system composed of: a Model 2360 Gradient Programmer (Isco, Lincoln, Nebraska); a Model 2350 HPLC Pump (Isco); a Knauer Differential-Refractometer (Rainin, Woburn, Massachusetts); and an Aminex HPX-87H Column (Bio-Rad, Richmond, California). The data were stored and integrated using the Dionex Advanced Computer Interface (Dionex, Sunnyvale, California).

The solvents used in the toxicity tests were donated by several different sources: Alamine 304, Alamine 308, and Alamine 336 (Henkel Corporation, La Grange, Illinois); Adogen 283-D (Sherex Chemical Company, Dublin, Ohio); Amberlite LA-2 (Rohm and Haas, Philadelphia, Pennsylvania).

The glacial acetic acid, sulfuric acid, and kerosene came from Fisher Scientific (Fair Lawn, New Jersey). The TOPO (Tri-n-octylphosphine oxide) was from Sigma Chemical Company (St. Louis, Missouri).

The batch hollow fiber system was operated in several different modes. First, the solvent phase, 20 weight percent TOPO in kerosene, was on the tube side of the membrane while the aqueous acid phase, 0.25M acetic and 0.25M propionic acid, was on the shell side. The flow rate (velocity) of the aqueous phase was maintained at a high flow rate. The solvent phase flow rate was then varied for each succeeding experiment. Each of the individual flow rate experiments lasted one hour. The concentrations were measured initially and at ten minute intervals for one hour. Next, the solvent phase flow rate was
maintained at a high flow rate while the aqueous phase flow rate was varied from experiment to experiment. The experiments were repeated after switching the solvent phase from tube to shell side and the aqueous phase from shell to tube side. The concentrations of the acids in the aqueous phase were determined by HPLC analysis of the aqueous phase samples. The toxicity tests were performed at 32°C using *P. acidipropionici*. Screw top flasks with an inoculum of 5 ml of *P. acidipropionici* seed culture were incubated for 5 hours. After the initial five hours, 10 ml of the solvent was added to the fermentation broth (105 ml). Absorbance readings (Spectronic 21, Milton Roy Company, Rochester, New York) at a wave length of 540 nm were then done for the next 3 days. The absorbance was measured with a Spectronic 21.

**THEORY**

The objective of using the hollow fiber module is to transport the desired solute from one phase to another phase. The solute is first transferred convectively from the bulk aqueous phase to the hollow fiber membrane wall. Next, the solute diffuses through the organic phase wetting the membrane. The solute is then convectively transferred to the bulk organic phase. The actual transport of the acid within the organic phase is accomplished chiefly as the TOPO-acid complex.

The distribution coefficient of an aqueous-acetic acid-propionic acid-kerosene system is < 0.001.9 However, when TOPO is added to the kerosene the distribution coefficient will increase significantly. The TOPO complexes with the acids thereby increasing the distribution of the acids in the solvent phase. The new distribution coefficient is defined as:

\[
H = \frac{C_{\text{org}}\text{acid} + C_{\text{org}}\text{TOPO-acid}}{C_{\text{aq}}\text{acid} + C_{\text{aq}}\text{coo-}}
\]  

The solute mass transfer across the liquid films on both the aqueous and organic sides of the membrane as well as the membrane itself. All three have an associated mass transfer coefficient. The inverse of the mass transfer coefficient is the resistance to transport across the respective layer. The total resistance (the inverse of the overall mass transfer coefficient) to acid transport is the summation of the individual resistances:

\[
\frac{1}{K} = \frac{1}{k_{\text{aq}}} + \frac{1}{k_{\text{mem}}} + \frac{1}{H^*k_{\text{org}}}
\]  

Now that the three individual resistances have been defined, it is desired to determine the resistance that is dominating. From the literature, the mass transfer coefficient inside the fibers is a function of the fluid velocity to the 0.33 power23,24. Also, the shell side mass transfer coefficient is a function of its fluid velocity to the 1.0 power21. The membrane
itself is unaffected by the fluid velocities on either the shell or the tube side of the fibers. The membrane resistance is a function of the membrane properties:

$$k_{\text{mem}} = \frac{D \cdot \varepsilon \cdot H}{X \cdot \tau}$$  \hspace{1cm} (3)

The method for obtaining the individual resistances is by performing flow rate, fluid velocity, experiments. One of the phases is set at a high velocity and several experiments at different velocities are performed with the other phase. Next, the process is reversed. For each experiment the overall mass transfer coefficient can be determined from the slope of a plot of the natural log of the concentration difference between the phases versus time (equations from Dahuron22, aqueous phase tube side):

$$\ln \frac{\Delta C}{\Delta C_0} = -\frac{t}{V_{\text{aq}}} \left( \frac{1}{V_{\text{aq}}} + \frac{1}{Q_{\text{aq}}} \right) \left( 1 - \exp \left[ \frac{-4 \cdot K \cdot L}{d \cdot V_{\text{aq}}} \left( 1 + \frac{Q_{\text{aq}}}{H \cdot Q_{\text{org}}} \right) \right] \right)$$  \hspace{1cm} (4)

$$\ln \frac{\Delta C}{\Delta C_0} = \ln \frac{C_{\text{aq}} \left( 1 + \frac{V_{\text{aq}}}{H \cdot Q_{\text{org}}} \right)}{C_{\text{org}}} - \frac{C_{\text{aq}} \left( \frac{V_{\text{aq}}}{H \cdot Q_{\text{org}}} \right)}{H}$$  \hspace{1cm} (5)

These equations must be modified slightly if the aqueous phase flows on the shell side. Next, by using the velocity correlations for the individual mass transfer coefficients with the overall mass transfer coefficients, the individual resistances can be determined by plotting the total resistance versus the inverse of the velocity correlation:

$$\frac{1}{K} = \frac{1}{k_{\text{aq}} \cdot V_{\text{aq}}^{0.33}} + B$$  \hspace{1cm} (6)

$$\frac{1}{K} = \frac{1}{H \cdot k_{\text{org}} \cdot V_{\text{org}}} + C$$  \hspace{1cm} (7)

The intercept for the first equation on a plot of $K^{-1}$ versus $V_{\text{aq}}^{-0.33}$ represents the sum of the membrane and shell side film resistances. The first term in either of the above equations represents the respective film resistance for each phase. The intercept for the second equation represents the membrane plus tube side resistance. Therefore, by extrapolating the plots of the above equations to their intercepts the individual film and membrane resistances can be determined.
RESULTS AND DISCUSSION

Table 1 shows the results of the toxicity tests. All the solvent systems were toxic to the *P. acidipropionici* except for the TOPO/kerosene system. The toxicity is being defined as constant absorbance reading at 540 nm for three succeeding time measurements (5 hours between sample measurements). Table 1 shows the time that each solvent system became toxic.

Table 1

<table>
<thead>
<tr>
<th>SOLVENT</th>
<th>Growth Time</th>
<th>OD&lt;sub&gt;540&lt;/sub&gt;</th>
<th>Growth Cessation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control$</td>
<td>45 hrs</td>
<td>6.98</td>
<td>NT@</td>
</tr>
<tr>
<td>Alamine 304</td>
<td>45 hrs</td>
<td>3.77</td>
<td>35 hrs</td>
</tr>
<tr>
<td>Alamine 308</td>
<td>45 hrs</td>
<td>0.69</td>
<td>20 hrs</td>
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<tr>
<td>Alamine 336</td>
<td>45 hrs</td>
<td>0.74</td>
<td>20 hrs</td>
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<tr>
<td>Adogen 283-D</td>
<td>45 hrs</td>
<td>1.52</td>
<td>40 hrs</td>
</tr>
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<td>Amberlite LA-2</td>
<td>45 hrs</td>
<td>1.08</td>
<td>15 hrs</td>
</tr>
<tr>
<td>Control$</td>
<td>30 hrs</td>
<td>5.46</td>
<td>NT@</td>
</tr>
<tr>
<td>20 Vol % Adogen 283-D in Dodecane</td>
<td>30 hrs</td>
<td>0.38</td>
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<tr>
<td>20 Vol % Amberlite LA-2 in Decane</td>
<td>30 hrs</td>
<td>0.47</td>
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<td>20 Vol % Alamine 308 in Kerosene</td>
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<td>0.50</td>
<td>20 hrs</td>
</tr>
<tr>
<td>20 Vol % Alamine 336 in Kerosene</td>
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<td>1.32</td>
<td>5 hrs</td>
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</tr>
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<td>Alamine 304</td>
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<td>0.63, 0.81</td>
<td>&lt;5 days</td>
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<tr>
<td>Control$</td>
<td>48 hrs</td>
<td>3.66</td>
<td>NT@</td>
</tr>
<tr>
<td>20 Vol % TOPO in Kerosene</td>
<td>48 hrs</td>
<td>6.53</td>
<td>NT@</td>
</tr>
</tbody>
</table>

$= $ run concurrently with solvent flasks but without any solvent exposure
#= period of time for bacterial growth, length of toxicity test
*= optical density (average of two separate flasks)
+= length of time until bacteria had a constant OD
@= OD still increasing at the end of the Growth Time, non toxic

Table 2 shows the results of the equilibrium experiments for determining the distribution coefficients of the acetic and propionic acid as a function of concentration in the aqueous-TOPO/kerosene system. Table 2 also shows the distribution coefficients of the mixed acids in the aqueous-TOPO/kerosene system. The distribution coefficients for both the acetic and propionic acid decrease as the initial acid concentration increases. The reason that the distribution coefficients decrease is that at higher initial concentrations the TOPO becomes saturated. Therefore, as the initial concentration passes the TOPO saturation point the distribution coefficient decreases. Note also that the propionic acid partitions into the organic phase much better than the acetic acid.
The qualitative effects of flow rates on the overall mass transfer coefficient are as follows:

<table>
<thead>
<tr>
<th>TUBE</th>
<th>SHELL</th>
<th>VARY</th>
<th>EFFECT ON K OF</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. AQUEOUS</td>
<td>SOLVENT</td>
<td>AQUEOUS</td>
<td>INCREASED FLOW</td>
</tr>
<tr>
<td>2. AQUEOUS</td>
<td>SOLVENT</td>
<td>SOLVENT</td>
<td>NO EFFECT</td>
</tr>
<tr>
<td>3. SOLVENT</td>
<td>AQUEOUS</td>
<td>AQUEOUS</td>
<td>INCREASE</td>
</tr>
<tr>
<td>4. SOLVENT</td>
<td>AQUEOUS</td>
<td>SOLVENT</td>
<td>EMULSION</td>
</tr>
</tbody>
</table>

The results of these four cases show that the solvent phase velocity is the factor that increases the overall mass transfer coefficient. By increasing the solvent phase velocity the thickness of the organic phase boundary layer is reduced. The reduction of this boundary layer results in a decreased resistance for the solute to pass through. The results of the effects of the velocity on the individual resistances are shown in Table 3. Figures 2-3 show preliminary results of the total resistances as a function of flow velocities. The standard deviations of the data points for the acetic acid are < 1.9x10^3 s/cm and < 3.3x10^3 s/cm for Figures 2 and 3, respectively. The standard deviations of the data points for the propionic acid are < 0.3x10^3 s/cm and < 0.2x10^3 s/cm for Figures 2 and 3, respectively.

Table 2

<table>
<thead>
<tr>
<th>Acid</th>
<th>Concentration</th>
<th>H</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetic</td>
<td>0.025 M</td>
<td>1.98</td>
</tr>
<tr>
<td>Propionic</td>
<td>0.025 M</td>
<td>6.95</td>
</tr>
<tr>
<td>Acetic</td>
<td>0.25 M</td>
<td>1.50</td>
</tr>
<tr>
<td>Propionic</td>
<td>0.25 M</td>
<td>13.69</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Acid</th>
<th>Concentration</th>
<th>H</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetic</td>
<td>0.025 M</td>
<td>2.16</td>
</tr>
<tr>
<td>Propionic</td>
<td>0.025 M</td>
<td>15.58</td>
</tr>
<tr>
<td>Acetic</td>
<td>0.25 M</td>
<td>0.86</td>
</tr>
<tr>
<td>Propionic</td>
<td>0.25 M</td>
<td>3.92</td>
</tr>
</tbody>
</table>

CONCLUSION

This study determined that the dominating resistance for the hollow fiber membrane system was the membrane itself. Organic film resistance was still significant so some reduction in the total resistance in the system could be achieved by increasing the organic fluid velocity. The overall mass transfer coefficient increased from 9.09x10^-5 cm/s to 1.08x10^-4 cm/s and 2.68x10^-5 cm/s to 3.64x10^-5 cm/s, respectively, for the propionic and acetic acid for a constant shell velocity of 82.9 cm/s and solvent velocities of 0.75 to 9.05 cm/s. The aqueous fluid velocity had a negligible effect on the resistance of the solute to mass transfer. At higher organic fluid velocities the total resistance is due almost solely to the membrane itself.
$1/K$ VERSUS $V_{aq}^{-0.33}$

AQUEOUS TUBE SIDE--ORGANIC SHELL SIDE $V_{org}=9.05$ CM/S

FIGURE 2

$1/K$ VERSUS $V_{org}^{-1.0}$

AQUEOUS TUBE SIDE--ORGANIC SHELL SIDE $V_{aq}=82.9$ CM/S

FIGURE 3
The secondary and tertiary amines that were experimented with were toxic to the \textit{P. acidipropionici}. Even when the amines were diluted in an organic solvent they were toxic. At present the TOPO/kerosene system is being used.

\textbf{Table 3}

\begin{tabular}{|l|c|c|c|}
\hline
\textbf{Acid} & \textbf{R\textsubscript{org}} & \textbf{R\textsubscript{mem}} & \textbf{R\textsubscript{aq}} \\
\hline
Acetic & 28.1-3.2 & 70.9-96.2 & 1.0-0.6 \\
Propionic & 16.6-1.7 & 76.6-93.7 & 6.8-4.6 \\
\hline
\end{tabular}

Note: Results for aqueous phase tube side and organic phase shell side

\textbf{Acknowledgements}

This study was funded by the Iowa Department of Transportation and the Iowa Corn Promotion Board.

\textbf{References}


EVALUATION OF A NEW ELECTROPHORETIC DEVICE FOR PROTEIN PURIFICATION

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Summary

A new electrophoretic separation device for protein purification, which potentially eliminates the drawbacks of commercially available equipment, was constructed and evaluated. The central feature of this device is that electrophoresis is done at the isoelectric point (pI) of the protein to be purified. A solution containing proteins flows through a chamber bounded by two microporous membranes, and a voltage gradient is applied transverse to the flow. The protein to be purified tends to remain in the chamber, while other proteins not at their pI tend to leave. A feed solution containing hemoglobin and alcohol dehydrogenase (ADH) was tested to obtain optimal operating conditions, and experiments using several microporous membranes were conducted. Very favorable results were obtained with one of the membranes: 99.3% removal of ADH and 98.2% hemoglobin purity after 3 hours of operation. There was evidence of concentration polarization at the surface of the membrane, which could be minimized by increasing the flow rate and pore size and by proper membrane selection.

Introduction

It is generally recognized that the most powerful methods for the analysis of protein mixtures are electrophoretic. Isoelectric focusing and SDS gel electrophoresis have become standard methods for protein analysis. Some progress has been made in the development of electrophoretic methods for large scale proteins separations. The approaches that have been taken have been reviewed recently by Ivory (1) and include thin-film continuous electrophoresis, stress-stabilized electrophoresis, and continuous effluent recycle electrophoresis. The thin-film apparatus has problems with instability, and the stress-stabilized device is expensive and can not be used at very large scale. In a version of the continuous recycle instrument developed by Bier and coworkers (2), expensive ampholytes are required that must be removed from the product.

The idea for the research presented here came from work reported by Goldman and Baptist (3). Their technique is based on performing the electrophoresis at the isoelectric point (pI) of the protein to be purified. Their apparatus consisted of a polyacrylamide gel placed
between two electrodes and containing a slot for the feed sample. Although this apparatus is relatively simple, it can not be scaled up directly.

In the present paper, results are presented for an apparatus capable of being scaled up based on the basic concept of Goldman and Baptist.

Materials and Methods

A diagram of the electrophoretic device that was constructed is shown in Figure 1. The apparatus consists of five cylindrical chambers with electrodes at each end. All cylinders except the middle one were made of Plexiglas with 3.5" O.D. x 1/8" thickness. The middle chamber, as shown in Figure 2, was made of a 3 mm Plexiglas sheet with a 58 mm square separation area and two pieces of specially designed inlet and outlet distributors.

The feed solution, buffered at the pI of the protein to be purified, flowed through the middle chamber bounded on each side by a microporous membrane. These membranes are used to retain the preferred protein in the middle chamber. The membranes are preferably oriented in the vertical position. The feed solution, contained in a beaker immersed in an ice bath, was recirculated through the system by means of a peristaltic pump.

On each side of these membranes was a 2" chamber containing the same buffer. During the electrophoresis run, the "impurity" protein would migrate through the membrane into one of these chambers due to its charged nature. Stainless steel cooling coils with 2°C water recirculated inside were present to dissipate the Joule heat generated by the electrical field. A magnetic stirring bar was put in each chamber to accelerate the heat dissipation. The volume of the buffer chambers (293 ml each) is much larger than for the separation chamber (10 ml) to keep the pH and temperature of the system stable during the operation.

A pair of 1" wide electrode chambers were mounted on opposing ends of the apparatus. Electrodes were made of 25 cm long x 0.254 mm diameter platinum wire and soldered to plug-type connectors mounted on the outside for connection to the power supply. The buffer chambers and electrode compartments were separated by ion-permselective membranes (Ionics, Inc.), which do not allow free passage of fluid while readily allowing passage of electric current. The cathodic chamber contained dilute sodium hydroxide (0.1 M) and the anodic chamber contained dilute sulfuric acid (0.1 M). By means of the electrodes, a constant voltage was applied. There was one vent hole on the top of each Plexiglas tube to eliminate bubble accumulation.

Six different membranes were tested in this study: two Fisher Magna Nylon 66 membranes with pore sizes of 5 and 20 μm; FSM (polyvinylidenefluoride) 2 μm and GRM (polysulfone) 2 μm membranes from DDS; Durapore (polyvinylidene difluoride) 5 μm membranes from Millipore; and Versapor (acrylic copolymer) 3 μm membranes from Gelman.

The feed was a binary protein solution that consisted of human hemoglobin (pI 7.4) and alcohol dehydrogenase (ADH, pI 5.4) from yeast. Human hemoglobin (2x crystallized and
lyophilized) was obtained from Sigma Chemical Co. ADH (2x crystallized and lyophilized) was obtained from Worthington Biochemical Corp. The feed was prepared using HEPES buffer at a concentration of approximately 0.5 g/l for each protein.

The hemoglobin concentration was determined by measuring the absorbance at 407 nm. The ADH concentration was determined by measuring the change in absorbance at 340 nm resulting from the reduction of NAD in the presence of ethanol (4). Total protein was measured using the BCA assay (Pierce).

Results and Discussion

The purpose of the electro-separation experiments was to determine the effect of the operating parameters and membrane type on the performance of the free-flow electrophoretic device. The first experiments were done to determine the buffer concentration at which to operate. Several buffer concentrations were tested with no proteins in the feed at pH 7.4 (the isoelectric point of hemoglobin), 30 V, flow rate of 5 ml/min, and Fisher Magna 5 μm membranes: it was found that the pH became unstable at less than 0.1 M buffer concentration. Thus, the remaining experiments were done at 0.1 M buffer concentration.

In the previous experiments it was noted that the applied field of 30 V gave slightly excessive foaming and bubble accumulation. Lowering the voltage to 20 V eliminated these problems, and thus this voltage was used for the remaining experiments.

Another set of experiments was carried out to determine the flow rate at which Joule heating becomes a problem. The temperature rise across the middle chamber was measured at 20 V for several flow rates below 10 ml/min. The temperature rise was the same at 5 and 10 ml/min (6.4°C) but rose to 8.0°C as the flow rate fell to 0.5 ml/min. It was concluded that the system could be operated at as low as 0.5 ml/min without much of a problem of protein degradation.

In the next set of experiments the effect of flow rate was determined for one type of membranes (Fisher Magna 20 μm). The results are shown in Figure 3 and Table 1. C and C₀ are concentrations of ADH at time t and time 0, respectively, and C/C₀ represents the fraction of ADH remaining in the feed. By far the best results were obtained at the highest flow rate, 20 ml/min. From these results it seems very probable that increasing the flow rate reduced the size of a layer of insoluble protein that had built up at the surface of the membrane, a phenomenon known as concentration polarization. Concentration polarization has been observed in crossflow microfiltration of lysed cells by Kroner et al. (5) and by Le and Atkinson (6). These results can not be due to a significant depletion of ADH as the feed moves through the chamber at the lower flow rates, since calculations indicate that at time 0 only 16% of the ADH entering the chamber leaves the system through the membranes at 2.5 ml/min, compared to 8% leaving at 20 ml/min.

The effect of pore size was studied at the 20 ml/min rate for the Fisher Magna membranes, and the results are shown in Figure 4 and Table 2. The fraction of ADH
remaining was significantly lower for the 20 \( \mu \text{m} \) membrane compared to the 5 \( \mu \text{m} \) membrane until 3 hours of operation, when the fractions of ADH remaining were nearly equal. Concentration polarization is again a probable reason for the differences in results since smaller pores would be more easily blocked by a concentration polarization gel layer.

A final set of experiments was performed to determine the effect of membrane type with membranes of similar pore size. The results are shown in Figure 5 and Table 3. The Millipore Durapore membranes with the 5 \( \mu \text{m} \) pore size were found to be the best compared to the four other membranes: the fraction of ADH remaining was lowest throughout the run, and the hemoglobin purity after 3 hours was the highest. The only negative aspect of the results with the Durapore membranes was the relatively large loss of hemoglobin (36%). It is notable that only the difference in material properties of the membrane has such a significant effect on the purification process. Of the membranes the polysulfone membrane by DDS (GRM) gave by far the poorest results. It is probable that different degrees of concentration polarization can be induced, depending on the material used for the membrane.

Conclusions

This electrophoretic device can be operated to give good purification of hemoglobin that originally contained ADH in as little as 3 hours. For all of the experiments, Joule heating was not a problem. There was evidence of concentration polarization at the surface of the membrane in the separation chamber, which could be minimized by increasing the flow rate and pore size and by proper membrane selection. Future work will be aimed at redesigning the device to enable higher flow rates to be used.

Acknowledgements

We are appreciative of support from the University of Oklahoma College of Engineering. We thank Professor Lloyd Lee for helpful discussions.

References


Table 1. Percentage of ADH and hemoglobin removed and final hemoglobin purity for the same membranes (Fisher 20 μm) for different flow rates (20 V, 0.1 M HEPES).

<table>
<thead>
<tr>
<th>Flow Rates (ml/min.)</th>
<th>% ADH removed</th>
<th>% Hem. removed</th>
<th>% of final Hem. purity</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.5</td>
<td>49.5</td>
<td>31.3</td>
<td>65.3 (for 2 hr)</td>
</tr>
<tr>
<td>10</td>
<td>93.5</td>
<td>28.6</td>
<td>94.0 (for 3 hr)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>77.1 (for 2 hr)</td>
</tr>
<tr>
<td>20</td>
<td>98.8</td>
<td>39.7</td>
<td>98.1 (for 3 hr)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>91.5 (for 2 hr)</td>
</tr>
</tbody>
</table>

Table 2. Percentage of ADH and hemoglobin removed and final hemoglobin purity for the same type membranes (Fisher Magna) with different pore sizes (20 V, 20 ml/min, 0.1 HEPES).

<table>
<thead>
<tr>
<th>Pore Sizes (μm)</th>
<th>% ADH removed</th>
<th>% Hem. removed</th>
<th>% of final Hem. purity</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>97.9</td>
<td>27.1</td>
<td>95.8</td>
</tr>
<tr>
<td>20</td>
<td>98.8</td>
<td>39.7</td>
<td>98.1</td>
</tr>
</tbody>
</table>
Table 3. Percentage of ADH and hemoglobin removed and final hemoglobin purity for different membranes with similar pore sizes (20 V, 20 ml/min, 0.1 M HEPES).

<table>
<thead>
<tr>
<th>Membrane</th>
<th>% ADH removed</th>
<th>% Hem. removed</th>
<th>% of final Hem. purity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fisher 5 μm</td>
<td>97.9</td>
<td>27.1</td>
<td>95.8</td>
</tr>
<tr>
<td>Millipore 5 μm</td>
<td>99.25</td>
<td>36.4</td>
<td>98.2</td>
</tr>
<tr>
<td>Gelman 3 μm</td>
<td>92.27</td>
<td>33.2</td>
<td>86.0</td>
</tr>
<tr>
<td>DDS FSM 2 μm</td>
<td>84.16</td>
<td>20.1</td>
<td>79.3</td>
</tr>
<tr>
<td>DDS GRM 2 μm</td>
<td>33.65</td>
<td>18.1</td>
<td>45.4</td>
</tr>
</tbody>
</table>
Figure 1. Diagram of the device.

A -- Electrode Chambers  
B -- Buffer Chambers  
C -- Separation Chamber  
D -- Electrodes  
E -- Ion-permselective Membranes  
F -- Separation Membranes  
G -- Cooling Coils  
H -- Stirrers

Figure 2. The separation chamber.
Figure 3. \( \frac{C}{C_0} \) (ADH) vs. time for different flow rates (Fisher Magna 20 μm, 20 V, 0.1 M HEPES).

Figure 4. \( \frac{C}{C_0} \) (ADH) vs. time for Fisher Magna membranes with different pore sizes (20 V, 20 ml/min, 0.1 M HEPES).
Figure 5. C/C_0 (ADH) vs. time for different membranes (20 V, 20 ml/min, 0.1 M HEPES).
CROSSFLOW MICROFILTRATION AND MEMBRANE FOULING FOR YEAST CELL SUSPENSIONS

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Department of Chemical Engineering University of Colorado Boulder, CO 80309-424

Abstract

A common technique for separating particles which are approximately one-tenth of a micrometer to a few micrometers in size from a suspending fluid is crossflow microfiltration. In this process the suspension to be filtered is forced tangentially through a rectangular or tubular channel with microporous walls. A decline in the flux with time due to membrane fouling is observed.

The characterization of the filter (ceramic) and the suspension (yeast cells in deionized water) system is based on two measured parameters: the specific cake resistance and the membrane resistance. Microfiltration experiments have been conducted under different operating conditions of transmembrane pressure, bulk suspension flowrate and particle concentration. High suspension flow rates, low particle concentrations and moderate pressures favor higher permeate fluxes.

A mathematical model incorporating the two measured parameters has been used to predict the steady state permeate flux. The steady-state theoretical flux was found to be in good agreement with the experimentally observed steady-state permeate flux.

1 INTRODUCTION

Crossflow microfiltration is a solid-liquid separation process in which the particles to be separated lie in the range from 0.1μm to 10 μm. This process is widely used in the biotechnology industries for microbial cell separations and high molecular weight protein purification. In this process the suspension to be filtered is made to flow axially under pressure through a tube or a channel with microporous walls. Typical pressures for microfiltration applications range from 5 psi to 40 psi. As the filtration is carried out, the fluid and smaller particles pass through the filter, and a concentration polarization layer of the rejected larger particles forms on the higher pressure side of the filter. This polarization layer causes a decline in the permeate flux with time. Unlike classical filtration (deadend filtration), wherein the permeate flux asymptotes to zero with time, crossflow predicts an almost steady state for the flux. At steady state, particles that are carried towards the filter are dragged along axially by the
shear stress exerted by bulk flow of the suspension. Hence this operation can be run for long intervals of time.

The steady-state flux that is observed in microfiltration operations is very small compared to the initial flux, i.e. there is a large flux decline. This is due to the formation of a stagnant cake layer formed on the inside of the filter. Early attempts to predict the steady state flux on the basis of the concentration polarization model for ultrafiltration gave values that were one or two orders of magnitude lower than the experimental ones (Blatt et al., 1970; Porter, 1972 a,b; Henry, 1972). This is because the values of the Brownian back-diffusion (mechanism used to explain the steady state reached) velocities for the particles in ultrafiltration are much higher than the ones for micron sized particles. Convective models developed by Leonard and Vassilieff (1984) and Davis and Birdsell (1987) treat the particle layer as a Newtonian fluid after the hydrodynamic shear exerted by the bulk flow causes the layer to flow tangentially. The effective viscosity depends on the concentration in the layer, which is not predicted in these models.

Davis and Leighton (1987) have used back-diffusion of particles away from the wall as a basis to explain the steady state reached, but instead of Brownian diffusion a new term "shear induced shear diffusion" has been introduced. Under the effect of a shear, the particles interact with those in the vicinity resulting in a net migration of particles from a region of high concentration to a low concentration. This process has been termed as "shear induced hydrodynamic diffusion". The order-of-magnitude of the shear-induced diffusivities as shown by Eckstein et al. (1977) are two to three times higher than the Brownian diffusivities. This explains the discrepancy observed in theoretical and experimental steady-state fluxes when Brownian diffusion was used to explain the steady state reached in crossflow microfiltration.

Romero and Davis (1988) have used a global model to predict the steady-state flux for crossflow microfiltration process based on hydrodynamic particle diffusion. In the present paper, the results of crossflow microfiltration experiments on a yeast-deionized water system are presented. Experiments have been conducted at different transmembrane pressures, bulk suspension flow rates and feed particle concentrations. The steady-state fluxes predicted by the global model of Romero and Davis are in good agreement with the experimentally observed steady-state fluxes.

# MATERIALS AND METHODS

The crossflow microfilter is made of Ceraflo® marketed by Norton Company, MA. The average pore size of the filter used is 0.2 μm. A tube bundle consisting of six to eight tubes was obtained. Each tube was used as a separate filter. The dimensions of one of the tubes are 0.24 cm inside diameter, 0.28 cm outside diameter and 28 cm length. Fleischmann’s dry yeast suspended in deionized water was the suspension filtered. The deionized water (resistivity of 18.2 ohm-cm) was prepared using the RO Pure and the NANO Pure filtering systems marketed by Barnstead Company.

The yeast suspension was forced into the filter using a Masterflex® peristaltic pump. The retentate coming out of the exit of the filter was recycled into the feed reservoir. Permeate was collected in a vessel kept on an electronic microbalance. The microbalance was directly connected to a computer that could receive and store the weight at regular intervals of time.
Pressure gauges were on either side of the filter. Copper and silicone tubing and brass connectors were used to complete the connection.

A single experiment consisted of passing deionized water through the filter for 60 minutes, followed by passing yeast suspension through the filter for another 60 minutes. Between experiments the filter was cleaned by backflushing (a process in which fluid is forced through the filter from outside to inside, resulting in lifting off of the cake layer) and backwashing (a process that follows backflushing and washes the lifted cake layer away) with deionized water. The filter was also mechanically cleaned using a plastic brush.

Crossflow microfiltration experiments were repeated on the filter under different operating conditions of varied transmembrane pressure, bulk suspension flow rate and feed particle concentration. Keeping two of the conditions constant, the third was varied over a wide range.

3 MODELING AND THEORETICAL ASPECTS

3.1 Steady-state theory

Consider a porous surface along which flows a Newtonian fluid containing neutrally buoyant particles. The positive \( y \) direction depicts the high pressure side of the filter and the bulk suspension flows in the positive \( z \) direction. A particle layer is formed on the filter with thickness \( \delta \). The pure fluid comes out of the filter with a velocity \( v_w(z) \). The pressure drop across the particle layer and the filter is \( \Delta P \). Our aim is to calculate \( v_w(z) \) and \( \delta(z) \) in terms of known quantities such as bulk suspension velocity, bulk concentration, transmembrane pressure and the hydraulic resistances of the membrane and the particle layer. The following theory applies to laminar flows only.

Darcy's law gives the expression for permeate flux (volume filtered per time per membrane area) at a given distance \( z \) from the filter entrance:

\[
J(z) = \frac{\Delta P}{\mu_o(R_m + R_c)},
\]

where \( \mu_o \) is the pure fluid viscosity, \( R_m \) is the membrane resistance and \( R_c \) is the cake resistance. For flat cakes the cake resistance is proportional to the particle layer thickness:

\[
R_c = \hat{R}_c \delta,
\]

where \( \hat{R}_c \) is the resistance per unit depth. For cylindrical filters the expression is modified to accommodate the decrease in the cake area with increase in \( y \):

\[
R_c = \hat{R}_c H_o \ln \left( \frac{H_o}{(H_o - \delta)} \right),
\]

where \( H_o \) is the inside radius of the clean cylindrical filter.

A polarization layer forms above the stagnant cake layer, and is assumed to offer no hydraulic resistance. At steady state, the rate of the particles in the polarization layer transported downstream must equal the rate at which particles enter the polarization layer.
in the $y$ direction everywhere upstream of that location. This implies that the cake thickness continuously increases with $z$.

