Modeling and Optimization of Microbial Desulfurization Processes

by

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B.S., Chemistry and Life Sciences
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ABSTRACT

The successful implementation of large-scale fossil fuel biodesulfurization depends in large part on the development of process models which can be used to optimize process design and operation \textit{a priori}. Attempts at implementing biodesulfurization must address two areas: biocatalyst production by fermentation, and process design for very high throughput fuel desulfurization. Demands from the extremely high volumetric throughput required in fuel processing coupled with the demands from sustaining viability of a living organism during the process make optimization of biodesulfurization a uniquely difficult problem. This work examines the production and use of \textit{Rhodococcus rhodochrous} as a biocatalyst for fuel desulfurization in a model system of dibenzothiophene in hexadecane.

Optimization of biocatalyst production by fermentation was carried out examining two areas of process performance: dynamic process demands and static process demands. Static demands include starting medium formulation, inoculum culture conditions at inoculation, initial fermentor conditions, and impact of substrates on subsequent desulfurization activity. Initial medium formulation was optimized by developing new experimental strategies based on multifactorial experimental design and Response Surface Methodology. Techniques borrowed from high-throughput screening also formed part of the medium optimization strategy. Model significance and predictive ability of the resulting response surfaces exceeded that for comparable studies of medium formulation given in the literature.

Dynamic fermentation demands include substrate uptake rates (i.e. for glucose, ammonium, and oxygen); process operating parameters such as agitation, aeration, pH control, and impact of substrate feeding strategies. Initial ammonium concentrations higher than 2 g/L were found to adversely affect productivity; however ammonium depletion is rapid. As a result, an ammonium feeding strategy is necessary in order to optimize fermentation of \textit{R. rhodochrous}. Optimization of the fermentation process as a whole was accomplished by combining information on dynamic and static demands obtained through experiment. Volumetric productivities of 0.36 g/L-h biomass under sulfur limited conditions were achieved. This value is well above that reported in the literature for comparable fermentation conditions.
Optimization of desulfurization processes was carried out by examining batch, semi-continuous, and continuous processes under a variety of operating conditions. Mathematical models for each system were also developed and used to arrive at theoretical predictions of optimal operating regions or specific conditions. One notable prediction which was confirmed experimentally is the optimal dilution rate for continuous desulfurization processes. Significant increases in specific activity and desulfurization productivity result from slight changes in dilution rate. Increases in dilution rate from 0.125 h$^{-1}$ to 0.25 h$^{-1}$ resulted in a three-fold increase in specific activity. This translates into a six-fold increase in desulfurization productivity. It is believed that the impact of the end product of the desulfurization pathway was mitigated by implementing these strategies. (The inhibitory effect of HBP is well documented.)

Theoretical models of glucose consumption were also developed. These agreed well with experiment and demonstrated the validity of the underlying assumptions and mathematical representations of metabolic and process phenomena. Derivations of optimal oil/water ratios were also carried out and form part of the overall optimization. A general scheme is presented for combining all the important process parameters in a complete process optimization.

Ideas for future work are developed in the last section. The concept of metabolic reconstruction is presented along with a realizable extension of the native dsz pathway with elements from the polychlorinated biphenyl degradation pathway. Work in the area of DNA shuffling and its implications for improvements in process performance are also discussed. This nascent technique holds enormous potential for the directed evolution of metabolic pathways and adaptation of native pathways to industrial processes.

Thesis Supervisor: Charles L. Cooney
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Kadir Abuchaibe, my closest friend and fellow engineer, has always provided me with sound advice and unwavering friendship. His integrity and dedication will always serve as an example to me.

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Chapter 1 Introduction

The presence of naturally occurring sulfur in fossil fuels has been the cause of serious environmental and health problems since the combustion of such fuels began en masse over two centuries ago. The causative agent in these problems is sulfur dioxide (SO₂); the combustion product of sulfur and a precursor of atmospheric sulfuric acid. The past three decades have seen the emergence of large efforts aimed at reducing the effects of sulfur dioxide emitted into the atmosphere. These efforts are motivated in part by one notable effect of SO₂ release -- acid rain. This acidified rainfall can bring about devastating ecological changes. One logical starting point in addressing this problem is to look for ways to remove sulfur from fossil fuels before combustion. As with many man-made problems, solutions can be found near the original source of the problem. In this case the solution is extracted, much as fossil fuels are, from the earth itself.

The work which follows describes a series of studies in microbial desulfurization. Also referred to as biodesulfurization, this area of study seeks to develop ways of removing sulfur from fossil fuels by using the unique and effective metabolic pathways that have developed in various microorganisms. The rich and varied genetic heritage of many of these organisms is a resource which has helped to address many existing problems and has already manifested itself in many commercial settings. Biodesulfurization in fuel refining is one such setting.

Desulfurization refers to any one of several techniques for the removal of sulfur from organic molecules which occur naturally in fossil fuels. The technological development of desulfurization has generally followed demands from regulatory agencies and has been limited by advances in catalyst technology. The current state-of-the-art in desulfurization is hydrodesulfurization (HDS). In this method a catalyst consisting of alumina-supported cobalt and molybdenum particles is exposed to the hydrocarbon (fuel) stream containing sulfur. This is carried out at temperatures ranging from 300 - 400°C and pressures from 0.7 - 5 MPa (hydrogen pressure).
This method is effective in removing most of the sulfur which occurs naturally; however there are several recalcitrant organic species which are resistant to sulfur removal. The most common among these are the benzothiophenes, examples of which are shown above.

In addition to being ineffective in desulfurizing these compounds, HDS can degrade the heating value (octane value) of the fuel by hydrogenating monoolefins at the higher temperatures and pressures needed to achieve deep desulfurization. Because of this harsher conditions are usually avoided. This results in average remaining sulfur levels which in many cases exceed legal mandates.

The shortcomings of conventional HDS combined with legal mandates have driven the investigation of new technologies for desulfurization. As an example, the Clean Air act of 1990 prohibits the sale, manufacture, or supply of diesel fuel with sulfur levels exceeding 0.05% wt (500 ppm) sulfur (McFarland, 1998). The average sulfur content after conventional HDS treatment of diesel can exceed 700 ppm. The shortfall between mandated levels and technologically achievable levels has prompted research efforts in the area of biodesulfurization.

This work describes the use of Rhodococcus rhodochrous IGTS8 as a biocatalyst for the removal of sulfur from dibenzothiophene and related compounds and contributes to a much larger effort in this area. The focus of this work has been on the development of fermentation processes for biocatalyst production; the development, modeling and optimization of continuous desulfurization processes; and on the determination of the efficiencies of substrate utilization during desulfurization.
Chapter 2 Background and Technical Aspects

This section describes the technical aspects which underly biodesulfurization and which will allow the reader to become more familiar with the chemistry and biology of microbial desulfurization. A brief description of biological sulfur utilization and a survey of various sulfur metabolism pathways will provide an overview sufficient for an understanding of the concepts which will follow.

Several classes of biological reactions exist for sulfur removal or metabolism. These constitute the sulfur cycle and are shown in Fig 2.0.

Figure 2.0 - The Sulfur Cycle (Senning, 1972)

Many of these reactions are carried out by specific metabolic pathways in plants, animals, and microorganisms. The diversity of sulfur-related metabolic pathways among prokaryotes is greater than for any other kingdom. Each class of reaction depicted in Fig. 2.0 can be carried out by at least one kind of prokaryote (Hopkins, 1993).

The concept of microbial desulfurization as investigated in this study is based on the dsz metabolic pathway of *Rhodococcus rhodochrous* IGTS8. This strain was first isolated by John Kilbane of the Institute of Gas Science and Technology. It is gram negative, slow growing ($\mu_{\text{max}} \sim 0.2 \text{ h}^{-1}$), and produces a characteristic red-orange
pigment. In general the genus *Rhodococcus* has been found to harbor many useful and unique pathways.

One key feature of the dsz pathway of *R. rhodochrous* is its ability to remove sulfur from dibenzothiophene (DBT) with subsequent production 2-hydroxybiphenyl (HBP) and sulfite as the end products. This is accomplished without subsequent destruction of the carbon backbone, a unique characteristic which makes the dsz pathway superior to other pathways for application in fuel refining. The dsz pathway is attributed to Omori (1992) and Gray (1998) and is shown in Fig. 2.1.

The first two steps in the pathway are carried out by the same enzyme, a monooxygenase (dsz C). In general it is unusual for multiple monooxygenations to be carried out by the
same enzyme. The third step is also carried out by a monooxygenase (dsz A). The first three monooxygenations are energy dependent and require a reduced flavin mononucleotide. The flavin reductase involved in this pathway is denoted frdA and was isolated and characterized by the molecular biology research group at Energy Biosystems (The Woodlands, TX). The last enzyme in the pathway is a desulfinase; (dszB), (2-(2’-hydroxyphenyl)benzene sulphinate desulfinase), which produces the end products sulfite and HBP. The end product HBP was found to have an inhibitory effect on enzyme activity of the dsz pathway (Hopkins, 1993) and is a well known germicidal/fungicidal agent (Dowicide 1 (Merck Index, 9th Ed., #7110)). The effect of this end product on enzyme activity and process performance will be discussed in more detail in subsequent sections.

Reviews of other pathways and organisms which metabolize sulfur are given in Hopkins (1993) as well as various web-based metabolic pathway databases (see Chapter 6 for a discussion of these databases).
Chapter 3 Materials and Methods

3.1 Materials

Chemicals

All chemicals used for media and spectrophotometric assays were reagent grade or better. Acetic acid, lactic acid, and gluconic acid assay kits were from Boeringher Mannheim, Germany. The D-glucose concentration in the aqueous phase was measured with an Accu-Chek glucose analyzer (Boehringer Mannheim, Germany). Ammonia concentration was determined by an ammonia test kit (171-A, Sigma Diagnostics, USA).

96-Well Plate Reader

A Molecular Dynamics 96-well plate reader was used to carry out optical density readings of *R. rhodochrous* cultivations in microplates at 595 nm. Auto-mixing was used to ensure homogeneity of the well contents at high cell density.

Fermentor and Control Systems

The fermentor used for all biomass production was a 15 L reactor (Biostat E, B. Braun Biotech International, Melsungen, Germany) with a working volume of 10L and a height/diameter ratio of 3. The reactor had four baffles and three Rushton turbines on the shaft at 1/4, 1/2 and 3/4 of the liquid level. All cultivation parameters were monitored by computer using Paragon (v 3.51). An OptoMax 22 interface was used to link the fermentor outputs and the computer. Control over pH, temperature, and agitation was carried out by internal control loops in the Braun Fermentor. Set points were set on the panel interface. Typical operating ranges are: T = 30°C, pH = 7.0 (adjusted with 2M NaOH and 2M HCl), N = 1000 min⁻¹, and the aeration at 10.0 standard L/min. Exhaust analysis of carbon dioxide, oxygen, nitrogen, and water during fermentation production was measured by mass spectrometer spectrometer (MGA 1600, Perkin-Elmer, USA). The carbon dioxide evolution rate (CER), the oxygen uptake rate (OUR), and the respiratory quotient (RQ) were evaluated from the gas phase material balance.
Centrifuge and Cell Storage Protocol

Cells were centrifuged in a Damon CRU-5000 centrifuge at 4500 min⁻¹ for 20 min at 4°C and washed with 0.156 M phosphate buffer at pH 7.0 and 4°C. The cell paste was stored in 0.156 M phosphate buffer (pH 7.5) at 4°C.

Orbital Shaker

Queue brand orbital shakers were used to carry out preliminary desulfurization studies. Typical operating conditions used were: Temperature 30°C and RPM: [100, 300].

Microbial Strains

All cultivations and biotransformations were carried out with *Rhodococcus rhodochrous* IGTS8, ATCC 53968. *E. coli* strains containing genes from the polychlorinated biphenyl pathway were obtained from Prof. Furukawa (Kyushu University, Kyushu, Japan) and Prof. Witholt (ETH, Zurich, Switzerland). The *E. coli* strains were used in preliminary studies of HBP conversion (see Chapter 7).

Biotransformation Reactor (BDS unit) and Protocols

Non-aseptic biotransformations were carried out in a 2L reactor (Setric, G.I., France) with a working volume of 1L. The reactor had four baffles and one Rushton turbine. A four headed pump system was used in the continuous process configuration (Masterflex, Cole-Parmer, USA). The working volume of the reactor was maintained constant at 1L.

Stored cell paste was diluted with 0.156 M phosphate buffer (pH 7.5) and homogenized on a standard vortex mixer before use. The ammonia concentration in the cell suspension was measured and amounted to less than 2 mg/L. Therefore, it is assumed that little cell growth could occur during the biotransformation process.

For biotransformations, 500 ml of cell suspension (OD₆₆₀ 220) was placed in the reactor and incubated at 30°C for one hour with 15 g/L glucose. 500 ml of hexadecane (3.5 - 4.0 g/l DBT in hexadecane) was added to the reactor. Parameters for biotransformation were set at T = 30°C, pH = 7.0 (adjusted with 2M NaOH), N = 650 min⁻¹, and aeration at 2.0 slpm. Dissolved oxygen (DO) and glucose concentration were
monitored. DO was monitored with an Ingold polarametric dissolved oxygen probe calibrated to 0% dissolved oxygen under nitrogen atmosphere.

Exhaust analysis of carbon dioxide, oxygen, nitrogen, and water vapor during biotransformation was carried out by mass spectrometer (MGA 1600, Perkin-Elmer, USA). The carbon dioxide evolution rate (CER), the oxygen uptake rate (OUR), and the respiratory quotient (RQ) were evaluated from the gas phase material balance.

Glucose was monitored using an Accu-Chek glucose analyzer (Boehringer Mannheim, Germany). Fresh cell suspension (OD$_{660} = 352$) with 15 g/L glucose was incubated for one hour in a shake flask at 30°C and then continuously fed into the reactor at a rate of 1.0 ml/min (Fig. 5.0). Fresh or recycled model fuel (fresh model fuel: 3.5 g/L DBT in hexadecane) was fed at 1.0 mL/min to maintain a constant oil/water ratio of R = 1:1 (v/v) in the reactor.

Bench-Scale Separator

Recycling of hexadecane during semi-continuous studies was conducted by continuous phase separation of the BDS unit effluent in a bench-scale settler (0.6 m length x 0.3 m height x 0.01 m width with 150 mL liquid working volume) (Fig. 6.9).

3.2 Analytical Procedures

Cell Density
Cell density (biomass concentration) absorbance measurements were carried out at 600 nm using a Hewlett Packard 8452A UV-vis spectrophotometer. Milli-Q (Millipore) water was used to carryout dilutions. Multiple readings (usually three) were taken to reduce measurement error caused by random variations. Conversions between absorbance and dry cell weight were taken from work done by Hopkins (1993); the conversion factor reported is 2.5 absorbance units at 100 nm for 1 g/L dry cell weight. In some cases dry cell weight was determined gravimetrically after centrifugation at 10,000 min$^{-1}$ for 20 min at 4°C, washing in 0.156 M phosphate buffer at pH 7.0 and drying at 42°C for 48h to 72 h. Results did not differ significantly from those reported by Hopkins.
Gas Chromatography

HBP and DBT concentrations during all experiments were monitored by gas chromatography of the hexadecane phase. 10 µL decane/mL hexadecane sample was used as an internal standard. The hexadecane and biomass containing aqueous phases were separated by centrifugation in 1.5 mL eppendorf tubes at 20,000 RPM for 10 min. Each sample was prepared by pipetting 1 mL from the top (hexadecane phase) into a clean eppendorf tube. The internal standard was then added.

A Hewlett Packard 5890A was used with the following conditions: oven temperature at 170°C, injection port temperature at 220°C, column flow rate 11.1 ml/min, injection volume 1 µL, and column head pressure 1.5 bar.

![GC HBP STD Curve](image1)

Figure 3.0 - HBP Standard Curve

![GC DBT STD Curve](image2)

Figure 3.1 - DBT Standard Curve
Standard curves generated using the aforementioned GC operating conditions are shown for HBP and DBT in Figs. 3.0 and 3.1, respectively.

**Growth Media**

The growth media used in this study varied greatly during the course of the fermentation development work. The nature of medium optimization required significant changes from time to time. What is shown in Table 3.0 is a typical medium formulation used during fermentation production runs. In all cases the trace metal composition remained constant.

Table 3.0 - Rr-100 Medium

<table>
<thead>
<tr>
<th>BLOCK I</th>
<th>100 X Trace Metal</th>
<th>BLOCK II</th>
<th>100 X Trace Metal</th>
<th>BLOCK III</th>
<th>100 X Trace Metal</th>
</tr>
</thead>
<tbody>
<tr>
<td>Na2HPO4</td>
<td>24.45 g/L</td>
<td>MgCl2 6H2O</td>
<td>0.64 g/L</td>
<td>Glucose</td>
<td>30 g/L</td>
</tr>
<tr>
<td>KH2PO4</td>
<td>15</td>
<td>EDTA</td>
<td>0.1 g/L</td>
<td>(NH4)6Mo7O24 4H2O</td>
<td>0.1 g/L</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CaCl2 2H2O</td>
<td>3.2 g/L</td>
<td>DMSO</td>
<td>1 mL/L</td>
</tr>
<tr>
<td></td>
<td></td>
<td>ZnCl2</td>
<td>3.2 g/L</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>FeCl2 4H2O</td>
<td>2.8 g/L</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Co(NO3)2 6H2O</td>
<td>0.07 g/L</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>MnCl2 4H2O</td>
<td>0.1 g/L</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>CuCl2 2H2O</td>
<td>1 g/L</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Na2B4O7 10H2O</td>
<td>0.07 g/L</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The initial formulation of Rr-100 was taken from Oldfied et al. (1998). Changes were made based on improvements in biomass yield (see Section 4.2, Response Surface Methodology).

The separation of the medium into five separate blocks was done to avoid unwanted cross reactions between medium components during autoclaving. In general it is wise to separate glucose from ammonium sources during autoclaving due to the formation of Mallard products which change the chemical nature of the substrates. Precipitation was observed when magnesium chloride and glucose were autoclaved together, as a result these two components were also separated. DMSO was always sterile filtered and added after autoclaving.
Chapter 4 Production of Biocatalyst

The production of biocatalyst is carried out by aerobic fermentation of *Rhodococcus rhodochrous*. The catalyst itself consists of a set of four enzymes (of the dsz/frdA operon) and is produced by constitutive expression of these enzymes under the appropriate conditions. (See Chapter 2 for a description of enzyme characteristics and derepression of these operons). The enzymes are present in the whole living cells and are functional only while the cell remains viable or while reducing equivalent in the form of NADH are supplied; therefore normal metabolic activities are required in order to sustain enzyme activity. Thus, the biocatalyst product is the *whole cell* harvested from the aerobic fermentation.

The following sections describe the production of catalyst by aerobic fermentation. A discussion of the operation of the fermentor and monitoring of the critical operating parameters is also included. The cultivation of this particular strain is carried out in either batch or fed-batch fermentation. Continuous culture did not form part of this study, although work in this area has been carried out by other groups. (Wang et al., 1996) and (Humphrey et al, 1996).

Results from each fermentation were used to improve subsequent fermentations in a trial and error method. However, medium development followed a more directed optimization path consisting of Response Surface Methodology, which is discussed in a subsequent section. The operation of the fermentor was carried out in all cases by computer control. Variables such as pH, dissolved oxygen tension (DO), agitation rate (RPM), and aeration rate (VVM) were monitored and controlled by computer.

4.1 Substrate Allocation Model

The characterization of biocatalyst production by fermentation must begin by examining the fundamental processes that are responsible for biomass production. Models which account for the use of nutrient substrates, biomass production, product formation and formation of metabolic wastes (such as CO₂) are referred to as *Substrate Allocation Models*. In this study only the production of biomass by aerobic fermentation is of concern since there is no product being formed other than biomass.
The fundamental governing equation for biomass growth by aerobic fermentation is given by

\[ CH_m O_n + aO_2 + bNH_3 \rightarrow cCH_a O_b N_d + dH_2 O + eCO_2 \]  \hspace{1cm} (4.0)

It is assumed that the carbon source is a carbohydrate. An elemental balance can be carried out in order to determine the coefficients of this stoichiometric equation. Immediately four balances can be established.

<table>
<thead>
<tr>
<th>Species</th>
<th>Balance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carbon</td>
<td>( c + e -1 = 0 )</td>
</tr>
<tr>
<td>Hydrogen</td>
<td>( m + 3b - c\alpha - 2d = 0 )</td>
</tr>
<tr>
<td>Oxygen</td>
<td>( n + 2a - c\beta - d - 2e = 0 )</td>
</tr>
<tr>
<td>Nitrogen</td>
<td>( b - c\delta = 0 )</td>
</tr>
</tbody>
</table>

Table 4.0

One additional constraint is needed in order to solve this system. This constraint comes from experimental observation and is based on measurement of the respiratory quotient (RQ).

\[ a\text{RQ} - e = 0 \]  \hspace{1cm} (4.1)

With these five constraints it is possible to solve for the stoichiometric coefficients. This is best carried out by solving the following matrix equation for \( a, b, c, d, \) and \( e \).

\[
\begin{bmatrix}
RQ & 0 & 0 & 0 & -1 \\
0 & 0 & 1 & 0 & 1 \\
0 & 3 & -\alpha & -2 & 0 \\
2 & 0 & -\beta & -1 & -2 \\
0 & 1 & -\delta & 0 & 0
\end{bmatrix}
\begin{bmatrix}
a \\
b \\
c \\
d \\
e
\end{bmatrix}
=
\begin{bmatrix}
0 \\
1 \\
-m \\
-n \\
0
\end{bmatrix}
\]  \hspace{1cm} (4.2)

By solving this matrix equation one can arrive at the stoichiometric representation of biomass growth and can assess how the various substrates (glucose, ammonia and oxygen in this case) are being allocated. The explicit solution to Eq. (4.2) is cumbersome and

---

1 This analysis is adapted from the approach outlined in Chapter 7 of Bioprocess Engineering (Michael L. Shuler and Fikret Kargi, 1992).
will not be represented here; however it will be used to determine the aforementioned coefficients for computer simulations of the fermentation process.

The determination of stoichiometric coefficients for the purposes of computer simulation is carried out by taking a representative value for RQ during a typical fermentation and solving Eq. 4.6. The results of this analysis with RQs in the range [1, 0.75] yield a generalized stoichiometric equation. This is given in Eq. 4.3.

$$C_6H_{12}O_6 + O_2 + 2.64NH_4 \rightarrow 5.29CH_{1.69}O_{0.29}N_{0.5} + 0.86CO_2 + 5.36H_2O$$  \hspace{1cm} (4.3)

With this equation in place it is possible to model the uptake of various substrates based on observed biomass growth rates. Also it should be apparent that shifts in RQ to values outside of the range [1, 0.75] would force reevaluation of the stoichiometric relationship given by Eq. 4.3.

### 4.2 Medium Development - Response Surface Methodology

The development of a nutrient medium that is fully optimized for aerobic fermentation of *R. rhodochrous* is a complex and ill-defined problem. In general the nutritional requirements of poorly characterized strains are not documented and the identification of all the chemical components needed to optimize growth may be impractical to carry out. Because prokaryotes generally do not have specific amino acid or vitamin requirements it is reasonable to expect that optimization with respect to basic substrates such as glucose, phosphate and nitrogen is a wise starting point.

Having identified a set of substrates around which to optimize, the problem becomes one of screening large numbers of combinations of substrate concentration for impact on cell growth in an accurate and efficient manner. The combinatorial nature of the problem makes it well suited for analysis via a statistical or experimental design. Specifically, one can implement an experimental design with the objective of identifying the combination of nutrient concentrations that will lead to the highest cell mass concentration while minimizing waste due to undepleted substrate.  

---

2 Typical values for cells grown on glucose are in the range of $RQ \sim 1$.
3 The assumption is made that higher the cell mass concentrations correspond to higher dsz enzyme
Choosing a specific type of experimental design is normally based on the objective of the study, the resources available, and the ease in obtaining data (Box 1978). Given the specific objective of this study and the cost of resources available to carry it out, the only remaining question is that of ease in obtaining data. Traditionally data on growth rates and cell density (as described in Chapter 3) is collected by taking samples from a fermentor or shake flask and measuring optical density (OD) at 600 nm. Additionally, the appropriate dilutions must be made in order to insure linearity of the optical density reading (also referred to as Beer's Law region), especially at high cell density. The problem with this traditional method is that the screening of large experimental designs is made difficult by the number of different flasks required, and the limitation of incubator size.

To get an idea of the size of the experimental space for this study it is necessary to look at the number of different variables along with the number of different levels at which these variables will be studied. Assume one wants to examine three substrates (the variables); glucose (carbon source), phosphate salts (phosphorous source), and ammonium chloride (nitrogen source), at three different concentration levels each. This gives $3^3 = 27$ different experiments for a full factorial design (FFD). Ideally one would run a FFD if time and resources permit. In this study the only limitation appears to be the ease of obtaining data.

The solution to this problem is found by looking for alternative means of obtaining data on cellular growth. One approach taken in this study is to carry out cell growth in sterile 96-well microplates. This approach allows one to simultaneously carry out up to 96 independent experiments per plate and to read the optical density of each well quickly and accurately in a microplate reader. In theory this method would allow one to carry out a FFD on three substrates at four different concentration levels each, with 15 wells left over for replication or blank. This effectively eliminates the problem of cumbersome data collection associated with the traditional method described above. Thus, a FFD is an acceptable experimental design for this study.

---

This assumption is supported by the fact that replacement of sulfate salts with dimethyl sulfoxide (DMSO) in the growth medium brings about derepression of the dsz operon, thereby allowing constitutive expression of these enzymes at catalytically significant levels.
A method of analyzing the data that will be generated must also be selected. An efficient way to carry out the data analysis is through the use of Response Surface Methodology (RSM). The use of RSM is usually carried out on data from a central composite design (CCD) experiment. Because the actual experiments are carried out as FFD, the data points which comprise a CCD are already contained in the experiment. This allows those points that belong to the CCD set to be selected from among the FFD set. The collection of data points in this manner allows a fuller exploration of the experimental space without compromising the analytical power of RSM or consuming an excessive amount of resources.

4.2.1 Fundamental Elements of Response Surface Methodology

Response surface methodology is a set of statistical and mathematical techniques which is used to model and analyze systems in which one seeks to optimize a response that is a function of several variables. In general a response surface is represented as,

\[ g = f(x_1, x_2, \ldots, x_n) + \epsilon \] (4.4)

where \( g \) is the response, \( x_n \) are the variables of interest, and \( \epsilon \) is the error or noise associated with the measurement. The response surface is therefore the surface generated by the function \( g \) (which in general is an \( (n + 1) \) dimensional surface).

A series of models can be used to represent the response surface, although the most common are first or second order polynomial models. In cases where the experimental space being explored is relatively large and where the surface generated is not expected to exhibit significant curvature one can use a first order model. This model is usually appropriate in cases where prior knowledge of regions which may contain an optimum does not exist. First order models take the form

\[ \hat{g} = \beta_0 + \sum_{i=1}^{k} \beta_i x_i + \epsilon \] (4.5)

where \( \beta_0 \) represents the intercept (or constant term) of the surface, while the \( \beta_i \) represent the slopes of the corresponding variables. Consequently the \( \beta_i \) give a measure of the impact that variations in a given variable will have on the overall response.
Experiments which begin to probe regions around an optimum must take into account the curvature which characterizes such regions. In these cases it is necessary to use a second order model in order to characterize the response. The general form for a second order model is given by

\[ \hat{g} = \beta_0 + \sum_{i=1}^{k} \beta_i x_i + \sum_{i=1}^{k} \beta_{ii} x_i^2 + \sum_{i<j} \beta_{ij} x_i x_j + \varepsilon \]  

Eq. 4.6 takes into account second order and interaction effects which allows more complicated behavior to be modeled. Fitting of these models is carried out by a least squares criterion. This yields the coefficients of the model as well as a measure of the intercept and the error.

Optimization of response can be achieved without knowledge of the underlying principles which govern the response. In the case of medium development no knowledge of complex metabolic interactions or nutrient demands is needed prior to carrying out experimental work. Only the response as a function of the variables is needed. This response is obtained experimentally.
4.2.2 Experimental Implementation of RSM

The response in this set of experiments is the biomass concentration, while the variables are the substrate concentrations. The growth of biomass is not monitored as a function of time, therefore the response is an endpoint assay. Measurement of the cell concentration is carried out with a microplate reader that measures the optical density of each well at 595 nm. The data collected are analyzed using Essential Regression®, a software package for RSM and statistical analysis.

The design of the experiment begins with the assignment of the variables and the concentration levels that will be tested. In this series of experiments the variables are the concentrations of glucose (G), ammonium (N), and phosphate (P). These are shown in Table 4.1 under the corresponding column headings in units of g/L.