The steady-state mass balance on particles in the concentration polarization layer gives (Romero and Davis, 1988):

\[ \int_{y_0}^{y + \delta} u(c - c_b)dy = \int_0^z v_w c_b dx, \]  

(3)

where $c_b$ is the particle concentration in the bulk suspension, $u$ is the velocity in the $z$ direction and $y$ is the distance measured from the membrane. For rectangular channels $v_w = J$, but for cylindrical tubes $v_w = J H_o / (H_o - \delta_c)$.

In typical crossflow microfiltration operations only a small fraction of the fluid is filtered in a single pass. A constant bulk flow rate with $z$ can be assumed. The shear stress exerted on the wall is given from Poiseuille's law:

\[ \tau_w = 3 \mu_b Q / (2W(H_o - \delta_c)^2), \]  

slit, (4.a)

\[ \tau_w = 4 \mu_b Q / (\pi(H_o - \delta_c)^3), \]  

tube, (4.b)

where $W$ is the width of the rectangular channel, $H_o$ the channel half-height or the tube radius, $Q$ is the bulk suspension flow rate and $\mu_b$ is the viscosity of the bulk suspension.

From the differential mass balance on a small element within the layer assuming no axial convection into the polarization layer (dilute suspension), we get (Romero and Davis, 1988):

\[ v_w c + D \frac{dc}{dy} = 0, \]  

(5)

where $D$ is the hydrodynamic diffusion coefficient.

The wall shear stress for thin polarization layers in tubes or channels is proportional to the shear rate, assuming that the suspension may be treated as an effective Newtonian fluid with a concentration-dependant viscosity, $\mu(c)$:

\[ \mu \frac{du}{dy} = \tau_w. \]  

(6)

After integrating this expression, changing the variable of integration to $c$ with equation (5) and substituting it in equation (3), we get (Davis et al., 1991):

\[ \frac{\tau_w}{v_w} \int_{c_b}^{c_e} \int_{c_b}^c \frac{D(c')dc'}{c' \mu(c')} \frac{(c - c_b)D(c)dc}{c} = \int_0^z v_w(x')c_b dx', \]  

(7)

where $c_b$ is the concentration of the particles in the bulk suspension and $c_b$ is the concentration of the particles at the edge of the stagnant cake layer. This expression does not apply for a region near the filter entrance where the shear prevents the formation of a particle layer, since the axial convection cannot be neglected there. This region $x_{cr}$ is determined from equation (7), where $v_w = J_m$ and $\tau_w = \tau_{wo}$:

\[ x_{cr} = \frac{\tau_{wo} D_o I_2}{J_m^3 \mu_b}, \]  

(8)
where \( J_m \) is the flux when there is no cake resistance and the dimensionless double integral \( I_2 \) is defined as:

\[
I_2 = \frac{(\mu_b/\mu_0)^3}{c_0} \int_{c_0}^{c} \int_{c}^{\infty} \frac{\tilde{D}(c') dc'}{c'} \frac{(c - c_0) \tilde{D}(c) dc}{c},
\]

and \( \tilde{\mu}(c) = \mu/\mu_0 \) and \( \tilde{D}(c) = \tau_\omega \tilde{D}/\tau_\omega D_\omega \) are dimensionless viscosity and diffusivity functions. \( D_\omega \) is the characteristic diffusivity of the particles and is given by \( D_\omega = a^2 \tau_\omega/\mu_b \) for shear-induced diffusion.

Equations (1), (2), (4) and (7) can be solved numerically to get steady-state profiles of permeate flux and stagnant cake layer thickness with filter length. The empirical corelations used for the dimensionless viscosity and the diffusion coefficient are given by Davis and Leighton (1987).

### 3.2 Determination of parameters

Two parameters that need to be determined from the yeast flux decline data are the membrane resistance \( (R_m) \) and the specific cake resistance \( (R_c) \). When a yeast suspension filtration was started, there was observed a rapid flux decline followed by a gradual flux decline. A complete theory of transient flux decline is given by Romero and Davis (1990), but it was found that the rapid flux decline observed at short times can be analyzed using deadend filtration theory. This can be explained by the similarity in deadend and crossflow filtration as the thin cake builds up quickly. The flux decline for deadend filtration is given by:

\[
J(t) = J_m \left( 1 + 2\kappa(t - t_o) \right)^{-1/2},
\]

where \( \kappa = R_c J_m \phi_b/R_m(\phi_b - \phi_c) \), \( t_o \) is the time at the start of the filtration, \( \phi_b \) is the volume fraction of the particles in the bulk suspension and \( \phi_c \) is the volume fraction of the particles in the stagnant cake layer. For simplicity, it is assumed here that \( R_m \) and \( J_m \) are constant.

A plot of \( J_m^2/J^2 - 1 \) versus \( t \) for short times is expected to yield a straight line with slope of \( 2\kappa \) and a \( t \)-intercept of \( t_o \). The membrane resistance can be calculated using Darcy's law for the initial flux observed (flux just prior to the start of yeast filtration).

### 4 RESULTS AND DISCUSSION

#### 4.1 Experimental results and parameter estimation

A typical flux decline curve is shown in Figure 1 for a transmembrane pressure of 35 psi, feed concentration of 0.01 g-dry/cm³ and a feed flow rate of 2.5 ml/sec. The temperature was 24.5°C. A flux decline was also observed for the water part, which was most likely due to the trace ions adhering to the low pressure side of the pore thus decreasing the flow rate (Errede, 1984). The initial flux for the yeast suspension is \( 3.8 \times 10^{-3} \) cm/sec and steady state flux is \( 0.80 \times 10^{-3} \). The theoretical curve for deadend filtration using equation (10) found from the transient drop in flux is also shown in Figure 1. As seen from Table 1, experiments were done varying pressures from 10 psi to 35 psi, flow rates from 0.5 ml/sec to 2.5 ml/sec and feed volume fraction from 0.003 to 0.06. Table 2 gives observations and
calculations from the experiments. The membrane resistance remained almost constant, ranging between $4.5-5.5 \times 10^{10}$ cm$^{-1}$. The specific cake resistance did not vary much over the pressure range: varying from $3.7-4.7 \times 10^{12}$ cm$^{-2}$. The steady state flux increased with increasing transmembrane pressures because the higher pressures force more fluid through the cake and membrane. The steady state flux increased as the bulk suspension flow rate was increased. This is because the shear stress is higher for higher flow rates, hence maintaining the back diffusion rate high and the stagnant cake layer small. As the feed particle concentration was increased, the steady state flux decreased. This is because as the bulk suspension concentration of the particles increases, the concentration difference that drives the hydrodynamic back-diffusion decreases and the stagnant cake thickness increases. It was found that at higher feed concentrations the flux rapidly declined to the steady state value due to the rapid build-up of the stagnant cake layer.

4.2 Comparison of the steady-state flux with theory

As seen from Figure 2, the predicted steady-state flux rises with an increase in the transmembrane pressure. The measured flux does not increase as much as predicted. This may be due to cake compaction at higher pressures. Figure 3 shows the variation of the steady-state flux with the feed particle concentration (volume fraction of the particles in the feed). The experimental observations are in good agreement with predicted values. At lower concentrations, the higher experimental value is because the flux did not reach a complete steady state in one hour. At higher concentrations the difference in the experimental and the predicted values is likely because in the calculation of the parameter $I_2$, it has been assumed to be inversely proportional to $c_0$, which from equation (9) can be seen to be valid only for dilute suspensions. From Figure 4, the steady-state flux is higher at higher suspension flow rates. Data from the experiments are in reasonable agreement with the theoretical curve.

5 CONCLUSIONS

The parameters involved in the model can be calculated by performing one or more microfiltration experiments on the target suspension at the concentration of feed. From the value of starting flux for the yeast suspension, the membrane resistance, $R_m$, can be calculated using Darcy's law. The transient fast drop in flux enables one to find the specific cake resistance, $R_c$. Using these parameters, the model then can predict the steady-state flux at different operating conditions of transmembrane pressure and bulk suspension flow rates.

Acknowledgements

This research was supported by the Center for Separations Using Thin Films at the University of Colorado, Boulder. The author would like to thank Dawn Downey and the University of Colorado's Undergraduate Research Opportunities Program for help in the experiments.
REFERENCES


Figure 1: Permeate flux decline for yeast suspension with $\phi_b=0.03$, $\Delta P=35$ psi and $Q=2.5$ ml/sec. The symbols represent the measured flux and the solid line is a fit from equation (10).

Figure 2: Variation of the steady-state flux with transmembrane pressure. The symbols represent measured fluxes and the solid line is the flux predicted by the model.
Figure 3: Variation of the steady-state flux with feed particle volume fraction. The symbols represent measured fluxes and the solid line is the flux predicted by the model.

Figure 4: Variation of the steady-state flux with shear rate at the wall. The symbols represent measured fluxes and the solid line is the flux predicted by the model.
### EXPERIMENTAL CONDITIONS

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<th>( \mu ) (cP)</th>
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Table 1: Experimental conditions for the experiments carried on a ceramic tubular filter with pore size 0.02 \( \mu m \).

### EXPERIMENTAL RESULTS FOR YEAST PART

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Table 2: Calculated initial flux, Steady-state flux, membrane resistance and specific cake resistance for experiments carried on a ceramic tubular filter with pore size 0.02 \( \mu m \).
The products of biotechnological processes are usually found in complex mixtures of rather dilute solutions and must be concentrated and purified in order to be used.

A new protein purification technique has emerged as a result of recombinant DNA technology developed during the 1980’s. In most cases, this is accomplished by adding DNA encoding an additional polypeptide (tail) to either the 5’ or 3’ end of the gene of interest. The expression of these gene fusions results in a protein fusion which can be purified using the properties of the attached tail. This technique has been used to facilitate affinity, ion-exchange, and hydrophobic separations (Sassenfeld, 1990).

Our research group proposes the use of recombinant DNA technology to create a fusion protein that can be recovered using starch-affinity interactions. The model fusion enzyme consists of β-galactosidase and the maltose binding protein.

The maltose binding protein (MBP) is a periplasmic protein of E. coli involved in the transport of maltose and maltodextrins across the bacterial envelope (Kellermann and Szmeleman, 1974).

Guan et al. (1988) developed the vectors producing MBP fusions to be purified by cross-linked amylose but did not address recovery and purification yields nor did they optimized the process of purification.

In the studies performed in our laboratory, affinity chromatography was used to understand better the interactions present in enzyme-starch complexes. In our studies we were interested in estimating the dissociation constant of the fusion enzyme-starch complex, and in determining the possibility of using starch for purification and recovery.

Kasai and Ishii (1975) proposed frontal analysis as a method for quantitative analysis of affinity chromatography. The dissociation constant $K_d$ is defined as the ratio of the free enzyme over the bound enzyme. Assuming that $C_0$ (initial protein concentration) is negligible compared to $K_d$, the dissociation
constant can be determined by using equation 1 (Kasai and Ishii, 1978):

\[ K_d = \frac{B_c}{(V_m - V_0)} \]  

where \( B_c \) is the total amount of the immobilized ligand, \( V_m \) is elution volume if interactions between the active site of the protein and the immobilized affinity ligand are allowed, and \( V_0 \) is the elution volume if no interactions are allowed.

The clarification, enrichment and high resolution steps can be combined in one if a specific and not expensive adsorbent is used. The objective of this work is to evaluate native starch for the recovery and purification of recombinant enzymes carrying a starch affinity domain. The direct recovery technique could be applied to affinity adsorption. If the interactions between starch and the starch binding domain are found to be too strong then starch could be used as carrier in an immobilized enzyme system.

MATERIALS AND METHODS

Enzyme Purification

The MBP-\( \beta \)-gal-fusion enzyme was purified using a modification of the procedures from Guan et al. (1988) and Steers et al. (1971). The fermentation part was performed following the recommended procedure for the fusion enzyme. The purification protocol followed the steps for purification of \( \beta \)-galactosidase reported by Steers et al. (1971).

Analytical Techniques

Total Protein and Enzyme Activity Assays

Protein concentration was determined using Bio-Rad Protein Assay (Bio Rad) based on Bradford's procedure (Bradford, 1976), with bovine serum albumin as a standard. The enzyme activity was determined by monitoring the release of o-nitrophenol from o-nitrophenyl-\( \beta \)-D-galactoside (ONPG) (Sigma) at 420 nm. One unit of \( \beta \)-galactosidase is defined as one nanomole of o-nitrophenol formed at pH 7.0 and 28 °C in one minute. The initial rate was estimated by linear regression of the change in absorbance as a function of time during the first three minutes of the reaction.

SDS-Polyacrylamide Gel Electrophoresis

The SDS PAGE was performed based on the Laemmli method (Laemmli, 1970). The gel consisted of a separating gel of 7.5% polyacrylamide at pH 8.8, and a stacking gel of 4.0% at pH 6.8.
as described for the Mini-Protean II Dual Slab Cell (Bio-Rad).

Western Blotting

Western blot of each gel was performed. The protein from the gel was transferred to a nitrocellulose membrane by electrophoresis. After the electroblotting, 10% milk diluent solution was used as blocking agent. The nitrocellulose membrane was then incubated with maltose binding protein antibody (New England Biolabs, Beverly, MA). The next day the excess antibody was removed, and the nitrocellulose membrane was incubated with protein A or Goat anti Rabbit IgG gold (Bio-Rad), depending on availability, until the bands were visible. The membrane was then washed, and it was dried at room temperature.

Column Chromatography

Purified protein was used in all the chromatography experiments. The protein solution concentration used was approximately 200 μg/mL, with an injection volume of 0.5 mL. All the experiments were performed at 4 °C with an ascending flow of 0.12 mL/minute. The proteins under study were pure bovine serum albumin, BSA (Bio-Rad), grade IX β-galactosidase (EC 3.21.23) from Sigma, and purified MBP-β-gal-fusion. Cross-linked amylose (Bio-Rad), raw potato starch, and Sephadex G-25 (Sigma) were used as resins.

The percent of the fusion with starch binding capacity was defined as the ratio of the protein bound to the total protein. The protein recovered was determined from the ratio of specifically eluted protein to total protein bound.

Frontal Analysis

Frontal analysis was performed with cross-linked amylose and raw potato starch. The enzyme concentration for frontal analysis was approximately 10 μg/mL. This solution was continuously loaded onto the columns with a flow rate of 0.12 mL/minute until the UV detector showed a constant enzyme concentration exiting from the column.

To determine Vₘ, the enzyme solution was prepared in the same column buffer as before. To determine V₀, the enzyme solution was prepared using the elution buffer, which contained 10 mM maltose. The amount of bound enzyme was determined from the difference of these elution volumes.

RESULTS

Affinity Chromatography

Affinity chromatography on cross-linked amylose and starch columns was used to obtain a better understanding of the interactions between the fusion enzyme and starch. Step-wise elution was used to determine the amount of fusion protein that
was specifically adsorbed to cross-linked amylose and/or starch. The same experiments were used to obtain the amount of recovery upon elution with 0.01 M maltose. In all the experiments elution was performed with the elution buffer containing 0.01 M maltose for consistency between experiments.

Figure 1 shows the chromatographic pattern of β-galactosidase and MBP-β-gal-fusion on cross-linked amylose. β-Galactosidase was used as a control to assure that maltose affects the elution of the maltose binding domain alone. About 70% of the total β-galactosidase did not bind to cross-linked amylose, while 30% of it could not be eluted with maltose. The fusion enzyme on cross-linked amylose showed 21% of inactivity towards cross-linked amylose. This amount that did not bind to the cross-linked column is probably a fusion protein that is lacking the maltose binding activity or its MBP domain is not properly folded to interact with cross-linked amylose.

Figure 1 confirms that there are specific interactions of MBP-β-gal-fusion with cross-linked amylose since 57% of the total enzyme can be eluted with 0.01 M maltose buffer in a sharp peak. The fusion enzyme had a 80% of binding protein, 70% of which could be recovered with 0.01 M maltose.

Because the goal of these studies is to use raw starch to purify fusion proteins, a raw potato starch column was prepared. The same experiments performed using cross-linked amylose were performed under the same conditions with raw starch.

The results of these experiments (Figure 2) also show that there is specific interaction of the MBP-β-gal-fusion enzyme and raw starch. Less enzyme (33%) was specifically eluted with maltose than in the case of cross-linked amylose column, but the same amount of fusion enzyme was bound. Figure 2 shows the chromatographic patterns for both β-galactosidase and MBP-β-gal-fusion on the raw starch column.

From the chromatographic patterns it was noted that not all the enzyme bound was eluted as a peak with the maltose buffer. In the raw starch system β-galactosidase was retained more than twice the amount that was retained on cross-linked amylose. The recovery of the fusion enzyme is lower, 41% from the raw starch column compared to 71% for cross-linked amylose. The fusion enzyme and β-galactosidase were slowly desorbed from the raw starch during the extensive washing with column buffer.

To understand better what was the cause for incomplete recovery of the fusion protein the same experiments were conducted using Sephadex G-25. Sephadex G-25 is a bead formed gel prepared by cross-linking of dextran with epichlorohydrin. It has α(1-6) glucosidic linkages, so there should not be any specific interactions between the MBP domain and the packaging material (Kellerman and Ferenci, 1982).

The chromatographic patterns for β-galactosidase and MBP-β-gal-fusion on Sephadex G-25 are presented in Figures 3 and 4, respectively. These experiments also showed retention of both enzymes on the Sephadex G-25 column. β-Galactosidase adsorbed more to Sephadex G-25 (41%) compared to cross-linked amylose.

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Approximately 55% of the MBP-β-gal-fusion adsorbed to Sephadex G-25, compared to 79% for cross-linked amylose column.

All the experiments described above were performed at the ionic strength of 0.5 M. This condition was recommended by New England Biolabs for purification of cytoplasmic enzyme fusions on cross-linked amylose. Since the enzymes were retained on the Sephadex G-25 column longer than expected, it was suspected that nonspecific interactions were the cause of this retention that was enhanced by high ionic strength. The chromatography experiments on Sephadex G-25 were repeated without the addition of NaCl in the buffers, thus, at ionic strength of 10 mM.

By lowering the ionic strength, the protein adsorption decreased to 16% for β-galactosidase and 26% for MBP-β-gal-fusion. There was some small percentage of enzyme that was eluted with maltose (Figure 5).

A new set of experiments were performed using the same conditions and columns, but with bovine serum albumin (BSA) to use as a control to confirm our hypothesis of nonspecific adsorption. BSA is often used in the protein adsorption systems to block high energy sites on the adsorbent, therefore, to reduce the amount of the nonspecific binding. BSA is a smaller protein than β-galactosidase and is considered to be rather hydrophobic protein.

BSA bound less in both cases (cross-linked amylose and potato starch) than β-galactosidase. On the cross-linked amylose column almost all of the BSA bound could be recovered, whereas β-galactosidase remained adsorbed. There was stronger binding to raw starch, for both proteins, than to cross-linked amylose. The amount of BSA bound to potato starch was 3.5 times higher than that on cross-linked amylose. BSA showed the same level of adsorption on Sephadex G-25 at both high and low ionic strengths.

**Frontal Analysis**

Frontal analysis was used to estimate the association constants of the enzymes under study with cross-linked amylose and raw starch. β-galactosidase, MBP-β-gal-fusion and BSA adsorption constants were determined using the method described by Kasai and Ishii (1978). The estimated adsorption constants give a quantitative measure of the strength of binding between the protein and the ligand, the higher the number the stronger the binding.

In the case of BSA and β-galactosidase V₀ and Vₘ should theoretically have the same values. Also, these values when compared to MBP-β-gal-fusion could be used to obtain information about the nonspecific interactions of the proteins with different adsorbents.

Table I presents the adsorption constants of the proteins on cross-linked amylose and raw starch. The adsorption constants were calculated using equation 1. Note that β-
galactosidase has approximately the same adsorption constant for both resins, while BSA exhibits 1.8 times stronger binding for raw starch than for cross-linked amylose. MBP-β-gal-fusion, containing the maltose binding domain shows the strongest binding for both resins. For cross-linked amylose the adsorption constant of MBP-β-gal-fusion is 65 times stronger than BSA and 28 times higher than that of β-galactosidase. The adsorption constant of MBP-β-gal-fusion on raw starch is 7.8 times higher than BSA and 6.6 times higher than β-galactosidase.

Table I. Adsorption constants (ml/g) at pH 7.0 and I= 0.5 M on cross-linked amylose and potato starch columns

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<th>Protein</th>
<th>Cross-linked Amylose</th>
<th>Potato Starch</th>
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<tr>
<td>BSA</td>
<td>0.21±0.01a</td>
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<tr>
<td>β-galactosidase</td>
<td>0.48±0.02a</td>
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<tr>
<td>MBP-β-gal fusion</td>
<td>14±0.25a</td>
<td>3.0±0.3a</td>
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*Standard error of the mean (n= 4).

**DISCUSSION**

Affinity chromatography was used to estimate the specificity of interactions between the fusion enzyme and starch. There are specific interactions of MBP-β-gal-fusion with cross-linked amylose since 57% of the enzyme loaded onto the column can be eluted with 10 mM maltose buffer in a sharp peak (Figure 1). This represents 71% of the enzyme bound that can be recovered using a biospecific eluant (maltose).

There seem to be some specific interactions between MBP-β-gal-fusion and raw potato starch as shown in Figure 2. About 50% of the enzyme that was bound was specifically eluted with maltose. Also, the adsorption constant for the fusion enzyme increases for both cross-linked amylose and raw starch when compared to the adsorption constants for β-galactosidase indicating a stronger binding due to the addition of the MBP domain (Table I).

β-Galactosidase was used as a control to assure that the binding is caused by the maltose binding domain alone. The adsorption constant of β-galactosidase on cross-linked amylose and raw starch given in Table I were comparable. Similarly, BSA has adsorption constants that are close for both these resins. This suggests that the interaction with the starch granule is mainly through the MBP domain.

There appears to be some other type of interactions present in the raw starch system since less of the fusion enzyme bound can be specifically eluted with maltose compared to cross-linked amylose. These interactions present in the starch system,
probably nonspecific in nature, did not enhance the binding to raw starch since the amount of fusion enzyme bound is the same on raw starch and on cross-linked amylose. The lower adsorption constant for MBP-β-gal-fusion on raw starch than on cross-linked amylose, suggest that the binding to starch is weaker than that to cross-linked amylose.

In the raw potato starch column, β-galactosidase binds more than in the cross-linked amylose column. Thus, there could be more nonspecific binding occurring on raw starch than on cross-linked amylose. The carbohydrate polar hydroxyl groups in carbohydrates, which are involved in hydrogen bonding, are more common than the hydrophobic groups. Dehydration of both the sugar and the binding site accompanies protein-carbohydrate complex formation. It is believed that mostly hydrogen bonds and Van der Waals (nonspecific) forces are present in these systems (Ross and Subramanian, 1981).

At high ionic strength (most of the experiments were performed at I = 0.5 M), the water molecules around the enzyme are probably tied up by the high ion concentration, causing a dehydration effect. This effect allows the ring structures of the amino acids present in β-galactosidase to interact with the crystalline regions which are present in the starch granule (Quirocho, 1988). This may possibly explain the nonspecific binding found on the starch column for β-galactosidase.

BSA bound less in both cases (cross-linked amylose and potato starch) than β-galactosidase. There was more binding to raw starch, for both proteins, than to cross-linked amylose. The fact that BSA binds more on the starch column than on the cross-linked amylose column gives more evidence that raw starch binds nonspecifically proteins.

Less MBP-β-gal-fusion interacted with Sephadex G-25 compared to the interactions with cross-linked amylose column. This was expected since Sephadex has α(1-6) glucosidic linkages and α(1-6) saccharides are known to interact very little ($K_c = 10^{-3}$) with maltose binding protein (Kellerman and Ferenci, 1982). Still 55% of the total protein adsorbed to Sephadex G-25 which is more than what was expected for normal chromatography losses.

β-Galactosidase adsorbed more to Sephadex G-25 (41%) compared to cross-linked amylose (30%) (Figure 3). The amount of protein that binds to Sephadex G-25 is similar for both β-galactosidase and MBP-β-gal-fusion. Moreover, none of the MBP-β-gal-fusion could be eluted with maltose from Sephadex G-25, meaning that the interactions present are not specific. These observations point the fact that β-galactosidase domain of the fusion enzyme is responsible for the nonspecific interactions with the resin.

When the experiments were repeated at low ionic strength (0.01 M), the nonspecific interactions were reduced for β-galactosidase and for MBP-β-gal-fusion on the Sephadex G-25 column, but not for BSA. It can be concluded that the nonspecificity is caused by the β-galactosidase domain's interaction with the resin.
BSA showed similar interaction for Sephadex G-25 at both high and low ionic strengths. If the interactions present were hydrophobic, there should be less nonspecific binding at lower ionic strength, and if the interactions were ionic, then there would be stronger binding at low ionic strength.

Sephadex G-25 contains some carboxyl groups, which at low ionic strength, could interact with charged solutes (Pharmacia). These effects can be eliminated by using solvents with ionic strength greater than 0.02 M (Pharmacia). The ionic strength used in our low-ionic-strength experiments is lower than that recommended to avoid ionic interactions. Therefore, it is possible that some ionic interactions caused the retention of BSA in these experiments.

The difference in cumulative elution volumes when the protein is inhibited and when is not inhibited is small. These small differences are probably caused by the nonspecific binding present on the systems. The nonspecific binding will cause a longer retention on the columns for the case when the protein is inhibited. The active binding site will be inhibited but the sites causing the nonspecific interactions will not be inhibited. The result is a longer elution volume (higher value of $V_o$) than if only specific affinity interactions are present and inhibited.

If the systems were ideal there should not be a difference between the elution volumes when β-galactosidase is inhibited and when it is not. The fact that in the presence of maltose, the elution volume of β-galactosidase is smaller than that with no maltose suggest that there are some protein-carbohydrate interactions between β-galactosidase and the packaging materials.

The two resins, raw starch and cross-linked amylose, can not be compared to each other since there are numerous differences between them and there is data that is unknown at this point. Raw starch has a solid-like surface, compared to the individual polymer chains of cross-linked amylose. Starch has crystalline regions that might participate in the nonspecific binding of proteins (Woodward et al., 1988). The exact number of sites of either cross-linked amylose or starch is unknown at the present time.

CONCLUSIONS

The cross-linked amylose resin is a good system to purify the MBP-β-gal-fusion since it does not cause large amounts of nonspecific binding and yields pure protein. But this system has also some problems. The resin has low capacity, it is compressible, and it is expensive compared to starch. In addition, it is very difficult to remove maltose from the maltose binding domain. This last complication seriously hinders the use of the system on a large scale.

Raw starch has some advantages over cross-linked amylose. Starch is inexpensive compared to cross-linked amylose, it is a
stable and environmentally safe, but it exhibits nonspecific binding for bovine serum albumin, β-galactosidase and the MBP-β-gal-fusion. Our results suggest that by lowering the ionic strength some of these interactions can be suppressed.

BIBLIOGRAPHY

Kasai, K., and Ishii, S., J. Biochem., Tokyo, 1978, 84, 1051.

Figure 1. Chromatographic patterns of β-galactosidase and MBP-β-gal-fusion at pH 7.0 and 4°C
Figure 3. Chromatographic patterns of β-galactosidase and HRP-
β-gal-fusion at pH 7.0 and 4°C

Figure 4. Chromatographic patterns of HRP-β-gal-fusion on
Sephadex G-25 and cross-linked amylose at pH 7.0 and
4°C

Figure 5. Chromatographic patterns of β-galactosidase and HRP-
β-gal-fusion on Sephadex G-25 at I = 0.5 M, pH 7.0 and
4°C
PREDICTING THE SOLUBILITY OF RECOMBINANT PROTEINS IN ESCHERICHIA COLI

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Summary

The cause of inclusion body formation in *Escherichia coli* grown at 37°C was studied using statistical analysis of the composition of 81 proteins that do and do not form inclusion bodies. Six composition derived parameters were used in this analysis; in declining order of their correlation with inclusion body formation the parameters are charge average, turn forming residue fraction, cysteine fraction, proline fraction, hydrophilicity, and total number of residues. The correlation with inclusion body formation is strong for the first two parameters but weak for the last four. This correlation can be used to predict the probability that a protein will form inclusion bodies using only the protein's amino acid composition as the basis for the prediction.

Introduction

A significant barrier to the full exploitation of recombinant DNA technology for protein production is the tendency for the targeted protein to form inclusion bodies. Although inclusion bodies have been observed mainly in *Escherichia coli*, evidence suggests they will also be a problem in other expression systems if yields start to approach that obtainable in *E. coli*. Inclusion bodies are *in vivo* agglomerations of proteins, appearing as large dense bodies in electron microscope pictures of *E. coli*. They require strong denaturants for dissolution such as urea or guanidine hydrochloride, behaving like proteins which have been irreversibly precipitated, as noted by Schein. Obtaining a usable product from a protein which has been expressed in inclusion bodies requires that the protein in the inclusion bodies be denatured and then refolded to the native form, a slow, difficult procedure which greatly reduces the net yield.

Inclusion bodies are rare in nature, with sickle cell anemia and other related blood diseases being some notable exceptions to the rule that proteins are always expressed in soluble form. The reason inclusion bodies frequently form in *E. coli* is basically their high level of expression, giving a concentration level rare in nature. Much recent work ties inclusion body formation to temperature sensitive denaturation and indicates lowering the expression temperature from 37°C to 30°C reduces their frequency.

Inclusion body formation has been the subject of reviews by Krueger *et al.*, Marston, Mitraki and King, and most recently, Schein. Schein, citing protein expression data for 22 proteins from the system of Nagai and Thogersen in which all factors were kept constant except protein sequence, concluded there must be some "solubilizing characteristics" in the six proteins.
expressed in soluble form. From this small number of proteins, however, Schein was not able to draw any firm conclusions on the nature of these "solubilizing characteristics". By expanding upon the Nagai and Thogersen system data using other proteins expressed under similar conditions, we obtained enough data to allow a thorough statistical analysis of a number of protein composition parameters in relation to inclusion body formation. The proteins used in this analysis are given in Table 1. All these proteins were expressed in *E. coli* at 37°C at expression levels at or above 3% of total cell protein.

**Data Modeling and Variable Selection**

Statistical analysis requires a large data base and an appropriate methodology. The methodology that can be used here is somewhat limited by the way *in vivo* solubility is reported in the literature. The only thing that can usually be ascertained from cloning and overexpression research reports regarding *in vivo* solubility besides the expression level is whether or not the protein forms inclusion bodies, that is, whether the protein fraction of interest after centrifugation appears chiefly in the supernatant or in the pellet fraction. This limits the analysis to what differentiates the group of soluble proteins from the group of insoluble proteins and if and how we can determine if a protein will form inclusion bodies by looking at its amino acid composition. Data in this form is suited to the technique of discriminant analysis. This technique statistically identifies and categorizes the difference between specified parameters for two sets of data, in this case *in vivo* soluble and insoluble proteins. The protein sequences for the 81 proteins are on data bases and the composition could be compared residue by residue, but the results of such an analysis might be difficult to interpret. A more tractable approach is to compare the two groups of proteins on the basis of physicochemical properties which are determined by protein composition and are hypothesized to be related to *in vivo* solubility based on *in vitro* solubility or some other aspect of proteins.

From studies of Phage P22 tailspike protein the formation of inclusion bodies has recently been explained as arising from the incorrect folding and the precipitation of folding intermediates. Analyses of the protein composition therefore focus on factors which relate to protein folding and protein solubility. Protein folding will be discussed first.

At least two aspects of protein composition can be shown to be related to its ability to fold correctly. First, an important difference between the expression of mammalian proteins in *E. coli* and in their native environment is the inability of *E. coli* to form disulfide bonds, due to the reducing environment of its cytoplasm. The fraction of cysteine in a mammalian protein expressed in *E. coli* therefore is a measure of the difficulty a protein may have in assuming its correct conformation in *E. coli*. In addition, one study suggested that disulfide bonds are present in some inclusion bodies, although this has been disputed. Another factor in the ability of proteins to fold correctly is the number of turns. Turns are the most difficult structures for proteins to form, so a high content of residues with a high Chou and Fasman index for forming turns, i.e. aspartic acid, asparagine, proline, glycine, and serine, may be indicative of a slow folding protein. The folding of proline in particular has been shown in some cases to be the rate limiting step for the folding of certain proteins. Therefore, three folding related parameters to be used are the following fractions: the cysteine fraction, the proline fraction, and the combined fraction of asparagine, proline, glycine, and serine (aspartic acid is omitted for statistical reasons to be discussed; see Statistical Methods).

The *in vivo* solubility, that is whether or not the protein forms inclusion bodies, is
different in at least one aspect from the *in vitro* solubility, being related more to the solubility of folding intermediates than to the solubility of the mature protein. However, when *comparing* the solubility *in vivo* of one protein to another, it is assumed that the same parameters which affect *in vitro* solubility can be used. Three parameters that are important in this respect are net charge, hydrophilicity, and size.