<table>
<thead>
<tr>
<th></th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
</tr>
</thead>
<tbody>
<tr>
<td>G</td>
<td>(0)</td>
<td>(011)</td>
<td>(010)</td>
<td>(01-1)</td>
<td>(001)</td>
<td>(000)</td>
<td>(00-1)</td>
<td>(0-11)</td>
<td>(0-10)</td>
</tr>
<tr>
<td>N</td>
<td>(0)</td>
<td>(101)</td>
<td>(100)</td>
<td>(10-1)</td>
<td>(-1-11)</td>
<td>(000)</td>
<td>(1-11)</td>
<td>(-101)</td>
<td>(-100)</td>
</tr>
<tr>
<td>P</td>
<td>(0)</td>
<td>(110)</td>
<td>(111)</td>
<td>(1-10)</td>
<td>(-111)</td>
<td>(000)</td>
<td>(11-1)</td>
<td>(-110)</td>
<td>(-1-1-1)</td>
</tr>
</tbody>
</table>

Table 4.1

The various combinations of concentration levels in each well are denoted by the coded number set. As shown in the coded-column heading the lowest concentration of each component is denoted by "-1", the median concentration is denoted by "0", and the highest by "1". The order of coded concentrations is given by (GPN). For example, (001) denotes [gluc] = 25 g/L, [ammonium] = 2 g/L, and [PO₄] = 22 g/L. Thus the
concentration of each nutrient in each well can be quickly and easily determined from the coded set.

The volume of a given nutrient stock that is to be dispensed into each well is determined by the initial concentration of the stock \([R]_i\), the desired final concentration \([R]_f\), and the total volume of liquid in the well, \(V_T\).

\[
V_{j,f} = \frac{[R]_f}{[R]_i} V_T 
\]  
(4.7)

In this study the total liquid volume was set at 100 \(\mu\)L. This volume was determined in part by considering the oxygen transport limitation that may occur at high cell concentrations. Because oxygen transport between the liquid and gas phases is mediated by interfacial surface area, the ratio of surface area of the liquid/gas interface to total liquid volume \((SA/V_T)\) was kept greater than 2.0 cm\(^{-1}\) (well diameter 0.6 cm diameter, liquid volume 0.1 cm\(^3\)). (Typical values for \((SA/V_T)\) for shake flasks are in the range of 0.5 - 20 cm\(^{-1}\), depending on agitation conditions.)

Microplate wells were charged with nutrients as indicated in Table 4.1. All solutions were autoclaved at 121\(^\circ\)C for 20 min, with the exception of DMSO which was sterile filtered. Glucose, phosphate, nitrogen and trace metal solutions were all autoclaved separately to avoid reactions between any of the medium components. Each well was inoculated with 3 \(\mu\)L of OD 2 cells (mid-log phase) and incubated for 6 days at 30\(^\circ\)C with agitation at 190 RPM in an orbital shaker. Readings of the plates were carried out at 3 and 6 days to monitor growth and to allow the oxygen in the well headspace to be replenished with sterile air. If necessary the appropriate dilutions were made into adjacent empty wells in order to insure linearity of the plate reader OD measurement.

The following section is an example of how the data are analyzed and the results that are generated. A statistical software package was used to carry out the multiple linear regression, ANOVA (analysis of variance), significance testing of the model and error, and significance testing of the coefficients. The model given in Eq 4.6 was subjected to response variable transforms of the following types: \(\exp(OD)\), \(\sqrt{OD}\), \(1/OD\), and \(\ln(OD)\). In most cases \(\ln(OD)\) resulted in the highest model predictive ability and
significance. Also, in most cases the linear ammonium concentration term did not seem to contribute to increases in model predictive ability or significance, hence this term was normally eliminated from the final model. This is commonly referred to as \textit{backward elimination}. The resulting response surface takes the following form.

\[
\ln(OD) = \beta_0 + \beta_1 C + \beta_2 C_p + \beta_3 C_g^2 + \beta_4 C_n^2 + \beta_5 C_p^2 + \beta_6 C_g C_p + \beta_7 C_p^2 C_n
\]

Data collected from a representative experiment were analyzed by RSM and are shown in Tables 4.2 - 4.3.

<table>
<thead>
<tr>
<th>Source</th>
<th>SS</th>
<th>SS%</th>
<th>MS</th>
<th>F</th>
<th>F Signif</th>
<th>dof</th>
</tr>
</thead>
<tbody>
<tr>
<td>Regression</td>
<td>7.0018</td>
<td>97</td>
<td>1.000</td>
<td>89.5348</td>
<td>3.646E-12</td>
<td>7</td>
</tr>
<tr>
<td>Residual</td>
<td>0.1899</td>
<td>3</td>
<td>0.0111</td>
<td>17</td>
<td>0.0111</td>
<td>17</td>
</tr>
<tr>
<td>LOF Error</td>
<td>0.1892</td>
<td>3</td>
<td>0.0118</td>
<td>16.47290</td>
<td>0.191483</td>
<td>16</td>
</tr>
<tr>
<td>Pure Error</td>
<td>0.000718</td>
<td>0</td>
<td>0.000718</td>
<td>1</td>
<td>0.000718</td>
<td>1</td>
</tr>
<tr>
<td>Total</td>
<td>7.2928</td>
<td>100</td>
<td></td>
<td></td>
<td></td>
<td>24</td>
</tr>
</tbody>
</table>

Table 4.2 - ANOVA

<table>
<thead>
<tr>
<th>Term</th>
<th>Coefficient</th>
<th>t Statistic</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Constant</td>
<td>-1.287</td>
<td>-12.60</td>
<td>4.776E-10</td>
</tr>
<tr>
<td>$C_g$</td>
<td>0.01979</td>
<td>3.241</td>
<td>0.00480</td>
</tr>
<tr>
<td>$C_p$</td>
<td>0.133</td>
<td>10.23</td>
<td>1.112E-8</td>
</tr>
<tr>
<td>$C_g^2$</td>
<td>-0.000496</td>
<td>-4.427</td>
<td>0.000369</td>
</tr>
<tr>
<td>$C_n^2$</td>
<td>-0.05758</td>
<td>-3.551</td>
<td>0.00246</td>
</tr>
<tr>
<td>$C_p^2$</td>
<td>-0.00502</td>
<td>-11.41</td>
<td>2.173E-9</td>
</tr>
<tr>
<td>$C_gC_p$</td>
<td>0.000562</td>
<td>3.089</td>
<td>0.00666</td>
</tr>
<tr>
<td>$C_nC_p$</td>
<td>0.01836</td>
<td>4.636</td>
<td>0.000236</td>
</tr>
</tbody>
</table>

Table 4.3

\(^4\) The negative effect of high ammonium concentrations on growth of \textit{R. rhodochrous} is well documented. Ammonium concentrations in excess of 3 g/L were found to be inhibitory to cell growth by Honda et al. (1999).
Table 4.3 gives the ANOVA for the model. As seen in Table 4.2 the model is highly significant, $F_{\text{significance}} = 3.64 \times 10^{-12}$, (small values indicate a high level of significance). This can also be seen by the F ratio which is 89.53; high values indicate a high level of model significance. Table (4.4) shows a summary output for the same data set. The values reported give an indication of the agreement between the model and the data and predictive ability of the model.

<table>
<thead>
<tr>
<th>Statistic</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>$R^2$</td>
<td>0.974</td>
</tr>
<tr>
<td>$R^2$ adjusted</td>
<td>0.963</td>
</tr>
<tr>
<td>Standard Error</td>
<td>0.106</td>
</tr>
<tr>
<td>PRESS</td>
<td>0.480</td>
</tr>
<tr>
<td>$R^2$ prediction</td>
<td>0.933</td>
</tr>
<tr>
<td>Durbin Watson</td>
<td>1.616</td>
</tr>
<tr>
<td>Autocorrelation</td>
<td>0.124</td>
</tr>
<tr>
<td>Collinearity</td>
<td>3.642E-5</td>
</tr>
<tr>
<td>CV</td>
<td>45.30</td>
</tr>
<tr>
<td>Precision Index</td>
<td>125.66</td>
</tr>
</tbody>
</table>

Table 4.4

The high values for $R^2$, $R^2$ adjusted, and $R^2$ prediction indicate that the model describes the distribution of data well (high $R^2$), that all terms are likely to contribute significantly to the model (high $R^2$ adjusted), and that the ability of the model to predict the response in repeated experiments is high (high $R^2$ prediction).

Substituting the regressed coefficients into Eq. 4.8 gives,

$$\ln(OD) = -1.287 + 0.01979C_g + 0.133C_p - 0.000496C_g^2 - 0.05758C_p^2 - 0.00502C_gC_p + 0.01836C_pC_n$$

The response surface that results from the data analysis can be plotted in three dimensions by fixing the nitrogen concentration and plotting $\ln(OD)$ vs. $C_g$ and $C_p$. (The ammonium concentration value was fixed at 3 g/L in order to reduce dimensionality by one.) A plot of the response surface for the data reported in this section is shown in Fig 4.0. It is worth noting that the second order model has adequately captured the curvature of the surface near the maximum. Fig. 4.1 shows the two dimensional contour plot of
the response surface. In this representation the glucose and phosphate concentrations that lead to the maximum response can easily be identified. These values can be found graphically by inspection of Fig. 4.1.
An analytical solution to find these values is also possible. The values of the regressor variables that give the maximal response are found by solving the following system for \( x_1, x_2, \ldots, x_n \),

\[
\begin{align*}
\frac{df}{dx_1} &= 0 \\
\frac{df}{dx_2} &= 0 \\
\vdots & \quad \vdots \\
\frac{df}{dx_n} &= 0
\end{align*}
\]

In the case of the preceding example the system that gives the regressor values that correspond to the maximum is

\[
\begin{align*}
\frac{d \ln(OD)}{d C_g} &= 0 \\
\frac{d \ln(OD)}{d C_p} &= 0
\end{align*}
\]

which upon solving gives, \( C_g = 30.0 \text{ g/L} \) and \( C_p = 16.7 \text{ g/L} \). Since the ammonium concentration is fixed, the set of optimized regressor values is \( \{ C_g = 30.0 \text{ g/L} , C_p = 16.7 \text{ g/L} , C_n = 3 \text{ g/L} \} \). (The selection of another value of ammonium concentration would produce slightly different values for \( C_g \) and \( C_p \); although due to the lack of significance of ammonium concentration in the model these differences are not significant.)

RSM techniques are very efficient in optimizing the response of most systems in which a response variable can be identified as a function of easily varied inputs. The ease of data interpretation and analysis combined with the ability to screen large numbers of combinations makes RSM a very powerful tool in process optimization. The results of this study can now be applied to the larger scale setting of a 10L fermentor. As described in the following section small changes in medium formulation can have a significant impact on the outcome of a fermentation.
4.3 Production of Biocatalyst by Fed-Batch Fermentation

Fermentation studies are used to characterize the cellular growth of *R. rhodochrous* under aerobic fermentation conditions. The objective is to optimize production of biocatalyst with respect yield. Ultimately the parameter one seeks to optimize is unit of desulfurizing activity per yield of biomass. The assumption is made that a linear correlation exists between specific enzyme activity and biomass concentration; therefore, the optimization will focus on increasing biomass yield. The threshold for commercial viability of a biomass production process is given as 30 g biomass/L in 48 hours or less.

Production of biocatalyst is carried out in a 10L Braun Biostat fermentor with internal controllers for pH, temperature, and dissolved oxygen. Continuous off-gas analysis was carried out by mass spectrometry. Typical operating conditions are shown in Table 4.5.

<table>
<thead>
<tr>
<th>Temperature (°C)</th>
<th>pH</th>
<th>DO (%)</th>
<th>Aeration (VVM)</th>
<th>Agitation (RPM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>30</td>
<td>6.8-7.2</td>
<td>&gt;10%</td>
<td>1-2</td>
<td>500-1300</td>
</tr>
</tbody>
</table>

Table 4.5

The ranges for these operating conditions are indicative of maxima and minima for each parameter.

Figures 4.2 - 4.6 show data for four separate fermentations. Each of these fermentations produced information on the growth characteristics and unique process requirements of *R. rhodochrous*. The information obtained by off gas analysis was valuable in determining changes in cellular physiology as a function of environmental conditions. Medium formulations used for each fermentation are listed in the Material and Methods Section.

Figure 4.2 shows data resulting from the first fed-batch fermentation (F-100) of *R. rhodochrous*. The growth rate during exponential growth phase was 0.035 h⁻¹. This

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5 Currently these are the conditions under which it is produced commercially.
6 Personal communication, Phil Degrazia and Daniel Monticello, Energy Biosystems Corp.
7 Among the unique process requirements is the need to use sulfur sources that do not contain sulfate or sulfur containing amino acids.
value is well below the value for $\mu_{\text{max}}$ given in the literature of 0.15 h$^{-1}$ (Honda et al. 1998). The cause for this likely lies in the fact that the fermentation medium does not contain sulfate or sulfur containing amino acids. As seen in Table 4.6 all fermentations exhibit a $\mu_{\text{max}}$ less than 0.15 h$^{-1}$, presumably for the same reason. The resultant productivity for F-100 was 0.11 g/L-h.

The glucose uptake profile for F-100 is plotted alongside the OD profile. As seen in the first plot of Figure 4.2 (upper left hand plot) the glucose consumption is nearly linear during the exponential growth phase. A plot of biomass/glucose versus time confirms this. This behavior is indicative of glucose uptake for cell respiration as opposed to cell growth. In order to determine if this is the case, data from off gas analysis is needed.

The data provided by the off-gas analysis for F-100 provide information regarding the utilization of oxygen and production of carbon dioxide. The medium is completely defined with glucose as the only carbon source, consequently the production of carbon dioxide as determined by the carbon dioxide evolution rate (CER) is indicative of carbon source utilization. Estimates of how much glucose is going into primary metabolism versus biomass formation or secondary metabolism can be made by comparing CER to GUR. As seen in the last plot of Figure 4.2 (lower right) glucose is consumed linearly (GUR is constant) from 75 h to 150 h while the CER undergoes significant changes over the same time period. The changes in CER are indicative of shifts in metabolism that bring about different utilization of glucose.

The RQ is used to monitor these changes easily. During exponential growth RQ is normally unity, provided no secondary metabolites are produced in significant quantities. The third plot of Fig. 4.2 (lower left) shows RQ as a function of time. RQ was 1.0 +/- 0.2 during the majority of the fermentation with the exception of the time period 85 h to 105 h and 125h to 140h. These time periods correspond roughly to the beginning and end of the exponential growth phase (85h - 145h), respectively. Shifts in metabolism are expected at the beginning and end exponential growth as substrate demands and allocation to different pathways changes.
Figure 4.2
There are two aspects of F-100 that are unusual. The first is the low absolute values for OUR and CER. Typically values for OUR and CER are on the order of 300 mmol/min (at >5-10 OD) whereas the values observed for F-100 are on the order of 1-2 mmol/min (at OD ~ 30). Low values for OUR and CER can be indicative of low biomass viability, low growth rates, or substrate inhibition. In the case of F-100, plate counts showed viability to be correlated with OD as expected (i.e. about 1E9 cells per OD-mL). Most likely the low values for OUR and CER resulted from the very low growth rate (0.035 h⁻¹). The unusually long lag time (0 h to 70 h) was most likely due to low inoculation density. Additionally, glucose depletion occurred at 50 h. This was unforeseen and may have contributed not only to a prolonged lag period but to lower growth rates. Nonetheless this provided information on glucose consumption that was used in subsequent fermentations.

Data from the second fermentation F-101 are depicted in Fig. 4.3. The aim of F-101 was to increase overall biomass productivity. Fig. 4.3 shows data on biomass concentration as a function of time. As seen in Fig. 4.3 the productivity was significantly increased. The growth rate was increased to μ=0.09 h⁻¹ and a biomass concentration of 27 g/L was reached in 72 hours, giving a productivity of 0.32 g/L-h.

![Figure 4.3](image)
Although this below the specified rage for commercial viability it is a significant improvement over F-100. The information gained during F-100 was used to develop a feeding strategy that would avoid glucose limitation early on in the fermentation. Long fermentations result in high glucose consumption; therefore a feeding strategy aimed at sustaining high cell growth rates for prolonged periods of time is required. Glucose pulses are shown in Fig 4.3 in g/L of broth. The amounts of glucose added were determined in part by predictions of GUR from previous experiments and by measurements of glucose concentration in the broth.

Data from the third fermentation (F-102) are depicted in Figs. 4.4 and 4.5. The results of the previous two fermentations were used to further optimize the feeding strategy and to increase productivity. The productivity for F-102 was 0.37 g/L-h; a small but significant increase over F-101. F-102 was also used to observe other cell characteristics under these optimized. As seen in Fig. 4.4, glucose utilization is fundamentally different from that observed in F-101.

There appears to be a biphasic glucose uptake pattern characterized by two distinct linear glucose concentration profiles. The transition between the two regions coincides with a divergence of the RQ from unity (i.e. CER/OUR deviates from unity). Normally shifts in
RQ are indicative of changes in metabolism; therefore it would be reasonable to assume that the shift in glucose uptake rate resulted from a change in metabolic state. However, as Fig. 4.5 shows this change does not appear to impact the growth rate. The glucose concentration is also plotted in Fig. 4.5 to indicate where the shift in utilization occurs.

![Graph showing OD (600 nm) vs Time (hrs)](image)

**Figure 4.5**

There are several difficulties associated with growth of *R. rhodochrous* that present a challenge in the optimization of productivity. The first of these is an apparent upper limit to cell concentration in the absence of any substrate limitations. Growth becomes non-exponential after a certain cell density is reached despite the fact that no known substrate is limiting (to include dissolved oxygen). Results from other fermentations (data not shown) indicate that OD 60 is an upper limit.

The second difficulty is associated with sulfur controlled substrates. Growth rates reported in the literature for sulfate containing media are in the range of 0.19 h⁻¹ (Honda et al., 1998). Although sulfate containing media would significantly increase biomass productivity, its use results in significantly lower desulfurization activity. Studies aimed at using sulfate-rich media without affecting desulfurization activity would
have the potential of significantly increasing the viability of large scale biodesulfurization processes.

An overall comparison of the three fermentations is shown in Fig. 4.6, which illustrates the significant improvement from the first fermentation to the second and third fermentation. Ideally one would attempt to shorten the lag time and increase the growth rate in order to increase the overall productivity.

Fig. 4.6

<table>
<thead>
<tr>
<th></th>
<th>$\mu$ (hr$^{-1}$)</th>
<th>$OD_{max}$</th>
<th>$\Delta T$ (hr)</th>
<th>$T$ (hr) - time to reach $OD_{max}$</th>
<th>Productivity (g/L-h)</th>
<th>Change from F-100</th>
</tr>
</thead>
<tbody>
<tr>
<td>F-100</td>
<td>0.03</td>
<td>35</td>
<td>75</td>
<td>150</td>
<td>0.11</td>
<td>Glucose pulse at 75 hrs</td>
</tr>
<tr>
<td>F-101</td>
<td>0.09</td>
<td>54</td>
<td>25</td>
<td>83</td>
<td>0.32</td>
<td>Fed batch strategy</td>
</tr>
<tr>
<td>F-102</td>
<td>0.07</td>
<td>65</td>
<td>50</td>
<td>90</td>
<td>0.36</td>
<td>Fed batch strategy</td>
</tr>
</tbody>
</table>

Table 4.6

Table 4.6 summarizes the results of the three fermentations and illustrates the progressive improvements that were achieved.
Chapter 5 - Biodesulfurization Process Modeling

This chapter describes a comprehensive and unified process model that incorporates many of the elements which impact the operation of a large-scale biodesulfurization process. The treatment covers topics such as enzyme decay, rates of biocatalysis, optimization of dilution rate, simultaneous optimization of throughput and catalyst use, derivation of optimal oil/water ratios, glucose uptake modeling, and the development of a generalized methodology for process optimization. The development begins with a balance of enzyme activity with decay for a continuously stirred tank reactor (CSTR).

The model does not depend on any empirical information or experimental results. Subsequent sections describe the agreement between the model developed and the data obtained. This usually entails comparison of actual experimental process performance with that predicted by the fundamental model corresponding to that process.

A schematic representation of the continuous desulfurization is shown in Fig 5.0. The primary components of the continuous desulfurization process include a CSTR, low temperature biocatalyst storage unit (held at 3⁰ C), a biocatalyst incubation vessel for reactivation, and a fuel storage vessel. The most important operating parameters include volumetric flow rates, vessel volumes, aeration rates, nutrient concentrations (primarily glucose), biocatalyst concentrations, temperatures and pH values that are controllable and which form the basis of engineering design calculations.
Process Flow Diagram for the Experimental Continuous Biodesulfurization Process

Except where noted otherwise the PFD depicted here represents the actual laboratory-scale setup used for all continuous desulfurization experiments.

Quantities listed with each unit operation are representative of the quantities used throughout the theoretical modeling developed in CH 5.

From Fermentation Production Stream

3° C Intermediate Biomass Storage Unit

Storage in 156 mM phosphate buffer, pH 7.2 with [Glu] > 10 g/L

From Fuel Middle Distillate or Crude Source Stream

Incubation Unit 30° C

$V_{inc}, D_{inc}, C_g^g(t)$

Sulfur-Rich (Sour) Fuel Storage Unit

Typical Sulfur Conc. [S] > 500 ppm

For experimental work [DBT] ~ 4 g/L

Primary BDS unit

$V_r = V_{oil} + V_{aq}$

$V_{oil} / V_{aq} = R_{o/w}$

$D_r, C_g^r(t), C_c(t), E(t)$

From Two-Phase treated fuel steam with emulsified biomass

Operating Conditions - BDS Unit

$T = 30^\circ C$

$pH = 7.2$

$DO > 10\%$

$[Glu] > 4 g/L$

Figure 5.0 - Process Flow Diagram
5.1 - Enzyme Activity Model

Modeling of the time dependence of the active enzyme concentration in the reactor can be done with a mass balance on the reactor. The flow terms accounting for flow of active enzyme in and out of the reactor are shown in Fig. 5.1 and Eq. 5.1. A term accounting for the kinetics of enzyme decay must also be included in the generalized balance.

Specific decay constants can be determined from batch-type experiments. Based on previous experimental data the enzyme decay approximately follows first order decay kinetics.

\[
\frac{dE(t)}{dt} = \frac{FE_0}{V_r} - \frac{FE(t)}{V_r} - kE(t) \quad (5.1)
\]

Rearranging we have,

\[
\frac{dE(t)}{dt} = \frac{FE_0}{V_r} - E(t) \left[ \frac{F}{V_r} + k \right] \quad (5.2)
\]

Solving equation (5.2) with \( E(0) = E_i \) yields.
\[ E(t) = \left( E_i - \frac{F E_o}{F+V_r k} \right) e^{\left( \frac{-F}{V_r k} \right) t} + \frac{F E_o}{F+V_r k} \]  \hspace{1cm} (5.3)

Where,

- \( E(t) \) is the concentration of active enzyme in the reactor at time \( t \) (g/L)
- \( E_o \) is the active enzyme concentration in the feed stream (g/L)
- \( E_i \) is the initial active enzyme concentration in the reactor (g/L)
- \( F \) is the feed stream flow rate (L/h)
- \( k \) is the first order enzyme decay constant (1/hr)
- \( V_r \) is the volume of the aqueous phase in the reactor

5.1.1 - Critical Operating Conditions

Eq 5.3 consists of both a transient and a steady state component. The duration of the transient behavior scales as \( t_{ss} \sim (F/V_r + k)^{-1} \) in (h). Additionally one can see that the long-term behavior is constant and is given by

\[ E(t) \rightarrow \left( \frac{F E_o}{F+V_r k} \right) \text{ in (g/L)}. \]

For certain critical ratios of \( (E_i/E_o) \), the enzyme concentration \( E(t) \) in the reactor undergoes no transient. By setting the coefficient of the exponential term in Eq. 5.3 equal to zero and solving for \( (E_i/E_o) \) one finds the set of enzyme ratios that will give a time invariant active enzyme concentration in the reactor.

\[ \left( E_i - \frac{F E_o}{F+V_r k} \right) = 0 \]  \hspace{1cm} (5.5)

\[ \left( \frac{E_i}{E_o} \right)_{crit} = \left( \frac{F}{F+V_r k} \right) \]  \hspace{1cm} (5.6)

By fixing the ratio of the initial enzyme concentration in the reactor to the feed stream enzyme concentration, as given in Eq. 5.6, a constant active enzyme concentration, \( E(t) \), can be maintained in the reactor starting from time zero. The
performance of the desulfurization system as judged by the specific enzyme activity can be expected to be constant as a result.\(^8\)

The change in cell concentration in the reactor over time can be modeled from this ratio given the restriction placed on the nature of the reactor flow conditions stated above. In order to conform to this condition the cell concentration profile will take the following form (see Eq. 5.29 for the differential equation leading to this result.)

\[ C_c(t) = (C_i - C_o) \left( e^{\frac{E}{V}} \right) + C_o \]  

where \( C_o \) is the cell concentration in the inlet feed stream and \( C_i \) is the initial cell concentration in the reactor.

Finding the operating condition given by Eq. 5.6 becomes important in large-scale settings where reactor volumes are 100,000 L or larger. One can see from the time scale for approach to steady state, \( t_{ss} \), that under certain operating conditions the time to reach steady state operation can be significant; (for example with \( F = 1000 \text{ L/h}, V_r = 1E5 \text{ L}, \) and \( k = 0.06 \text{ h}^{-1}; t_{ss} \approx 14 \text{ h} \)). This can represent a significant portion of the start-up and cycle times for semi-continuous operation of large-scale biodesulfurization units.

**5.2 - Dimensionless Scaling Parameters**

Further analysis of the critical enzyme ratio given in Eq 5.6 reveals that a simple manipulation of this quantity may yield useful information. Dividing the numerator and denominator by flow rate (\( F \)) and defining the dilution rate as \( D = F/V \) yields,

\[
\left( \frac{E_i}{E_o} \right)_{crit} = \frac{D}{D+k} = \frac{1}{1+k/D} \]  

The quantity \( (k/D) \) is a dimensionless number which represents the relative ratio of the rates of enzyme dilution to enzyme degradation in a continuous flow desulfurization reactor. This quantity is properly interpreted as the *reaction Damkohler number* for

\(^8\) This result is confirmed experimentally and is discussed in Chapter 6.
enzyme degradation, denoted $Da_d$. The more common representation of this quantity involves the space time, $\tau$ [hrs], which is defined as $1/D$. This representation yields,

$$\left( \frac{E_i}{E_o} \right)_{crit} = \frac{1}{1 + \tau k}$$

thus,

$$Da_d = \tau k$$

(5.10)

This result indicates that as the space time diminishes the critical enzyme ratio goes to unity, as expected. Process design calculations aimed at optimizing overall performance of continuous desulfurization processes should incorporate this number as one of the critical operating parameters in addition to the usual collection of operating parameters.

5.3 - Simultaneous Optimization of Biocatalyst Use and Throughput

The competing demands of efficiency of catalyst use and extent of desulfurization have to be weighed against each other in an optimization of the desulfurization process. The result obtained in Eq. 5.9 is not sufficient to guarantee optimal operation of the desulfurization process, therefore this condition is supplemented with a constraint on dilution rate.

The optimization of a continuous desulfurization process relies heavily on the identification of flow conditions which lead to a maximum use of biocatalyst without compromising throughput. The objective is to identify the operating conditions which will optimize the extent of desulfurization while minimizing the extent of enzyme degradation. This will insure the most cost effective use of biocatalyst as well as the most time efficient use of capital resources devoted to desulfurization (reactors, fermentors and other capital equipment).

The process of enzyme degradation is first order in enzyme concentration, while at the DBT concentration ranges used in this study the catalytic desulfurization rate is zero order in substrate; therefore it is possible to carry out a mathematical optimization a priori without relying on other empirical information. Following the development given
in Section 5.2 the zero order catalytic reaction Damkohler number $D_{a_r}$ can be defined as

$$D_{a_r} = \frac{\tau k_{cat}}{C_{DBT}}$$  \hspace{1cm} (5.11)

With the Damkohler numbers defined for each competing process it is possible to evaluate the extent to which each process could be expected to progress as a function of the dilution rate. This extent of reaction, or conversion, can be defined for each process as follows,

$$X_d = \frac{D_{a_d}}{1 + D_{a_d}}$$ \hspace{1cm} (5.12)

$$X_r = \frac{D_{a_r}}{1 + D_{a_r}}$$ \hspace{1cm} (5.13)

Where $X_d$ represents the extent of enzyme degradation and $X_r$ represents the extent of desulfurization. Both values are normalized between zero and unity, with unity representing an extent of reaction of 100\%.\(^9\) Figure (5.2)\(^10\) illustrates the dependence of $X_d$ and $X_r$ on dilution rate.