Protein solubility is predicted for low ionic content solutions by the Debye-Hückel equation\(^1\), with the log of protein solubility being proportional to the square of the net protein charge. Protein solubility thus increases with increasing net charge, positive or negative. At neutral pH's the net charge of a protein is very close to the difference between the number of basic and acidic residues, that is the number of arginines and lysines minus the number of aspartates and glutamates. This quantity divided by the total number of residues approximates the average charge per residue in a protein and is called the approximate charge average. *In vivo*, the approximate charge average should be slightly more electronegative than the actual charge average. The reason for this is the effects of the slightly basic pH of *E. coli* (7.5-7.9)\(^4\) and of the ionic double layer\(^5\), which each have a slightly electronegative effect. The exact amount of this effect is unknown. The effect of charge can therefore be estimated by the parameter approximate charge average - $a$, where "$a$" is the difference between the approximate charge average and the actual charge average, "$a$" being a constant obtained by modeling the data (see Modeling Results).

The Debye-Hückel equation also contains a size related term, the protein radius\(^13\). While the size of a protein cannot be directly determined from the protein composition, being structure dependent, the number of residues in a protein is roughly related to molecular size and can be easily measured.

Hydrophilicity or hydrophobicity affects proteins in a different way. The repulsion between hydrophobic aliphatic and aromatic side chains on amino acid residues and water has been shown by Kauzmann\(^16\) to be a key factor stabilizing protein structure. Although many of the hydrophobic groups in proteins are buried within the molecule to minimize the contact area with water, some do contact water, and these hydrophobic interactions affect protein solubility (see Arakawa and Timasheff\(^7\)). The Hopp and Woods formula\(^18\) provides a convenient way of calculating average hydrophilicity, assigning negative values to aliphatic residues like valine or aromatic residues like tyrosine and positive values to ionized residues like arginine, and is used in this model.

To summarize, the 81 proteins listed in Table 1 can be compared using six easily computed composition related parameters, cysteine fraction, proline fraction, turn forming residue fraction, approximate charge average - $a$, number of residues, and hydrophilicity, through the technique of discriminant analysis. This technique is used to maximize the statistical differentiation between the insoluble and soluble proteins, using a composite parameter of the individual parameters called the canonical variable, or CV. This composite parameter consists of the sum of the individual parameters multiplied by their respective adjustable coefficients, or $\lambda$'s, as given by the equation:

$$\text{CV} = \sum_{i} \lambda_i x_i$$

where
\[ \lambda = \text{adjustable coefficient} \]
\[ x = \text{composition related parameter} \]
\[ n = \text{number of parameters} \]

The \( \lambda' \)'s were calculated from the two sets of data using standard statistical techniques described by Fisher\(^{19} \). The protein compositions were obtained from the University of Wisconsin Genetic Computer Group programs\(^{20} \).

### Modeling Results

Various statistical parameters for the modeling using the 81 proteins are shown in Table 2 and Table 3. The effectiveness of a parameter in determining membership in a group can be estimated by analyzing the parameter \((\mu_{\text{insol}} - \mu_{\text{sol}})/\sigma_{\text{pooled}}\), or the difference in means divided by the pooled group standard deviation. Discriminant analysis optimizes the \( \lambda' \)'s so that \((\mu_{\text{insol}} - \mu_{\text{sol}})/\sigma_{\text{pooled}}\) is as large as possible, and thus maximizes the possibility of correctly identifying a protein on the basis of these parameters alone as being soluble or insoluble. An initial evaluation can therefore be made on a parameter on the basis of \((\mu_{\text{insol}} - \mu_{\text{sol}})/\sigma_{\text{pooled}}\), before using it in the discriminant analysis calculations. The value for \((\mu_{\text{insol}} - \mu_{\text{sol}})/\sigma_{\text{pooled}}\) for the size parameter was 0, so it was not used in the computer evaluation. The quantity 0.03 for "a" gives close to the highest value of \((\mu_{\text{insol}} - \mu_{\text{sol}})/\sigma_{\text{pooled}}\) for approximate charge average - al and is within the expected range predicted by isoelectric point calculations and double layer theory\(^{15} \). The actual parameter used to measure charge is therefore approximate charge average - 0.03.

Values for other statistic parameters calculated for the individual parameters are also shown in Tables 2 and 3, with the first three rows representing values calculated on the individual composition parameters and the last two rows representing calculated values for the discriminant analysis equation. In Table 3 the turn fraction for the purposes of analysis is split into two parts, the proline fraction and the fraction of the other three turn-forming residues, to determine if the proline fraction, as has been suggested\(^{8} \), has any especially strong correlation with \textit{in vivo} solubility as compared with the other three turn inducing residues used in the turn fraction compilation.

The F value calculated by the discriminant analysis computer program is used, as is \((\mu_{\text{insol}} - \mu_{\text{sol}})/\sigma_{\text{pooled}}\), to estimate the statistical difference between groups. An F value of 1.0, as does a value for \((\mu_{\text{insol}} - \mu_{\text{sol}})/\sigma_{\text{pooled}}\) of zero, means there is no statistical difference between groups. The F value may be used with the F distribution to statistically evaluate the significance of a given parameter's between group differences (see Statistical Methods).

The non-normalized \( \lambda' \) 's as stated were used directly in the CV equation. The normalized \( \lambda' \) 's give an estimation of the weighted contribution of each term in the equation, \textit{i.e.}, in terms of the magnitude of each \( \lambda \) adjusted for the size of its sequence parameter, by multiplying each \( \lambda \) by its parameter’s pooled within-group variance. These results for normalized \( \lambda \) are consistent with the results for \((\mu_{\text{insol}} - \mu_{\text{sol}})/\sigma_{\text{pooled}}\) F, and the level of significance.
In Table 2 the proline fraction and the remaining turn fraction were combined into one parameter, as the proline fraction as seen in Table 3 contributes no more than its proportional share to the weighted λ relative to the other three residues in the turn fraction. As the separate consideration of proline does not add any accuracy and complicates the computer calculation, it is the Table 3 values which are used for the results that follow.

Values of \((\text{CV-} \text{CV})\), where \(\text{CV}\) is the value of the discriminant (1.15), are shown in Figure 1. The value of \(\text{CV}\) is used to determine the statistical probability that a given protein will be soluble or insoluble, as shown in Figure 2. High values of \(|\text{CV-} \text{CV}|\) mean the protein can be determined to be soluble or insoluble with a high degree of confidence. Low values of \(|\text{CV-} \text{CV}|\) mean the protein is in the borderline region of solubility and has a roughly comparable chance of being either soluble or insoluble. Using the percentage probabilities to classify proteins as soluble or insoluble, discriminant analysis successfully classifies 22 of the 27 soluble proteins and 49 of the 54 insoluble proteins, for an overall accuracy of over 88%.

Discussion

The values for the \(\lambda\)'s in the canonical variable equation as determined by discriminant analysis give the optimum formula for estimating the in vivo solubility for proteins. The probable solubility of other proteins which have not yet been expressed in E. coli can be evaluated if the amino acid composition is known by calculating the parameters as described above and inserting them into the discriminant analysis equation. The resulting value of \(\text{CV}\) may be used to obtain the probability of in vivo protein solubility or insolubility using Figure 2. \(\text{CV}\) values may also provide a good relative comparison of solubility for proteins expressed under different conditions than those specified for the Table 1 proteins; for instance if a protein expressed under a given set of conditions is insoluble, another protein with a higher \(\text{CV}\) expressed under the same conditions would likely also be insoluble.

These probability calculations concerning the chances of inclusion body formation should be helpful to researchers in microbial protein production. It should be possible after estimating the solubility of a cloned protein by calculations of the above type to plan the cloning procedure, especially promoter selection, fusion protein partner, and expression temperature accordingly to produce soluble or insoluble protein. For instance, a highly electronegative, low \(\text{CV}\) fusion protein partner for targeted proteins in lieu of the commonly used protein β-galactosidase (for which \((\text{CV-} \text{CV}) = -0.39\)) would likely be more efficient in producing soluble proteins.

In general, discriminant analysis adjusts the \(\lambda\)'s so that the terms with the strongest correlation with membership in the different groups, in this case solubility or insolubility, have the strongest effect on the canonical variable or \(\text{CV}\), while terms with a weaker correlation have a lesser effect on \(\text{CV}\). The weighted \(\lambda\)'s give a close estimate of the relative importance of each parameter in the \(\text{CV}\) equation. As can be seen from Table 2, the turn fraction and approximate charge average - 0.03 make up most of the value of \(\text{CV}\), the contribution of the other terms being small, indicating these two terms largely determine in vivo solubility. This is in line with their much stronger individual correlation with in vivo solubility as seen by their high \(F\) values.
and significance levels. The F values and significance levels for the cysteine fraction and hydrophilicity are low indicating a low level of correlation, and their contribution to the CV is correspondingly small.

It is interesting to relate these results to a recent model proposed by Mitraki and King\textsuperscript{7} for inclusion body formation:

\begin{equation}
\text{translation} \rightarrow P_f^\text{ref} \leftrightarrow P_m \rightarrow \text{native} \\
\uparrow \\
P^\text{ref} \\
\downarrow \\
\text{inclusion body}
\end{equation}

where: $P_f^\text{ref}$ is a soluble partially folded early intermediate $P_m$ is the intermediate competent to form into the monomer $P^\text{ref}$ is the species that generates the aggregate (i.e. inclusion body)

From this model it is clear how the \textit{in vivo} solubility of proteins is affected both by folding rates and solubility factors. As an example of the effect of folding rates on solubility, a protein with many turn forming residues folds more slowly, hence has a higher concentration of folding intermediates, which increases the probability of these intermediates precipitating to form inclusion bodies. As an example of the effect of solubility factors, the folding intermediates of a highly electronegative protein should be more soluble than average and therefore less likely to precipitate to form inclusion bodies than less electronegative, less soluble proteins.

**STATISTICAL METHODS**

Statistical methods used in this analysis assume a standard Gaussian distribution, which however is not a critical assumption if the data is not too badly skewed. The discriminant analysis technique used assumes the individual parameters act independently, which for these parameters is substantially true. The value of the turn forming residue fraction does not include aspartatic acid to eliminate interference with the charge average, which also contains aspartic acid.

The pooled standard deviation combines the standard deviations from two different but statistically similar populations by adding their variances, \textit{i.e.}, for two populations 1 and 2 of size $N_1$ and $N_2$ and means of $\mu_1$ and $\mu_2$ respectively:

$$
\sigma_{\text{pooled}} = \left( \frac{\sum(x_1 - \mu_1)^2 + \sum(x_2 - \mu_2)^2}{(N_1 + N_2 - 2)} \right)^{1/2}
$$
The F value, like \( \frac{(\mu_1 - \mu_2)}{\sigma_{\text{pooled}}} \), provides a test of the null hypothesis (that is, that there is no statistically significant between-group difference) but in a statistically quantifiable manner. It does so by comparing the between-group variance to the within-group variance. F values for \( n \) degrees of freedom are tabulated in standard statistical tables. If the calculated F value is larger than the tabulated value for a given confidence level, the between-group differences are statistically significant at that confidence level. If \( \mu_r \) equals the combined group mean, the F value is given by the following equation:

\[
F = \frac{[N_1(\mu_1 - \mu_r)^2 + N_2(\mu_2 - \mu_r)^2](N_1 + N_2 - 2)}{(N_1 - 1)\sigma_1^2 + (N_2 - 1)\sigma_2^2}
\]

The discriminant analysis computer calculations were performed by the discriminant analysis program written by BMDP Statistical Software, Inc. In addition to calculating the CV for a protein the computer also calculates the posterior probability that the protein will be soluble by using ratios called the Mahalanobis distances, which are derived from the covariance matrices used in the discriminant analysis calculations. Basically, if the CV for a protein is close to the CV value of 1.15, the posterior probability that the protein will be soluble or insoluble will be close to 0.5. If on the other hand the CV for a protein is far from the CV value of 1.15, the posterior probability that the protein is soluble or insoluble will be near unity. These computer calculated values were used to prepare Figure 2.

Acknowledgments

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References

Table 2  Statistical model: Results for four parameters.

<table>
<thead>
<tr>
<th>Statistic Parameter</th>
<th>Fraction</th>
<th>Hydrophilicity Index</th>
<th>Approximate Charge Average</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>((\mu_0 - \mu_1))</td>
<td>0.98</td>
<td>-0.26</td>
<td>-1.37</td>
</tr>
<tr>
<td>(\sigma_{pooled})</td>
<td>0.54</td>
<td></td>
<td></td>
</tr>
<tr>
<td>&quot;F&quot; Value</td>
<td>17.19</td>
<td>2.03</td>
<td>32.78</td>
</tr>
<tr>
<td>Level of Significance</td>
<td>100%</td>
<td>85%</td>
<td>100%</td>
</tr>
<tr>
<td>Adjustable Coefficients ((\lambda))</td>
<td>13.40</td>
<td>0.80</td>
<td>-33.25</td>
</tr>
<tr>
<td>Normalized or Weighted (\lambda)</td>
<td>0.50</td>
<td>0.20</td>
<td>-0.81</td>
</tr>
</tbody>
</table>
### Table 3: Statistical model: Results for five parameters.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Statistic</th>
<th>Fracton Turns (w/o Pro)</th>
<th>Prolines</th>
<th>Cysteines</th>
<th>Hydropilicity Index</th>
<th>Approximate Charge Average</th>
</tr>
</thead>
<tbody>
<tr>
<td>(d-c) pooled</td>
<td>F Value</td>
<td>0.88</td>
<td>0.35</td>
<td>0.54</td>
<td>-0.26</td>
<td>-1.37</td>
</tr>
<tr>
<td>&quot;F&quot; Value</td>
<td>Level of Significance</td>
<td>15.20</td>
<td>4.05</td>
<td>9.36</td>
<td>0.80</td>
<td>-33.25</td>
</tr>
<tr>
<td>Adjustable Coefficients ($\lambda_\alpha$)</td>
<td>0.50</td>
<td>0.08</td>
<td>0.15</td>
<td>0.20</td>
<td>-0.81</td>
<td></td>
</tr>
</tbody>
</table>

### Table 1: Proteins studied.

<table>
<thead>
<tr>
<th>Insoluble Proteins</th>
<th>Soluble Proteins</th>
</tr>
</thead>
<tbody>
<tr>
<td>(ci fusion proteins)</td>
<td>(ci fusion proteins)</td>
</tr>
<tr>
<td>1. human $\alpha$-globin</td>
<td>17. human interferon $\alpha_2$</td>
</tr>
<tr>
<td>2. human $\beta$-globin</td>
<td>18. human interferon $\gamma$</td>
</tr>
<tr>
<td>3. pancreatic ribonuclease A</td>
<td>19. human basic fib. growth factor</td>
</tr>
<tr>
<td>4. human myoglobin</td>
<td>20. ricin A</td>
</tr>
<tr>
<td>5. human c-myc</td>
<td>21. insulin growth factor #1</td>
</tr>
<tr>
<td>6. chicken $\beta$-actin</td>
<td>22. diphtheria toxin</td>
</tr>
<tr>
<td>7. chicken myosin light chain</td>
<td>23. diphtheria twn./melanocyte hormone</td>
</tr>
<tr>
<td>9. Xenopus histone 2B</td>
<td>25. mx protein</td>
</tr>
<tr>
<td>10. Xenopus TFIIB</td>
<td>26. bovine prochymosin</td>
</tr>
<tr>
<td>11. tobacco mosaic virus coat</td>
<td>27. bovine prochymosin N-terminal</td>
</tr>
<tr>
<td>12. yeast mating $\alpha_1$</td>
<td>28. phage p22 tail prot.</td>
</tr>
<tr>
<td>13. N-end human gelosin</td>
<td>29. sv40 small t antigen</td>
</tr>
<tr>
<td>14. yeast SWI-5</td>
<td>30. $\gamma_6$ resolvase</td>
</tr>
<tr>
<td>15. caltrin</td>
<td>31. $\beta$-galactosidase/HSV-1</td>
</tr>
<tr>
<td>16. pre-prosubtilisin</td>
<td>32. $\beta$-gal/penicillin frag</td>
</tr>
<tr>
<td>35. B. subtilus $\varphi$29 Prot.13</td>
<td>36. Leishmania major DHR-TS</td>
</tr>
<tr>
<td>37. CAT/ANF PTCAN-11</td>
<td>38. CAT/ANF PTCAX-42</td>
</tr>
<tr>
<td>39. phage $\lambda$O protein</td>
<td>40. phage T4 protein 23</td>
</tr>
<tr>
<td>41. interleukin-2</td>
<td>42. interleukin-4</td>
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<tr>
<td>44. prorenin</td>
<td>46. human macrophage colony</td>
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<tr>
<td>45. prorenin/linker fusion</td>
<td>47. H-ras, N-terminal</td>
</tr>
<tr>
<td>51. salmon growth hormone</td>
<td>52. $\gamma$ heavy chain</td>
</tr>
<tr>
<td>53. bovine growth hormone</td>
<td>54. HGH/$\beta$-lactamase</td>
</tr>
<tr>
<td>55. human tropomyosin</td>
<td>56. human gelosin</td>
</tr>
<tr>
<td>57. human gelosin, C-term.</td>
<td>58. tropomini T</td>
</tr>
<tr>
<td>59. chicken tropomin C</td>
<td>60. CGN4 Ca$^+$ binding dom</td>
</tr>
<tr>
<td>61. thioredoxin</td>
<td>62. maltose binding protein</td>
</tr>
<tr>
<td>63. T7 RNA polymerase</td>
<td>64. $\beta$-galactosidase</td>
</tr>
<tr>
<td>65. T4 DNA ligase</td>
<td>66. T7 gene 19 protein</td>
</tr>
<tr>
<td>67. CAT/ANF PTCAX-11</td>
<td>68. CAT/ANF PTCAX-92</td>
</tr>
<tr>
<td>69. $\beta$-gal/hirudin</td>
<td>70. CAT/ANF PTCAX-82</td>
</tr>
<tr>
<td>71. B. subtilus $\varphi$29 Prot.13</td>
<td>72. B. subtilus $\varphi$29 Prot.10</td>
</tr>
<tr>
<td>73. T3 Protein 18</td>
<td>74. T4 RNA ligase</td>
</tr>
<tr>
<td>75. human growth hormone</td>
<td>76. human interferon $\alpha_1$</td>
</tr>
<tr>
<td>77. H-ras</td>
<td>78. H-ras, C-terminal</td>
</tr>
<tr>
<td>79. $\alpha$-antitrypsin</td>
<td>80. murine TNF</td>
</tr>
<tr>
<td>81. human TNF</td>
<td>82. H-ras, C-terminal</td>
</tr>
</tbody>
</table>
Figure 1  Canonical variable (CV) minus discriminant canonical value (CV = 1.15) for the proteins studied. Soluble proteins are shown with solid bars and insoluble proteins with open bars.

Figure 2  Predicted probability of protein solubility or insolubility based on canonical value of protein. CV = 1.15. If (CV-\bar{CV}) is positive, use dashed line for protein insolubility. If (CV-\bar{CV}) is negative, use solid line for protein solubility.
EVOLUTION OF A PHASE SEPARATED GRAVITY INDEPENDENT BIOREACTOR

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ABSTRACT
The evolution of a phase-separated gravity-independent bioreactor is described. The initial prototype, a zero head-space manifold silicone membrane based reactor, maintained large diffusional resistances. Obtaining oxygen transfer rates needed to support carbon-recycling aerobic microbes is impossible if large resistances are maintained. Next generation designs (Mark I and II) mimic heat exchanger design to promote turbulence at the tubing-liquid interface, thereby reducing liquid and gas side diffusional resistances. While oxygen transfer rates increased by a factor of ten, liquid channeling prevented further increases. To overcome these problems, a Mark III reactor was developed which maintains inverted phases, i.e., media flows inside the silicone tubing, oxygen gas is applied external to the tubing. This enhances design through changes in gas side driving force concentration and liquid side turbulence levels. Combining an applied external pressure of four atmospheres with increased Reynolds numbers resulted in oxygen transfer intensities of 232 mmol O₂/l/h (1000 times greater than first prototype and comparable to a conventional fermenter). A 1.0 liter Mark III reactor can potentially deliver oxygen supplies necessary to support cell cultures needed to recycle a 10 astronaut carbon load continuously.

INTRODUCTION
Food supplies ultimately determine the length of space travel. Long excursions in space are not presently possible without replenishing food reserves. If long term space travel is to become a reality an onboard carbon recycle system must be developed. Recycling a fixed organic carbon supply ensures a constant food supply. Earth has a fixed amount of carbon that is recycled by a host of microorganisms on a slow but continuous basis. The same logic can ultimately be applied in space. However, growing microorganisms in space offers many challenges. For example, oxygen-containing gas bubbles injected into liquid media do not rise in microgravity environments. This inhibits transfer of oxygen to the media. Also, bubbles tend to coalesce with the liquid and become the dispersed phase while the gas becomes the continuous phase due to the dominance of surface tension forces.
Presently, we have no knowledge of a functional gravity-independent bioreactor designed to grow high cell density aerobic cultures in space. We are aware of the cell culture mammalian bioreactor currently being tested at Johnson Space Center and the reactor constructed at the Jet Propulsion Laboratory. However, low oxygen delivery capacities of these reactors will not allow growth of oxygen demanding aerobic carbon-recycling microorganisms.

The variable to be maximized is oxygen transfer intensity (mmol $O_2$/hr/l). This describes the reactor's capability to replenish oxygen to an actively metabolizing culture of microorganisms. Poor oxygen solubilities in fermentation broths limit aerobic growth. Reactors that transfer oxygen efficiently will support high density cultures. This is illustrated in Figure 1. Solid lines represent unrestricted exponential growth at various doubling times from 1 to 4 hours. The lines with circles represent the growth curves developed from various reactors with differing oxygen transfer intensities, i.e., when oxygen is limiting growth follows the lines with circles. High transfer intensities result in high cell densities and yields, thus this becomes the principal design parameter.

Cell Growth, doubling times & oxygen transfer intensity

Phase-separated bioreactors have the distinct advantage of operating under bubble-free conditions. This circumvents gas-liquid disengagement problems that develop in microgravity environments. However, designing a silicone-based phase-separated bioreactor with high oxygen transferring capabilities, suited for oxygen intensive microbial growth, has proven to be a challenge.

Petersen et al. designed and constructed a phase-separated membrane bioreactor. The reactor consisted of a silicone tube zero head-space plexiglass fermenter. Conclusions drawn from the study include:

i) At infinite stirrer speed the mass transfer coefficient becomes infinite, implying that the external fluid resistances are extremely high compared to resistance from the oxygen diffusion across the membrane.

ii) Oxygen limitation dominated under most conditions and should thus be the focus of future studies.
Overall oxygen mass transfer resistance is a summation of individual gas, liquid, and membrane resistances. This can be expressed as: 

\[ \frac{1}{K} (\text{overall mass transfer resistance}) = \frac{1}{k_1} + \frac{1}{k_2 \cdot m^*} + \frac{1}{R}. \]

Where \( m^* \) is the slope of the gas-liquid equilibrium line. An application of this expression follows. Figure 2.a was developed by holding air velocity constant while liquid velocities are varied. Figure 2.b results when the gas-film resistances are subtracted from Figure 2.a and air rate is varied while liquid flowrate is held constant. The regression line intersects near the origin indicating little or no membrane resistance. If the regression line had passed above the origin on the y axis this would indicate a detectable membrane resistance existed.

The development of the three CSU phase-separated bioreactors were based on conclusions derived from the JPL study. Oxygen transfer intensities became the focus of design. Lowering fluid resistances would increase oxygen transfer intensities. In order to lower fluid resistances a different design approach seemed appropriate. CSU reactors are modeled on heat exchanger design.

**APPARATUS AND METHODS**

Both the Mark I and Mark II reactors mimic the design illustrated in Figure 3. Media flows countercurrent external to oxygen-containing silicone tubes. The geometry of the two reactors is essentially the same. Working volume of Mark II is 14.5X greater than the 25 ml Mark I. It also has 3 times more tubing per unit volume. Both reactors were constructed out of silicone tubing and plexiglass.

The Mark III does not resemble either the Mark I or II reactors with respect to performance or design. The phases are inverted, air is applied external and the water is run internal with respect to the tubing. Therefore, the silicone tubing, contained in a pressure vessel, becomes the reactor. Construction material consists of steel and silicone tubing. Table 1 gives the tubing specification for the different reactors.
Table 1 Tubing Specification

<table>
<thead>
<tr>
<th></th>
<th>Mark I</th>
<th>Mark II</th>
<th>Mark III</th>
</tr>
</thead>
<tbody>
<tr>
<td>tubing ID mm</td>
<td>0.5</td>
<td>1.57</td>
<td>3.35</td>
</tr>
<tr>
<td>tubing OD mm</td>
<td>0.93</td>
<td>3.17</td>
<td>4.65</td>
</tr>
<tr>
<td>tubing wall thickness mm</td>
<td>0.215</td>
<td>0.80</td>
<td>0.65</td>
</tr>
<tr>
<td>tubing length cm</td>
<td>30</td>
<td>1101</td>
<td>1400</td>
</tr>
<tr>
<td>m² / reactor volume</td>
<td>0.32</td>
<td>5.46</td>
<td>3.03</td>
</tr>
</tbody>
</table>

**OXYGEN TRANSFER MEASUREMENTS**

Gassing-out and sulphite oxidation techniques are the traditional means by which oxygen transfer measurements are determined in a fermenter. Unfortunately both techniques possess major drawbacks. Bell and Gallo\(^3\) demonstrated that minor amounts of surface active contaminants (such as amino acids, proteins, fatty acids, esters, lipids, etc.) could have major effect on the accuracy of the sulphite oxidation technique. The accuracy of a gassing-out experiment is completely dependent on the response time of the dissolved oxygen probe. Discussions by Taguchi and Humphrey,\(^4\) Heineken\(^5\) and Wernau and Wilkie\(^6\) point out the necessity to incorporate a probe response correction factor.

Conducting steady state experiments is achieved by passing deoxygenated water through a reactor at constant velocities and monitoring relative changes in dissolved oxygen before and after the water is exposed to the reactor see figure 3.

**Experimental Design**

![Diagram](image)

Figure 3
RESULTS AND DISCUSSION

The design concepts are based on the fundamental equation for mass transfer. Namely, rate of mass transport = mass transfer coefficient \times surface area \times driving force. Several steps in the design of the Mark III follow naturally from this.

Step 1. Surface area for mass transfer is maximized by maximizing the surface area of the silicone tubing.

Step 2. Driving force can be increased by switching from air (21% O\textsubscript{2}) to compressed oxygen (95-100% O\textsubscript{2}). The mass flux of O\textsubscript{2} will rise linearly with O\textsubscript{2} concentration in the gas phase. Increasing the absolute pressure of the gas phase from 1 to 5 atmospheres will likewise linearly increase mass flux to the extent that Henry's law holds.

Step 3. The overall mass transfer coefficient (whose reciprocal is a resistance) can be resolved into three major components: liquid film, gas film and membrane resistances. The two fluid resistances will be controlled by the Reynolds number (Re) of the flow according to an equation of the form Sh = f(Re, Sc) where

\[ Re = \frac{ud}{v}, \quad Sh = \frac{hd}{D}, \quad Sc = \frac{v}{D}. \]

The exponent on the Reynolds number is typically 0.5 for laminar flow and 0.8 for turbulent flow. The Sherwood number, Sh, can be viewed as the film side mass transfer coefficient made dimensionless by the rate of mass transfer by diffusion alone (D/d). It needs to be maximized, making the flow as turbulent as possible is the logical way to do this i.e. also maximize the Reynolds number. The Reynolds number, however, contains two design variables for us; the fluid velocity (u) and the tube diameter (d), both of which appear in the numerator. Laminar flow predominates even at Reynolds numbers of 4,000 because we are limited in small scale simulations to using nominal diameter tubing.

Liquid resistances are smallest if turbulent conditions are maintained inside the tubing. Typically, this condition in pipes is achieved at Reynolds numbers greater than 2300. However, in the coiled tubing arrangements of the Mark III apparatus, secondary flow stabilizes the onset of turbulence which is still not seen at Reynolds number of 4560. This was determined experimentally by plotting friction factor versus Reynolds number, Figure 4. To promote the transition from laminar to turbulent flow, larger diameter straight tubes and higher flow rates are necessary.

![Friction factor vs Reynolds number](image)
Our experimental data show no detectable film resistance on the gas side of the membrane. Water flow rate was held constant as air velocities fluctuated from a Reynolds number of 89 to 3450. Increases in dissolved oxygen were nondetectable. A logical explanation of this observation lies in the diffusivities of oxygen in air, silicone and water. Oxygen diffusives 4 orders of magnitudes faster in air than in silicone or water. Therefore, concentration gradients exterior to the tube is negligible. This is illustrated in Figure 5.

As \( \frac{1}{K_1} = \frac{1}{k_1} + \frac{1}{(k_s \cdot m^n)} + \frac{1}{R} \), and given that the objective is to make both \( \frac{1}{k_s} \) and \( \frac{1}{k_1} \) as small as possible, the membrane resistance will eventually become important. It is equal to the oxygen permeability in silicone which is 6.86 times higher than oxygen in water at 25°C.

In our experiments, the fraction of gas utilization was not measured. However, the Mark III has the inherent potential of utilizing a large percentage of the gas entering the reactor. Due to the importance of this parameter it will receive more attention in future studies.

**MARK I**

The small working volume made evaluation of the Mark I reactor extremely difficult. Under nearly all experimental conditions the dissolved oxygen probe was forced to function at very low concentrations. This was attributed to the low surface area per unit volume. However, experimental data collected from this reactor revealed oxygen transfer intensities 10 times higher than previously reported.²

**Mark II**

**WATER FLOW PATTERN**

Increasing total volume and percent tubing while holding geometry constant seemed to be a logical step in design development. The percent tubing was increased 3 fold in order to increase the membrane surface area. This increased dissolved oxygen concentrations well above the detectable range the of the probe. Initial evaluations indicated that massive channeling predominated the Mark II reactor. This was evidenced by the decreasing intensities as water velocities increase. Mass transfer should increase as flow rate increases. However, the converse of this may be seen if channeling exists. Subsequent dye injection studies substantiated the presence of severe channeling. Dye rushed past either side of the tubes, see Figure 6. To minimize channeling the top and sides of the reactor were packed with foam. This substantially reduced channeling. Now as the external water velocities increased, oxygen transfer intensities also increased.

Figure 5

Figure 6
From a biological viewpoint this reactor has major drawbacks. Growing yeast or any other microbe in a silicone/foam packed plexiglass reactor presents two major problems.

i) Cells would accumulate and adhere to the foam.
ii) Sterilization is quite difficult if not impossible.

**MARK III**

These problems combined with relatively poor oxygen transfer intensities prompted construction of the Mark III reactor. Unlike other CSU reactors the Mark III operates under inverted phases i.e. liquid flows inside the tube and oxygen is applied externally. Phase inversion addresses channeling, sterility and cell adhesion problems. Channeling is minimized in a pipe. Silicone tubing maintains sterile integrity after multiple steam sterilization cycles. Silicone resists microbial adhesion if turbulent conditions are maintained at the silicone interface. This configuration also allows for the driving force and surface area to be increased substantially. Table 2 gives a summary of results.

**TABLE 2. SUMMARY OF RESULTS**

<table>
<thead>
<tr>
<th></th>
<th>JPL</th>
<th>Mark I</th>
<th>Mark II</th>
<th>Mark III</th>
</tr>
</thead>
<tbody>
<tr>
<td>intensity (mmol O₂/l/h)</td>
<td>0.22</td>
<td>3.33</td>
<td>2.57</td>
<td>232 @200 psig O₂</td>
</tr>
<tr>
<td>Kₐ (m/h)</td>
<td>0.0619</td>
<td>0.0199</td>
<td>0.776</td>
<td>0.6919</td>
</tr>
<tr>
<td>Kₐ (1/h)</td>
<td>8.1</td>
<td>0.0199</td>
<td>0.151</td>
<td>9.4</td>
</tr>
<tr>
<td>flux (mmol O₂/hr.cm²)</td>
<td>1.74e⁻³</td>
<td>0.0033</td>
<td>0.0131</td>
<td>0.01705</td>
</tr>
<tr>
<td>Sherwood #</td>
<td>16.5</td>
<td>10.6</td>
<td>135</td>
<td>257.5</td>
</tr>
<tr>
<td>economy (mmol O₂/watt h)</td>
<td>0.376</td>
<td>2.27</td>
<td>1.0</td>
<td>136</td>
</tr>
<tr>
<td>% tubing</td>
<td>7.5</td>
<td>5.0</td>
<td>17.4</td>
<td>30%</td>
</tr>
<tr>
<td>intensity/% tubing (mmol/l/h/% tubing)</td>
<td>0.293</td>
<td>0.666</td>
<td>0.147</td>
<td>7.73</td>
</tr>
</tbody>
</table>

Conventional fermenters claim to maintain oxygen transfer intensity capabilities of 200 mmol O₂/l/h. However, under experimental conditions this is rarely obtained. Power economy of a typical fermenter is approximately 3 times lower than the Mark III. If gas utilization is used as an efficiency parameter the Mark III again rates highest. Typical fermenters utilize less than 20% of the incoming gas. The Mark III utilizes 100% of the incoming gas.

**APPLICATION TO CELSS ENVIRONMENT.**

The results obtained with the Mark III bioreactor can now be put into perspective by calculating the size of reactor necessary in a CELSS application.

**ASSUMPTION I:** Rate of aerobic microbial growth in a reactor is only limited by the poor solubility of oxygen in media.
STEP (1) Determine daily carbon recycle load for ten astronauts. As an approximation, healthy adults produce 16.2 g carbon every 24 period, 14 g of which are derived from feces and 2.2 g from urine. Thus the reactor must be able to handle 162 g carbon per 24 hour period.

STEP (2) Determine grams of dry biomass needed to address the daily 162 g carbon load. Assuming 50% yield (g biomass produced per g substrate consumed), the weight of dry biomass needed is 81 g per 24h period.

STEP (3) Determine grams of oxygen needed to support 81 g biomass with the following equation;

\[
\frac{g_{\text{oxygen}}}{g_{\text{biomass}}} = 16 \left[ \frac{2C + H/2 - O'}{\gamma \times M} \times \frac{O'}{1600} + \frac{C'}{600} + \frac{N'}{933} - \frac{H'}{200} \right]
\]

where C, H, and O represent the number of atoms of carbon, hydrogen, and oxygen, respectively, in each molecule of carbon source; C', H', O', and N' represents the percentage of carbon, hydrogen, oxygen, and nitrogen, respectively, in the cells; Y represents the yield of cells based on carbon source, gram cells per gram carbon source consumed; and M represents the molecular weight of the carbon source.

ASSUMPTION II: Methanol is the available fermentable carbon substrate. Complex forms of carbon are easily oxidized to carbon dioxide by heat. Carbon dioxide is then converted to consumable methanol catalytically.

ASSUMPTION III: Yeast are to be grown in the reactor. Yeast contain less nitrogen per unit mass relative to prokaryotic bacteria. The lower nitrogen levels are more amenable to human consumption.

YEAST ELEMENTAL COMPOSITION: O 31%, C 47%, N 7.5%, H 6.5%.

Utilizing equation I: a value of 1.66 g oxygen is required to produce 1 g of biomass. Multiplying this value by 81 g biomass equals 134 grams of oxygen which is the total oxygen needed to recycle a 10 astronaut 24 hour carbon load. Therefore, the reactor must deliver 4206 mmol O₂ per 24 hour to the media. The Mark III delivers approximately 200 mmol O₂/l/hr. Thus the total oxygen transferred per 24 hour period is 4800 mmol O₂. Therefore, a one liter vessel run continuously has the potential to recycle all the carbon produced by 10 astronauts every 24 hours. Based on the above assumptions a ONE LITER BIOREACTOR has the ability to recycle a ten astronaut carbon load continuously.

CONCLUSIONS

First attempts at building a silicone phase-separated gravity-independent bioreactor were associated with large liquid film resistances and channeling. Both the liquid film resistances and channeling are minimized through inversion, i.e., liquid flows inside the silicone tubing (Mark III). Inversion maximizes turbulence at the silicone-tubing interface and controls channeling. This improves the economy, intensity, Sherwood number, Kᵢ, Kᵢᵃ, and flux, refer to table 2.

Oxygen flux through water, silicone and air differ dramatically due to varying permeabilities and solubilities. Relative rates of oxygen transfer in silicone and air are much higher than in water. This results in minute gas boundary layers with extremely small gas side Reynold numbers. Inherently high gas utilizations ensue.
High oxygen transfer intensities result in small reactor vessel volume. A one liter Mark III, tested abiotically and terrestrially, has the potential to replenish a ten astronaut food supply. Original prototypes require over 200 liters to maintain the same number of organisms.

Future work will focus on liquid side turbulence. Even at Reynolds numbers of 4500 the Mark III is still working under laminar conditions as shown in Figure 4. Increasing both the tubing ID and velocities will promote turbulence and increase mass transfer. We intend to design a turbulent flow reactor by adjusting these parameters.


REFERENCES


A STRATEGY FOR DECONTAMINATION OF SOILS CONTAINING ELEVATED LEVELS OF PCP

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Abstract

Photodegradation of pentachlorophenol (PCP) has been investigated with a view to develop processes for remediation of contaminated soils. Soil films, about 0.5 mm thick, were exposed to UV-irradiation in order to simulate photodegradation in soil beds. Both upper horizon (high organic carbon) and lower horizon (low organic carbon) soils have been used. Photodegradation in soil slurries has been studied to evaluate reactor treatment possibilities. Soluble as well as total PCP have been measured in kinetic experiments to study aqueous phase - solid phase interactions. Photodegradation in the aqueous phase after mobilizing PCP from soil has also been investigated.

A PCP mixed culture has been developed from the local wastewater treatment plant sludge. This culture has been used to study biodegradation kinetics in the irradiated soil slurry and the irradiated soil wash. An integrated scheme for decontamination of soils with high PCP concentrations has been presented.

Introduction:

Pentachlorophenol (PCP) is a general biocide, and has been widely used as a wood preservative (Crosby \textit{et al.}, 1981). PCP has been listed by the U.S. EPA as a priority pollutant and its use is presently restricted in the U.S.A. because of its negative environmental effects (Federal Register, Jul., 1984). Many PCP-contaminated sites exist in Missouri and in other parts of U.S. Most of these sites are located in wood treatment facilities.
PCP is rapidly photolyzed in aqueous solutions via a number of pathways depending on several factors including pH, major ion composition, dissolved oxygen, presence of organic proton donors and presence of trace metals. The first step in the direct photolysis of PCP in distilled water solutions is a photonucleophilic substitution of hydroxide for chloride (Wong and Crosby, 1981). Mechanism of photolysis in soil are poorly understood due to the complex processes of quenching, photosensitization, effects of presence of organic matter, soil type and light attenuation in soils (Miller et al., 1989).

In several studies on biodegradation of PCP in soils, it has been reported that the microbial activity becomes inhibited at high PCP levels (Watanabe, 1973; Crawford and Mohn, 1985; Rao, 1990; Hsieh, 1991). Watanabe (1973) isolated a *Pseudomonas* species from rice paddy soil capable of growth using PCP as a sole carbon and energy source. Growth of this organism was inhibited at 100 mg PCP/Kg soil, and ceased completely at 200 mg/Kg. Similar concentration effects have been found by others as well. Alternative methods are required for remediation of contaminated sites as high PCP concentrations are frequently encountered.

The purpose of this study was to investigate the possibility of using photodegradation in treatment methodologies for PCP contaminated soils. Photodegradation on soils with PCP levels at which biodegradation is difficult to achieve, was carried out to reduce the PCP concentrations. Since complete mineralization of PCP by photodegradation would take a long time to occur, the soils were irradiated to reduce PCP concentrations, and were then inoculated with a PCP acclimated mixed culture to degrade the residual PCP. Moreover, PCP photoproducts have been reported to be easily metabolized (Hwang et al., 1986). Thus, PCP contaminated soil or soil wash may first be subjected to photolysis, followed by biodegradation to treat any residual PCP or its photoproducts.

**Materials and methods:**

PCP (99.3% purity) was obtained from Sigma Chemical Company, St. Louis, MO. Methylene chloride, hexane, and isopropanol (analytical grade) were obtained from Fisher Scientific Co. All other chemicals used were of laboratory and/or analytical grade.
A Rayonet photoreactor, Model RPR-208 (Southern New England Ultraviolet Company, Hamden, CT), was used for irradiating the samples with UV light. The reactor was fitted with RUL 3500Å lamps. Eight lamps were used which gave a total output of 100 watts of 'black light phosphor' UV. The core of the reactor was cooled to room temperature with a forced air draft. Glassware used in irradiation experiments, were of borosilicate glass because it does not transmit light of wavelength lower than 300 nm. As sunlight reaching the earth surface does not have light of wavelength lower than 295 nm, the conditions of irradiation were justifiable as pertinent to natural conditions (Lemaire et al., 1982). Light intensity in the center of the reactor was measured by potassium ferrioxalate chemical actinometry (Calvert and Pitts, 1966) and was found to be $1.0497 \times 10^{16}$ quanta/sec which was approximately three times the intensity of sunlight at noon on a sunny day (August 1, 1991) in Columbia, MO.

Aqueous solutions of PCP were made in 0.05N NaOH solution. PCP is slightly soluble in water; with NaOH it forms sodium pentachlorophenate (PCP-Na) which increases it solubility to a great extent (the pKa of PCP @ 25°C is 4.7; at a pH of 6.7 it is almost 99% ionized). pH of solutions were adjusted as required with concentrated sulphuric acid. The solutions were irradiated in capped test tubes filled to the top 0.5cm. The test tubes were placed in a special stand used for irradiation in the reactor.

Soil films, of about 0.5 mm thickness were made by using an aqueous soil-slurry containing 0.7gm of dried soil and spreading it uniformly over a 5.6 cm circular aluminum foil in a glass Petridish. Menfro silt loams, containing 0.36 and 1.38% organic matter were used. The pH of the slurry was 6.5. The slurry was dried to the required moisture content and 1 ml of 2.5 g/L PCP-methylene chloride solution was applied over the film, resulting in a PCP loading of about 3600 mg/Kg of soil. Methylene chloride was allowed to evaporate at room temperature, leaving PCP on the soil. The Petridish was covered and sealed carefully. The sealed cell was placed in the reactor to be irradiated. Similar techniques have been used by Hautala (1978).

Soil samples after irradiation were washed off the aluminum foil with methylene chloride and collected in a centrifuge tube, shaken for at least 2 hours, and centrifuged at 15000 rpm for 20 min. The supernatant was concentrated into 2ml of isopropanol in a rotary evaporator and then
analyzed with a gas chromatograph.

Soil slurries were made using PCP adsorbed soils. 30 gm of soil (Menfro silt loam, 1.38% organic matter), and 150 ml of distilled water was autoclaved in a conical flask at 121°C and under 15 psi pressure for 40 minutes to sterilize the soil. After cooling, pH was adjusted to 7 by adding phosphate buffers (5.8 g/L of KH₂PO₄ and 4.5 g/L of K₂HPO₄). Six milliliters of a 5000 ppm Na-PCP stock solution was added to the flask. The flask was shaken in a rotary shaker at 200 rpm for 48 hr (equilibrium adsorption time was found to be approximately 40 hr). The supernate was then decanted and the soil dried at 30°C. 120 ml of distilled water was added to the soil to make a slurry containing 20% solids. PCP concentration in the slurry was approximately 700 mg PCP/Kg of soil. The slurry was irradiated in the photoreactor in a 250 ml borosilicate glass flask. The flasks were continuously shaken with a wrist shaker throughout the period of irradiation to maintain homogeneity of the slurry. For PCP analysis, slurry samples (4 ml) were extracted with 25 ml hexane for 25 hr. The hexane fraction was then concentrated into 2 ml isopropanol in a rotary evaporator. The extractant was changed from methylene chloride to hexane to prevent contamination of the extracted samples with humic substances, which caused problems with chromatographic analysis.

Soil wash was prepared by shaking the preadsorbed soil with 120 ml of pH 7 buffered water for 48 hr. The slurry was then centrifuged, and the supernate collected. The supernate was irradiated in the photoreactor in flasks similar to that used with the slurries. Soil wash samples were extracted in a similar fashion as the slurry samples.

Aqueous samples were analyzed using GC after extracting a 10 ml sample in methylene chloride. Methylene chloride was then evaporated in a rotary evaporator and then finally concentrated into 2 ml of isopropanol as per EPA method 604 (Federal Register, Oct., 1984). Extraction efficiency for the aqueous, soil slurry, soil wash and soil films were 95% or higher.

A Perkin Elmer, Model 8500 Gas Chromatograph fitted with a NON-PAKD 15m X 0.53 I.D. Altech column with RSL-200 coating, was used. The oven temperature was maintained at 75°C for 2 min., and then increased at a ramp rate of 8°C/min. to 180°C. Helium (15 ml/min) was used as the carrier gas. A flame ionization detector was used.
Some aqueous samples were analyzed also with a Perkin Elmer, Model 250 HPLC to verify the results obtained with GC. Isocratic program was run with acetonitrile/acetic acid (100:1) solution as the mobile phase. Flow rate was 0.5 ml/min. A C-18 reversed phased column was used.

For biodegradation studies, a mixed culture was developed from a sludge sample from the local wastewater treatment plant. The sludge sample was acclimated to a substrate containing glucose and PCP as carbon sources. Glucose level was maintained at 1000 mg/L during the entire acclimation process. PCP level was gradually increased from 10 mg/L to 100 mg/L. The culture showed poor activity in the absence of glucose, indicating that PCP was used as a cometabolite. Biodegradation studies were conducted in a Hach Model 2173B respirometer where oxygen uptake was measured.

COD (Chemical Oxygen Demand) analyses were done by the micro-digestion method. Spectrophotometric measurements were made on a Spectronic 21 spectrophotometer (Milton Roy Company).

Results and Discussion:

Irradiation of soil films. The degradation of PCP in soil films was studied at moisture contents of 20, 40 and 60 % and at two different levels of organic matter in soil (0.36% and 1.38%). After irradiation for 12 hr in the photoreactor, approximately 40% PCP degradation was observed in all three different moisture levels with the low organic matter soil. The results are shown in figure 1. Control experiments where the films were kept in dark in the irradiation chamber, showed less than 5% loss of PCP from low organic matter soils, indicating photolysis as the major cause for PCP disappearance, and volatilization and biodegradation of PCP was insignificant. Earlier, experiments were conducted (not reported here) to evaluate volatilization of PCP from aqueous samples at pH 7.0 at 30°C and 40°C. These experiments showed no volatilization losses of PCP under these experimental conditions. No effect of moisture content on the extent of PCP loss was observed. These results agree well with those of Hautala (1978) who reported no meaningful change in photodegradation of parathion with change in moisture
content. However, the literature concerning the effect of moisture content is very inconclusive. Burkhard and Guth (1979) found that photodegradation losses of the organophosphorous insecticides profenos and diazinon was higher in moist soils (12% moisture content) compared to dry (oven dried soils). Hautala (1978) reported enhanced photodegradation of 2,4-Dichlorophenoxyacetic acid adsorbed on soils when excessive amounts of water was added to form slurries, perhaps due to the solubilization of the compound in the aqueous phase. With the pesticide Sevin, the same author reported decrease in photodegradation rate with increasing moisture content. Thus the effect of moisture content on photodegradation rate appears to be specific for each soil and chemical, and generalizations cannot be made.

Irradiation of films of high organic matter soil, resulted in approximately 30% PCP loss both in the irradiated and 'dark samples' at the three moisture levels. The equal amount of PCP loss in the dark sample eliminated the possibility of photodegradation losses. Repeated experiments confirmed these findings. One possible explanation of this phenomenon is that PCP was irreversibly bound to the organic matter and complexed with humic substances, thus making it unavailable for photolysis as well as extraction. Boyd et al. (1989) have reported such irreversible adsorption due to oxidative coupling or formation of covalent bonds with the long-chain humic acids. Wei (1990) also observed irreversible adsorption of PCP on Menfro silt loam. Similar photodegradation experiments were also carried out on soil form which part of the organic matter was extracted out with 0.5N NaOH. A 30% PCP loss in the irradiated sample and 17% PCP loss was observed in the dark control. This supported the hypothesis that organic matter present in the soil was irreversibly adsorbing PCP and making it unavailable for photolysis.

Dupont et al., (1990) studied the photodegradation of PCP in soil layers (2 to 3 mm thick) under UV and simulated sunlight over a period of 50 days. PCP was one of the compounds in a creosote mixture applied on the soil. They found degradation rates constants ranging from 0.067±0.009/day to 0.007±0.004/day for different soil types and in the presence of different soil amendments. However, none of the amendments (hydrogen peroxide, riboflavin, methylene blue, peat moss) significantly affected the photodegradation rates. Degradation rates varied significantly with different soils. This was attributed to the internal reflectance characteristics of the soils. Color of soil is a physical manifestation of the internal reflectance characteristic of a
solid media. Korfinacher et al., (1980) and Behymer and Hites (1985) concluded that color, or relative internal reflectance affected solid phase photolysis reaction rates.

Photodegradation rates in soil films and slurries are much slower than those in aqueous solutions. This was confirmed from the photodegradation experiments with aqueous PCP solutions. A PCP solution in distilled water, initial concentration approximately 100 mg/L, at pH 6 was irradiated in the photoreactor. About 90% degradation was observed in 2.5 hr (figure 2). Dark controls showed no PCP loss. In comparison, 40% loss of PCP was observed with the 0.36% organic matter soil in 12 hr. This attenuation in photodegradation rate is probably due to the loss of excitation energy of photons to pigments in the soil. Hautala (1978) reported absence of fluorescence of carbaryl when it was adsorbed on soils. A contributing factor may also be the inner filter effect (Yokley et al., 1986). When the chromophores are trapped between soil particles they are shielded from light, although they may be only a soil particle deep from the surface. However, the relative contribution of each process is unknown.

Soils absorb light strongly, and only the fraction of compounds exposed to the radiation undergo direct photolysis. For this reason, the term half life of photolysis in soils is misleading (Miller et al., 1989). Based on this rationale, no attempt was made to calculate the reaction rates on soil.

Since photolysis of PCP in soils with higher organic matter posed a problem, the possibility of treating contaminated soils in slurry batch reactors was evaluated.

Irradiation of soil slurries. Soil slurries were prepared with the higher organic matter soil (1.38% organic matter). PCP was preadsorbed on soil as mentioned before. The initial concentration of PCP in the slurry corresponded to about 600 mg/Kg of soil. Approximately 50% degradation was observed after 12 hr of irradiation. The total PCP and soluble phase PCP concentrations are plotted in figure 3. A plot of total COD and soluble phase COD is shown in figure 4. A constant value of the total COD indicates that no organic carbon loss occurred by volatilization or by biodegradation. An increasing trend in the soluble COD of the irradiated slurry suggests the formation of soluble photoproducts. The slight initial increase in the soluble COD was perhaps due to the desorption of PCP and organic matter of the soil. No PCP loss was
observed in the 'dark' controls. A notable point in the study with soil slurries is that the adsorbed PCP could be photodegraded even in the higher organic matter soil.

Biodegradation of the photolyzed products from soil slurries and soil wash.

Soil slurries, irradiated for 12 hours in the Rayonet photoreactor, with or without supplementation by 500 mg/L glucose, were inoculated with about 1 gm of the mixed culture cells per Kg of soil. An identical 'dark' control (where the soil slurry was covered with aluminium foil during irradiation) was also run. The plot of oxygen uptake with time is shown in figure 5. Higher oxygen uptake was observed in the irradiated slurry compared to that in the 'dark' slurry, indicating that the PCP as well as its photoproducts were easily metabolized. In the absence of glucose, considerably little oxygen uptake was observed in both the irradiated and the dark samples (figure 5), indicating that even the photoproducts were utilized by the cells as cometabolites.

The concentrations of PCP over the 12 hour irradiation period and the subsequent biodegradation phase are presented in figure 6. While irradiation resulted in the usual 50% loss in the concentration of PCP, notable PCP reduction was observed after the 12 hr irradiation period in the slurry system containing glucose. The initial PCP concentration of 720 mg/Kg soil was reduced to 350 mg/Kg soil at the end of the irradiation period. Subsequent biodegradation for 50 hr resulted in a PCP concentration of 60 mg/Kg soil. In the irradiated slurry where no glucose was added, very little PCP reduction was observed during the biodegradation period. This further indicates that the culture degraded PCP only as a cometabolite. Both the 'dark' slurries showed little change in PCP concentration. The reduced rates of oxygen uptake in the 'dark' slurry with glucose indicates that the cells were inhibited by PCP concentrations present in this system.

An identical experiment was carried out with irradiated and 'dark' soil wash solutions (prepared by equilibrating PCP contaminated soil with buffered water, pH = 7.0). PCP concentrations in the soil wash was significantly lower, as expected, as only a part of the adsorbed PCP was mobilized into the aqueous phase. The oxygen uptake values are shown in figure 7. The difference in the oxygen uptake was approximately 60 mg/L with or without the presence of
glucose. The corresponding PCP concentrations are shown in figure 8. PCP levels decreased to less than 5 mg/L, from the 17.5 mg/L initially present, during the biodegradation phase in the irradiated soil wash containing glucose. PCP level in the dark sample decreased from 52.5 mg/L to 29 mg/L, indicating that the culture metabolized PCP below its acclimation level, in the presence of glucose. In the absence of glucose, PCP was degraded in the irradiated sample. It is likely that the culture could degrade PCP when present in very low levels, in the absence of glucose. Some oxygen uptake occurred in the 'dark' slurry and in the soil wash, in the absence of glucose, probably because the culture metabolized a small fraction of the soluble organic matter extracted from soil. This could not be pursued further since radioactively -tagged PCP was not used in these experiments. Abiotic controls showed no oxygen uptake, nor any significant PCP loss.

**Conclusions:**

Although, photodegradation of PCP in soil systems are considerably slower than in aqueous phase, it is significant. High concentrations of PCP show potential toxicity even to acclimated stains of microorganisms. Photodegradation being an abiotic process, can be applicable to any levels of contamination. Photodegradation can reduce the potentially toxic levels of PCP to levels which can be easily bioremediated. Moreover, the photoproducts formed have been shown to be easily assimilable by PCP acclimated cultures, thus facilitating total decontamination.

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Administrative review and, therefore, may not necessarily reflect the views of the Agency and no official endorsement should be inferred.

Mass spectral determinations were performed by the University of Missouri-Columbia Center for Mass Spectrometry, supported by the National Science Foundation under Grant No. PCM-8117116.

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FIG. 1  PCP DEGRADATION IN SOIL FILMS AT DIFFERENT MOISTURE CONTENTS (LOW ORGANIC MATTER SOIL)

FIG. 2  AQUEOUS PCP SOLUTION PHOTODEGRADATION KINETICS AT pH 6.0

First order rate constant, 
\[ k = 1.128/\text{hr.} \]
FIG. 3  KINETICS OF PCP PHOTODEGRADATION IN SOIL SLURRY
FIG. 4a  TOTAL COD VS. TIME FOR IRRADIATED
AND DARK SOIL SLURRY

FIG. 4b  COD OF SOLUBLE PHASE IN IRRADIATED
AND DARK SOIL SLURRY
FIG. 7 OXYGEN UPTAKE IN IRRADIATED AND DARK SOIL WASH

FIG. 8 PCP CONCENTRATIONS IN PHOTO-BIO DEGRADATION OF SOIL WASH
FIG. 5 OXYGEN UPTAKE IN IRRADIATED AND DARK SOIL SLURRY

FIG. 6 PCP CONCENTRATIONS IN PHOTO-BIODEGRADATION OF SOIL SLURRY
Practical Considerations for Implementation of a Field Scale 
In-situ Bioremediation Project 

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SUMMARY 

Bioremediation projects often require a unique approach. Project management, site 
characterization, treatability studies and field application all require activities that are 
vital to successful implementation. The paper examines staffing, budget, schedule, 
project objectives and other management considerations. Site characterization data 
requirements necessary to adequately model the dynamic chemical and physical 
situation at the site are detailed. The need for treatability studies is examined along 
with a close look at treatability study objectives. Implementation obstacles are 
examined, including nutrient and oxygen availability, and transport limitations. 

INTRODUCTION 

There has been a great deal of discussion recently regarding the effectiveness of 
traditional extraction methods for remediation of contaminated ground water. Much of 
this discussion was sparked by the release of three reports, two from the Environmental 
Protection Agency (EPA), and one from the Office of Technology Assessment (OTA). 
The EPA reports are titled "Performance Evaluations of Pump-and-Treat 
Remediations" and "Evaluation of Ground Water Extraction Remedies." The OTA 
report is titled "Cleanups and Cleanup Technology." As a result of these three 
reports, the perceived effectiveness of extraction remedies for ground water 
remediation is questionable. Bioremediation has been suggested as an alternative to 
pump and treat technology for remediation of sites with contaminated ground water. 
This paper examines the practical problems of implementing bioremediation and 
analyzes some of the transport processes that control the rate and limit its effectiveness. 

PROJECT MANAGEMENT 

The first challenge in completing a successful bioremediation project is management 
philosophy. Management of a bioremediation project is not fundamentally different 
from managing any other engineering project, but some unique factors must be
considered. When project planning incorporates appropriate consideration of these details, the project should proceed smoothly.

In any remediation project, source control should be a high priority. Removing organic contamination from soil is a difficult and expensive process. Allowing sources to continue to contaminate soil and water only increases the difficulty and the cost. Source control measures should, therefore, be implemented expeditiously. Possible source control measures include removal of drums or underground storage tanks, dredging of sludge ponds or trenches, and removal of organic floaters on the water table or sinkers pooled on the surface of lower confining units.

A team effort is needed to investigate and remediate a contaminated site. For example, the members of a team could include a soil scientist such as a soil chemist or soil microbiologist, a civil engineer with specialization in hydrogeology and ground water flow, a geologist with experience in site characterization, and a biochemical engineer. Each of these specialists provides necessary expertise in completing a successful bioremediation project. In-situ bioremediation involves the dynamic interaction of ground water, geologic formations, microbial life, and man-made chemicals not ubiquitous to the environment. It is, therefore, not surprising that the successful bioremediation project team is competent in the areas of hydrogeology, soil and water chemistry, microbiology, transport phenomena, and traditional engineering.

Bioremediation project managers must be ready to adjust traditional budget and scheduling requirements. Since considerably more information is necessary to adequately design an in-situ remediation scheme than a traditional excavation, the feasibility study and design of the project will require more time and money. Most costs and delays will come at the beginning of the project (during the site characterization and feasibility study phases) as opposed to the end of the project. Project managers must have the foresight to correctly evaluate the potential for successful application of bioremediation at a particular site. Once this potential has been established, the decision maker must decide that it is appropriate to spend more money at the inception of the project for a savings to be realized in the long term.

Bioremediation projects will require many parameters that are not examined in detail for more traditional remediation schemes. Some of this information gathering would benefit other remediation projects as well. These data requirements will be discussed in detail in the next section.

Cost and schedule will not only be affected by the additional data gathered, but also by the structure of the data gathering phase. Since many parameters vary seasonally, the data gathering phase probably should continue for a minimum of one year. Understanding the dynamics of the ground water and contaminant behavior is critical to proper design of a ground water system. For example, if the primary contaminant is a floater, the material will have saturated the soil matrix between the high and low points of the water table. This can be confirmed when pollutant concentrations rise and
fall with the water table, or by careful core sampling. If only one sampling event is
scheduled, the project team will be unable to screen injection/extraction wells at the
proper depth or artificially maintain the water table at optimum depth. Without a
commitment of adequate budget and schedule, a bioremediation project may fail,
because not enough effort was allocated in the data gathering phase.

DATA OBJECTIVES

The project team should meet at the beginning of the project to thoroughly discuss the
data quality objectives. If some members of the project team are excluded, vital
information may be overlooked. This will cause unnecessary delays and cost
overruns. All team members must be included up front in the data gathering process.

In order for the decision maker to commit adequate resources and time to a potential
bioremediation project, it may be necessary to provide an initial screening study. This
project can be limited in scope, but generally should include: the type and amounts of
compounds likely to be involved; a relative ranking of their biodegradation potential;
an understanding of regional (local) ground water flow and geological formation; and
possibly soil and water samples for bench scale tests. This screening process may or
may not involve actual field work depending upon the types and amount of regional
information that is available.

The traditional site characteristics will be needed, including an understanding of the
chemical pollutants present in the soil and ground water, and the extent of their
distribution. This will include hydrogeological investigations to learn the
characteristics of aquifers, including their flow and matrix characteristics. This type
of information is commonly gathered for hazardous waste site characterization and is
useful in planning a bioremediation project as well. The various methods available for
gathering this data include a wide variety of intrusive and non-intrusive techniques.
Selection of individual investigative techniques goes beyond the scope of this paper, but
the project team should give careful consideration to investigative methods.

Various other data essential to a properly designed bioremediation project are often
overlooked during site characterization. Soil chemistry, water chemistry, fate and
transport, physical soil properties and seasonal variations all play an important role in
in-situ remediation design. If organic contaminants strongly adsorb to the surface of
soil particles, the remediation scheme must take this fact into account.

Soil physical properties, including hydraulic conductivity, permeability, bulk density
and porosity, are commonly determined at hazardous waste sites. Additional
parameters include heterogeneity, moisture content, and morphology.

Water chemistry plays a similar role. Beyond understanding the contaminants' volatilite,
solubility, and diffusivity, one also must consider the effects of pH, total
dissolved solids, BOD, COD, dissolved oxygen, dissolved nutrients, and total organic
carbon.
Knowledge of soil and water chemistry along with the chemistry of the individual compounds will assist in understanding the fate and transport of the contaminants through the environment. In the natural environment, the contaminants will undergo many natural chemical and physical processes that control where a compound goes, and in what form. It is a priority to understand the phase distribution of each contaminant. A compound may exist in a variety of phases: sorbed onto the solid soil particles; a free phase organic liquid; dissolved into the aqueous phase; and as a vapor in the vadose zone. Second, the project team must decide which physical (transport) processes control contaminant movement. If a compound is highly soluble, it probably will move with ground water. If sorption is prevalent, the compound will bind to the soil. If diffusion is the primary driving force, the compound probably will migrate slowly.

Seasonal variations play an important role in designing a bioremediation project. Water table and ground water flow patterns may change significantly over the course of a year. If these fluctuations are not adequately defined and carefully monitored, the design may prove faulty. Depending upon the size of the project, ground water elevations probably should be taken monthly from wells screened at different depths and locations across the site. Ground water sampling probably should occur at least quarterly during the data gathering phase.

All of this data gathering will help the project team in performing an accurate mass balance on the contaminants. The data will suggest in what phase and at what location the contaminants tend to concentrate, and during which seasons. This information is all required for proper design.

It may be necessary before actual field implementation to include bench or pilot studies to determine the actual effectiveness of the remedy. Good cost and performance data are presently not available on in-situ bioremediation projects. This may cause difficulty in implementing the remedy.

**THEORY**

To better understand the rate limiting aspects of in-situ bioremediation, a single pore model was developed. For simplicity this model considers a cylindrical pore filled with a hydrocarbon substrate. The conceptual model is shown in Figure 1.

Several important assumptions made in the model are detailed as follows:

1. Microbial growth is limited by the oxygen diffusing through the stagnant water in the pore. It is always in pseudo steady-state.

2. The microbial mass at the interface is responsible for the remediation and does not affect oxygen diffusion.
3. The substrate is consumed at the interface; thus, it does not diffuse out of the cylindrical pore.