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\(^9\) It is important to keep in mind that this representation of extent of reaction does not make any reference to the actual total desulfurizing capacity of the particular system under study. In this context it permits direct comparison of reaction rates of different processes under continuous flow conditions.

\(^{10}\) Figures 5.2 and 5.3 were generated using $k_d \sim 0.06$ hr and $k_r \sim 120$ mol/(L-hr) in order to illustrate the method and results. Actual values for $k_r$ are likely to vary with time and with different strains.
It is evident that the reaction extent of desulfurization is fairly insensitive to changes in dilution rate in the operating range $0 - 10 \text{ hr}^{-1}$; whereas the extent of enzyme degradation is very sensitive to dilution rate between $0 - 1 \text{ hr}^{-1}$. This immediately suggests that an optimum operating dilution rate exists that both maximizes $X_r$ and minimizes $X_d$. This is best observed by plotting the new function, $X_{opt} = X_r - X_d$, which is the difference between the two extents of reaction. This new function is plotted in Figure 5.3. The maximum can be found by solving the following equation derived from Eqs 5.12 and 5.13 for $D_r$,

$$\frac{d(X_r - X_d)}{dD_r} = \frac{k_d (C^0_{DBT} D_r + k_r)^2 - C^0_{DBT} k_r (D_r + k_d)^2}{(C^0_{DBT} D_r + k_r)^2 (D_r + k_d)^2} = 0$$

Solving for the optimal dilution rate gives,

$$\left(D_r\right)_{opt} = \sqrt{\frac{k_d k_r}{C^0_{DBT}}}$$

which is simply the geometric mean of the rate constants for these two competing processes divided by the inlet DBT concentration. Data collected on the rates of enzyme decay under typical operating conditions indicate that $k_d \approx 0.06 \text{ hr}^{-1}$ while published data give values for $k_r$, (Gray et. al 1998). 

![Figure 5.3](image-url)
With these values the optimum dilution rate can be calculated for a reactor system in which enzyme catalysis is first order; however, for the substrate concentration ranges used in this study, enzyme catalysis is zero order. Additionally Eq. 5.15 has been derived under the assumption that desulfurization is zero order. The optimal dilution rate for first order enzyme decay and first order catalysis is given by the geometric mean of the rate constants,

$$D_{r}^{opt} = \sqrt{k_d k_r}.$$  

Equation 5.15 gives an operating condition that is unique for this specific system; in other words, it is uniquely determined by experimentally observed rate constants for each of these competing processes.\(^{11}\) In general the optimization of the dilution rate will depend on the specific activity of the biocatalyst. Equation 5.15 can be made more general by accounting for differences in activity, biocatalyst concentration, and inlet DBT concentration. This is carried out by replacing the reaction rate constant by the specific activity and including the appropriate conversion factors. This results in,

$$\left(D_{r}\right)_{opt} = \frac{k_d \cdot SA(t) \cdot C_e}{\sqrt{C_{DBT}^{n} \cdot \left(\frac{M_{HBP}}{M_{DBT}}\right)}}$$  \hspace{1cm} (5.15b)

where SA has units of (g HBP produced/(g DCW-hr)). This result assumes first order enzyme decay and makes no assumption regarding the order of desulfurization. Because the specific activity is time dependent it will take into account shifts in rates of biocatalysis due to depletion of one or more substrates (i.e. DBT, glucose, or oxygen). Thus the order of the desulfurization reaction is no longer a concern in optimization of flow through reactor systems, provided that specific activity can be measured as a function of time.

\(^{11}\)Incidentally, at this dilution rate only 2% of the desulfurizing capacity at the original rate of \(k_{ca}\) is used, as a result, optimization of the use of biocatalyst will rely on the design of an alternative biodesulfurization process based on multiple CSTRs or recycle with concentration (see Appendix A5 for derivations of enzyme activity models for recycle with concentration processes.)
5.4 - Optimization of Oil/Water Ratio

Another aspect of optimization of throughput and catalyst use is the derivation of an optimal oil/water ratio, $R_{o/w}$. This ratio represents the ratio of organic volume to aqueous volume in the BDS unit. The aqueous fraction contains the biomass and nutrients to carry out the desulfurization reaction, while the organic fraction contains only the fuel to be treated along with DBT-x as well as the HBP-x produced during the bioconversion.

Several quantities need to be defined before a derivation of optimal $R_{o/w}$ can be carried out. The first is the molar volumetric productivity (MVP) (in mol HBP/(L-hr)),

$$MVP = \frac{SA \cdot C_c}{M_{w,HBP}}$$  \hspace{1cm} (5.16)

This quantity describes the amount, in moles, of HBP that is produced in the BDS unit per liter per hour. The next quantity is the molar productivity (MP) (in mol HPB/hr),

$$MP = \frac{SA \cdot C_c \cdot V_{aq}}{M_{w,HBP}}$$  \hspace{1cm} (5.17)

This quantity gives the total number of moles of HPB produced by the BDS unit per hour. The MP is in effect a production rate$^{12}$ for desulfurization and is readily varied by controllable operating conditions such as $V_{aq}$ and $C_c$. The next step in the derivation involves a molar balance of incoming DBT and outgoing DBT plus HBP. This takes the following form (on a molar basis)$^{13}$,

$$DBT_{in} - DBT_{out} = HBP_{out}$$  \hspace{1cm} (5.18)

The stoichiometry of the desulfurization reaction determines the molar balance for each component in the reactor streams. Using this balance another relationship between

---

$^{12}$ This rate of production should not be interpreted as a kinetic rate, rather as a measure of the desulfurizing capacity of the BDS unit per unit time.

$^{13}$ Eq. 5.18 assumes no intermediate or secondary product formation.
the amount of DBT entering the BDS unit and the DBT conversion capacity can be derived. This DBT conversion capacity is essentially the molar productivity (MP) defined above. The combination of these quantities gives the following,

\[ F_{oil} \cdot DBT_{in} \cdot Q = \frac{SA \cdot C_c \cdot V_{aq}}{M_w_{HBP}} \]  \hspace{1cm} (5.19)

Where \( F_{oil} \) is the fuel flow rate into the BDS unit in (L/hr), \( DBT_{in} \) is the concentration of DBT in the fuel inlet stream (mol/L), and \( Q \) is the fractional conversion of DBT to HBP. Now a maximum HBP inlet concentration can be derived based on the biocatalytic capacity of the system, as defined by system operating parameters, and the extent of desulfurization desired, as given by \( Q \).

\[ (DBT_{in})_{max} = \frac{SA \cdot C_c \cdot V_{aq}}{Q \cdot M_w_{HBP} \cdot F_{oil}} \] \hspace{1cm} (5.20)

Now that this quantity has been defined, an oil/water ratio can be defined for the continuous flow system. Normally the oil/water ratio defines a ratio of organic to aqueous volumes; however, in a continuous flow system it is possible to define the \( R_{o/w} \) as the ratio of organic to aqueous feed flow rates through the BDS unit.\(^{14}\)

\[ R_{o/w} = \frac{F_{oil}}{F_{aq}} \] \hspace{1cm} (5.21)

Substituting this quantity into Eq 5.20 and making use of the definition of the aqueous fraction space time \( \tau_{aq} \) one arrives at the following

\[ (DBT_{in})_{max} = \frac{SA \cdot C_c \cdot \tau_{aq}}{Q \cdot M_w_{HBP} \cdot R_{o/w}} \] \hspace{1cm} (5.22)

\(^{14}\) The assumption is made that the initial ratio of organic to aqueous volume fractions in the BDS unit is identical to the ratio of organic to aqueous volumetric flow rates; otherwise one would have to take into account the time dependence of \( R_{o/w}(t) \) in the BDS unit.
As discussed in Section 5.3 an optimum dilution rate can be found based on the experimentally determined rate constants for enzyme decay and biocatalysis. As a result, if the optimum dilution rate is used then the quantity $\tau_{aq}$ will reflect this. The resulting $R_{o/w}$ will correspond to the optimum oil/water volumetric flow rate ratio.

\[
(R_{o/w})_{opt} = \frac{SA \cdot C_c \cdot (\tau_{aq})_{opt}}{Q \cdot M_{HBP} \cdot (DBT_{in})_{max}}
\] (5.23)

This optimum ratio is one of the critical operating conditions needed to fully optimize the operation of a continuous BDS unit. Figures 5.4 and 5.5 illustrate the dependence of $R_{o/w}$ on Q with varying SA.\textsuperscript{15}

Figure 5.4 depicts a family of curves for biocatalyst with 180, 90, and 60 times the SA of the native strain used in this study. The reason for plotting this particular family of curves lies in the projected and as yet unpublished results of current work being carried out with genetically modified strains of \textit{R. rhodochrous} where specific activities of 180 times that observed in wild strains have been observed.

As illustrated in Figure 5.4, relatively favorable $R_{o/w}$ ratios are predicted for all curves at all fractional conversions. By \textit{favorable} one is to infer that the amount of biocatalyst required per unit amount of oil is low with respect to existing alternatives. The situation for wild strains is not as favorable however, as shown in Figure 5.5. As indicated in Figure (5.5) the optimal $R_{o/w}$ predicted for the wild strain with a fractional conversion of 25% is approximately 1.25. Coincidentally, all desulfurization experiments in this study were carried out at a $R_{o/w}$ close to unity. Additionally, as noted Chapter 6, \textit{Characterization of Desulfurization Kinetics}, typical fractional conversions for most desulfurization experiments were on the order of 25%.

\textsuperscript{15} Figures (5.4) and (5.5) were generated from Eq (5.23) using $C_c = 20$ g/L and DBT\textsubscript{in} = 100 $\mu$M.
Typical operating range for this study
5.5 - Glucose Uptake Model

Modeling of the glucose consumption rate in the biodesulfurization (BDS) unit requires an understanding of processes requiring glucose and the extent to which glucose utilization can be accounted for by each of these processes. The description which follows is for a fully continuous desulfurization process as depicted in Figure 5.0. The assumption is made that there is no cellular growth and that basal metabolic respiration is the only metabolic activity occurring outside of desulfurization. (This is confirmed by control experiments where basal metabolic rates are measured under no-desulfurization and under no-growth conditions, see Section 6.2).

Glucose consumption can be accounted for by two processes which occur simultaneously during the desulfurization process, namely cell metabolism and desulfurization. The stoichiometric relationship for each of these processes is given below for desulfurization and basal cell metabolism, respectively.

\[ 3C_{12}H_{8}S + C_{6}H_{12}O_{6} + 10.5O_{2} \rightarrow 3H_{2}SO_{4} + 6CO_{2} + 3C_{12}H_{10}O \]  \hspace{1cm} (5.24)

\[ C_{6}H_{12}O_{6} + 6O_{2} \rightarrow 6CO_{2} + 6H_{2}O \]  \hspace{1cm} (5.25)

Each of these equations sets the stoichiometry for glucose consumption intracellularly. The realization that two significant but separate processes compete for glucose in cells carrying out desulfurization allows one to construct a model of glucose consumption from first principles. The extent to which glucose is channeled along each pathway, (i.e. metabolism versus desulfurization) cannot be predicted theoretically; however a measure of the channeling ratio must be included in any model attempting to explain glucose consumption in such a system. The ratio appears in the model which follows as two coefficients of the respective terms representing cellular metabolism and desulfurization.

The glucose concentration profile in the BDS unit, \( C_{g}'(t) \), can be described mathematically by accounting for all processes in which glucose is removed from or added to the BDS unit. The primary differential equation describing this quantity
Glucose → Glycolysis → TCA cycle → 6 CO₂

10 NADH + 2 FADH₂

O₂ + 2NADH + 4H⁺ → 2H₂O + 2NAD⁺

O₂

RQₜ = \begin{cases} 0.5, 0.6 & \psi < \zeta \\ 0.5 & \psi \geq \zeta \end{cases}

α + β = 1
ψ + ζ = 1

DBT + 3FMNH₂ + 3.5O₂ → HBP + H₂O + H₂SO₄ + 3FMN

Glycolysis

Oxidative Phosphorylation

TCA cycle

Desulfurization

α, β, ψ, ζ are mole fractions
contains three terms that account for flow, cell metabolism as given in Eq. (5.25), and desulfurization as given in Eq. (5.24). This balance is represented in Eq. (5.26). This equation also contains quantities which are time dependent and which have separate differential equations describing the behavior of each. These are the BDS unit inlet stream glucose concentration, \( C_{g}^{in}(t) \); the cell concentration in the BDS unit, \( C_{c}(t) \); and the active enzyme concentration in the BDS unit, \( E(t) \).

The following system of four equations includes all the pertinent differential equations that describe the behavior of each of the aforementioned quantities and accounts for all processes that impact glucose concentration in the BDS unit, both metabolically and macroscopically.

\[
\frac{dC_{g}^\prime(t)}{dt} = \frac{F_{r}}{V_{r}} [C_{g}^{in}(t) - C_{g}^\prime(t)] - \alpha C_{c}(t) - \beta E(t)
\]

\textit{Principal Equation} \hspace{1cm} (5.26)

\[
\frac{dC_{g}^{in}(t)}{dt} = \frac{F_{inc}}{V_{inc}} [C_{g}^{i}(t) - C_{g}^{in}(t)] - \lambda C_{c}^{inc}
\]

\textit{Incubation Unit} \hspace{1cm} (5.27)

\[
\frac{dE(t)}{dt} = \frac{F_{r}E_{o}}{V_{r}} - E(t) \left( \frac{F_{r}}{V_{r}} + k \right)
\]

\textit{Enzyme Balance} \hspace{1cm} (5.28)

\[
\frac{dC_{c}(t)}{dt} = \frac{F_{r}}{V_{r}} [C_{o} - C_{c}(t)]
\]

\textit{Cell Balance} \hspace{1cm} (5.29)

The coefficients \( \alpha \) and \( \beta \) are the specific glucose uptake rates for cell metabolism and desulfurization, respectively.\(^{17}\) Experimental determination of these quantities is described in Section 6.3.

The active enzyme concentration and the cell concentration in the BDS unit as a function of time and in terms of system operating parameters have already been solved.

\(^{16}\) See Fig. 5.0 for an illustration of the incubation unit as part of the overall process. The incubation unit was used to bring the cells to 30°C for one hour before being used to desulfurize.