4. All surface effects are neglected.

Fick's Law for diffusion of the oxygen yields the rate of oxygen delivery to the oil/water interface:

\[ R_O = \frac{D_O C_O \pi r^2}{z} \]  

(1)

The rate of accumulation of biomass is given by:

\[ \frac{dX}{dt} = \left( \frac{D_O C_O \pi r^2}{z} \right) Y_O \]  

(2)

The rate of hydrocarbon biodegradation is given by:

\[ \rho_s \frac{dz}{dt} = \left( \frac{D_O C_O}{z} \right) \frac{Y_O}{Y_s} \]  

(3)

Equation (3) is variable separable and gives the time required for complete remediation:

\[ t = \frac{\rho_s Y_s z^2}{2D_O C_O Y_O} \]  

(4)

Following a similar vein of logic, the time required for pump and treat remediation through hydrocarbon diffusion out of the same pore volume can be described by the following equation:

\[ t = \frac{\rho_s z^2}{2D_s C_s} \]  

(5)

Endogenous metabolism can be included in the model with first order kinetics of the form:

\[ R_{em} = k_d X \]  

(6)

The rate of oxygen consumption for endogenous metabolism is:

\[ R_{oem} = \frac{8k_d X \sigma_b \gamma_b}{12} \]  

(7)
Subtracting equation (7) from equation (1) gives the amount of oxygen available for growth:

\[ R_{\text{growth}} = \frac{D_0 C_0 \pi r^2}{z} - \frac{8 k_d X \sigma_b \gamma_b}{12} \]  

(8)

Since growth is assumed to be oxygen limited, the net biomass growth rate is found from equation (8) and the growth yield on oxygen less the mass lost to endogenous metabolism.

\[ \frac{dX}{dt} = \left( \frac{D_0 C_0 \pi r^2}{z} - \frac{8 k_d X \sigma_b \gamma_b}{12} \right) Y_o - k_d X \]  

(9)

The rate of hydrocarbon degradation is given by:

\[ \rho_s \pi r^2 \frac{dz}{dt} = \left( \frac{D_0 C_0 \pi r^2}{z} - \frac{8 k_d X \sigma_b \gamma_b}{12} \right) \frac{Y_o}{Y_s} \]  

(10)

The variables may be put into dimensionless form through the following substitutions:

\[ \xi = \frac{z}{r} \quad \tau = \frac{t D_0}{r^2} \quad M = \frac{X}{X_0} \quad \text{where} \quad X_0 = \frac{12 C_0 \pi r^3}{8 \sigma_b \gamma_b} \]

This will result in the following dimensionless equations:

\[ \frac{dM}{d\tau} = \left( \frac{1}{\xi} - \phi^2 M \right) \frac{1}{W_2} - \phi^2 M \]  

(11)

\[ \frac{df}{d\tau} = \left( \frac{1}{\xi} - \phi^2 M \right) \frac{1}{W_1} \]  

(12)

The dimensionless groups are the Thiele Modulus \( \phi^2 \), the oxygen supply factor \( W_1 \) discussed by Wu et al.4, and the dimensionless yield factor \( W_2 \); that is,

\[ \phi^2 = \frac{k_d r^2}{D_0} \quad W_1 = \frac{Y_s \sigma_s}{Y_o \sigma_o} \quad \text{and} \quad W_2 = \frac{X_0}{Y_o C_0 \pi r^3} = \frac{12}{8 Y_o \sigma_b \gamma_b} \]

RESULTS

Equation (4) was evaluated and compared to the results given by equation (5) for pump and treat remediation and by equations (9) and (10) for bioremediation with endogenous metabolism. The results are shown in Table 1 and Figure 2. For equations (4), (9), and (10), the following values were used:
\( \rho_s = 0.775 \text{ g/ml} \), \( \tau^2 = 0.01 \text{ cm}^2 \), \( D_O = 2.5 \times 10^{-5} \text{ cm}^2/\text{s} \), \( C_O = 7.6 \times 10^{-6} \text{ g/ml} \)

\( Y_O = 0.466 \), \( Y_S = 1.00 \), \( \sigma_b = 0.462 \), \( \gamma_b = 4.291 \)

\( k_d = 2.78 \times 10^{-7} \text{ s}^{-1} \), \( z(0) = 0.001 \text{ cm} \), and \( X(0) = 3 \times 10^{-6} \text{ g} \)

For equation (5), octane was used as a substrate. The following values were used:

\( \rho_s = 0.703 \text{ g/ml} \), \( D_S = 7.0 \times 10^{-6} \text{ cm}^2/\text{s} \), and \( C_S = 2.0 \times 10^{-6} \text{ g/ml} \). Figure 2 is a plot of the position of the hydrocarbon interface, which is a measure of the amount of hydrocarbon removed from the pore vs. time. The quantity of biomass for the case with endogenous metabolism is also shown.

Equations (11) and (12) were solved for various values of \( \phi^2 \) using the Runge Kutta technique. The results are shown in Figure 3.

**DISCUSSION**

The results in Figure 2 give the calculated remediation times associated with the three models. Curve 1 shows the calculated time for extraction of octane based on diffusion out of the pore. Octane was chosen due to its higher solubility (than longer chain hydrocarbons) in the aqueous phase. The extraction model is sensitive to the solubility of the hydrocarbon. Less soluble hydrocarbons (decane) show almost no removal over the first 250 days. Curves 2 and 3 are the bioremediation results for oxygen limited microbial growth at the interface with and without endogenous metabolism, respectively. Curve 4 shows that the quantity of biomass passes through a maximum and then decreases because the rate with which oxygen is supplied to the interface depends on the square of the distance.

The bioremediation models are not as sensitive to hydrocarbon solubility, and for contaminants less soluble than octane, should allow remediation to be completed in a much more rapid time-frame. For the data used in this analysis, removal of the hydrocarbon proceeded at a moderate pace. The problems of delivering enough oxygen to the oil/water interface is the limiting factor here. Diffusion and the low solubility of oxygen in water are the time controlling factors. Aqueous solubility of oxygen becomes less of a factor in the vadose zone; however, pores of the type considered here may be found in the vadose zone near the water table.

The effect of the Thiele modulus on biomass production is clearly seen in Figure 3. However, from the values selected, remediation time does not appear to be affected significantly by the Thiele modulus. The oxygen supply factor \( (W_1) \) and the dimensionless yield factor \( (W_2) \) for this model should not vary greatly in the field, and therefore, a sensitivity analysis was not done on these factors.
CONCLUSIONS

The simple models used in this paper demonstrate some of the complex transport problems that are critical to successful application of in-situ bioremediation. The importance of understanding these concepts for successful project management is clear. In any event, depending upon the nature of a particular site and the compounds present, complete remediation can take a very long time.

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Although the research described in this article has been funded in part by the U.S. Environmental Protection Agency under assistance agreement R-815709 to the Hazardous Substance Research Center for U.S. EPA Regions 7 & 8 with headquarters at Kansas State University, it has not been subjected to the Agency's peer and administrative review and therefore may not necessarily reflect the views of the Agency and no official endorsement should be inferred. This research was partially supported by the Kansas State University Center for Hazardous Substance Research.

REFERENCES


Table 1. Time required to remediate hydrocarbon phase trapped within a pore.

<table>
<thead>
<tr>
<th>Depth of Hydrocarbon Removed (cm)</th>
<th>Pump &amp; Treat (Octane)</th>
<th>Bioremediation</th>
<th>Bioremediation with Endogenous Metabolism</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.01</td>
<td>120 days</td>
<td>5 days</td>
<td>4 days</td>
</tr>
<tr>
<td>0.05</td>
<td>2 years</td>
<td>120 days</td>
<td>210 days</td>
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<tr>
<td>0.10</td>
<td>8 years</td>
<td>1.4 years</td>
<td>2.3 years</td>
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<td>0.50</td>
<td>200 years</td>
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<td>56 years</td>
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<tr>
<td>1.00</td>
<td>800 years</td>
<td>140 years</td>
<td>225 years</td>
</tr>
</tbody>
</table>

Figure 1. Soil Pore Filled with Hydrocarbon.
Figure 2. Remediation Time in a Pore filled with Hydrocarbon, (1) Extraction of Octane, equation 5; (2) Bioremediation with endogenous metabolism; (3) Bioremediation, equation 4; (4) Biomass with endogenous metabolism.

Figure 3. The Effect of the Thiele Modulus on Remediation Times. \( \phi^2 = 1 \times 10^{-4} \) (1) Biomass, (A) Depth, \( \phi^2 = 1 \times 10^{-5} \); (2) Biomass; (B) Depth, \( \phi^2 = 1 \times 10^{-6} \); (3) Biomass; (C) Depth: For all curves, \( W_1 = 200,000 \) and \( W_2 = 1000 \).
PARAMETRIC SENSITIVITY STUDIES OF
*Rhizopus Oligosporus* SOLID SUBSTRATE FERMENTATION

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SUMMARY

In this work the effects of environmental parameters on the performance of solid substrate fermentation (SSF) for protein production are studied. These parameters are: (i) Air flow rate, (ii) The inlet air relative humidity, (iii) The inlet air temperature, (iv) The heat transfer coefficient between the outer wall of the fermenter and the air in the incubator. The air flow is supplied to effect cooling of the fermented mass by evaporation of water. A dynamic model is developed, which permits estimation of biomass content, total dry matter, moisture content and temperature of the fermented matter. The model includes the effect of temperature and moisture content on both the maximum specific growth rate and the maximum attainable biomass content. Uniformity of conditions is assumed throughout the reactor volume.

The results of the simulation are compared with actual experimental data and show good agreement with them. The most important conclusions are: (i) The evaporative cooling of the biomass is very effective for temperature control, (ii) The effect of lowering the inlet air temperature is similar to that of decreasing the air relative humidity, (iii) The air flow rate and the heat transfer coefficient have strong effects, but they affect the biomass morphology and are not controllable easily. The ultimate goal is to implement the determined effects of the environmental parameters on the SSF biomass production and the temperature and moisture variation profiles to effectively control the SSF and optimize the biomass production.

INTRODUCTION

In solid substrate fermentation (SSF) the microbial growth and product formation occur on the surface of solid material without the presence of excess water. SSF is not widely used because: (i) The types of substrate are limited, (ii) Substrates are inhomogeneous throughout the fermentation time, (iii) Microbial heat generation and heat transfer limitations cause heat build-up, (iv) It is difficult to measure parameters such as pH, water content, DO,
cell content and substrate concentration in the solid state. The SSF process still has the advantages of superior productivity, simpler techniques, reduced energy requirements, low waste water output and improved product recovery [4, 3, 11]. The greater limitation is the difficulty of the heat removal, due primarily to the low thermal conductivity of the fermented matter.

Several conventional methods have been tried to remove excess heat [10, 8, 5, 6]. Convective heat transfer from the reactor wall has limited effects due to the internal heat transfer limitation. The method of evaporative cooling with inserting partially saturated air at a lower temperature is the most promising, provided that an effective system of uniform aeration exists. The air flow causes variation of water content profile and therefore it has an effect on biomass production and substrate consumption. The need of an adequate model, which can predict the effects of environmental parameters on SSF is evident. A model adapted from [9] and modified to include varying substrate content with time was used in this study. The influences of changing environmental parameters such as air flow rate, air relative humidity, air inlet temperature and overall heat transfer coefficient were determined. The overall heat transfer coefficient was used as an estimation of the heat transfer between the fermented matter and the incubator.

Within the limitations of the model an understanding of the behavior of the system and the trends of various control actions will be helpful before a generalized estimation of the parameters changing with time is applied. The results of the simulation, whenever possible, are compared with experimental data baring in mind that deviations from reality might be expected, as physiological factors, such as morphology cannot be quantified in the model.

**MATERIALS AND METHODS**

The microorganism used was *Rhizopus oligosporus* (NRRL 2710). Flour-free yellow corn grit (# 25 mesh particle size) was used as the substrate. The mineral medium used had the following composition (in g/100 ml): (NH₄)₂SO₄, 7.59; urea, 4.09; NaH₂PO₄, 1.726; MgSO₄•7H₂O, 0.059; KCl, 0.159; CaCl₂, 0.059; FeSO₄•7H₂O, 0.137; alanine, 0.045. 400 g of substrate were soaked for 3–4 h with 400 ml of mineral medium. The wet material was cooked for 40 min at 110°C in an autoclave to form a sticky dough. This material was kept in a refrigerator at 4°C for 3 h. Then it was crunched and sieved through a #25 screen to obtain granular homogeneous substrate. The granular material was adjusted to 40–45% moisture content with tap water pH 3.5 and reautoclaved for 3 min at 110°C to evenly distribute water within the substrate. The substrate was inoculated on a 10% w/w basis to compensate for the nonsterile condition of the SSF. The fermentations took place in a rocking reactor made of acrylic plastic.

The rocking vessel has inside and outside voids to provide uniform water supply and aeration. Air and water were supplied from the inner void part through fine holes. Gravity causes the water to permeate through then substrate. The reactor was twisting via a forward-backward rotation sequence. Temperature of the incubator and the inlet air was controlled by an independent on-off loop, using an electrical heater along with a fan and a heated water bath respectively. Total air flow rate and air relative humidity can be controlled by splitting the air in two streams, one of which is totally humidified and the other is dried by
passing through a silica gel column. Two mass flow meters controlled by a computer were established. The total air flow can be controlled as the sum of the dry and the wet air flow rate and the relative humidity from the wet air flow rate.

**Analytical methods**

Samples from the reactor were taken in a radial direction with a cork borer. An 1 g portion of the sample was diluted with 20 ml of 0.5% (w/v) NaCl solution and mixed in a shaker. This diluted sample was centrifuged at 3000 rpm for 10 min to separate the liquid from the solids. The liquid was removed and used for the analysis of sugar and pH. Glucose and reducing sugar were measured by a glucose analyser (Yellow Springs Instruments, Model 27) and the DNSA method, respectively. Water content of the wet sample was determined by a gravimetric method based on the weight loss after placing samples in an oven at 105°C for 24 h. True protein concentration was determined by the micro-Kjeldahl method after treating the sample with 10-15 ml of 30% trichloroacetic acid (TCA) in a vortex-mixer to precipitate the protein nitrogen [9].

**MODEL**

The dynamic behavior of the SSF system is described by unsteady state energy and material balances. For the cell growth a logistic type of model was used [7]. In this model two parameters are used, the maximum specific growth rate \( \mu_M \), and the maximum attainable cell content \( x_M \). The equation for substrate consumption includes catabolism and anabolism terms. The substrate is treated as dry matter, which includes both substrate and biomass. The substrate consists mostly of starch and its consumption for maintenance and growth is modelled using simple stoichiometric formulas for anabolism and catabolism respectively, approximating the substrate as consisting of starch and NH\(_3\) as nitrogen source. The stoichiometric coefficients were determined using CO\(_2\) evolution data and material balances of H, C, O and N. The molecular formula of the dry biomass was CH\(_{1.78}\)O\(_{0.63}\)N\(_{0.079}\) [1].

The equation for the variation of moisture content includes the water production for maintenance and growth as well as the water exchange with the inlet air. Steady state of air flow and saturation of the air at the exit temperature are assumed. These assumptions are reasonable as the air residence time in the reactor is less than 1 min and good conduct of air with the solid substrate is achieved. The exiting air is also assumed to be in thermal equilibrium with the fermented matter at any time. The thermal balance takes into account the evaporative heat loss and and the heat transfer with the air in the incubator. In all equations homogeneity of conditions (substrate composition, water content, biomass content and temperature) is assumed. Basic modifications from the Ryoo's model [9] are: (i) The inclusion of the contributions from the variation of dry matter in the equations (2) to (4), (ii) The calculation of the humidity and enthalpy variations of the air in terms of the dry air. (iii) The effect of the variable heat capacity \( c_P \) was taken into account in the thermal balance equation. The model equations are:

\[
\frac{dx_1}{dt} = \mu_M \left(1 - \frac{x_1}{x_M}\right) x_1
\]
\[
\frac{dx_2}{dt} = \frac{Y_{S/X}}{1 - Y_{S/X}} x_1 x_2 + m x_1 \quad (2)
\]

\[
\frac{dx_3}{dt} = \{ F(H_{in} - H_{out}) + Y_{H_2O/X} \left( x_1 \frac{dx_2}{dt} + x_2 \frac{dx_1}{dt} \right) \\
+ m_{H_2O} x_1 x_2 - x_3 \frac{dx_2}{dt} \} / x_2 \quad (3)
\]

\[
\frac{dx_4}{dt} = \{ hA(x_4 - T_{sur}) + F(E_{in} - E_{out}) + Y_{Q/X} \left( x_1 \frac{dx_2}{dt} + x_2 \frac{dx_1}{dt} \right) + m Q x_1 x_2 \\\n- x_4 x_3 c_p \frac{dx_3}{dt} \} / \{ x_2 (c_p + c_p x_3) \} - \frac{x_4}{x_2} \frac{dx_2}{dt} \quad (4)
\]

**Model parameters**

- \( hA \): Overall heat transfer coefficient (Kcal/h°C)
- \( T_{sur} \): Temperature in the incubator (°C)
- \( E_{in} \): Enthalpy of the inlet air (Kcal/gr dry air)
- \( E_{out} \): Enthalpy of the outlet air (Kcal/gr dry air)
- \( Y_{Q/X} \): Energy yield coefficient (Kcal/gr biomass)
- \( m_Q \): Energy maintenance coefficient (Kcal/gr biomass·h)
- \( x_1 \): Biomass content (gr biomass/gr dry matter)
- \( x_2 \): Total dry matter (gr)
- \( x_3 \): Moisture content (gr water/gr dry matter)
- \( x_4 \): Temperature of the fermented matter (°C)
- \( \mu_M \): Maximum biomass specific growth rate (h⁻¹)
- \( x_M \): Maximum attainable biomass content (gr biomass/gr dry matter)
- \( H_{in} \): Absolute humidity of the inlet air (gr water/gr dry air)
- \( H_{out} \): Absolute humidity of the outlet air (gr water/gr dry air)
- \( Y_{H_2O} \): Yield coefficient for water (gr water/gr biomass)
- \( m_{H_2O} \): Maintenance coefficient for water (gr water/gr biomass·h)
- \( F \): Dry air flow rate (gr air/h)
- \( m \): Maintenance coefficient (gr dry matter/gr biomass·h)
- \( Y_{S/X} \): Yield coefficient (gr dry matter/gr biomass)
- \( c_p \): Average heat capacity of the fermented matter (Kcal/gr°C)

The calculation of the absolute humidity of the air was made by taking an Antoine expression for the partial pressure of water in the air and taking total pressure of 676.2 mmHg—due to the elevation. The air flow rate measured in lt/min of humidified air is converted to (gr dry air/h) presuming ideal gas behavior. Also the pressure of the air is supposed to be constant. The variation of \( \mu_M \) and \( x_M \) with temperature and moisture content is given by:

\[
\mu_M = \sqrt{\mu_M T \mu_M W} \quad (5)
\]

\[
x_M = \sqrt{x_M T x_M W} \quad (6)
\]

where subscripts \( T \) and \( W \) denote temperature and moisture dependence respectively. \( \mu_M W \)
and \( x_{MW} \) are expressed as polynomials with respect to moisture content, while \( \mu_{MT} \) and \( x_{MT} \) are expressed as exponentials with respect to inverse absolute temperature \( T^{-1} \). \( \mu_{MT} \) increases with \( T \), till \( T=37^\circ C \), where it gets its maximum value. After this point \( \mu_{MT} \) decreases with \( T \). Analogously behaves \( x_{MT} \), and the maximum occurs at \( T=39^\circ C \). At a temperature of \( 43^\circ C \) the fungal biomass is assumed to have a zero maximum specific growth rate. Thus this temperature is supposed to be lethal for the fungi. Therefore, once the biomass reaches \( 43^\circ C \) it dies out, and although temperature drops, the biomass doesn't grow any more. Also, the maintenance coefficients for heat and water are set to follow an exponential decrease after that thermal shock in an attempt to simulate the gradually dropping temperature profile after 17 h, measured experimentally in an uncontrolled fermentation. No effect of substrate on specific growth rate is considered. In other words it is assumed that no substrate constituent becomes limiting during the course of the fermentation.

The heat capacity of the fermented matter is calculated as a weighted average of the heat capacity of dry matter and the heat capacity of the water:

\[
c_p = \frac{c_{p_1} + c_{p_2}x_3}{1 + x_3}
\]  

(7)

where \( c_{p_1} \) and \( c_{p_2} \) are the specific heats of the dry matter (substrate and biomass) and water respectively—considered as constants. Finally, for the simulation study the parameter \( h_A \) was set equal to 1.6902 Kcal/h\(^\circ C\). This value is calculated as a combination of the conductive resistance of the outer wall of the fermenter and a convective heat transfer coefficient for free convection between the reactor wall and the air in the incubator:

\[
\frac{1}{h_A} = \frac{1}{h_{out}A} + \frac{\Delta x}{kA}
\]

where \( h_{out} \) : Convective heat transfer coefficient of the outer wall (Kcal/hm\(^2\)\(^\circ C\)), \( A \) : Outer wall surface area, \( \Delta x \) : Thickness of the outer wall of the fermenter (m\(^2\)) and \( k \) : Thermal conductivity of the fermenter outer wall (Kcal/h \cdot m\(^\circ C\)).

When \( h_{out} \) is within the limits of free convection, the overall \( h_A \) varies between 0.4541 and 2.8385 Kcal/h\(^\circ C\). In the simulation \( h_A \) is varied between 0.8451 and 4.2255 Kcal/h\(^\circ C\). As shown in Figure 2 the employment of high \( h_A \) values doesn't seem to further improve biomass production.

**RESULTS AND DISCUSSION**

The system equations were integrated for 50 h fermentation time using the following set of initial conditions for the variables \( x_1, \ldots, x_4 \): \( x_1 = 0.007, x_2 = 340, x_3 = 1.5, x_4 = 37 \), and each time varying one of the environmental parameters from their basis values: \( F = 7 \) lt/min, \( h_A = 1.6902 \) Kcal/h\(^\circ C\), \( RH \) (relative humidity of inlet air) = 100\% , \( T_{inlet} = 37^\circ C \). The relative humidity doesn't show up in the model equations, but is used to calculate the enthalpy and absolute humidity of the inlet air. The following variations in the environmental parameters were studied:

- \( F \) : 2, 3, 7, 13 lt/min
- \( h_A \) : 0.4541, 1.6902, 2.1128, and 4.2255 Kcal/h\(^\circ C\)
The effects of varying the environmental parameters on the biomass content, $x_1$, are shown in Figure 2 for $F$, in Figure 3 for $hA$, in Figure 4 for $RH$ and in Figure 5 for $T_{inlet}$. In Figure 6 the assumption of exponentially decaying maintenance coefficients for energy and water -after the presumed death of the microorganisms- is applied in an attempt to simulate the experimentally determined temperature profile of an uncontrolled fermentation.

The comparison of the simulated biomass content and total biomass profiles at $37^\circ C$ and $60\%$ moisture content shows that the biomass production can be predicted reasonably well by the model. The substrate consumption estimate is, however conservative. This can be attributed to the assumption that the substrate was homogeneous. For the fermentations at $41^\circ C$ and $60\%$ moisture content the predictions are better. This shows that the predictions are sensitive to the accuracy of determining the various constant coefficients and the success of the temperature and moisture content dependence representation for $x_M$ and $\mu_M$.

The basic observations on the behavior of the SSF system in changes of environmental conditions are:

1. The effect of decreasing the air inlet temperature is similar to the effect of decreasing its relative humidity. This implies that the sensible heat effect, produced by temperature difference between the air and the fermented matter is very small compared to the efficiency of heat removal by evaporation of water.

2. The biomass production shows a maximum at a specific relative humidity or temperature (Figures 10 and 12). Decreasing $T_{inlet}$ there is a drastic increase in biomass production because the biomass temperature doesn’t reach the supposedly deadly temperature of $43^\circ C$. Further reduction of $T_{inlet}$ from the optimum effects cooling better, but adversely affects biomass production. This is due to the decrease of the moisture content to values where both $x_M$ and $\mu_M$ have lower values. The reduction of $RH$ produces the similar results.

3. The increase in air flow rate produces a smoother effect on biomass production (Figure 2). The SSF performance improves at high air flow rates, but (i) the biomass production doesn’t get very high because there is a drop in water content and (ii) the effect of the high air flow rate on the physiological factors of the culture has not been considered.

4. Increasing the overall heat transfer coefficient also improves biomass production and it seems to be a better way of controlling the process combined with saturated air flow (Figure 3). This result is in question because the moisture content rapidly increases with increasing $hA$, and $x_{MW}$ extrapolated to such high moisture contents has higher values. The increase of $hA$ beyond the limits of free external convection doesn’t noticeably improve the total biomass production, though it delays the appearance of the maximum. Furthermore an increasing $hA$ cannot be reliably used as the overall effect of heat transfer coefficients as it doesn’t include the heat transfer resistance of the fermented matter.

5. The temperature and moisture content dependence of $\mu_M$ and $x_M$ is affecting the SSF because both temperature and moisture are allowed to vary. Thus, any uncertainties
in the estimation of these parameters can cause uncertainty in the prediction of the model.

The simulated temperature profile agrees with the experimental results at late stages of the fermentation as can be seen in Figure 6. Thus, the assumption of exponential decline of maintenance coefficient for heat and water production after the lethal shock of the biomass is well justified. Another possibility would be to set an exponential decline of $\mu_M$ at the same conditions. The rapid decline of temperature after about 17 h of fermentation may be also be explained by the existence of large temperature gradients inside the fermented matter that cause decline in the metabolic activity of the cells well before a local temperature measurement exceeds 43°C. The model prediction is poor at the early stages of the fermentation, where it predicts rapid increase of the temperature, while a slowly increasing temperature is measured experimentally. Inhomogeneities and improper contact of the air with the substrate might be the reason for this.

CONCLUSIONS

Overall, the results demonstrate the effectiveness of the temperature control by evaporative cooling of the fermented matter and the feasibility of an optimum temperature and water content policy for improved biomass production. One has to consider the results with the following limitations. The model doesn’t account for:

i) Imperfect mixing of air with the substrate

ii) Inhomogeneous substrate

iii) Heat and mass transfer between air and the substrate

iv) Variations of temperature and water content in the SSF fermenter.

Another difficulty with the model is the extrapolation of existing data for the prediction of $\mu_M$ and $x_M$.

ACKNOWLEDGEMENT

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References


Figure 2: Effect of inlet air flow rate $F$ on biomass content variation. $RH = 100\%$, $T_{inlet} = 37^\circ C$, $hA = 1.6902$ Kcal/h°C. 1: 2 lt/min, 2: 5 lt/min, 3: 7 lt/min, 4: 13 lt/min.

Figure 3: Effect of overall heat transfer coefficient $hA$ on biomass content variation. $RH = 100\%$, $T_{inlet} = 37^\circ C$, $F = 7$ lt/min. 1: 0.8451, 2: 1.6902, 3: 2.1128, 4: 4.2255 Kcal/h°C.
Figure 4: Effect of inlet air relative humidity RH on biomass content variation. \( h_A = 1.6902 \text{ Kcal/h°C}, T_{\text{inlet}} = 37^\circ\text{C}, F = 7 \text{ h/min}. \) 1: 85%, 2: 90%, 3: 95%, 4: 100%.

Figure 5: Effect of inlet air temperature \( T_{\text{inlet}} \) on biomass content variation. \( h_A = 1.6902 \text{ Kcal/h°C}, T_{\text{inlet}} = 37^\circ\text{C}, RH = 100\%. \) 1: 34°C, 2: 35.5°C, 3: 36.5°C, 4: 37°C.

Figure 6: Simulation of the temperature profile in an uncontrolled fermentation. \( T_{\text{inlet}} = 33.5^\circ\text{C}, h_A = 0.8451 \text{ Kcal/h°C}, F = 7 \text{ h/min}; RH = 100\%. \) 1: Maintenance coefficients \( m_Q \) and \( m_{H_2O} \) set to 0, 2: Maintenance coefficients \( m_Q \) and \( m_{H_2O} \) following exponential decay, Δ: Experimental results.
PRODUCTION OF ACETYL-XYLAN ESTERASE
FROM ASPERGILLUS NIGER

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ABSTRACT: Aspergillus niger ATCC no. 10864 was used to study the production of the enzyme acetyl-xylan esterase. The enzyme was assayed using chemically acetylated xylan; acetic acid was detected by HPLC. The fungi were grown in 200 ml shake flasks cultures at 30 °C and 200 rpm using different combinations of xylan, cellulose and sucrose as carbon source. The medium that consisted of 1 percent of each Solka-Floc, sucrose and Oat spelt xylan produced higher titers of enzyme than any other combination. Lower pH values were associated with enzyme productivity in shake flask culture.

INTRODUCTION

Plant fiber consists of 40% cellulose, 35-48% hemicellulose, small amounts of pectin and lower but variable amounts of lignin. One of the major hemicelluloses found in cell walls of hardwoods, grasses and some cereals and trees is xylan. According to its origin, xylan is substituted to various degrees with acetyl, arabinosyl and glucuronyl residues and is cross-linked via esterified phenolic acids, these phenolic acid esters, primarily ferulic acid, are thought to anchor lignin chains to xylans. It was suggested that this substitution of xylan is one of the main resistances of plant cell wall to degradation, as the complete xylan hydrolysis requires a combination of enzymes capable of hydrolyzing every substitute. Several such microbial enzyme activities have been reported. One of these, acetyl xylan esterase [E.C.3.1.6] which removes acetyl groups from the xylan backbone has been the most extensively studied activity.
In a study on hardwood hemicellulose preparation\textsuperscript{3} it has been concluded that the acetyl substitutes are located on C-2 and/or C-3 of the xylopyranose ring of the 1,4-\beta-linked linear xylan chain. The degrees of substitution varies e.g. in hardwood and mature grasses every second xylose residue, and in beech leaves during maturation almost all the xylose residues are substituted\textsuperscript{16}. Acetylation could greatly impede enzymatic hydrolysis by microorganisms and even be an important factor influencing digestibility of the plant cell wall in ruminant\textsuperscript{17}. These claims were based on results of digestibility tests, carried out in the rumen, that have shown that the degree of acetylation greatly impedes their hydrolysis\textsuperscript{18}. As a consequence the enzymatic deacetylation of xylan may be a prerequisite for its breakdown. The enzymes that are involved in this deacetylation are acetyl-xylan esterases and liberation of acetic acid is expected to be seen during their activity. This indicates a way to detect the enzymes presence.

Esterases that deacetylate acetylated xylan have been shown to be present in cellulotic systems of several fungi, streptomyces, plants and animals. Esterases present in fungal systems are found to exhibit much higher activities against acetylated xylan than any esterase of nonfungal origin\textsuperscript{19}.

Enzymic attack of acetylated xylan would be expected to be involved in a number of instances of agricultural importance, such as the attack of plants by pathogens, the breakdown of plant residues in soil, or the digestion of plant cell walls in the rumen\textsuperscript{20}. It can also be of interest in the technological applications such as the bioconversion of the water soluble hemicellulose fraction obtained by steaming wood\textsuperscript{21} which can contain acetylated xylooligosaccharides.

This paper uses the fungi \textit{Aspergillus niger} to study acetyl-xylan esterase activity production in different media.

**METHODS AND MATERIALS**

**Preparation of inoculum:**
For the preparation of inoculum a basal medium \textsuperscript{22} composed of 3.0 g/l NaNO\textsubscript{3}, 1.0 g/l K\textsubscript{2}HPO\textsubscript{4}, 0.5 g/l MgSO\textsubscript{4}·7H\textsubscript{2}O, 0.5 g/l KCL, 0.1 g/l FeSO\textsubscript{4}·7H\textsubscript{2}O\textsubscript{2}, 5 ml/l trace element solution and 2.5 ml/l biotin solution\textsuperscript{23} (see table 1) and 0.2% Tween 80 (used by Mandels \textit{et al} \textsuperscript{29}). The medium was supplemented with 10 g/l sucrose and 1 g/l bactopeptone and dispensed in 500 ml Erlenmeyer flasks (200 ml per flask) and sterilized. The flasks were inoculated with spores from a petri dish of \textit{A. niger} ATCC no. 10864 and incubated at 30 °C temperature and 200 rpm shaking.

**Preparation of chemically acetylated xylan:**
Chemically acetylated xylan was used as a substrate in assaying for the acetyl-xylan esterase enzyme. It was prepared according to Mitchell \textsuperscript{25}. In brief, oat
spelt xylan (Sigma) was swelled in formamide (10 w/v) with constant stirring of 18-24 hrs. Once sufficiently swelled 1.38 ml of pyridine per ml of formamide was added followed by 1.35 ml of acetic anhydride per ml of formamide to achieve acetylation. The mixture was left to react at ambient temperature for 4-6 hrs with continuous stirring. To quench the reaction and precipitate the water-insoluble, acetylated xylan fraction, the mixture was poured in 1 l of deionized water, stirred for 0.5 hrs and centrifuged for 10 mins in 8000g. The resulting supernatant was washed with 2 l of deionized water and 1 l of methanol by repeated batch centrifugations. The resulting xylan was dried slowly in a vacuum oven at room temperature, ground and stored at room temperature.

Table 1:

<table>
<thead>
<tr>
<th>Trace element solution</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>distilled water</td>
<td>95 ml</td>
</tr>
<tr>
<td>citric acid, 1H₂O</td>
<td>5.00 g</td>
</tr>
<tr>
<td>ZnSO₄·7H₂O</td>
<td>5.00 g</td>
</tr>
<tr>
<td>Fe(NH₄)₂(SO₄)₂·6H₂O</td>
<td>1.00 g</td>
</tr>
<tr>
<td>CuSO₄·5H₂O</td>
<td>0.25 g</td>
</tr>
<tr>
<td>MnSO₄·H₂O</td>
<td>0.05 g</td>
</tr>
<tr>
<td>H₃BO₃ (anhydrous)</td>
<td>0.05 g</td>
</tr>
<tr>
<td>Na₂MoO₄·2H₂O</td>
<td>0.05 g</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Biotin solution</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>distilled water</td>
<td>50 ml</td>
</tr>
<tr>
<td>Biotin (Merck)</td>
<td>5 mg</td>
</tr>
</tbody>
</table>

Enzyme preparation:

For enzyme preparation A. niger was cultivated in basal media containing a combination of carbohydrates shown on table 2. The media were also supplemented with 5 ml/l of trace element solution, 2.5 ml/l of biotin solution, 0.2% Tween 80 and 0.1 % bactopeptone. These tests were carried out in 500 ml Erleameyer flasks, each containing 200 ml of the desired medium and incubated with shaking (200 rpm) at 30 °C. 5% v/v of a 2-3 days old culture was used as inoculum. Samples were taken at approximately every 3 days for assay of acetyl-xylan esterase and determination of the pH.

Enzyme Assay:

Acetyl xylan esterase activity was measured using chemically acetylated xylan as a substrate. In this procedure 1% w/v solution of the substrate in 0.4 M phosphate
buffer pH 6.5 was used. The reaction mixture contained 2:1 volume ratio of substrate solution and enzyme preparation. The procedure included: incubation for 1 hr at 30 °C, centrifugation for 10 mins and filtration. The released acetic acid was quantified by HPLC using a Biorad HPX-87H column maintained at 43 °C. Samples were eluted with 0.02 N H₂SO₄ at a flow rate of 0.6 ml/min. The resulting peaks were detected with the Waters R401 refractometer. The retention time for acetic acid was 16.00 min under these conditions.

<table>
<thead>
<tr>
<th>Flask code</th>
<th>Carbon source</th>
</tr>
</thead>
<tbody>
<tr>
<td>B</td>
<td>1% Solka-Floc + 1% oat spelt xylan</td>
</tr>
<tr>
<td>C</td>
<td>1% Solka-Floc + 1% oat spelt xylan + 1% sucrose</td>
</tr>
<tr>
<td>D</td>
<td>1% oat spelt xylan</td>
</tr>
<tr>
<td>E</td>
<td>1% oat spelt xylan + 1% sucrose</td>
</tr>
</tbody>
</table>

RESULTS-DISCUSSION

All enzyme preparations obtained from the A. niger cultures deesterified chemically acetylated xylan. However, the extent of deesterification varied widely from culture to culture. The acetyl-xylan esterase enzyme levels and production profiles varied with carbon source (Figure 1). Generally, acetyl-xylan esterase was present in the inoculum. The rate of inactivation varied. In the cultures containing only xylan as the carbon source (flask D), 80 % of the enzyme activity was lost in the first 3 days of fermentation. After that, there was only a small change in the activity. When xylan was supplemented with cellulose (flask B) a steady increase in the enzyme activity was observed up to the 9th day. Cellulose was probably a good inducer for the formation of acetyl-xylan esterase. When xylan was supplemented with cellulose and sucrose (flask C) the results were even more impressive. This culture gave the smoothest enzyme activity profile with a maximum at 3 days. The combination of xylan and sucrose (flask E) did not show any considerable enzyme activity. From these observations, xylan could be considered as an inducer of the formation of the enzyme, although not as good an inducer as cellulose. Sucrose served as a readily available carbon source for cell growth.

The pH profiles for all cultures are reported in Figure 2. Lower pH were associated with enzyme productivity. There is a downward trend in culture pH in all studies. From HPLC analysis, lactic acid was observed. Maximum enzyme activity was always found to be in the pH range of 2.8-3.2. This was interesting, knowing that fungi usually grow above pH values of 4.0.
Cellulose, sucrose and xylan will be used as combined substrates in 14-L fermenters to study parameters associated with the effects of culture conditions on the acetyl-xylan esterase synthesis. For the optimization of enzyme productivity, temperature, agitation rate and aeration rate will be studied.

REFERENCES

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Fig. 1. Acetyl-xylan esterase activity as a function of fermentation time.

B: 1% Solka-Floc + 1% xylan, C: 1% Solka-Floc + 1% xylan + 1% sucrose,
D: 1% xylan, E: 1% xylan + 1% sucrose.
Fig. 2. PH changes as a function of fermentation time.

- B: 1% Solka-Floc + 1% xylan,  
- C: 1% Solka-Floc + 1% xylan + 1% sucrose,  
- D: 1% xylan,  
- E: 1% xylan + 1% sucrose.
Biological and Latex Particle Partitioning in Aqueous Two-Phase Systems

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1 Summary

Aqueous two-phase extraction is a technique which can be used to isolate particulate biological materials. However, in order for this technique to be used effectively the motion of particulates within the phase system must be understood. Here, biological particles and latex spheres have been partitioned in order to begin developing an understanding of their motion in a phase system.

2 Introduction

Separation processes tend to be the most expensive and time consuming steps in the production of biotechnology products¹². Many separation methods have been studied and characterized for the preparation of soluble products, such as proteins, and large particulate products, such as cells. However, fewer studies have focused on evaluating separation methods for the recovery of intermediate-sized particles such as ribosomes and vesicles. One method of isolating intermediate-sized particles is aqueous two-phase extraction.

Separation by extraction exploits the fact that different particles and solubles partition differently between two immiscible phases. Traditionally, extraction is performed by combining organic and aqueous solutions, which are highly immiscible. Antibiotics, such as penicillin, and amino acids can be purified using organic/aqueous two-phase extraction²³. However, organic/aqueous systems cannot be applied to most biological products. One reason for this is that many biological materials, such as proteins, obtain their tertiary structure from hydrophobic and hydrophilic interactions. Thus, when a biological material is contacted with an organic phase, it tends to denature and lose its activity. Aqueous two-phase extraction, however, overcomes these obstacles.

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In aqueous two-phase extraction, two structurally distinct polymers, or a polymer and a salt, are combined at sufficiently high concentrations with water and buffers to form two immiscible aqueous phases. One of the two phases is enriched in one polymer, and the other is enriched in the other polymer or salt. Aqueous two-phase systems generally have a water content greater than 85%, which makes the systems gentle enough to be used with biological materials. Figure 1 shows a two-component phase diagram for a polyethylene glycol and dextran aqueous two-phase system. Two phases for when the polymer concentrations lie to the right of the binodial curve, which separates the single phase and two phase regions. The points at which tie-lines, which connect top and bottom phase compositions at equilibrium, intersect the binodial curve indicate the compositions of the top and bottom phases for various overall system compositions. The location of the binodial curve is determined by the molecular weights of the polymers used, types of polymers used, salts used, temperature and pH. In addition, high concentrations of other species, for example proteins and nucleic acids, shift the binodial curve.

Partitioning, which is the separation of a solute between two or more phases, is fully described at equilibrium by the partition coefficient, \( K \), in the case of solubles. The partition coefficient, as shown in Equation (1), is defined as the concentration of a solute in the top phase divided by its concentration in the lower phase.

\[
K = \frac{C_{\text{top}}}{C_{\text{bot}}}
\]  

However, this relationship is not sufficient to describe the partitioning of particulate material. Particulates tend to partition to one of the phases, and to the interfacial region between the phases. As this is the case, particulate partitioning in this paper is described by the percent recovery in each phase and at the interface.

Aqueous two-phase extraction was first applied to particulate partitioning by Per-Åke Albertsson. In 1955, Albertsson found that chloroplasts partition strongly to the polyethylene glycol phase of a polyethylene glycol/potassium phosphaste phase system. Since that time, three approaches have been taken to study the partitioning of biological particulates. In one of the approaches, the results of partitioning cells are applied to the partitioning of particulates. Hofsten studied the partitioning of \( E. coli \) in polyethylene glycol/dextran systems. He showed that the partitioning of cells is dependent on the medium in which the cells are grown. In addition, it was found that there are vast differences in the partitioning behavior for different \( E. coli \) strains. Miheeva studied the partitioning of human erythrocytes in phase systems composed of polyethylene glycol/dextran, polyethylene glycol/ficoll and dextran/ficoll. In general, she found that the cells partition to the ficoll phase in the polyethylene glycol/ficoll systems and into the dextran phase in the polyethylene glycol/dextran systems. She also found that the ionic conditions affect the partitioning in dextran/ficoll systems so that the cells could be partitioned to either phase. The ionic conditions also affect the electrostatic potential between the phases, and Gascoine and Fisher studied the effect that this factor has on the partitioning of cells. It was found that the recovery to the top phase for red blood cells from several animal species increases as the electrostatic potential between the phases is increased.

Another approach to particulate partitioning is to study the partitioning of representative biological particles. Smeds and Enfors studied the partitioning of chromatophores from \( Rhodospirillum rubrum \) and found that the addition of polyethylene glycol-palmate to
a polyethylene glycol/dextran system forces the chromatophores into the polyethylene glycol rich phase. De Ley found that ribosomes partition strongly to the top phase of a sodium dextran sulfate/methylcellulose system. In addition, Pestka was able to recover 90% of E. coli ribosomes in the dextran phase of a polyethylene glycol/dextran system, and in the ficoll phase of a dextran/ficoll system. We have partitioned ribosomes and vesicles derived from Serratia marcescens. These have been shown to increase natural killer cell activity, and are components of ImuVert®, a biological response modifier produced by Cell Technology, Inc. ImuVert® is an example of a particulate biological product for which purification could be enhanced by aqueous two-phase extraction. Currently the product is isolated using ultracentrifugation, which is labor intensive. It is believed that aqueous two-phase extraction could be a less labor intensive isolation step.

The third approach to studying the partitioning of particulates is to use model particles. Eriksson studied the partitioning of liposomes made with specific phospholipids and found that the characteristics of the phospholipid affect the partitioning of the liposomes. We have chosen, however, to work with latex spheres as a model system. They are readily available in a wide range of sizes, with various surface characteristics, and exhibit interesting partitioning behavior.

It is important to note that all of the studies mentioned previously were primarily concerned with understanding the thermodynamics of particulate partitioning. This includes the effects on equilibrium partitioning of thermodynamic factors such as pH, ionic conditions, polymer choice, etc. A few studies, however, have looked at the effects of factors which relate to the fluid dynamics and mechanics of particle partitioning, such as time of phase separation, particle size, and interfacial area available. The goal of this research is to develop an understanding of how particulate materials partition in an aqueous two-phase system.

3 Materials and Methods

3.1 Assembly of Phase Systems

All phase systems were prepared from stock solutions. The appropriate amount of each of the stock solutions was mixed with water, leaving enough of the total weight available for the sample to be added. Typically systems were prepared to give a final mass of 5 g. Systems were then used immediately, or frozen for future use. Polymers and salts were obtained from Sigma. Phase systems were made with a phase volume equal to unity along the tieline including the point in Table I.

3.2 Samples

Two types of samples were used: ribosomes and vesicles derived from Serratia marcescens, and latex spheres. The ribosomes and vesicles were prepared by Cell Technology, Inc. using ultracentrifugation, and both types of particles were present in the samples. The ribosomes have a diameter of approximately 0.25 μm, while the average vesicle size is approximately 0.250 μm. The latex spheres were obtained from Interfacial Dynamics Corporation. Both
Table I—composition of phase systems

<table>
<thead>
<tr>
<th>Sample</th>
<th>wt% Dextran*</th>
<th>wt% PEG#</th>
<th>Salt Content (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ImuVert®</td>
<td>7.6</td>
<td>6.0</td>
<td>40.0 MgSO₄, 37.5 (NH₄)₂SO₄</td>
</tr>
<tr>
<td>Latex Spheres</td>
<td>7.0</td>
<td>5.0</td>
<td>100 K₂HPO₄</td>
</tr>
</tbody>
</table>

*the dextran molecular weight was 229,000 for both systems
#the PEG molecular weight was 3350 for the ImuVert® system and 8000 for the latex sphere system

sets of the spheres used in these experiments have carboxylated surfaces. The spheres have diameters of .297 μm and 2.22 μm.

3.3 Experimental Procedure

The systems were prepared as described above, or they were removed from the freezer and thawed. Samples were added to the phase systems, which were then vortexed thoroughly. At this point, the systems were allowed to settle for a specified length of time. The systems which contained vesicles and ribosomes were kept at 4 °C at all times. Systems with latex spheres as the sample were tested in triplicate and kept at room temperature.

Once the settling time was complete, samples were removed from the systems. Top and bottom phase samples were obtained using a syringe. The interface sample consisted of the interface and the residual top and bottom phases. All phase volumes were recorded.

3.4 Sample Analysis

Analysis of all samples was completed using a spectrophotometer. The wavelength used for analysis depended on the sample. Typically the absorbance of the biological particles was read at 260 nm and 280 nm, and that of the latex spheres at 350 nm for the smaller spheres and 700 nm for the larger spheres.

3.5 Recovery Calculations

The recovery was calculated as given by Equation (2). The recovery at the interface was determined using Equation (3).

\[ R = V_{ts} \cdot C_{ts} + V_{bs} \cdot C_{bs} + V_{ts} \cdot C_{is} \]  
\[ IR = R - V_{top} \cdot C_{ts} - V_{bot} \cdot C_{bs} \]

The interfacial recovery (IR) was the portion of the total recovery (R) which could not be attributed to either the top or bottom phase. Note: the samples did not exhibit an affinity for the walls of the tubes or for the top phase/air interface. The percent recovered was then calculated using the total recovery and the recovery for each phase and the interface.


4 Results

The results are shown in Figures 2, 3 and 4. In all cases, a phase boundary was visible at the first time at which systems were sampled [15 minutes after mixing was stopped]. The figures exhibit several interesting features. First, the latex spheres exhibit an affinity for the interface, as do the ribosomes and vesicles. Also, the larger latex spheres partition to the interface more strongly than do the smaller latex spheres. In addition, the rate at which the larger latex spheres partition to the interface appears to be significantly faster than that of the smaller latex spheres, but slower than that of the ribosomes and vesicles. Lastly, all of the particles partition strongly to one phase and the interface, leaving essentially no particulate material in the other phase. The latex spheres partition to the top phase and the interface, whereas the ribosomes and vesicles partition to the bottom phase and the interface.

5 Discussion

The affinity of particulates for the interface due to thermodynamic considerations has been described previously. In this situation, the particulate material decreases the interfacial energy by adsorption to the interface. However, the partitioning of the particles is a rate process, as seen in Figures 2, 3 and 4, in contrast to soluble materials which reach equilibrium partitioning before the phases separate. Particulate materials achieve thermodynamic equilibrium very slowly when compared to solubles. As this is the case, the time and method of phase separation [centrifugation or not] must be considered when partitioning particulates.

In order to consider the time dependence of particulate partitioning, the mechanism for the motion of the particulates from one phase to the other or to the interface must be understood. This is the problem we seek to understand. Currently, it is known that single particle sedimentation and Brownian motion are not responsible for this motion. Equations (4) and (5) give the estimated time for a particle to reach the interface from the opposite end of a phase for sedimentation and Brownian motion, respectively.

\[ t_s \approx \frac{[9\mu L]}{[2ga^2\Delta \rho]} \quad (4) \]

\[ t_b \approx \frac{[6\pi \mu a L^2]}{[kT]} \quad (5) \]

The longest sedimentation time allowed in these experiments was of the order of \(1 \times 10^5\) seconds, while quantities representative of the ribosomes and vesicles produce a \(t_s\) on the order of \(3 \times 10^8\) seconds and a \(t_b\) of \(1 \times 10^9\) seconds. In making these estimates, the typical values of \(L = 3\) cm, \(\mu = .2\) g/cm-s, and \(\Delta \rho = .1\) g/cm\(^3\) were chosen. The \(t_s\) above is the proper order of magnitude for the latex spheres, but \(\Delta \rho\) for the spheres is negative. Thus, the latex spheres have a net upward force on them due to gravity. Therefore, both of these mechanisms are too slow to describe the particle motion.

As sedimentation and Brownian motion of single particles cannot account for the observed motion of the particles, it is proposed that the particulates adhere to the surface of phase droplets and are then carried with the droplets to the interface. Adhesion of particulates to phase droplets has been observed, but the net motion of the particles has not been analyzed. It is this net motion which we intend to examine in future studies.
6 Acknowledgements

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7 Nomenclature

\begin{tabular}{lcl}
\text{a} & \text{particle diameter [\( \mu \text{m} \)]} & \text{\( t_s \)} & \text{required sedimentation time [s]} \\
\text{C} & \text{concentration [moles/l]} & \text{\( t_b \)} & \text{required Brownian motion time [s]} \\
\text{g} & \text{981 cm/s}^2 & \text{T} & \text{temperature [Kelvin]} \\
\text{IR} & \text{interfacial recovery} & \text{V} & \text{volume [cm}^3\text{]} \\
\text{k} & \text{Boltzman’s constant [1.381 x 10^{-16} erg/K]} & \mu & \text{viscosity [g/cm-s]} \\
\text{K} & \text{partition coefficient} & \Delta \rho & \text{density difference [g/cm}^3\text{]} \\
\text{L} & \text{distance of motion [cm]} & & \\
\text{R} & \text{total recovery} & & \\
\text{subscripts} & & \text{bot} & \text{bottom phase} \\
\text{top} & \text{top phase} & \text{bs} & \text{bottom phase sample} \\
\text{ts} & \text{top phase sample} & & \\
\text{is} & \text{interfacial sample} & & \\
\end{tabular}
8 References

Figure 1 - Example of an Aqueous Two-Phase System, Courtesy of Steven Snyder

Figure 2 - Recovery vs. time for ImuVert® partitioning
Figure 3 - Recovery vs. time for 0.297 µm latex partitioning

Figure 4 - Recovery vs. time for 2.22 µm latex partitioning
Introduction

Sulfur in coal is a pervasive source of air pollution and acid rain generated by power plants. Since coal always contains organic sulfur, pyrites, and sulfates, several coal cleaning methods have been developed for removing sulfur contents from coal prior to combustion. Although inorganic forms of sulfur can be removed by physical means, organic sulfur requires chemical or biological action in order to break C-S bonds within the hydrocarbons of the coal itself (Rameshwar, 1989). The methodology which this work focused on involves microbial desulfurization. A group of bacteria and yeast has been isolated from a coal environment and shown to extract sulfur from the thiophenic linkages which are common in coal. One bacterium and one yeast have been identified as the most efficient (Mattoon, 1990).

A novel coal bioreactor with cell recycle is being designed for microbial desulfurization of pulverized Illinois no. 6 coal particles containing 1 w% pyritic, 2.4 w% organic sulfur, and 0.2 w% sulfate sulfur (Detz, 1979). This bioreactor includes an inclined settler which is capable of separating the microorganisms from the desulfurized coal particles. This work reports the theory for an inclined settler operated under steady-state conditions with the possibility that the bidisperse suspension of microorganisms and coal particles suspension is highly concentrated. Growth kinetics on minimal media containing coal particles as a sulfur source have been determined in order to provide a rational choice for the flow rate of coal particles and nutrients to the bioreactor. The flow rate of the underflow stream, which contains cleaned coal particles from the inclined settler, was also determined by this method.

Inclined Settler Theory for a Bidisperse Suspension

Particles can be classified according to their differences in settling velocities in an inclined settler. The basic principle is that larger particles settle more quickly than smaller particles of the same shape and density. An inclined settler may be used to classify a feed suspension into an underflow fraction and an overflow fraction, as shown in Figure 1. Continuous steady-state operation of a single inclined settling channel is considered first. It is assumed that the suspension contained two discrete sizes, for which species 1 particles are larger particles (like coal), and species 2 particles are smaller particles (like cells).

A bidisperse suspension of negatively buoyant particles in an inclined settler forms a clear fluid region on top, two suspension regions below it, and a sediment layer at the bottom of the settler. Due to the continuity of the particle flux, the bottom region of suspension contains both of the particle species in concentrations equal to the original feed suspension concentrations. The upper region contains only the slower settling particle species, because the faster settling particles have settled out of this region. The concentration in
Figure 1: Schematic diagram of continuous inclined settler for steady-state particle classification.

This region must also satisfy the particle flux continuity equation for the interface separating the two regions (Smith, 1966 and Davis and Acrivos, 1985)

$$\phi_{2,2}(v_{1,1} - v_{2,1}) = \phi_{2,1}(v_{1,1} - v_{2,1})$$

(1)

The left-hand side eq 1 is the rate at which particles of species 2 enter the bottom of region 2, and the right-hand side is the rate at which they leave the top of region 1. The first subscript refers to the particle species, and the second subscript refers to the region; for example, $\phi_{2,1}$ is the volume fraction of species 2 in region 1, and $v_{2,1}$ is the average sedimentation velocity of species 2 in region 1. The settling velocity is calculated with the assumption that all particles are sphere. The settling velocity of each particle species present is:

$$v_{i,k} = u_{i,0}f_{i,k}$$

(2)

where $f_{i,k}$ is the hindered settling function for particle species i in region k, and $u_{i,0}$ is the Stokes settling velocity for an isolated particle of species i. The latter is given by Stokes law:

$$u_{i,0} = \frac{kD_i^2(\rho_i - \rho)g}{18\mu}$$

(3)

where $D_i$ is the particle diameter, $\rho_i$ and $\rho$ are the density of the particles and Newtonian fluid, respectively, $\mu$ is the viscosity of the fluid, $g$ is the gravity acceleration constant, and $k$ is a correction factor to account for shape effects, wall effects, and any calibration differences between the measured diameter and the effective Stokes diameter.

A new hindered settling function is proposed for the settling velocity of particles:

$$f_{i,k} = (1 - \Phi_k)^n[1 + \sum(C_{i,j} + n)\phi_{j,k}]$$

(4)

where $\Phi_k$ is the total volume fraction of particles in region k, and $\phi_{j,k}$ is the volume fraction of species j in region k. The dimensionless sedimentation coefficients, $C_{ij}$, were calculated numerically by Batchelor and Wen (1982) as functions of the size ratio ($D_j/D_i$) and the reduced density ratio ($\rho_j/\rho_i$). The value of the $n$ is set equal to $-C_{ii}$, which is approximately 5.6 at low Reynolds number for noncolloidal particles (Davis and Birdsell, 1988). This hindered settling functions agree with the theory of Batchelor and Wen (1982) for dilute polydisperse suspensions. It also reduces to the Richardson and Zaki (1954) correlation for monodisperse suspensions.
Three different cases shown in Figure 2 were considered for using the inclined settler, consisting of (2a) the overflow containing only a clear fluid, (2b) the overflow containing small particles along with fluid, and (2c) the overflow consisting of both types of particles and fluid. The theory predicts overflow and underflow concentrations, given the feed concentration and feed and overflow rates.

If the particle concentrations and settling velocities in each region are known, these may be used together with macroscopic mass balances and the inclined settling rate to predict the composition of the product streams during the use of an inclined settler for classification of particles by size and/or density. A feed suspension with a particle volume fraction of coal, $\phi_{1,f}$, and cells, $\phi_{2,f}$, is fed into the settler at the volumetric rate of $Q_f$, and underflow and overflow suspensions are removed from the settler with volumetric flow rates of $Q_u$ and $Q_o$, respectively. Steady-state mass balances about the entire settler for total suspension, particles of a species 1, and particles of species 2 are, respectively (Davis et al., 1989)

\[ Q_f = Q_o + Q_u \]  
\[ Q_f \phi_{1,f} = Q_o \phi_{1,o} + Q_u \phi_{1,u} \]  
\[ Q_f \phi_{2,f} = Q_o \phi_{2,o} + Q_u \phi_{2,u} \]

The clarification rate, $S(v)$, in an inclined channel may be determined by using PNK theory (Ponder, 1925 and Nakamura and Kuroda, 1937). $S(v)$ is the volumetric rate at which material crosses the interface separating regions from each other. According to the theory of inclined settling (Davis et al., 1982), this is equal to the vertical settling velocity of a given species multiplied by the horizontal projection of the interface separating regions. For the rectangular geometry of Figure 1, this is given by

\[ S(v_{i,k}) = v_{i,k} w (L_k \sin \theta + b \cos \theta) \]  

where $w$ is the width of the channel, $b$ is the spacing between the inclined walls, $\theta$ is the angle of inclination of the channel from the vertical, and $L_k$ is the length of the channel from its bottom up to the top of region $k$. The value of $L_k$ depends on the overflow rate. Since an increased overflow rate reduces the hold-up time of the particles in the settler, the species start to reach the overflow instead of settling out of suspension. As a result, $L_k$ increases as $Q_o$ is increased. Subscripts $i$ and $k$ refer to the species and regions, respectively.

Depending on the magnitude of the overflow rate, the theoretical equations may be derived for three possible cases as described above.
Case 1 (shown in Figure 2a):

\[ Q_o < S(v_{2,2}) < S(v_{1,1}) \]  
\[ (9) \]

All of the particles settle out of the suspension before reaching the overflow (\( \phi_{1,o} = 0 \) and \( \phi_{2,o} = 0 \)). The particle mass balances eq 6 and 7 become:

\[ Q_f \phi_{1,f} = Q_u \phi_{1,u} \]  
\[ (10) \]

\[ Q_f \phi_{2,f} = Q_u \phi_{2,u} \]  
\[ (11) \]

Case 2 (shown in Figure 2b):

\[ S(v_{2,2}) < Q_o < S(v_{1,1}) \]  
\[ (12) \]

All of the coal particles settle out of the suspension before reaching the overflow, so \( \phi_{1,o} = 0 \). Equation 10 is valid for coal particles, and eq 7 is valid for the cells for this case.

Equation 7 has two unknowns, \( \phi_{2,u} \) and \( \phi_{2,u} \), so another mass balance is required to determine either the underflow or overflow concentration of the cells. This final mass balance is the mixing balance on the particles reaching the underflow at the bottom of the vessel. The volumetric rate due to feed short-circuiting into the underflow without sedimentation and the volumetric rate of cell sedimentation in region 2 will determine the volumetric rate at which cells report to the underflow:

\[ Q_u \phi_{2,u} = (Q_f - Q_o) \phi_{2,f} + v_{2,2} \phi_{2,2} (L_2 - L_1) \sin \theta \]  
\[ (13) \]

Case 3 (shown in Figure 2c):

\[ S(v_{2,2}) < S(v_{1,1}) < Q_o \]  
\[ (14) \]

Some of the coal particles and all cell particles reach the overflow. Equations 1-7 and the mixing point balances for each type of particles are used to calculate the \( \phi_{1,u} \), \( \phi_{1,o} \), \( \phi_{2,o} \) and \( \phi_{2,u} \). The underflow mixing point balances for coal particles and cells are, respectively

\[ Q_u \phi_{1,u} = (Q_f - Q_o) \phi_{1,f} + S(v_{1,1}) \phi_{1,f} \]  
\[ (15) \]

\[ Q_u \phi_{2,u} = (Q_f - Q_o) \phi_{2,f} \]  
\[ (16) \]

Experimental Measurements

In order to provide numerical values to use in the inclined settling theory, the volume-average sizes of coal particles, yeast and bacteria cells had to be determined. The volume-average diameters of the coal, yeast and bacteria as measured by a Coulter Multisizer, are 37 \( \mu \)m, 3.6 \( \mu \)m and 1.2 \( \mu \)m, respectively. The coal, yeast and bacteria densities were determined to be 1.313 g/mL, 1.078 g/mL and 1.025 g/mL, respectively, by a neutral buoyancy technique. The operating temperature was chosen as 30°C. The density and viscosity of the working fluid were nearly equal to those of pure water (\( \mu = 0.0082 \) P and \( \rho = 0.9956 \) g/mL.)

Since the theory requires the Stokes settling velocities, the correction factors for coal, yeast and bacteria in eq 3 were determined by directly measuring the median sedimentation velocity in dilute samples (0.5 v\%) with the sedimentation/light extinction method described by Davis and Birdsell (1988) and Davis and Hassen (1988) and then using the median particle diameters of coal, yeast and bacteria as measured by a Coulter Multisizer in eq. 3 to solve for the correction factor \( k \). The values for coal, yeast and bacteria

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are $k = 0.88, 0.92$ and $0.89$, respectively. Since the shape of the yeast is nearly spherical, the factor $k$ was found to be closest to unity. Because of the irregular shape of coal and cylindrical shape of bacteria, the correction factors for bacteria and coal were found to be lower.

Underflow rates from the inclined settler were determined by equating the dilution rate to the specific growth rate of yeast and bacteria. Several shake flask experiments were performed to determine the specific growth rate of yeast or bacteria on minimal medium which had coal as a sulfur source. The specific growth rate of yeast and bacteria are, respectively, $0.212 \pm 0.035 \text{ h}^{-1}$ and $0.223 \pm 0.007 \text{ h}^{-1}$, with 90% confidence limits. The working volume of the fermentor is planned to be 1 L. Since the overflow is recycled to the reactor, the underflow rate from the inclined settler is equal to the feed flow rate to the reactor: $Q_u = 3.53 \text{ L/min}$ for yeast-coal experiments and $Q_u = 4.22 \text{ L/min}$ for bacteria-coal experiments.

**Results and Discussions**

The new hindered settling functions was used to define the hindered settling velocity of the particles. Equation 4 may be written for bidisperse suspensions:

$$v_{1,1} = u_{1,0}(1 - \phi_{1,1} - \phi_{2,1})^n(1 + (C_{1,2} + n)\phi_{2,1})$$  \(17\)

$$v_{2,1} = u_{2,0}(1 - \phi_{1,1} - \phi_{2,1})^n(1 + (C_{2,1} + n)\phi_{1,1})$$  \(18\)

$$v_{2,2} = u_{2,0}(1 - \phi_{2,2})^n$$  \(19\)

The prediction of the model are compared with the previously published experimental results of the interface velocities. Due to space limitation, only the comparison of the model predictions with the experimental data of Al-Naafa and Selim (1989) is presented here. Figure 3 and 4 shows that the new model of the hindered settling function does an excellent job in predicting the settling velocities of small light and large dense particle species.

The sedimentation coefficients reported by Batchelor and Wen (1982) are negative. In this case, the ratio of $D_{\text{coal}}/D_{\text{cell}}$ was very large, so a negative velocity for the cells was calculated in the first region of the inclined settler. As a result, the theory indicates that cells may move upwards owing to the upswelling or backflow of the suspending fluid caused by the sedimentation of coal particles.

After defining the experimental parameters which are presented above, the method used to provide a solution started with eq 1, where the volume fraction of each particles species is at its specified initial value for the first region (Davis et al., 1982). Equation 1 is then solved for the cell volume fraction in the second region. DNEQNF in IMSL was used to solve eq 1 using the Levenberg-Marquardt algorithm and a finite difference approximation to the Jacobian. Since the particle volume fractions and settling velocities in each region are then determined, overflow and underflow volume fractions may be solved by using the equations for the three different possible cases. Case 2 is the most important because the main goal is to separate the cells from coal particles as much as possible and recycle the cells to the bioreactor. The numerical results of the inclined settler theory for a bidisperse suspensions are shown in Figures 5-10. The dimensions of the inclined settler are $L = 40 \text{ cm}$, $w = 4 \text{ cm}$, $b = 0.5 \text{ cm}$, and $\theta = 45^\circ$.

First, the coal was chosen to be concentrated (20 v%), and the concentration of cells was varied within the range 1 to 15 v%. Second, the concentration of cells was kept at 20 v%, and the concentration of coal was varied within the range 1 to 15 v%, in order to determine the hindered settling effect with increasing concentrations of particles. In Figures 5 and 8, the fractional recovery of the cells are shown as functions of the dimensionless overflow rate $Q_o/S(u_{2,0})$, where

$$S(u_{2,0}) = u_{2,0}w(L\sin\theta + b\cos\theta)$$  \(20\)

The concentration of cells in the overflow stream increases as the dimensionless volumetric overflow rate increases. This is due to the fact that when the overflow rate is high, cells will not have time to settle.
out of the suspensions in the settler. The same reason causes a reduction in the amount of cells in the underflow stream, as shown in Figures 6 and 9. The concentration of the coal in the underflow increases with increasing overflow rate (Figures 7 and 10). This is because all of the coal is recovered in the underflow, and the underflow rate is held fixed while the overflow rate increases with increasing feed rate. The recovery of the cells in the overflow also increases as a function of increasing cell volume fraction in the feed stream at a fixed dimensionless overflow rate. Increasing the volume fraction of particles in the feed decreases the settling velocity of the particles in the settler because the hindered settling effects become more important with increased volume fraction.

Figure 3: Comparison of model predictions with the experimental data of Al-Naafa and Selim (1989) for binary suspensions with particles of different sizes and different densities in diethylene glycol with \( \rho = 1.117 \text{ g/mL} \) and \( \mu = 0.2872 \text{ Pa} \) (\( D_1 = 274 \mu m, D_2 = 81 \mu m, \rho_1 = 2.88 \text{ g/mL}, \rho_2 = 2.5 \text{ g/mL}, \phi_1 = 0.2201 \).)

Figure 4: Comparison of model predictions with the experimental data of Al-Naafa and Selim (1989) for binary suspensions with particles of different sizes and different densities in ethylene glycol with \( \rho = 1.113 \text{ g/mL} \) and \( \mu = 0.1736 \text{ Pa} \) (\( D_1 = 274 \mu m, D_2 = 81 \mu m, \rho_1 = 2.88 \text{ g/mL}, \rho_2 = 2.5 \text{ g/mL}, \phi_1 = 0.2201 \).)
Figure 5: The fractional recovery of cells in the overflow vs. dimensionless overflow rates, $\phi_1 = 0.2$ (5a) and $\phi_2 = 0.2$ (5b).