\(^{17}\) The units of \( \alpha \) and \( \beta \) are \((g \text{ Glucose consumed})/(g \text{ DCW} - \text{hr})\).
for and yield the results given in Eq. 5.3 and Eq. 5.7, respectively. What remains is to solve for $C_{g}^{\text{in}}(t)$, the incubator unit glucose concentration. Solving Eq. 5.27

with $C_{g}^{\text{in}}(0) = C_{g,\text{ini}}^{\text{in}}$ yields,

$$C_{g}^{\text{in}}(t) = \left( \frac{\lambda V_{\text{inc}}}{F_{\text{inc}}} C_{e}^{\text{inc}} + C_{g,\text{ini}}^{\text{in}} - C_{g}^{i} \right) e^{\left( \frac{F_{\text{inc}}}{V_{\text{inc}}} \right) t} + C_{g}^{i} - \frac{\lambda V_{\text{inc}}}{F_{\text{inc}}} C_{e}^{\text{inc}}$$

(5.30)

This quantity is a function of time because glucose consumption in the incubation unit is time dependent. The coefficient $\lambda$ is the glucose uptake rate for cells undergoing incubation. This quantity would naturally be expected to differ from the basal glucose uptake rate, $\alpha$, due to the fact that the cells undergo a temperature transition from $3^\circ\text{C}$ to $30^\circ\text{C}$ in the incubator.

Estimation of this parameter is analogous to that for $\alpha$ and $\beta$.

The solved expressions for active enzyme concentration and cell concentration in the BDS unit, and glucose concentration in the BDS inlet stream are now substituted into the principal equation, Eq. 5.26. Before solving this equation a number of simplifications can be made in order to make interpretation of the solution more tractable.

The first simplification involves the coefficient of the exponential term in the enzyme consumption term. As discussed in Section 5.1, the operation of the BDS unit under the condition that $(E_{i}/E_{\text{in}}) = (1/\left[1 + k/D_{e}\right])$ ensures a steady state enzyme concentration, thus effectively eliminating the exponential term from the active enzyme concentration equation, Eq. 5.3. Upon substitution of a dilution rate for each unit operation, such that $(F_{\text{in}}/V_{\text{n}}) = D_{n}$, Eq. 5.26 becomes,

$$\frac{dC_{g}^{i}(t)}{dt} = D_{n} \left[ \left( \frac{\lambda C_{e}^{\text{inc}}}{D_{\text{inc}}} + C_{g,\text{ini}}^{\text{in}} - C_{g}^{i} \right) e^{-D_{n}t} + C_{g}^{i} \right] - \lambda \left[ \frac{E_{g}}{1 + k/D_{e}} \right] - \alpha \left[ (C_{g}^{i} - C_{e}^{(i,D)}) e^{-D_{n}t} \right] + C_{e}$$

(5.31)

The next simplification involves the cell metabolism and enzyme consumption terms. Given that the BDS unit will operate under conditions leading to steady state enzyme concentration an assumption regarding the ratio of enzyme concentration to cell

18 The units of $\lambda$ are $(\text{g Glucose consumed})/(\text{g DCW - hr})$. 

51
concentration can be made; namely, that the ratio of active enzyme concentration to cell concentration is constant for all times.\textsuperscript{19} This assumption allows the terms accounting for glucose consumption due to cell metabolism and enzyme activity to be grouped into one term accounting for all chemical consumption of glucose in the BDS unit. Applying this assumption to Eq. 5.31 results in the following,

\[ E(t) = \gamma C_c(t) \] (5.32)

\[
\frac{dC^r(t)}{dt} = D \left[ \left( \frac{\lambda C_r^{inc}}{D_{inc}} + C_{x,inc} - C_f \right) e^{(-D_{inc} t)} + C^r_f - \frac{\lambda C_r^{inc}}{D_{inc}} - C^r_f(t) \right] - (\alpha + \beta \gamma) \left( C_i - C_r e^{(-\alpha t)} \right) + C^r \] (5.33)

A solution of Eq. 5.33 would produce a result that does not sacrifice generality for simplicity; however, for the purposes of most of the experimental work carried out in this study it will be unnecessary to work with the full general solution. Further modifications to Eq. 5.33 will render it more tractable and amenable to testing without sacrificing accuracy. Eq. 5.33 is left for the interested reader to solve and investigate the full range of interesting behavior which its solution predicts.

Depending on what conditions are imposed on the system during experimental work it is possible to make a series of simplifications which are fully justified on a physical basis. The first is the elimination of changes in cell concentration. This condition is applicable when the critical enzyme ratio given by Eq. 5.6 is close to unity. Because the critical enzyme ratio is coupled to the ratio of inlet biomass to initial biomass concentration in the BDS unit, (as described by Eq. 5.32), this simplification will apply to Eq. 5.33.

Another simplification is possible when the variation of the incubator outlet glucose concentration stabilizes rapidly. A measure of the duration of the incubator unit glucose concentration transient (the exponential term in Eq. 5.30) is given by the inverse of the dilution rate, $1/D_{inc}$. During most experiments $1/D_{inc}$ is on the order of 1 h. If this

\textsuperscript{19} The assumption is made that the measured cell concentration accurately represents the actual concentration of respiring cells and not a combination of dead biomass and respiring cells. Correct experimental protocol in handling of harvested biomass increases the likelihood of this assumption.
condition holds it is possible to eliminate the time dependence of the BDS unit inlet glucose concentration.

After applying these two simplifications Eq. 5.33 can be recast in the following form,

\[
\frac{dC'_g(t)}{dt} = D_r (C'^{\text{in}} - C'_g(t)) - (\alpha + \beta \gamma) C_o \tag{5.34}
\]

\[
C'_g(0) = C'_{g,\text{ini}} \tag{5.35}
\]

The solution of Eq. 5.34 with the initial condition given results in,

\[
C'_g(t) = \left[ C'_{g,\text{ini}} - C'^{\text{in}} + \left( \frac{\alpha + \beta \gamma}{D_r} \right) C_o \right] e^{-D_r t} - \left( \frac{\alpha + \beta \gamma}{D_r} \right) C_o + C'^{\text{in}} \tag{5.36}
\]

which is substantially more manageable than the full solution to Eq. 5.33. At first glance one will notice that the dependence on the incubation unit dilution rate has been eliminated, as well as the dependence on variations in biomass concentration.

Preliminary verification of this glucose consumption model can be carried out by comparing the prediction of the model with the actual outcome from an experiment taken at random. As noted earlier, all of the quantities included in Eq. 5.36 are either set by experimenter (such as dilution rates and initial concentrations), or determined by cellular physiology (such as specific glucose uptake rates and dsz enzyme to biomass ratios). It is important to keep in mind that Eq. 5.36 has been derived specifically for CSTR-based systems which follow the unit-operation relationship depicted in Fig. 5.0. Figure 5.6 shows the surprisingly good agreement that exists between the model developed and the data obtained. It is important to keep in mind that the agreement between the model and the data is not the result of curve fitting or empirical parameter estimation. The prediction of the theory is based solely on first principles. The parameters shown in Eq. 5.36 are representative of the actual operating conditions used in experimental process settings.
A more comprehensive evaluation of this glucose uptake model will be the subject of future work. The evaluation of the accuracy of the model under various operating conditions requires that it be subjected to comparison with all glucose concentration profiles observed, provided that the simplifying assumptions discussed before hold. Otherwise a comparison with the full solution to Eq. 5.33 may be required.

Figure 5.6

Section 6.2 describes the experimental determination of the parameters $\alpha$, $\beta$, and $\gamma$ which are required before the model given by Eq. 5.36 can be used to predict glucose uptake in continuous desulfurization processes.

5.6 - Prediction of Total Glucose Uptake

With a fairly good glucose uptake model in place it is possible to proceed to the application of the model in predicting the total glucose uptake rate that might be observed during a continuous desulfurization run. Because the model is based on a number of operating parameters that are known to impact the rate of desulfurization as well as the rate of glucose uptake, the mathematical descriptions of the two processes are consistent within the context of a given process. What follows is a derivation of a theoretical glucose uptake rate for the BDS unit during the course of a desulfurization run.
The derivation of a glucose uptake rate for the BDS unit begins with a simple glucose balance around the unit. The glucose uptake (GU) is defined as the amount of glucose in (g/L) that is consumed in the BDS unit.

\[
GU = C_{g}^{in} - C_{g}(t)
\]  

(5.37)

This is a mathematical statement of the glucose balance in the BDS unit. The glucose uptake rate (GUR in g/L-hr) is therefore defined as follows,

\[
GUR = D_r \left( C_{g}^{in} - C_{g}'(t) \right)
\]  

(5.38)

The total amount of glucose consumed in the BDS unit between the time of initiation or commencement of desulfurization (denoted \( t_i \)) and the end of the run (denoted \( t_f \)) is given by,

\[
TGU = \int_{t_i}^{t_f} D_r \left( C_{g}^{in} - C_{g}'(t) \right) dt
\]  

(5.39)

Eq. 5.39 can be used to determine the glucose demand that would be expected during a given desulfurization run. Solving Eq. 5.39 with Eq. 5.36 yields,

\[
TGU = \left[ \frac{C_{g,ini}^{r} - C_{g}^{in} + \left( \frac{\alpha + \beta \gamma}{D_r} \right) C_o}{D_r} \right] \left[ e^{\left( -\frac{\alpha + \beta \gamma}{D_r} \right) t_f} - 1 \right] + \left( \frac{\alpha + \beta \gamma}{D_r} \right) C_o \cdot t_f
\]  

(5.40)

It should be immediately evident that the longer the process continues, the less significant the transient contribution is to the total TGU.\(^{20}\) The determination of how long the desulfurization process would have to continue before the exponential contribution can be neglected is determined by comparing the order of magnitude of the inverse of the reactor dilution rate with the time length of the process \( t_f \). In general

\(^{20}\) The transient contribution is given by the exponential term.
neglecting the transient is justified if $t_f >> 1/D_r$. If this condition holds it is possible to simplify Eq. 5.40 as follows,

$$
TGU \equiv \left( \frac{\alpha + \beta \gamma}{D_r} \right) \cdot C_o \cdot t_f + \left( \frac{C_{g_{ini}}^{in} - C_{g_{ini}}^{r}}{D_r} - \left( \frac{\alpha + \beta \gamma}{D_r^2} \right) \right) C_o
$$

(5.41)

With this result it is possible to devise strategies for minimization of TGU.

### 5.7 - Optimization of Glucose Loading

Because $C_g'(t)$ represents the BDS unit outlet glucose concentration, any glucose leaving the BDS-unit is wasted; therefore, one would like to determine how to minimize this quantity. The objective of the TGU minimization strategy is to keep the outlet BDS unit glucose concentration as low as possible without compromising performance.

Eq. (5.41) shows the total glucose uptake expected during the course of a relatively long-lived desulfurization process. Because the long-term behavior is dominated by a term that is first-order in time, an effective TGU minimization strategy must focus on minimization of either the slope or the intercept of the equation given in Eq. (5.41). Unfortunately both of these quantities contain parameters that are determined by cellular physiology. These quantities cannot be changed easily. The only remaining parameters that are controllable are the biomass concentration, $C_o$, and the BDS unit dilution rate, $D_r$.

Because of the restriction placed on $D_r$ by Eq. 5.15, it is not possible to vary this parameter in order to minimize TGU without compromising system performance in other respects. Cell loading appears to be fixed as well; however, this quantity has no restrictions placed on it so far. This quantity would appear to impact the total capacity of a BDS unit and not other operational aspects. Theoretically one is limited in how much the biomass concentration can be reduced without compromising performance. In light of this, any excess biomass that does not contribute significantly to the desulfurizing capacity of the system should be reduced to a minimum in order to minimize TGU. Therefore an optimization of biomass concentration will necessarily lead to minimization of TGU.
5.8 Summary of Biodesulfurization Process Optimization Scheme

The preceding sections have outlined the theoretical basis of a process optimization strategy for large-scale biodesulfurization. Based on the required sequence of derivations for the most pertinent operating parameters it is possible to prescribe a generalized strategy for a process design and optimization strategy. This is illustrated in Figure 5.6.

![Optimization Flow Diagram for Continuous](image)

The steps highlighted with bold lined boxes are considered by the author to have the greatest impact on the outcome of the optimization strategy and should be afforded a greater degree of consideration during the experimental and scale-up phases of a process development scheme.
List of Quantities

The following table summarizes the variables and constants used throughout the derivation given in Chapter 5.

<table>
<thead>
<tr>
<th>Quantity</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>$C_{gr}^r(t)$</td>
<td>Glucose concentration in the BDS unit (g/L)</td>
</tr>
<tr>
<td>$C_{g}^{in}(t)$</td>
<td>Glucose concentration in the BDS unit inlet stream (also the glucose concentration in the incubator unit outlet stream) (g/L)</td>
</tr>
<tr>
<td>$C_g^i$</td>
<td>Glucose concentration in the incubator unit inlet stream (also the glucose concentration in the 3°C unit outlet stream) (g/L)</td>
</tr>
<tr>
<td>$C_{gi}^r$</td>
<td>Initial Glucose concentration in the BDS unit (g/L)</td>
</tr>
<tr>
<td>$C_c(t)$</td>
<td>Cell concentration in the BDS unit (g-DCW/L)</td>
</tr>
<tr>
<td>$C_{g}^{inc}$</td>
<td>Cell concentration in the incubator unit (g/L)</td>
</tr>
<tr>
<td>$C_o$</td>
<td>Cell concentration in the BDS unit inlet stream (normally equal to $C_{inc}$) (g/L)</td>
</tr>
<tr>
<td>$E(t)$</td>
<td>Active Enzyme Concentration in the BDS unit (g/L)</td>
</tr>
<tr>
<td>$E_o$</td>
<td>Active enzyme concentration in the BDS unit inlet stream (g/L)</td>
</tr>
<tr>
<td>$E_i$</td>
<td>Initial active enzyme concentration in the BDS unit (g/L)</td>
</tr>
<tr>
<td>$F_r$</td>
<td>Inlet flow rate of aqueous fraction to BDS unit (L/hr)</td>
</tr>
<tr>
<td>$F_{inc}$</td>
<td>Inlet flow rate of aqueous fraction to incubator unit (L/hr)</td>
</tr>
<tr>
<td>$V_r$</td>
<td>Volume occupied by aqueous fraction in BDS unit (L)</td>
</tr>
<tr>
<td>$V_{inc}$</td>
<td>Volume occupied by fluid in incubator unit (L)</td>
</tr>
</tbody>
</table>
5.9 Determination of Oxygen and Glucose Channeling Ratios

The objective of the following derivation is to determine the channeling ratios for glucose, \((\alpha, \beta)\), and oxygen, \((\psi, \zeta)\) in terms of measurable quantities. These ratios determine the allocation of glucose and oxygen between desulfurization and basal metabolism, as shown in Fig. 5.7. Determining these ratios is necessary in order to assess the efficiency of glucose and oxygen utilization for desulfurization. Ideally these ratios should be related to easily observable quantities, such as OUR and GUR.

\[
O_2 + 2NADH + 4H^+ \rightarrow 2H_2O + 2NAD^+
\]

RQT \[
\begin{cases} 
0.5 & \text{if } 0.5 \leq \psi \leq 0.6 \\
< 0.5 & \text{if } \psi \geq 0.6
\end{cases}
\]

\[
\alpha, \beta, \psi, \zeta \text{ are mole fractions}
\]

Figure 5.7

The derivation begins by observing that \(\alpha, \psi\) and \(\beta, \zeta\) are related stoichiometrically by the respective metabolic reactions, as shown in Fig 5.7. Two additional quantities need to be defined; \(A_{NADH}\) and \(A_{O2}\), which represent the total NADH and O2 produced (or delivered) during a given time period. These quantities are necessarily a function of time and can be related to the GUR and OUR as follows,

\[
A_{NADH} = 10 \int_{t_i}^{t_f} GUR(t) \, dt \tag{5.42}
\]
\[ A_{O_2} = \int_{t_i}^{t_f} OUR(t)dt \quad (5.43) \]

Two main assumptions must hold in order for Eqs. 5.42 and 5.43 to hold; the first is that the consumption of glucose results in 10 mol of NADH by way of glycolysis and the TCA cycle, and the second is \( OUR(t) = OTR(t) \). With the equations given in Fig. 5.7 it is possible to relate \( \alpha \) and \( \psi \), and \( \beta \) and \( \zeta \) to each other through the respective stoichiometries as follows,

\[ \frac{\alpha}{\psi} = \frac{2A_{O_2}}{A_{NADH}} \quad (5.44) \]

\[ \frac{\beta}{\zeta} = \frac{3.5A_{O_2}}{3A_{NADH}} \quad (5.45) \]

Combining Eqs. 5.42 - 5.45,

\[ \frac{\alpha}{\psi} = \left( \frac{20}{35} \right) \frac{\int_{t_i}^{t_f} GUR(t)dt}{\int_{t_i}^{t_f} OUR(t)dt} = \phi_1 \quad (5.46) \]

\[ \frac{\beta}{\zeta} = \left( \frac{35}{3} \right) \frac{\int_{t_i}^{t_f} GUR(t)dt}{\int_{t_i}^{t_f} OUR(t)dt} = \phi_2 \quad (5.47) \]

Because the branching ratios are mole fractions the following additional constraints hold,

\[ \alpha + \beta = 1 \quad (5.48) \]

\[ \psi + \zeta = 1 \quad (5.49) \]
Thus there are four equations in four unknowns. Simplifying the above set of equations,

\[
\begin{align*}
\alpha &= \phi_1 \psi + \beta = 1 \\
\beta &= \phi_2 \zeta + \psi + \zeta = 1
\end{align*}
\]

solving this system gives,

\[
\begin{align*}
\alpha &= \frac{\phi_1 (1 - \phi_2)}{\phi_1 - \phi_2} \\
\beta &= 1 - \frac{\phi_1 (1 - \phi_2)}{\phi_1 - \phi_2} \\
\psi &= \frac{(1 - \phi_2)}{\phi_1 - \phi_2} \\
\zeta &= 1 - \frac{(1 - \phi_2)}{\phi_1 - \phi_2}
\end{align*}
\]

This result is remarkable in that by simply measuring the GUR(t) and OUR(t) one can arrive at a measure of how much glucose (reducing equivalents) and oxygen is consumed by either desulfurization or cellular respiration. This gives the glucose and oxygen utilization efficiencies directly.

The derivation shown above would not be valid in systems where glucose is metabolized to secondary metabolites or other species. If alternate glucose metabolism pathways were in effect then the stoichiometry of these alternate pathways would have to be taken into account. The most typical deviations from the stoichiometry given in Eq. 5.24 result when cells produce organic acids. Production of acetic, lactic, or gluconic acids would result in different amounts of reducing equivalents per mol of glucose, thus changing the stoichiometry. The effect of organic acid production can be taken into account and incorporated into the channeling ratios given above, but this would require monitoring of additional species (i.e. the production rates of specific organic acids).
Chapter 6 - Characterization of Desulfurization Kinetics

Characterization of the desulfurizing activity of *R. rhodochrous* under simulated large-scale conditions requires the development of several experimental and analytical techniques. The most pertinent techniques involve the development of a method for carrying out desulfurization under controlled conditions, measuring the progress of desulfurization and calculating the specific activity based on a model of product formation.

The development of a generalized method for carrying out desulfurization is accomplished by establishing a set of operating conditions that can be standardized and repeated across several experimental trials. Because control over operating parameters is simplified in closed systems, the determination of enzyme decay kinetics is greatly simplified. Likewise the standardization and repeatability of runs is easier to insure in closed systems. Preliminary characterization of enzyme decay kinetics was carried out in a batch-type system consisting of a stirred tank reactor with agitation, aeration and temperature control. Initial characterization experiments were carried out in 2 L shake flasks using an organic to aqueous volume ratio of 1:1. Cell concentrations were typically in the range of 44.0 g/L DCW (or OD$_{600\text{nm}}$ of ~220). The results of these
experiments indicate that the kinetics of enzyme decay are first order. The constant of degradation determined from these experiments was on the order of 0.06 hr\(^{-1}\) during the initial 24 hours of monitoring. A typical enzyme activity profile during this period of monitoring is shown in Figure 6.1.

Monitoring of the extent of desulfurization was carried out by measuring the concentration of dibenzothiophene (DBT) and 2-hydroxybiphenyl (HBP) by gas chromatography. The concentration profiles of these components were monitored and analyzed in order to arrive at a measure of enzyme decay kinetics. Since the change in concentration of each of these components is directly related to enzyme activity the concentration of these components was used to determine SA(t). A sample concentration profile used to derive the SA is shown in Figure 6.2.

![Figure 6.1](image)

The derivation of a SA for a given experimental run was carried out by fitting a suitable model of HBP production to the HBP concentration time profile. One such model is given by
\[ HBP(t) = A(1 - e^{(-kt)}) \]  

Where \( A \) represents the asymptotic value of the HPB concentration (in g/L) that the system approaches as time progresses, and \( k \) is the first order decay constant (in h\(^{-1}\)). Eq. 6.0 is a first order decay model and is typical of most enzyme decay behavior. The coefficient, \( A \), represents the long-term or steady-state HBP concentration. One would expect the HBP concentration to approach a limiting or asymptotic value in a closed system undergoing exponential decay of enzyme activity. Figure 6.2 illustrates a typical HPB concentration profile. A fit of the data using this model is also shown.\(^{22}\)

![Figure 6.2](image)

It is possible to define \( SA \) in terms of Eq 6.0. Given that HBP concentration is indicative of enzyme activity and that it is relatively easy to measure it is reasonable to use HBP generation models as a basis for specific enzyme activity. Using Eq 6.0 it is possible to define \( SA \) as follows,

\(^{21}\) See Material and Methods section.
\(^{22}\) The fit was carried out using a least squares fitting method on the two-parameter model given in Eq 6.0.
All experiments resulted in good agreement between model and data; as indicated \( R^2 > 0.98 \).
\[ SA(t) = \frac{\alpha \cdot A}{C_c} e^{(-\alpha t)} \]  

(6.2)

This result indicates that SA is a time dependent quantity, as expected. This result is also confirmed experimentally in the literature (Gray, 1996).

**6.1 Batch Desulfurization Results**

The results of a typical batch desulfurization in shake flasks were presented in the previous section along with the model developed to explain the production of HBP as a function of time. These experiments allowed the desulfurization kinetics to be characterized and based on the kinetics of enzyme decay. The following section outlines the characterization of desulfurization kinetics in a CSTR with an outlet stream recycle ratio of one, (i.e. a continuously and completely recycled CSTR). This is the continuous process analogue of a closed or batch system. This process is depicted in Fig 6.3.

The kinetics of HBP production and enzyme decay in the closed CSTR system are very similar to that observed in shake flask experiments. This is expected given the closed nature of these processes. However, differences in rate of desulfurization would be expected given the differences in oxygen transport brought about by enhanced agitation and aeration. In general all the shake flask and closed CSTR experiments exhibited initial activities of approximately 0.05 g HBP /L-h.

Figure 6.3 illustrates the full recycle CSTR system used to characterize the performance of the biocatalyst under flow conditions. Normally differences in performance are observed when scaling up processes to larger volumes, this underlies the rationale for using this type system to characterize the performance of a batch biodesulfurization process versus the use of shake flasks. The typical shake flask experiment is less than 500 mL in volume, whereas the process depicted in Fig. 6.3 uses a 1 L reactor (BDS unit) to carry out the biodesulfurization.
One surprising feature of the kinetics of HBP production in the closed CSTR system is in the duration of biocatalyst activity. (A measure of the duration is given by the half life of the catalyst. This is calculated by dividing ln(2) by first order enzyme decay constant, k.) The half-life of biocatalyst in shake flask systems is generally on the order of 6-7 hours; whereas in the closed CSTR system the half-life is usually greater than 9 hours. This increase is a small but significant improvement in the longevity of catalyst activity from one system to another. This improvement has implications for scale up operations where catalyst longevity is a critical design parameter. Estimates given by Energy Biosystems predict that a half life of greater than 24 hours is needed in order to achieve commercial feasibility for large scale biodesulfurization (personal communication).

Another interesting feature of these systems is that the maximum HBP concentration observed in a closed process is the same for either the shake flask or closed CSTR system. This is most likely due to enzyme inhibition caused by HBP. Evidence
for this is given by Hopkins (1993) and Gray (1996). This limitation appears to have a significant impact on the overall desulfurizing capacity of the process; although at low DBT inlet concentrations the inhibitory effect is not expected to play a role since the maximum HBP concentration that could be observed under such circumstances is small relative to $K_I$. This effect is discussed in more detail in Section 6.2.2.

Figures 6.4 - 6.7 illustrate typical concentration profiles for DBT and HBP in a closed CSTR system, SA(t) is also depicted. Fig. 6.4 shows the apparent mass balance which exists between HBP and DBT (the mass balance was first confirmed by Hopkins (1993)). Upon closer examination one can see that there is a significant deviation from the mass balance. The data obtained for HBP and DBT concentration as a function of time were subjected to the following balance,

$$C_{DBT}(0) - \left( C_{DBT}(t) + \frac{M_{DBT}}{M_{HBP}} C_{HBP}(t) \right) = 0$$

(6.3)

Deviations from this balance indicate that either HBP or DBT are not being accounted for due to losses or conversion to other species. Possible causes for the deviations are a high solubility of either species in the aqueous phase, or a buildup of intermediates along the dsz pathway without complete conversion to HBP. Given the low solubility of DBT and HBP in aqueous phases (partition coefficients of 1E-4 and 0.02 in C-16 aqueous systems (Hopkins 1993)), the buildup of intermediates is most likely. This view is supported by experimental evidence of that inhibitory effect of HBP is on the last enzyme(s) in the dsz pathway.

In spite of the non-stoichiometric conversion of DBT to HBP, the activity model based on HBP production Eq. 6.0 is still the most appropriate because deviations such as the one just noted allow the effect of environmental conditions on specific enzymes or sets of enzymes in the system to be investigated more fully. For example, one can see from the deviation shown in Fig. 6.7 that the enzymes responsible for HBP production (i.e. either dszA or dszB) are more affected than dszC. Whether the impact is from inhibition or increased susceptibility to degradation is not clear; however this identifies the weak link in the bioconversion process.
6.2 Semi-Continuous and Continuous Desulfurization

One of the primary objectives of this study was to develop a laboratory scale continuous biodesulfurization process capable of carrying out a high level of desulfurization for extended periods of time. The demonstration of such a laboratory-scale process has promising implications for the development of large scale biodesulfurization processes.

Fig. 6.8 is the process flow diagram for the laboratory-scale continuous biodesulfurization process used in this study. As seen in the diagram biomass was stored in a low temperature storage unit at 3°C before incubation. Due to the extended time required for a typical continuous desulfurization run, the volume of biomass required was in excess of 2.5 L of OD 250 cells. Cells were stored in 156 mM phosphate buffer at pH 7.0.
The incubation unit was used to bring the cells to 30°C for one hour before being used to desulfurize. The mean residence time for cells in the incubator unit was one hour. During this time the cells are able to begin utilizing glucose and reestablishing metabolic processes. Typical glucose concentrations in the inlet to the incubator unit were in the range 10 - 20 g/L.

Cells are pumped continuously from the incubation unit into the BDS unit. Model fuel (hexadecane, 4 g/L DBT) is pumped into the BDS unit continuously at the same volumetric flow rate as the cells, thus the volume ratio $R_{w/v}$ was approximately 1.0 during all experiments. The mean residence time of fuel and cells in the BDS unit ranged from 4 h to 12 h depending on the experiment. Residence times were not varied within any given experimental run. Conditions in the BDS unit were typically within the following ranges; pH = [6.8, 7.9], agitation = [500, 650] RPM, aeration = [1, 2] VVM, and temperature = 30°C ± 0.2°C. The liquid volumes of each vessel are as follows: storage unit, 2.5L; incubation unit, 60 mL; BDS unit 1 L.

### 6.2.1 Semi-Continuous Desulfurization

A semi-continuous desulfurization process was carried out before carrying out fully continuous desulfurization experiment. This was done in order to assess the performance of the pumps, the effect of the incubation unit, and the impact on the biocatalyst of recycling HBP-rich fuel into the BDS unit. Semi-continuous experiments were run as shown in Fig. 6.8, with the exception of the BDS outlet stream which is processed through a settler. A diagram of the settler is shown in Fig. (). After being processed through the settler, the fuel is continuously recycled into the BDS unit while the spent biocatalyst is sent to a waste reservoir.