Figure 6: The fractional recovery of cells in the underflow vs. dimensionless overflow rates, $\phi_1 = 0.2$ (6a) and $\phi_2 = 0.2$ (6b).

Figure 7: The fractional recovery of coal in the underflow vs. dimensionless overflow rates, $\phi_1 = 0.2$ (7a) and $\phi_2 = 0.2$ (7b).
Figure 8: The fractional recovery of cell in the overflow vs. dimensionless overflow rates, \( \phi_{1,j} = 0.2 \) (8a) and \( \phi_{2,j} = 0.2 \) (8b).

Figure 9: The fractional recovery of cell in the underflow vs. dimensionless overflow rates, \( \phi_{1,j} = 0.2 \) (9a) and \( \phi_{2,j} = 0.2 \) (9b).

Figure 10: The fractional recovery of coal in the underflow vs. dimensionless overflow rates, \( \phi_{1,j} = 0.2 \) (10a) and \( \phi_{2,j} = 0.2 \) (10b).
Future Work

In this study, we have demonstrated the steady-state theory of inclined settling for a bidisperse settling suspension. An inclined settler may be used to separate coal particles and yeast or bacteria cells using a continuous reactor. In order to compare the theoretical separation ability of the inclined settler with experimental data, several steady-state experiments will be carried out without and then with growth of the cells in the reactor by using the experimental apparatus shown in Figure 1. This theory will later be expanded to cover a wide range of sizes of coal particles and cells.

References

EFFECT OF PLANTS AND TREES ON THE FATE, TRANSPORT AND BIODEGRADATION OF CONTAMINANTS IN THE SOIL AND GROUNDWATER

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SUMMARY

This research examines a new method of biodegradation of hazardous chemicals leaking from landfills. The low level contamination situation under study may make conventional physical, chemical and biological treatments cost ineffective. The potential of using plants in aiding the remediation of contaminated soil and groundwater is being investigated. Factors such as plant species, type of contaminant, and the role of diffusion, transport, transpiration and metabolism of the plant system (soil, root, shoots and stem) are considered. Models are being developed to describe the fate of the contaminants in the soil, roots and plant systems.

INTRODUCTION

There are a number of techniques available to remediate soil and groundwater contaminated with organic compounds. These generally include physical containment, withdrawal and treatment, and in situ treatment. A possible cost effective bioremediation technology has been suggested by Schnoor and Licht that involves the use of deep-rooted poplar trees planted as a buffer zone at the edge of the contaminated site. Plants may stimulate the removal of the hazardous organic substances by uptake and accumulation, metabolism, and microbial biotransformation in the rhizosphere. This could prove to be an efficient treatment technique to remediate soil and groundwater if effective planting and management strategies can be developed.

The following formula for microbial growth, with the organic contaminant as the substrate, defines the oxygen requirements for aerobic biodegradation.

\[ \text{CH}_m \text{O}_l + a\text{NH}_3 + b\text{O}_2 \rightarrow y\text{CH}_p \text{O}_n \text{N}_q + z\text{CH}_r \text{O}_s \text{N}_t + \text{CH}_2\text{O} + d\text{CO}_2 \]

Contaminant (substrate) Biomass Extracellular product

In order to degrade contaminants at low concentration levels in the soil or groundwater the limiting factors will probably be the supply of oxygen and substrate. In situ biodegradation depends on the rhizosphere microbial communities, root exudates which act as
supplemental substrates, oxygen transfer to the soil, and the rate of microbial degradation. This paper examines how plant roots are able to support a viable microbial population which in turn is available to biodegrade contaminants.

Some of the contaminants may be taken up by the roots into the plant before they are broken down by microbes in the rhizosphere. The movement of the contaminants in the soil and ground water is affected by the flow of water through the root zone. A soil and root water transport model and a plant uptake model are presented. A fuller treatment of the topics covered here can be found in an article by Shimp et al. 4

IN SITU BIODEGRADATION

Rhizosphere Microbial Communities

The rhizosphere, the region immediately surrounding the root of a plant, serves as an enrichment zone for increased growth of certain bacteria. Different species of plants support different bacterial flora, via a complex interaction of growth enhancers such as sugars, and inhibitors such as phenolic compounds. Some approaches to understanding this complexity have been reviewed by Bazin et al. 5 Any complete model for the impact of plants on bioremediation must consider not just the plant, but the microbial communities that it supports in different soil zones and different soil types.

Plant-microorganism interactions have been known and studied for a long time. There is a vast body of literature concerning rhizosphere biology, and various aspects of root microorganisms and their interactions with plants have been reviewed. 6 But the structure, dynamics and function of microbial communities on roots and the influence of plant deposits and exudates on rhizosphere microorganisms are still regarded as great challenges to microbiologists, biochemists and engineers.

Plants can serve two different functions in bioremediation. They may act themselves to take up and either emit or degrade compounds, or they may facilitate growth of microorganisms that are proficient in degradation of those compounds. For example, bacteria of the genus Rhizobium have been shown to be proficient in degradation of a herbicide glyphosate. 7 The bacteria are also specifically associated with root nodules of legumes and their numbers in soil may be increased by the growth of legumes. In this way legumes could enhance the degradation of glyphosate indirectly. If one could do a similar selective enrichment or engineering for bacteria that degrade more challenging compounds one might have a significant impact on bioremediation processes.

Experimental work with plants has been mostly limited to studies of herbicides, pesticides and some heavy metals. While Hatzios and Penner 8 discuss metabolism and accumulation of herbicides in plants, there is little evidence that plants can metabolize a wide variety of hydrocarbon compounds. Considerably more work has been done with microbes; some of the effective ones
due to the porosity so that the growth of microbes is enhanced.

**Microbial Degradation Models**

Conley et al.\(^2\) models the reduction in biological oxygen demand (BOD) of wastewater treated by the root zone method which employs wetland plants to oxidize organic contaminants in the rhizosphere and promote microbe growth. First-order kinetics and plug flow are assumed.

\[
\frac{C_e}{C_r} = e^{(-K_f \theta)}
\]

where the hydraulic retention time, \(\theta\); is defined as

\[
\theta = \frac{V_v}{Q} = \frac{\epsilon V}{Q}
\]

The volume of the bed, derived from the previous equations is

\[
V = \frac{Q(\ln C_r - \ln C_e)}{K_f \epsilon}
\]

where

- \(C_f\) = influent concentration (mg/L)
- \(C_e\) = effluent concentration (mg/L)
- \(V_v\) = void volume in root zone bed
- \(K_f\) = temperature dependent rate constant (d\(^{-1}\))
- \(Q\) = wastewater flow rate (m\(^3\)/d)
- \(\epsilon\) = porosity of media bed
- \(\epsilon V\) = void volume in root zone bed
- \(V_v\) = volume of root zone bed (m\(^3\))

In another degradation model for the contaminants in the saturated root zone, a tanks-in-series approach is used where the ground water flows through a network of roots. The microbial growth follows the Monod kinetic model and depends on the concentration of oxygen (\(C_o\)), biomass (\(C_b\)), organic contaminants (\(C_s\)) and root exudates (\(C_r\)). The microbes live in the pore spaces (\(\epsilon V\)) and on the soil particles (\(\rho q_{b, i}\)).

Under steady state flow conditions

\[Q_{i-1} = Q_u + Q_i\]

the contaminant balance is:

\[
eV_i \frac{dC_{s, i}}{dt} + pV_i \frac{dq_{s, i}}{dt} = Q_{i-1} C_{s, i-1} - Q_i C_{s, i} - Q_u C_{s, i}
\]

where the last term includes biodegradation in the pore space and biodegradation on soil particles. The notation is
TRANSPORT IN SOIL AND PLANTS

Water Transport

The root-soil water movement is a major component of the subsurface hydrologic system. A quantitative understanding of water movement in the root-soil environment is needed. Microscopic analyses of the root extraction process have been presented by a number of authors; see, for example, Cushman. This study considered the radial flow of water to a single root, which was modeled as an infinitely long cylinder of constant radius that absorbs water from the soil matrix. However, studies on this scale are impractical for use in field-scale agricultural studies of soil-moisture transport. Thus, many investigators have used a macroscopic representation to describe the water extraction process by a crop's root system. For example, some results have been presented by Bresler.

A major drawback in using this model is that the extraction of soil-water by a root system is simulated by using a sink term in the soil-water flow equation. Thus, the vertical movement of water through the root system is neglected. However, the resistance to the vertical flow of water through a root system can affect the distribution of soil-water extraction by the roots. Marino and Tracy, and Tracy and Mariano developed a coupled root-soil water flow model that was shown to provide a more realistic and accurate description of the movement of water through the root-soil environment.

In their study the soil-water movement in the vertical and horizontal direction of a nonhomogeneous variably-saturated soil can be described as:

\[
\frac{\partial}{\partial x_i} \left[ K_{s_i} \frac{\partial}{\partial x_i} (\psi_s + x_2) \right] - S_w R_d (\psi_s - \psi_r + \psi_o) = (\beta S_s + S_y) \frac{ds_e}{d\psi_s} \frac{d\psi_s}{dt}
\]

Water movement in the root in the vertical and horizontal directions can be described as;

where
\( x_1, x_2 \) = horizontal and vertical direction, respectively
\( K_{s_i} \) = hydraulic conductivity of the soil in the \( x_i \) direction
\[
\begin{align*}
\frac{\partial}{\partial x_i} [K_{ri} R_d \frac{\partial}{\partial x_i} (\psi_x + \psi_w)] + S_w R_d \Gamma (\psi_s - \psi_x + \psi_w) &= R_d \frac{\partial W C_r}{\partial t} + W C_r \frac{\partial R_d}{\partial t}
\end{align*}
\]

\( K_{ri} \) = hydraulic conductivity of the root in the \( x_i \) direction  
\( R_d \) = root density  
\( S_e \) = effective saturation  
\( S_s \) = specific storage of a soil  
\( S_w \) = degree of soil-water saturation  
\( S_y \) = specific yield of a soil  
\( t \) = time  
\( W C_r \) = water content in a root  
\( \Gamma \) = root permeability factor  
\( \psi_o \) = osmotic pressure head of soil-water  
\( \psi_r \) = root-water pressure head  
\( \psi_s \) = soil-water pressure head  
\( \beta = 0 \) if \( \psi_s \leq 0 \)  
\( \beta = 1 \) if \( \psi_s > 0 \)  

The equations can be solved numerically using a Galerkin finite element method with appropriate boundary and initial conditions.  

**Uptake by Plants**

A good review of water and solute movement in plants can be found in Devlin and Witham.\(^ {27} \) Plants generally translocate water through the xylem and solutes through the phloem. The phloem is the inner bark while the xylem is the woody part of the tree. The distance from the leaves where photosynthesis occurs to the roots of the plant are great. The phloem transports solutes through tissue called sieve tube elements. Solute translocation rates can vary from 40 cm/hr for pumpkin to 290 cm/hr for straight-necked squash. The only tree listed was the willow which had a phloem translocation rate of 100 cm/hr; for a metabolite to travel 16 cm, it had to go through 1600 to 2000 sieve plates. The main role of xylem is to transport water. The xylem consists of vessel elements which are open ended, tracheids which are elongated and have open pits on the sides, and fibers. It is through the tracheids and vessels that the plant is able to move large amounts of water.\(^ {27} \)

While extensive research has taken place on the uptake of chemicals by plants, modeling this behavior is a newer development. There are currently two approaches to the modeling of plant uptake. One is from the soil science perspective where the concern is the uptake of pesticides. The other approach arose out of an attempt to understand solute flow in plants. Trapp et al.\(^ {28} \) uses the pesticide approach.

One model of chemical behavior in plants based on plant anatomy was made by Boersma et al.\(^ {29} \) A refinement of this model includes interaction of the root with the soil which allows for coupling with existing models for chemical behavior in the soil. Chemical uptake is further modeled by using relationships which include the partition coefficient (the chemical's log \( K_{oc} \)) and the plant uptake rate. The root concentration factor (RFC) and the
transpiration stream concentration factor (TSCF) are included. The model also takes into account biodegradation in the reactive environment of plasmalemma cells of the symplastic system.

The soybean plant was modeled where compartments were assigned to the soil, the phloem and xylem of root, stem and leaf. The root has a storage compartment assigned to it. The leaf and root phloem compartments were designed to interact with their respective apoplast and symplast compartments. The compartments were separated by physical and chemical barriers designated by reflection coefficients, partition coefficients and hydraulic conductivity. The compartments were defined by volume, contact area with other compartments, sorption coefficient and a coefficient for first order loss processes.

UTAB (Uptake, Translocation, Accumulation, and Biodegradation) is a three leaf, three stem, one root adaptation of the model described in the previous article. Here Boersma et al. makes further refinements with three leaf compartments, three stem compartments and one root compartment. With these modifications, each compartment has two transport compartments; one each for the phloem and the xylem, and a storage compartment. The soil was assigned two compartments for the root outside the Casparian strip: one for the free space in the soil and another for the cell volume. In the leaves and the root the xylem and phloem are allowed to interact, while in the stem the xylem and phloem are separated by the storage compartment.

With this latest model by Boersma et al. the fate of contaminants in water taken up by the tree can be modeled whether the chemical is transpired, accumulated in plant tissue or is biodegraded. This approach also has an advantage over the soil science based model in that the transport of oxygen from the stem through the roots to the soil can be taken into account.

CONCLUSIONS

Plants and trees are capable of assisting in the bioremediation of contaminated soil and groundwater. They provide a favorable subsurface environment by supplying oxygen and additional substrate that promotes microbial growth. Although much is yet to be learned about microbial degradation in the rhizosphere and plant transport mechanisms, the models derived for ground water movement, plant uptake and microbial growth should provide the basis for the design of shelter belts capable of decontaminating soil and groundwater.

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the views of the Agency and no official endorsement should be inferred. This research was partially supported by the Kansas State University Center for Hazardous Substance Research.

REFERENCES


Sound Production by Interfacial Effects in Airlift Reactors

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Summary

An experimental investigation of gas-liquid interfacial phenomena in an airlift fermentor has been carried out with macrovideography and acoustic signals generated at both the sparger and the free surface. Several liquid media, including distilled water, distilled water with electrolyte (NaCl), and solutions of glycerol in distilled water, were used to investigate the effects of electrolyte and viscosity. Sound spectra and frequencies from the disengagement region and from the sieve plate region are quite different over a wide range of superficial gas velocities. In addition, changes in the bubble formation pattern (number and location of active holes in sieve plate) were studied as a function of superficial gas velocity using videography and light scattering. This phase of the investigation has revealed some significant transitions that may contribute to loss of cellular viability in some sparged reactors.

Introduction

Airlift reactors, which have a number of advantages over more traditional stirred tank reactors, have been increasingly used for aerobic fermentations. Because airlift reactors provide adequate agitation at low average shear stresses, they have been shown to be viable bioreactors for large scale suspension cultivation of animal and plant cells. However, the shear sensitivity of these cells is still a major concern in the application of airlift fermentors, and an understanding of the cause of loss of cellular viability is a necessary forerunner to successful design and appropriate operation of airlift reactors for the culture of plant and animal cells.

Some recent speculations in the literature suggest that gas-liquid interfacial phenomena have played prominent roles in observed instances of loss of viability in both insect and animal cell cultures. However, there is no consensus regarding the effects of bubble formation, detachment, translation, coalescence, breakage, and disengagement upon cellular viability in cultures with direct sparging. Tramper et al.¹ considered that bubble rising and bursting could be a source of damage to cells from the results of insect cell suspension cultures. They also concluded² that bubble formation in the region of air injection might be primarily responsible for loss of cell viability. Handa et al.³ reported experimental evidence for their hypothesis that cell death in sparged systems was associated only with the region of bubble disengagement from the free...
liquid surface. Handa-Corrigan et al.\textsuperscript{4} presented their experimental observations and proposed two mechanisms of cell damage in the region of bubble disengagement: damage due to rapid oscillations caused by bubble bursting and shearing in draining liquid films in foams. Kunas and Papoutsakis\textsuperscript{5} determined apparent growth rates for hybridoma cultures in agitated reactors with and without bubble entrainment. Their results indicated entrained bubbles interacting with a freely moving gas-liquid interface caused significant cell damage.

In studies of bubbles as sources of ambient noise\textsuperscript{6-8}, it has been shown that some sounds produced in water are related to bubble dynamic behavior, e.g., bubble oscillations. Consequently, we have initiated a study of gas-phase acoustic signals produced by an airlift fermentor with the expectation that certain "dangerous" phenomena might leave characteristic earmarks.

Experimental Apparatus

All the results were obtained with an acrylic plastic, 3-liter, split column airlift reactor, which is depicted in Figure 1. The reactor had optically-flat faces, but the interior corners were filleted to minimize possible dead-region effects. Air was introduced through 14 1-mm holes in a sieve-plate sparger and the apparatus could be operated at superficial gas velocities ranging from 0.3 to 25 cm/s.

A standard acoustic microphone was employed for detection of sounds produced at the top and bottom of the column. The signal from the mic was sampled with a Nicolet 4094A digital storage oscilloscope (DSO) using time intervals of 100 to 500 \textmu s and the data were transferred to an IBM-compatible PC for further processing (mean and root-mean-square, autocorrelation coefficient and Fourier transform). A commercial software package was used to create graphic images of the spectra.

Results and Discussion

An example of output from the microphone (when positioned at the top of the column) is provided in Figure 2 to illustrate the different sound frequencies from the sparger and the free surface. It is obvious that the bubble formation sounds at the sieve plate (the first part of this record) have lower frequencies than that of the bubble disengagement noises which appear at 0.046 s. Here, the noises accompanying rising bubbles are negligible due to their very low intensities.\textsuperscript{8,9}

The root-mean-square (RMS) values measuring the intensities of sound signals increase with increasing superficial gas velocities \((u_g, 0.3 \text{ to about } 25 \text{ cm/s})\). The RMS data with different liquids and at the different positions are summarized in Figure 3. The influence of superficial gas velocity on the RMS values for the glycerol solution is larger than it is on those for water. The variation of RMS values from the mic located at the bottom outside the column is insignificant when \(u_g\) increases.

The spectra in Figure 4 (distilled water) also show the effects of the superficial velocity on the noises produced. With increasing superficial gas velocity, the spectral peak for bubble disengagement near 1000 Hz exhibited a maximum around \(u_g = 1.425 \text{ cm/s}\) and a secondary peak at 1200 Hz emerges at slightly larger gas rates. Bubble disengagement does not appear to be a major source of sound at either very low or very high superficial velocities. Comparing Figure 4 with Figure 5, significant differences are observed for the peaks in the disengagement region.

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with variation of liquid viscosity. Some peak shift is apparent in Figure 5, where increased media viscosity appears to result in more persistent noise levels at 1000 to 1300 Hz; the increased acoustic energy suggests that increasing viscosity may actually cause cellular damage in the disengagement region. The addition of electrolyte gave almost identical results (Figure 6) with those in Figure 5, except that no clear maximum in detected signal energy was found over the experimental range of superficial velocities, $0.782 < u_g < 6.00 \text{ cm/s}$.

Bubble formation pattern transitions at the sieve plate were also investigated. Typical findings for both distilled water and aqueous glycerol solution are illustrated by Figure 7. The frequencies of change of bubble formation pattern show several maxima accompanying some violent motions in this region. The frequency declined steadily at higher gas rates, effectively reaching zero at about $u_g > 7 \text{ cm/s}$, when all 14 sieve plate holes began to bubble actively.

Conclusions

The spectra obtained clearly show the importance of the bubble formation and disengagement processes with respect to sound generation. These processes produce the dominant spectral features in nearly all of the recorded cases. The results have led us to further consideration of an earlier hypothesis that bubble disengagement phenomena, particularly bursting, are especially harmful to cellular entities. At the same time, possible cellular enrichment at the gas-liquid interface\textsuperscript{10} may have an amplifying effect upon the importance of gas disengagement phenomena in sparged cultures. Solely on the basis of the acoustic data, we believe the bubble formation process at the sparger is energetic enough to warrant additional attention.

Increasing superficial gas velocity will prominently change the noises produced at the disengagement region. Enhanced disengagement noises appear for gas rates less than about 1.5 to 4 cm/s in the distilled water system and 2.5 to 7 cm/s in the 61\% glycerol system. In contrast, disengagement noises seem to decrease with the increase of $u_g$ in the electrolyte solution system. The dominant spectral features detected at the top of the column suggest disengagement and flow over the divider as important sound producers. Increased electrolyte content and viscosity of liquid do not have significant effects upon bubble formation noises, but do appear to affect those of bubble disengagement, especially increasing the peak levels of spectral densities for bubble disengagement.

Acknowledgement

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References


Figure 1. Photograph of the 3-liter, split-column, airlift reactor operating with distilled water at $u_g=0.8$ cm/s. The sieve-plate sparger had 14 1-mm diameter holes.
Figure 2. Typical signal obtained from microphone positioned above the disengagement region using distilled water with $u_g=0.69$ cm/s. Water level was elevated to eliminate noise produced by flow cascading over the divider.

Figure 3. Variation of RMS voltages with superficial gas velocity for distilled water and aqueous glycerol systems. Note the large values obtained for mic located in the gas space under the sieve plate.
Figure 4. Effect of superficial gas velocity on spectra obtained with distilled water.
Figure 5. Effect of superficial gas velocity on spectra obtained with 61\% glycerol.
Figure 6. Effect of superficial gas velocity on spectra obtained with 0.117 M NaCl solution.
Figure 7. Frequency of bubble formation pattern changes observed per minute for distilled water and 50 percent glycerol systems at superficial velocities ($u_g$'s) ranging from 0 to 7 cm/s.
SOY YOGURT FERMENTATION OF RAPID HYDRATION HYDROTHERMAL COOKED SOY MILK

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ABSTRACT

Soy yogurt made from rapid hydration hydrothermal cooked soy milk (RHHTC) was studied. The effect of heat treatment through the steam infusion unit on RHHTC soy milk was monitored chemically and microbiologically. Concentrations of sucrose, raffinose, and stachyose are significantly reduced due to the effect of steam infusion heating at 290, 300, 305, 309, and 314°F. At temperatures ranging from 290 to 314°F, no microbial survivors were detected if the sterilization of the process equipment before treatment is achieved. The yogurt was successfully fermented. The final pH of all samples treated with different temperature levels falls in the range of 4.5 to 4.7. RHHTC soy yogurt treated at 305°F gives the highest viscosity (259.38 cp). Sucrose in soy milk is readily metabolized by yogurt bacteria.

INTRODUCTION

Research on the fermentation of soy milk using different kinds of lactic acid bacteria has been conducted. The methods of production, sensory analysis of the products, and utilization of soybean carbohydrate have been investigated. Raffinose and stachyose can be successfully utilized by some lactic acid bacteria, and the authors reported that the bacteria have α-galactosidase enzyme to hydrolyze these sugars. Soy milk has been efficiently produced by using direct steam infusion in the Rapid Hydration Hydrothermal Cooking (RHHTC) system developed in Kansas State University. The soy milk shows reasonably good characteristics to be used as an ingredient for making soy yogurt because of its high solid content and viscosity. The
curdling pH (pI) is about 4.75 which is close to the pH of good bovine yogurt (4.5). The yogurt fermentation of hot grind soy milk was conducted previously at Kansas State University. The author reported that the conventional soy milk needs to be fortified in order to obtain good quality soy yogurt. Thus, it seems possible to use RHHTC, an effective processing method, to make soy milk, for yogurt production. The process is a new way to stabilize soy protein. Basic research is needed to manufacture soy yogurt with consistent quality using soybean ingredients.

Soybeans are less expensive and more plentiful than bovine milk. Moreover, soy milk has no cholesterol. Soy based products have already been developed which compete favorably with dairy products. Yogurt is the fastest growing American cultured dairy product. The consumption per capita increased 211% from 1973 to 1983. Frozen soy yogurt can command a market on the strength of being cholesterol free. Thus, the economic incentive exists to develop a soy yogurt and frozen soy yogurt, in which soy can be utilized and used as a major ingredient. The fundamental knowledge for the production of such products is needed. This research is designed to develop basic information on the fermentation process. In the future work, process variables that may increase the acceptability to the product, biochemically and sensorily will be studied.

MATERIALS & METHODS

I. SOY MILK PREPARATION

Soy milk was prepared by using the rapid hydration hydrothermal cooking (RHHTC) method (Figure 1). RHHTC method was as described elsewhere.

II. TREATMENT OF SOY MILK

Soybean flour was prepared as described elsewhere. It was heated in the RHHTC unit at temperatures ranging from 290 to 314°F for 30 to 35 seconds. The holding tube was a 37 foot folded tube. Cell count (standard plate count) of the RHHTC soy milk was used to determine its sterility. Soy milk released from the heat treatment process was analyzed for changes in the concentrations of sucrose, raffinose and stachyose by HPLC. Ten grams of sample were weighed and diluted with isopropanol in a 25 mL volumetric flask. The suspension was mixed well and let stand for 20 min.; then it was centrifuged to get rid of protein solids. The supernatant was filtered twice with 7-cm Whatman no.50 and Waters C-18 (Sep-pak) solid phase
extraction cartridges. The final liquid portion was injected to Varian 5000 HPLC operating at room temperature (25°C) to analyze for sugar concentrations. The HPLC was equipped with an Amino-5 column (Bio-system); HPLC grade acetonitrile/water (75/25) was used as a solvent. The Waters refractometer detector was adjusted at 32X attenuation. Flow rate of the solvent was fixed at 0.8 mL/min. Viscosity, and total solids of the RHHTC soy milk were analyzed by standard AOAC methods. The quality of treated soy milk as a substrate for yogurt fermentation was tested as well. The microbial growth, stachyose and raffinose concentrations, and acid production were measured.

III. SOY YOGURT FERMENTATION

The starter culture was propagated for about 16 hours in soy milk medium before use. Five percent by volume of starter culture inoculum was added into each 200 mL of soy milk sample. The mix was stirred and incubated at 44°C for 6 hours. The basic characteristics of yogurt, sugar concentrations, pH, acidity, and cell concentration were monitored as described in Section II and IV.

VI. MICROBIAL COUNT AND IDENTIFICATION

Viable cell counts of soy slurry, and soy milk were made using standard plate count method. For bacterial identification, 300 colonies from each petri dish were randomly selected. Gram negative bacteria were tested for their oxidase activity. Oxidase positive isolates were identified by Oxi/Ferm tube test kits (Hoffman-La Roche Inc., Nutley, NJ 07110), while oxidase negative bacteria were tested by Micro ID test kits (Organon Technika Inc., Durham, NC 27704). Gram positive bacteria are identified by their morphological and biochemical properties.

RESULTS AND DISCUSSION

Figure 2 shows that the sterilization of soy milk using RHHTC process is achieved at temperatures above 270°F for time above 29 sec. The initial microbial numbers in soy slurry are about 10⁴ to 10⁵ cells/mL. The RHHTC soy milk treated at 290 to 314°F is sterile and can be kept for months without precipitation. The predominant Gram negative bacteria in soy slurry are Pseudomonas sp., Serratia liquifaciens, S. rubidaea, and Enterobacter cloacae, while the Gram positive bacteria are Bacillus sp. The only bacteria which survives RHHTC steam infusion at temperatures lower than 270°F for 29 sec. is a spore forming Bacillus sp. (Figure
2). Consequently, temperatures of 290 to 314°F are chosen to produce the sterile RHHTC soy milk in this study.

The characteristics of soy milk are shown in Table 1. The neutral pH (6.8) is observed from all soy milk samples treated with different temperatures, 290 to 314°F. The soy milk produced at 290°F has the lowest viscosity while those of 305 and 309°F treatments are as high as 19.5 and 17.5 cp, respectively. The 290°F soy milk has the highest solids content, and the solids content decreases as the treating temperatures increases (Table 1). RHHTC soy yogurt samples produced from these soy milk samples have the properties as shown in Table 2. The pH values of soy yogurt are in the range of 4.5 to 4.7, which are close to the pI of soy protein (4.75). Thus, the curds have a very good texture characteristic; viscosities are 187, 167, 259, and 197 cp, respectively for 290 to 309°F treatments. Acidity values of the yogurt samples ranged from 0.6 to 0.8%, as lactic acid, respectively. Treatment at 300°F gave the highest lactic acid production.

Sugars concentrations are reduced due to the steam infusion heat treatment by RHHTC process (Tables 2, 3). In Table 3, sucrose, raffinose, and stachyose concentrations are significantly reduced ($\alpha = .05$) by 28.87%, 12.7%, and 14.79%, respectively; the soy milk concentrations are 0.345, 0.103, and 0.461 g/100mL for the respective oligosaccharides. Sucrose is readily metabolized by yogurt bacteria; after fermentation, the final sucrose concentrations ranged from 0.066 to 0.088 g/100mL. There are no significant differences among the final sucrose contents in the soy yogurt samples treated with different temperatures. Treatment at 305°F gives the lowest final stachyose concentration, 0.418 g/100mL; however, no significant difference among these concentrations was found. There are no significant effects of soy milk preparation temperature on raffinose concentration in the soy yogurt for the data in Table 2. Comparing the sugars concentration in soybean slurry, RHHTC soy milk, and soy yogurt, sucrose concentrations are significantly reduced. The concentrations decrease significantly from soybean slurry to RHHTC soy milk and soy yogurt (Table 3). The final sucrose concentration is low, which shows a vital activity of the cultures. On the other hand, the concentration of stachyose is significantly reduced due to the effect of heat treatment (Table 3). However, the difference of stachyose concentrations before and after yogurt fermentation are not significant ($\alpha = .05$). The results show that *S. thermophilus* and *L. bulgaricus* can readily utilize sucrose, but
they do not metabolize significant quantities of the raffinose and stachyose present in soy milk.

CONCLUSIONS

The rapid hydration hydrothermal cooking process has high efficiency in the production and sterilization of soy milk. At a process time of 29 sec., 270°F is required for the complete destruction of cells and spores in the slurry. RHHTC soy milk from 305°F sample is the best substrate for yogurt fermentation with good utilization of sugars and good texture. Sugar concentrations are significantly reduced after heat treatment and fermentation. Sucrose concentration is 84.12% less than that of the original soy slurry, but raffinose and stachyose reductions are only 28% and 17%, respectively.

REFERENCES


### TABLE 1. The Characteristics of RHHTC Soy Milk Treated with Different Temperatures

<table>
<thead>
<tr>
<th>TEMP. (°F)</th>
<th>pH</th>
<th>SOLIDS (%)</th>
<th>VISCOSITY (cp)</th>
<th>SUGAR CONCENTRATION (g/100mL)</th>
<th>SUCROSE</th>
<th>RAFFINOSE</th>
<th>STACHYOSE</th>
</tr>
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<tbody>
<tr>
<td>290</td>
<td>6.85</td>
<td>9.64</td>
<td>5.5</td>
<td></td>
<td>0.377</td>
<td>0.127</td>
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### TABLE 2. The Characteristics of RHHTC Soy Yogurt from Different RHHTC Soy Milk

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<th>TEMP. (°F)</th>
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<th>VISCOSITY (cp)</th>
<th>SUGAR CONCENTRATION (g/100mL)</th>
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NT=Not Tested

### Table 3. The Comparison of Per Cent Solids and Sugars Concentrations in Soybean Slurry, RHHTC Soy Milk, and Soy Yogurt

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* The same letter indicates no significant difference.
Figure 1. Modified Penick and Ford Jet Cooker and Cooler

Figure 2. Surviving Bacterial (%) in RHHTC Soy Milk after Steam Infusion Heat Treatment for 28 to 35 sec.
ANALYSES

1. PCP in soil slurry
   The well mixed soil slurry was sampled by 10 mL sterilized wide mouth pipet. The sample was separated into clean aqueous phase and sedimentary soil by centrifugation at 15000 rpm for 10 minutes. The concentration of PCP in clean aqueous phase was determined by HPLC. PCP in sedimentary soil was extracted by 10 mL 2-propanol in 50 mL Teflon capped tubes. After shaking for 2 hours, the mixture was centrifuged and the supernatant was collected. The soil was extracted a second time again with 2-propanol. The two supernatants were mixed and filtered through a 0.2 µm filter. The analysis of PCP in the supernatant was conducted with Perkin Elmer Model 250 HPLC involving a C-18 column and a UV detector at 254 nm. The solvent used was a 100:1 mixture of acetonitrile and acetic acid at a flow rate of 0.5 mL/min.

2. PCP in soil beds
   Soil was sampled by a sterilized spoon, and extracted with 2-propanol in 50 ml teflon capped tubes. The procedure of extraction and PCP determination were same as the one described above.

EXPERIMENTS

1. Growth of Phanerochaete chrysosporium and PCP degradation in soil beds
   The soil beds for growth were prepared by adding carbon source and basal salts solution (except veratryl alcohol, and Tween 80) into 200 g soil in 300 ml flasks. Different amounts of PCP were added to soil. After sterilization, either mycelia or spores were used to inoculate in different flasks. The incubation temperature was 30 °C. Moisture content of soil was 17% to 23% for the duration of experiments. The growth of Phanerochaete chrysosporium was observed qualitatively by the white conolies that appeared in the soil beds. Initial and final extractable concentrations of PCP in soil were monitored. Several control flasks containing only the soil with and without cells were also set up.

2. PCP degradation in soil slurry
   The soil slurries were inoculated either with mycelia or with spores inoculum. The spore inoculum consisted of 2 mL spore suspension (5X10⁷ spores per ml) into 170 mL soil slurry. The mycelial inoculum was obtained from homogenized mycelia which were grown in nutrient broth containing 20 mg/L of PCP. Initial pH values of the culture media were between 5.8-6.2. Sponge stoppers were used to allow exchange of air. Sample were taken by wide mouth pipet to prevent blockage by mycelium pellet and soil particals. The soil slurry was made up by adding soil (30%, w/w), Tween 80 (0.1%, v/v), and veratryl alcohol (1 mM) in nitrogen-limited nutrient medium in 1000 ml unbaflled Erlenmeyer flasks at 30 °C. The carbon sources for cell growth were 2g/L glucose, or 10g/L glucose, or 10g/L peat moss. PCP in soil slurry was extracted and monitored by HPLC.

RESULTS AND DISCUSSION

1. Effect of carbon source on growth of Phanerochaete chrysosporium in PCP contaminated soil beds
   The colonies of Phanerochaete chrysosporium showed up on the surface and on the walls of the flask around the soil. The experimental observations related to growth are presented in
Little growth of the cells was observed in control soil-beds that were not supplemented with carbon source and basal salts. The observations in flasks supplemented with glucose and inoculated with spores are shown in Table 1. In these experiments, a spore suspension containing about $5 \times 10^7$ spores was added to the top of the soil bed but the bed was not disturbed. In this case, addition of basal salts resulted in improved growth of cells. Flasks containing basal salts and glucose ($10 \text{ g/Kg soil}$) showed the best overall growth. These observations are in general agreement with those of Lamar et al. (1990) who reported that the fungi would not grow in soil without addition of some organic matter. In the flasks containing no PCP, the first colonies were observed by third day and the growth appeared to peak by the sixth day.

Addition of PCP to the soil inhibited the growth potential in the soil beds also. The concentrations of 30 mg extractable PCP/Kg-soil and above increased lag phase substantially. In flask containing 60 mg extractable PCP/Kg-soil, no colonies (reflecting germination of spores) were observed even after 15 days. In fact, none were observed after 25 days when the experiments were terminated.

Experimental observations from flasks supplemented with peat moss ($10 \text{ g/Kg-soil}$) are presented in Table 3. In this experiment, the concentrations of extractable PCP ranged between 30 - 150 mg/Kg-soil. The flasks were inoculated with either spore suspension ($5 \times 10^7$ spores) or growing mycelia (10% v/v) but the soil bed was not disturbed after inoculation. As a result, the colonies appeared faster than when the beds were mixed, perhaps because of higher inoculum densities on the surface of soil. Again, the colonies appeared first on the surface and then spread around all the surfaces. A major difference in these observations from those made in flasks supplemented with glucose, is in the growth of cells at higher PCP concentrations. In the presence of peat moss, the spores germinated even at 60 mg extractable PCP/Kg-soil and the mycelia tolerated even 150 mg extractable PCP/Kg-soil. Still, the spores were inhibited to a greater extent than the mycelia. Mycelial inoculations showed more exuberant growth also. The reasons for the beneficial effects of peat moss are not very clear. The addition of peat moss increases the level of available carbon, potassium, and to some extent nitrogen. The presence of peat moss as organic carbon source also influences the adsorption and desorption of PCP. It has been noticed that solvent-extractable PCP from soils containing peat moss is significantly higher than that from soils without peat moss. This may have some impact upon the cells attached to the soil matrices as these will be exposed to lower PCP concentrations.

2. PCP degradation in soil-beds

The concentration of PCP was measured in soil samples taken on the first day and on the 25th day after incubation at 30 °C in dark. The experiments involved two types of inocula (spore and mycelial), two carbon sources (glucose and peat moss) and two levels of glucose ($2 \text{ g/Kg-soil}$ and $10 \text{ g/Kg-soil}$). On the basis of results presented in Figures 1 to 3, no significant difference was observed between flasks inoculated with mycelia and those inoculated with spores, when supplemental carbon was absent. Addition of supplemental carbon resulted in an increase in the amount of extractable PCP that disappeared from the soil. When the concentration of PCP (extractable) was increased above 60 mg/Kg-soil, the soil containing peat moss still resulted in significant reduction in extractable PCP. Reductions in glucose concentrations from $10 \text{ g/Kg-soil}$ to $2 \text{ g/Kg-soil}$ did not significantly alter the extent of PCP removal from soils. Peat moss ($10 \text{ g/Kg-soil}$) could be used to substitute glucose as a carbon source, without any increase in extractable PCP after 25 days. This observation may suggest peat moss as a more desirable carbon source. Similar observations have been reported also by Zytner et al. (1989) who found that peat
moss can absorb up to eight times its own weight of perchloroethylene. Up to 80% reduction in PCP concentrations was observed even in flasks (supplemented with peat moss and inoculated with mycelia) containing 60 mg extractable PCP/Kg-soil initially.

3. PCP degradation in soil slurry

The soil slurry was made up by adding 30% (w/w) soil, 0.1% (v/v) Tween 80, and 1 mM veratryl alcohol with nitrogen-limited nutrient media in 1000 mL un baffled erlenmeyer flask at 30°C. Either glucose (2 and 10 g/L) or peat moss (10 g/L) was used as carbon source. Several different concentrations of PCP were used in these experiments. In each case, an abiotic control was also incorporated. Incubations were conducted at 30°C and 200 rpm in a rotary shaker. Samples were collected at regular intervals and analyzed for PCP concentrations after extractions with 2-propanol. Figures 4 to 6 show the results of these analyses from soil slurry experiments.

The results comparing glucose and peat moss as carbon sources are presented in Figure 4. In this case, a mycelial inoculum was used. In all cases, analysis of aqueous phase showed no detectable PCP. Extractions of soil phase with 2-propanol showed a rapid disappearance in the concentration of extractable PCP in flask containing peat moss, but little loss in abiotic flask as well as in the one containing 10 g/L glucose over a period of 20 days. Glucose has been known to cause catabolite repression of ligninase system in Phanerochaete chrysosporium. Peat moss, being a very complex substance, apparently does not activate the repression in this system. These results are in agreement with those of Lamar et al. (1990).

Figure 5 shows the results of experiments involving two different levels of glucose in the soil slurry inoculated with spores. The kinetics of these measurements suggest that PCP disappears more rapidly in the flask containing lower level of glucose. After 22 days, even the flask with 10 g/L glucose starts to show PCP disappearance. Unfortunately, the experiment was terminated on day 30. Hence, this aspect could not be pursued any further. These findings appear to be in disagreement with the results obtained from soil beds where virtually no effect of carbon source was observed. However, in soil beds, only two widely spaced (timewise) samples were analyzed and, therefore, no kinetics was investigated. Here, even though the two carbon sources show different kinetics, degradation is predicted for long periods of time.

Results from flasks containing 60 mg extractable PCP/Kg soil (Figure 6), show virtually no reduction in PCP concentration over 30 days. Apparently, this concentration of PCP is very toxic to the cellular metabolism. Unfortunately, cell concentration could not be measured from these samples. Hence, it is not possible to say whether this persistence of PCP is due to inhibition of growth or due to inhibition of PCP metabolism. Lamar et al. (1990) have suggested that mycelia can tolerate PCP concentrations as high as 500 mg/L, but the spores are significantly more sensitive. Since the spores were used to inoculate the flasks for this set of experiments, the results obtained here are in agreement with those of Lamar et al. (1990).

CONCLUSIONS

1. Supplementation of soil with additional carbon source is desirable to enhance cell growth.
2. Supplementation with peat moss may be more desirable due to higher tolerance of cells to the concentration of contaminant.
3. The spores were able to germinate in soil containing up to 60 mg PCP/Kg soil when 10 g peat moss was added to the soil.
4. Mycelia can tolerate higher concentration of PCP than do the spores.
5. In presence of peat moss, the highest extracted concentrations of PCP that could be metabolized in soil-beds were 60 mg/Kg-soil.

6. In kinetic studies in soil-slurries, a clear catabolite repression by glucose was observed. Peat moss did not invoke the repression and resulted in rapid removal of up to 30 mg extractable PCP/Kg-soil.

REFERENCES


Table 1. Effect of Glucose and Basal Salts on Growth of *Phanerochaete chrysosporium* in Sol-beds with Inoculum of Spores

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Table 2. Effect of PCP on Growth of *Phanerochaete chrysosporium* in Soil-beds with Inoculum of Mycelia. 10 g/Kg-soil Glucose as Supplemental Carbon Source

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Table 3. Effect of Type Inoculum (Mycelium vs. Spores) on Growth of *Phanerochaete chrysosporium* in Soil-beds. 10 g/Kg-soil Peat Moss as Supplemental Carbon Source

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202
Figure 1 PCP Degradation in Soil-beds (30 mg extractable PCP/Kg-soil)

Initial conc. of PCP

Final conc. of PCP

0-PCP in soil
1-9: Extractable PCP in soil
1. Abiotic
2. Peat moss
3. Peat moss+mycelium
4. Spore
5. Mycelium
6. Glucose(10 g/Kg)+spore
7. Glucose(10 g/Kg)+mycelium
8. Glucose(2 g/Kg)+spore
9. Glucose(2 g/Kg)+mycelium

Figure 2 PCP Degradation in Soil-beds (60 mg extractable PCP/Kg-soil)

Initial conc. of PCP

Final conc. of PCP

0-PCP in soil
1-9: Extractable PCP in soil
1. Abiotic
2. Peat moss+spore
3. Peat moss+mycelium
4. Spore
5. Mycelium
6. Glucose(10g/Kg)+spore
7. Glucose(10g/Kg)+mycelium
8. Glucose(2g/Kg)+spore
9. Glucose(2g/Kg)+mycelium
1-6: Extractable PCP in soil
1- Abiotic
2- Peat moss+spore
3- Peat moss+mycelium
4- Mycelium
5- Glucose(10g/Kg)+mycelium
6- Glucose(2 g/Kg)+mycelium

Figure 3 PCP Degradation in Soil-beds (150 mg extractable PCP/Kg-soil)

![Bar chart showing PCP degradation in soil-beds.](image)

- Initial conc. of PCP
- Final conc. of PCP

Figure 4. Effect of The Type of Supplemental Carbon Source on PCP Degradation in Soil Slurry with Mycelial inoculum (Extractable PCP 30 mg/Kg-soil). ◆ Abiotic Control;
◆ 10 g/L Glucose; □ 10 g/L Peat Moss

![Graph showing PCP degradation over days.](image)
Figure 5. Effect of the Concentration of Glucose on PCP Degradation in Soil Slurry with Spore Inoculum (Extractable PCP 30 mg/kg-soil). □ Abiotic Control; ■ 10 g/L Glucose; □ 2 g/L Glucose

Figure 6. Effect of the Concentration of Glucose on PCP Degradation in Soil Slurry with Spore Inoculum (Extractable PCP 60 mg/kg-soil). □ Abiotic Control; ♦ 10 g/L Glucose; ◇ 2 g/L Glucose
CELLULAR RESPONSES OF INSECT CELLS SF-9 TO HYDRODYNAMIC STRESSES

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Department of Chemical Engineering,
University of Missouri, Columbia, MO 65211

ABSTRACT

Cellular responses of insect cell Spodoptera frugiperda to well-defined shear stress are studied. Anchoradge independent Sf-9 cells were subjected to lamimiar shear stresses up to 10 dynes/cm² in a Brookfield viscometer. Cells exposed to shear resulted in 30% decreases in intracellular calcium ion concentration. Effect of non-ionic surfactant F-68 upto 0.2% (w/v) did not cause significant change in Ca²⁺, but 40% increase in Ca²⁺ was observed in addition of 1.0% (w/v) F-68. Population distribution of Sf-9 cells in cell cycle was shifted after exposure to sublethal shear of 5 dynes/cm² for 90 min.

INTRODUCTION

Microbial, plant, and animal cells are frequently exposed to environmental stress in bioengineering and processes, including dissolved oxygen concentration, pH, nutrients, and hydrodynamic stresses. Effect of hydrodynamic stress on various eukaryotic cells have been widely reported in biomedical literature. But the results are diverse and contradicting. The disparity of cell damage mechanism may arise from the hydrodynamic parameters in bioreactor and model of cell responses.

The term shear sensitivity of a specific cell line is often used in biotechnology in terms of cell viability, which is measured by dye exclusion method. More subtle measurements of cellular responses to hydrodynamic stress is required to elucidate cell damage mechanism in sublethal range.

It is well known that plasma membrane of cells plays a vital role in cellular response to environmental stress. Intracellular calcium ion Ca²⁺ is recognised as one of the universal second messengers involved in protein secretion, energy metabolism, and growth and differentiation (Rasmussen, et al., 1985). Levesque et al. (1989) studied the effect of shear stress on endothelial cells and link cellular response to arachidonic acid mechanism (Fig.1). This signalling pathway is controlled by Ca²⁺ via membrane phospholipid metabolism (Fig.2). It is postulated that Ca²⁺ is a subtle indicator to plasma membrane integrity in response to extracellular hydrodynamic stress.

Cell growth can be monitored by cell cycle analysis. Mitosis are phase of DNA replication. Prior to M phase is denoted as G₂ phase including events of nuclear membrane breakdown and rearrangement of cell surface and cytoskeleton. S phase is denoted for DNA replication and G₁ phase is for protein synthesis. In general it takes 12-18 hr for a complete cell cycle. Effect of hydrodynamic stress on cell growth can be expressed in terms of population distribution of different phases in cell cycle.

In this study, anchorage-independent insect cells were used. Effect of hydrodynamic stress on cellular responses were studied using intracellular calcium ion concentration and flow cytometric measurements.
MATERIAL AND METHODS

Cell Culture

Insect cell line Spodoptera frugiperda (Sf-9, passage 18) was obtained from ATCC (Rockville, MD) and used for experiments between passage 22-40 in this work. Sf-9 cells were grown on stationery T-175 culture flasks (Falcon, PA) at 28°C. TNM-FH medium was supplemented with heat inactivated 10% fetal bovine serum (Sigma, MO). The initial osmolarity and pH of the medium were 355 mOsm/l and 6.2, respectively. Sf-9 cells were subcultured twice a week.

Cell Counts

Cell counts were measured using hemacytometer. Viabilities were determined by trypan blue exclusion method. An average cell number was taken from 4 cell counts from each of 2 independent samples.

Shear Experiment

Confluent cultures of Sf-9 cells on T-175 flasks were removed by gentle pipetting. Dissociated cells were counted and used in shear experiments. Brookfield viscometer of small sample adaptor was used. A computer interface was installed to control larinar flow field.

Calcium Ion Measurement

To load cells with fura-2, a fluorescent calcium-binding probe, a 2 ml aliquot of the cell suspension of 2 x 10^6 cells was incubated with 2 μM fura-2AM (Sigma, MO) for 20 min at 28°C. The hydrolysis of the membrane permeant fura-2AM by nonspecific cytoplasmic esterases traps fura-2 inside the cell. Fura-2 loaded cells were washed twice with 2 ml of balanced salt solution (BBS, pH 6.2) and incubated for 5 min at 28°C. The cell sample was transferred to a quartz cuvette and placed in a temperature regulated sample-chamber of a dual-excitation spectrophotometer (SPEX, Edison, NJ) for Ca^{2+} measurements. After 2 min of equilibration period, the cells were sequentially illuminated with 340 and 380 nm light. Fluorescence intensity measurements were converted to calcium ion concentrations according to the calibration equation described by Grynkiewicz et al. (1985).

Flow Cytometric Analysis

Flow cytometric analysis was run on an EPICS S Flowcytometer. Sf-9 cells were resuspended in 0.2 ml TNM-FH medium containing 10% FBS at a concentration of 10^6 cells/ml. 0.8 ml ice-cold 70% ethanol was added to cell suspension. After incubate in the dark for 30 min at 4°C, the cells were washed twice in BBS and then resuspended in Krishan stain solution. Samples of 10^6 cells were analysed by flowcytometer at excitation wavelength 380 nm.

RESULTS AND DISCUSSION

Animal cells exhibited wide range of shear sensitivity with respect to the stress level required to cause overt cell lysis. It is proposed cell damage mechanism can be tackled by studying the second messenger system. After cell harvest at exponential growth phase, Ca^{2+} of Sf-9 cells was measured to be 178 nm, which is comparable to the range of Ca^{2+} for mammalian cell lines reported in literature. In Table 1, cytoplasmic Ca^{2+} of 6 eukaryotic cell lines was presented.

Nonionic surfactant F-68 is widely used in medium preparation in replacement of serum. It is proposed Pluronic F-68 had protective effect on animal cells in agitation and sparging.
Murhammer and Goochee (1990) proposed F-68 formed a sheath layer around cell membrane for its protective mechanism. In our study, adding F-68 into the medium caused an increase of \( \text{Ca}^{2+} \) (Fig. 3A). It was found Pluronic F-68 concentration is proportional to the increase of base level of \( \text{Ca}^{2+} \) (Fig. 3B). Up to 0.2% (w/v) F-68 only increase 5% \( \text{Ca}^{2+} \) base level. But higher concentration of F-68 and other surfactant such as tween-80 had advert effect on cell growth which might be due to disturbance of cell membrane.

To study the effect of shear stress, cell cultures were exposed to stress of 10 dynes/cm\(^2\) for 30 min. Cell viability test showed no difference in cell samples after shear, Thus this level of shear belonged to sublethal range. After loaded with fura-2, decrease of 30% base line of \( \text{Ca}^{2+} \) was observed (Fig. 3). This might suggest that sheared cells might be more responsive to extracellular agonist such as hormones or presented in serum. Further work was ongoing on this aspect.

To test the effect of shear stress on cell cycle kinetics, Sf-9 cells taken from late exponential phase were exposed to laminar shear stress of 5 dynes/cm\(^2\) for different exposure time. After shearing for 90 min, the population distribution of the cell culture was shifted to G\(_1\) phase (Fig. 5a). Similar results were observed for S-phase cell culture. It might suggest oxygen limitation in the viscometer. If these cells can reenter cell cycle under shear by nutrient stimulation is under investigation.

**SUMMARY**

Effects of laminar shear stress used in this study so far was in sublethal range. Yet, it could be detected by flow cytometry in terms of shifts in population distribution. Even in sublethal range, cell responded to environmental stress in terms of intracellular calcium ion concentration. Low shear stress resulted in a 30% drop of \( \text{Ca}^{2+} \), and nonionic surfactant F-68 was shown to have no significant effect on \( \text{Ca}^{2+} \) up to 0.2% (w/v) in medium.

**REFERENCES**


Rasmussen, H., Kojima, J., Barrett, P., (1985) Information flow in the calcium messenger


Table 1. $[\text{Ca}^{2+}\_i]$ of different eucaryotic cell lines.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>$[\text{Ca}^{2+}_i]$ (nM)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neuroblastoma (NG108)</td>
<td>65</td>
<td>Snyder, 1990</td>
</tr>
<tr>
<td>Neuroblastoma (NIE115)</td>
<td>129</td>
<td>Hoover, 1990</td>
</tr>
<tr>
<td>Platelet</td>
<td>192</td>
<td>Michell, 1989</td>
</tr>
<tr>
<td>Endotheliel cell (CCL209)</td>
<td>150</td>
<td>Kelvin, 1990</td>
</tr>
<tr>
<td>Fibroblast (3T6SJ)</td>
<td>116</td>
<td>Erb et al., 1990</td>
</tr>
<tr>
<td>Spodoptera frugiperda (Sf-9)</td>
<td>178</td>
<td></td>
</tr>
</tbody>
</table>
Figure 1. Arachidonic acid cascade. 
$[\text{Ca}^{2+}]$ plays a role of modulation of enzymatic function. (Levesque, et al., 1989)
Figure 2. Role of intracellular calcium ion.
Extracellular $[\text{Ca}^{2+}]$ is about 1 mM compared to intracellular $[\text{Ca}^{2+}]$ of order 100 nM. (Weisman, et al, 1984)

$[\text{Ca}^{2+}_{\text{ext}}] = 1 \text{ mM}$

$[\text{Ca}^{2+}_{\text{int}}] = 100 \text{ nM}$
Figure 3. Effect of F-68 on [Ca\textsuperscript{2+}].

(a) Comparison of 0.1% and 1.0% F-68 on [Ca\textsuperscript{2+}].

(b) Effect of F-68 concentration on [Ca\textsuperscript{2+}] percent increase.
Figure 4. Effect of shear on $[\text{Ca}^{2+}_i]$. Base line of $[\text{Ca}^{2+}_i]$ decrease from 178 nM to 114 nM after shearing at 10 dyne/cm$^2$ for 30 min.
Figure 5. Effect of shear on cell cycle.

Cells in G1 phase (a) and S phase (b) show a population distribution shift after shearing at 5 dynes/cm² for 90 min.

(a) Effect of low shear stress on G1-phase cells

(b) Effect of hydrodynamic stress on S-phase cells
A mathematical model for ripening of Cheddar cheese

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Columbia, MO 65201

ABSTRACT

A model to explain the observed kinetics in Cheddar cheese ripening is developed on the basis of homogeneous enzyme-catalyzed reactions taking place inside the cheese block. It is assumed that cheese components are uniformly distributed, there is no concentration gradient of enzymes and heat and mass transfers are absent. The proteolytic reactions are assumed to follow the Michaelis-Menten kinetics.

The model successfully describes the variation in the amounts of peptides and amino acids that play an important role in establishing the cheese flavour. The cheese ripening process is accelerated by starter dipeptidases which generate more amino acids into the cheese matrix.

INTRODUCTION

In the conventional process of cheese ripening, cheese acquires its characteristic flavour, body and texture over a period of several months at low temperatures (2-15°C). This period of cheese ripening starts with Cheddar-cheese curd in which microorganisms are entrapped. A small amount of lactose is also entrapped in the curd. In the phase I of cheese ripening which lasts between 2-4 weeks, microorganisms grow at the expense of the lactose. Some lysis of cells may also occur. In phase II, most of the ripening occurs over several months. In this phase, lysis of cells releases dipeptidases in the cheese matrix and peptides formed by proteolysis of casein are converted into amino acids [1].

To shorten the ripening process and to eliminate the bitter taste of the cheese, mixed cultures of the cheese starters have been used [2]. prt mutants produce significantly
less bitterness than the parent cells (prt+) do. Many prt- cells also have lac- (non-lactose fermenting) character during curd formation and produce much less lactic acid. Thus, a mixture of the parent cells with lac-prt- cells is a better starter culture in the cheese making. With the mixed starters, commensalism occurs between the parent cells, lac+prt+ (lactose-fermenting), and lac-prt- cells [3].

So far no mathematical model for the cheese ripening has been published. Here, a mathematical model for phase II of the cheese ripening has been proposed.

**CHEESE RIPENING MODEL**

The model involves the following four assumptions:

1. The cheese components (cells, proteins, fat and moisture) are uniformly distributed in the cheese block [4].
2. Only homogeneous enzyme-catalyzed reactions take place.
3. There are no enzyme concentration gradients.
4. There is no mass or heat transfer between the inside and the outside of the cheese block.

Thus, in essence, the local enzymatic reactions dominate and the effects of diffusion are neglected in this model.

The cheese considered here is a Cheddar cheese which is made using only starter bacteria (streptococci) [5]. Proteinases present in milk, rennet and starter bacteria break down casein to peptides and peptidases break down peptides further to amino acids. However, this model considers only the microbial proteinases and dipeptidases because peptides and amino acids are formed during cheese ripening mainly as a result of activities of those enzymes [6, 7]. The proteinases are present on the surface of starter cells while the dipeptidases are intracellularly located. Thus, dipeptidases act on the peptides mainly when they are present in the cheese matrix after cell lysis.

The ripening reactions are sequential, are dependent on enzymes, and are represented by:

\[
\text{Proteinases} \quad \text{Dipeptidases} \\
\text{Casein} \longrightarrow \text{Dipeptides} \longrightarrow \text{Amino acids}
\]
Kinetic Equations:

There are six kinetic equations which govern the ripening reactions of phase II. Since no lactose is present in this phase, only cell lysis is considered. In our experimental work, as well as from the work of others [8], it has been found that there is no significant difference between dipeptidase components in the prt+ and prt- cells. Since proteinases are bound at the cell wall, their components are not affected by cell lysis. Hence, in this model, no distinction has been made between different types of cells.

Cells: \( \frac{dX}{dt} = -k_t X \), \( X(0) = X_0 \)

Proteinases: \( \frac{dE_1}{dt} = -k_1 E_1 \), \( E_1(0) = E_{1,0} \)

Dipeptidases: \( \frac{dE_2}{dt} = k_f a_2 X - k_2 E_2 \), \( E_2(0) = E_{2,0} \)

Casein: \( \frac{dA}{dt} = -V f E_1 \left[ \frac{A}{(A + K_m)} \right] \), \( A(0) = A_0 \)

Dipeptides: \( \frac{dB}{dt} = -(1.08)*dA/dt - (1/1.08)*dC/dt \), \( B(0) = B_0 \)

Amino acids: \( \frac{dC}{dt} = V_b \left[ \frac{B}{(B + K_m')} \right] E_2 \), \( C(0) = C_0 \)

Initial conditions are designated by the subscript 0. The stoichiometric coefficients 1.08 and 1/1.08 in the dipeptide balance equation were calculated based on weight.

Since the enzymes result from cellular activity, their concentrations are often reported as units/ c.f.u. instead of units/ g cheese as in the above. Hence, the enzyme activities have been normalized as follows:

\( \tilde{e}_1 = \frac{E_1}{X e_{1,0}} \) and \( e_2 = \frac{E_2}{X} \)

Here \( e_{1,0} \) is the initial specific activity of proteinases in cheese matrix. The normalized equations are the following.

\( \frac{dX}{dt} = -k_t X \), \( X(0) = X_0 \)

\( \frac{d\tilde{e}_1}{dt} = -((k_1/k_2) k_2 - k_f \tilde{e}_1) \), \( \tilde{e}_1(0) = 1.0 \)

\( \frac{de_2}{dt} = k_f a_2 + (k_f - k_2) e_2 \), \( e_2(0) = e_{2,0} \)

\( \frac{dA}{dt} = -V f e_{1,0} \left[ \frac{A}{(A + K_m)} \right] X \tilde{e}_1 \), \( A(0) = A_0 \)

\( \frac{dB}{dt} = -(1.08)*dA/dt - (1/1.08)*dC/dt \), \( B(0) = B_0 \)

\( \frac{dC}{dt} = V_b \left[ \frac{B}{(B + K_m')} \right] X e_2 \), \( C(0) = C_0 \)

Experimental data to estimate model parameters were obtained from the published papers [5, 9, 10, 11, 12, 13, 14, 15, 16, 17]. None of these data sets was complete in the different variables considered in this model. Hence, the missing values of variables in the data sets were assumed to be average values for quality Cheddar cheeses reported in the literature [18].
RESULTS AND DISCUSSION

Model parameters were estimated in order to fit all the available experimental data for the fixed values of \( K_m = 0.207 \) [19] and \( K_m' = 1.15 \) [20] in mg/g of cheese, respectively. \( V_f* e_{1,0} \) was estimated as a single parameter. A total of six parameters were estimated using Marquardt’s algorithm [21] and 52 experimental data points. The estimated values for the model parameters are shown in Table 1.

Table 1. Estimation of parameter values (95% confidence limits).

<table>
<thead>
<tr>
<th>Parameters:</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>( k_1 )</td>
<td>( 8.56E-03 \pm 1.11E-03 ) [1/day]</td>
</tr>
<tr>
<td>( k_2 )</td>
<td>( 2.35E-02 \pm 2.29E-03 ) [1/day]</td>
</tr>
<tr>
<td>( a_2 )</td>
<td>( 8.72E-09 \pm 4.84E-10 ) [units/c.f.u.]*</td>
</tr>
<tr>
<td>( k_1/k_2 )</td>
<td>( 1.00E-04 \pm 1.19E-04 ) [-]</td>
</tr>
<tr>
<td>( V_f* e_{1,0} )</td>
<td>( 4.46E-08 \pm 6.81E-09 ) [mg/c.f.u./day]</td>
</tr>
<tr>
<td>( V_b )</td>
<td>( 3.88E+00 \pm 2.89E-01 ) [mg/units/day]*</td>
</tr>
</tbody>
</table>

* one units is \( \mu \text{mole of product liberated min}^{-1} (\text{ml extract})^{-1} \).

The model equations describe the degradation of casein and dipeptides and the formation of amino acids very well. The ratio \( (k_1/k_2) \) was estimated to be very small. This indicates that the cell-wall bound proteinases are much more stable than dipeptidases, which is also reported by Law et al. [5], because proteinases remain attached to the cell wall after cell lysis while dipeptidases are released in the cheese matrix as free enzymes. This is also in agreement with the report that immobilized enzymes (cell-wall proteinases) have longer half life than suspended enzymes (free dipeptidases) [22].

Several calculated profiles and experimental data have been shown in Fig. 1-4. Solid lines in Fig. 1-4 are the results of simulations corresponding to the initial conditions of each investigation. Experimental data, where available, are shown as stars [5, 15, 17]. Some of the other figures show more scatter but indicate similar trends.

Sensitivity tests of the model for each parameter and their initial values were also made. Parameters \( k_1, k_2 \) and \( a_2 \) and the initial values of \( X(0) \) and \( e_2(0) \) were found to significantly affect the results while the ratio of \( k_1/k_2 \) does not (see Fig. 5-16). The parameters \( a_2, X(0) \) and \( e_2(0) \) can be manipulated by use of different amounts of starter
cultures. If a higher concentration of starter cultures, \( X(0) \), is used, more amino acids are produced. This is deemed to be good because the amino acids are the precursor of good cheese flavour [23]. An imbalance of proteinases and peptidases can, however, result in formation of too much bitter peptides. This results in a degraded cheese flavour. The use of mixed cultures of \( \text{prt}^+ \) and \( \text{prt}^- \) cells has been suggested as a means to correct this problem [2]. Manipulations of \( \text{prt}^+ \) and \( \text{prt}^- \) cells in the phase proceeding phase II of ripening, influence the relative proportions of proteinases and peptidases. This factor must be considered in the phase I when \( E_1 \) is synthesized since the control of bitterness is essentially the control of total proteinase activity in the cheese matrix.

The ripening process can be accelerated by starter dipeptidases. However, lower amounts of surface-bound proteinases are required for better cheese flavour. These enzymatic reactions are well described in the model proposed.

**NOMENCLATURE**

\[ A: \text{Casein concentration [mg/g cheese]} \]
\[ a_2: \text{Fraction of intracellular dipeptidases that are released after cell lysis [units/c.f.u.]} \]
\[ B: \text{Dipeptide concentration [mg/g cheese]} \]
\[ C: \text{Amino acid concentration [mg/g cheese]} \]
\[ E_1: \text{Activity of proteinases [units/g cheese]} \]
\[ e_{1,0}: \text{Initial specific activity of proteinases in cheese block [units/c.f.u.]} \]
\[ E_2: \text{Activity of dipeptidases [units/g cheese]} \]
\[ e_2: \text{Specific activity of dipeptidases [units/c.f.u.]} \]
\[ k_1: \text{Rate constant of proteinase degradation [1/day]} \]
\[ k_2: \text{Rate constant of dipeptidase degradation [1/day]} \]
\[ k: \text{Rate constant of cell lysis [1/day]} \]
\[ K_m: \text{Michaelis-Menten constant of casein degradation [mg/g]} \]
\[ K_m': \text{Michaelis-Menten constant of amino acid formation [mg/g]} \]
\[ V_b: \text{Maximum velocity of amino acid formation by dipeptidases [mg/units/day]} \]
\[ V_f: \text{Maximum velocity of casein degradation by proteinases [mg/units/day]} \]
\[ X: \text{Viable cell count [c.f.u./g cheese]} \]
REFERENCES

ESTIMATION OF PARAMETERS

Biomass

Fig. 1 [#15]

E2

Fig. 2 [#5]

Peptides

Fig. 3 [#15]

Amino acids

Fig. 4 [#17]
MODEL SENSITIVITY - Parameters

Fig. 5 Peptides

Fig. 6 Amino acids

Fig. 7 Peptides

Fig. 8 Amino acids
MODEL SENSITIVITY - Parameters

Peptides

Amino acids

Fig. 9

Fig. 10

Peptides

Amino acids

Fig. 11

Fig. 12
MODEL SENSITIVITY

Peptides

Amino acids

Fig. 13

Fig. 14

Peptides

Amino acids

Fig. 15

Fig. 16