The purpose of the settler is to allow the biomass to separate from the fuel through phase-separation. This process is mediated by the density difference between the biomass/aqueous phase ($\rho_{aq} = 1.05$ g/mL) and the hexadecane fuel ($\rho_{C-16} = 0.77$ g/mL). Contrary to expectations the biocatalyst did not form a stable emulsion with the fuel, thereby allowing a gravity driven separation process to be employed.\(^\text{23}\)

---

\(^{23}\) Stable emulsions were observed only in batch systems where contact time between fuel and cells exceeded 12 h.
Data collected from a representative semi-continuous experiment are shown in Figs. 6.10 - 6.13. These data show the concentration profiles of DBT and HBP; the specific activity versus time; and the mass balance between HBP and DBT, which is expected to hold at all times. The duration of the experiment was 24 hours (approximately 3 residence times for the BDS unit). The critical enzyme ratio given by Eq. 5.9 was used to determine the inlet to initial biomass concentration ratios that should be used in order to insure constant specific enzyme activity. As seen in Fig. 6.12, the prediction of constant SA made in Section 5.1.1 is confirmed experimentally. The first eight hours (approximately one residence time) show a nearly linear HBP production rate. This is indicative of constant specific enzyme activity. The activity model given by Eq. 6.2 was used to derive the specific activity as a function of time based on the HBP generation data. The SA is plotted versus time in Fig 6.13.
The slope of the fitted line to SA indicates a small but significant decrease in activity with time. This deviation from theoretical constant activity is most likely due to the inhibitory effect of HBP on one or more of the enzymes of the dsz pathway (Hopkins 1993 and Gray 1996). Hopkins confirmed the onset of an inhibitory effect starting at fuel HBP concentrations of 0.5 g/L with complete inhibition of activity at approximately 0.8 g/L. This result is confirmed by the behavior of the SA in Fig 6.11. Assuming these values indicate ranges within which the onset of inhibition would be observed then the apparently immediate cessation of activity at 8 hours in Fig. 6.11 would be explained. (The fuel HBP concentration at 8 hours is approximately 0.8 g/L). The partition coefficient for HBP between a hexadecane/aqueous system is approximately 0.02. This would result in an aqueous phase HBP concentration of 0.016 g/L at 0.8 g/L in the fuel and 0.01 g/L aqueous at 0.5 g/L in the fuel. From this value one can infer that the inhibitory threshold for cessation of enzymatic activity lies near 0.01 g/L aqueous phase HBP concentration. This is an important value to consider when designing processes in which the fuel concentration of HBP might exceed 0.5 g/L. Based on these data one should avoid processes in which the fuel stream HBP concentrations are expected to exceed 0.5 g/L.
6.2.2 Continuous Desulfurization

Fig. 6.14 shows the process used for all continuous desulfurization experiments. Because the system is fully continuous the settler was no longer necessary and was not used in subsequent experiments. The demonstration of a successful continuous desulfurization process is one of the objectives of this study. In addition to this the identification of critical operating parameters that impact the performance of continuous systems is also an objective.

Interesting behavior resulted from the implementation of continuous desulfurization process designs. The most notable impact on process performance was an increase in SA and, consequently, desulfurization productivity with increases in dilution rate. The impact of these changes is shown in Fig. 6.14.

![Figure 6.14](image)

As seen in Fig. 6.14, increases in dilution rate (i.e. shorter residence times) resulted not only in higher productivity due to higher throughput, but higher cell specific activity. A three-fold increase in SA was observed in the $D = 0.25 \text{ h}^{-1}$ continuous process over the batch case. This equates to a six-fold increase in desulfurization productivity (3X from...
increased SA and 2X from doubled dilution rate). An explanation of this counterintuitive result is given by the theoretical derivation of optimal dilution rate (Section 5.3).

The competing demands of various rate processes must be taken into account in order to understand how such a result, as shown in Fig. 6.14, is possible. The bioconversion process is reasonably assumed to be zero order in substrate (at $C_{DBT} \sim 4.0$ g/L the aqueous phase concentration greatly exceeds $K_m$), whereas the enzyme degradation process is first order in nature. Both of these rate processes are taking place simultaneously in both batch and continuous systems. However in continuous processes a dilution rate is imposed on the system; this dilution rate is also a rate process in that it has a time scale associated with it. Therefore in a continuous desulfurization process one has at least three competing rate processes. The interaction of these processes leads to interesting and in many case counterintuitive behavior, especially when the order of each process differs, as it does here.

In order to account for the impact of each process in a context that allows direct comparison, a non-dimensionalization of the quantities representing each process needs to be carried out. The most common non-dimensional group associated with continuous systems in which rate processes (i.e. typically chemical reactions) take place is the Damkohler number. This dimensionless group allows direct comparison of the time scales for rates of reaction versus rates of dilution. Eqs. 5.10 and 5.11 give the Damkohler number for each process in terms of the rate parameters for each process.

Fig. 5.3 illustrates the trend toward higher desulfurization in the region $D = 1.3$ h$^{-1}$. This is certainly in agreement with the trend observed in Fig. 6.14. Future studies should be aimed at confirming experimentally the theoretically predicted optimum dilution rate.

The following sections describe another aspect of desulfurization which is as critical to successful performance as the specific activity; glucose consumption. Studies carried out by Energy Biosystems (and confirmed in this study) indicate that consumption of glucose or another similar carbon source during desulfurization accounts for a large fraction of the cost of desulfurization (Dan Monticello, personal communication). As a result experimental determination of glucose utilization efficiencies is an important process parameter.
6.3 - Determination of Glucose Uptake Rates

Experimental determination of glucose uptake rates for cell metabolism and desulfurization were carried out in 2 L shake flasks under standard operating conditions.24 The objective was to determine the allocation of glucose between the competing processes; cellular respiration and desulfurization. Determining these values was necessary in order to complete the glucose uptake model developed in Section 5.5.

Two sets of two 2 L flasks were charged with respiring biomass and incubated for one hour with a limiting amount of glucose. One set was used to probe non-desulfurizing biomass, while the other set was used to track glucose during desulfurization. Previous experiments allowed estimation of glucose uptake rates so that a limiting amount of glucose could be fed at time zero, such that the glucose would be nearly depleted after one hour. This was done to ensure that the cells had reached a metabolic steady state, given that previous to the experiment the cells had been stored at 4°C in phosphate buffer for several hours. Figure 6.15 shows the kinetics of glucose utilization for *non-desulfurizing* OD$_{600nm}$ 125 cells, *respiring* at 30°C. It is important to remember

\[
\text{Resting Cell Assay post pulse}
\]

\[
y = -4.9062x + 28.336 \\
R^2 = 0.9999
\]

Figure 6.15

---

24 Standard operating conditions: 30°C, 250 RPM, and 1 atm.
that the cells are respiring but that no growth is occurring, so that any glucose consumption is due only to basal metabolic processes.\textsuperscript{25}

The depletion of glucose for respiring cells followed a linear profile, as expected in the absence of growth. From the slope of the fitted line, \((m)\), it is possible to derive the specific glucose uptake rate (SGUR) for cells under these conditions. In general the glucose uptake rate (GUR) is the slope of the fitted line, while the SGUR is given by,

\[ SGUR = \frac{m}{C_c} \]  \hspace{1cm} (6.4)

Using this relation the SGUR is found to be equal to \(-0.196 \pm 0.04\) (g glucose/(g DCW-hr)). This quantity also represents \(\alpha\) in Section 5.5 - Glucose Uptake Model. The next set of data are taken from cells carrying out desulfurization under the same conditions.

Figure 6.16 shows the glucose utilization kinetics for cells carrying out desulfurization.

The exponential decay of this glucose concentration profile is as expected, given that the enzyme decay kinetics also follow an exponential decay profile. As discussed earlier glucose consumption is tied to desulfurization by way of NADH consumption which is effected by the flavin reductase (frdA).\textsuperscript{26} The profile of this curve contains glucose

\textsuperscript{25} Steps are taken during the harvesting procedure to insure that only trace amount of nutrients remain from the original spent fermentation broth. Several washings with 156 mM phosphate buffer were carried out prior to GUR experimental runs.

\textsuperscript{26} The flavin reductase (frdA) consumes one NADH equivalent per FMNH\textsubscript{2} produced. Reduced flavin
consumption information for both basal cell metabolism and desulfurization. Consequently it is necessary to separate out each contribution individually.

The analysis of the data presented in Figure 6.16 is somewhat different from that carried out for respiring cells. Here the slope of the glucose concentration profile is time dependent; however, because one is only interested in glucose consumption rates for desulfurizing cells the initial rate can be taken as representative of the GUR of fully desulfurizing cells. Taking the derivative of the fitted curve, evaluating it at time zero, and dividing by the cell concentration gives the SGUR for desulfurizing cells. Based on the data presented in Figure 6.16 the maximum SGUR is $-0.63 \pm 0.03 \text{ (g glucose/(g DCW -hr))}$. The SGUR for desulfurizing cells includes both basal cell metabolism and desulfurization. In order to find a SGUR for desulfurization only it is necessary to separate out the contribution due to cell metabolism. SGUR data are available for purely respiring cells, as seen in Figure 6.15; therefore, finding the SGUR contribution from desulfurization alone reduces to

$$\text{SGUR}_{\text{desulf}} = \text{SGUR}_{\text{desulf + meta}} - \text{SGUR}_{\text{meta}}$$

(6.5)

Applying Eq 6.3 to the previously calculated values for SGUR for each case yields, $\text{SGUR}_{\text{desulf}} = -0.43 \pm 0.05 \text{ (g glucose/(g DCW -hr))}$. This value represents the amount of glucose consumed due only to desulfurization processes. It also represents $\beta$ in Section 5.5 - Glucose Uptake Model.

It is possible calculate the amount of channeling of glucose into either basal metabolism or desulfurization. These values are summarized in Table 6.1. The values for each SGUR also are tabulated. Based on these data there would appear to be approximately twice the rate of glucose consumption in cells carrying out desulfurization than those simply respiring. Surprisingly, only a small fraction of this glucose

---

27 This method is analogous to the method of initial rates used to determine chemical kinetics parameters. (see Fogler (1992), Chapter 3).

28 Experimental evidence suggests that there is significant loss of glucose due to futile cycles or other losses occurring during desulfurization.
consumption can be accounted for by desulfurization as evidenced by HBP production rates.

<p>| | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>$SGUR_{\text{meta+desulf}}$</td>
<td>-0.63</td>
<td>±0.03</td>
</tr>
<tr>
<td>$SGUR_{\text{meta}}$</td>
<td>-0.196</td>
<td>±0.04</td>
</tr>
<tr>
<td>$SGUR_{\text{desulf}}$</td>
<td>-0.43</td>
<td>±0.05</td>
</tr>
<tr>
<td>Metabolism</td>
<td>31%</td>
<td>±5.00</td>
</tr>
<tr>
<td>Desulfurization</td>
<td>68%</td>
<td>±5.00</td>
</tr>
</tbody>
</table>

Table (6.1)

6.3.1 - Glucose Utilization Efficiency

In addition to allocation this information can be used to give an indication of glucose utilization efficiency during desulfurization. The efficiency of desulfurization can be assessed by comparing the amount of glucose consumed due to non-metabolic processes with the amount needed to carry out desulfurization.

The calculation of the desulfurization efficiency is done to determine what fraction of the glucose allocated to non-metabolic processes (i.e. desulfurization related phenomena) is used to convert DBT to HBP. From the data presented in Figures (6.1) and (6.2) it is possible to calculate this quantity as follows

$$TGU_{\text{desulfurizing cells}} - TGU_{\text{respiring cells}} = TGU_{\text{non-metabolic processes}}$$  \hspace{1cm} (6.6)

Where $TGU$ is total glucose uptake during a given time period.\textsuperscript{29} As seen on Figures (6.1) and (6.2) the $TGU$ for the same time period (1.5 h to 7.5 h) for respiring cells and desulfurizing cells is 20 g/L and 40 g/L, respectively. This gives a $TGU_{\text{non-metabolic processes}}$ of ~ 20 g/L. The maximum productivity of HBP for desulfurizing cells during this same time period was 0.35 g/L HBP (data not shown). Based on the reaction stoichiometry for desulfurization, the amount of HBP that could have been produced based on the calculated $TGU_{\text{non-metabolic processes}}$ is 57 g/L HBP. This results in an apparently low glucose utilization efficiency. It would appear that, ~99% of the glucose channeled to

\textsuperscript{29} Non-metabolic refers to processes not associated with basal metabolic respiration, in this case these processes are associated with desulfurization.
desulfurization pathways is not used to desulfurize. The production of secondary metabolites would change the interpretation of this low efficiency.

6.3.2 Carbon Balance about Reactor

A preliminary analysis of glucose uptake rates for desulfurizing cells in closed systems has been established. With this information it is possible to establish a framework within which to analyze glucose uptake rates for desulfurizing cells in open systems (as depicted in Figure 5.0). The analysis begins by noting the following reaction, which takes place in the cells by way of glucose metabolism.30

$$C_6H_{12}O_6 + 6O_2 \rightarrow 6CO_2 + 6H_2O$$

(6.7)

The following balance on carbon can be established for a continuous flow reactor system at constant volume. Another assumption is that there is no cellular growth, otherwise there would be a channeling of carbon into building of cellular structures in addition to metabolism. (This is verified experimentally for all desulfurization experiments.) A balance based on measurable quantities such as carbon dioxide evolution rate (CER) and glucose concentration would take the following form,31

$$CER = \frac{6F_{cells}}{M_{WG}} \left( C_{g_{feed}} - C_{g_{exit}} \right)$$

(6.8)

Violations of this balance would be indicative of one or more processes in which carbon is being consumed but which are not accounted for by the generalized balance on the reactor system. The most common causes would be cellular growth or removal of dissolved CO$_2$ in the liquid exit stream. It is important at this point to emphasize the difference between cellular metabolism and cellular growth.

In the case of cellular metabolism all the CO$_2$ resulting from complete combustion of glucose would eventually be released and measured; whereas in cellular

---

30 This assumes complete metabolism of glucose through glycolysis and the TCA cycle. If glucose is used for the production of organic acids then the balance given in Eq. 6.1 would need to be modified.

31 The factor (6) in the balance arises from the stoichiometry of the metabolic reaction shown in Eq 5.25.
growth part of the carbon consumed would go to form part of cellular structures (i.e. protein, polysaccharides, etc.) and hence would not be released as gaseous CO$_2$.

Carbon balances for all experiments carried out on open systems have been confirmed, hence all carbon entering and leaving these systems has been accounted for using Eq. 6.7. The production of acetic acid, lactic acid and gluconic acid were monitored during additional experiments. It was found that these species were not produced in significant quantities.
Chapter 7 Future Work

7.1 Native Pathway Augmentation & Metabolic Reconstruction

The ability of various bacterial species to degrade benzo-thiophenic compounds is well documented and has been observed to take place along one of two metabolic pathways (one reductive and one oxidative). Degradation of dibenzothiophene through the oxidative pathway is of greater biochemical interest due to the removal of the sulfur moiety and preservation of the carbon backbone. The pathway (shown in fig 1) is coded for by the dsz operon.

![Native dsz Pathway](image)

The end product of this pathway is 2-hydroxybiphenyl; an effective germicidal and fungicidal agent marketed under the name Dowicide 1®. In addition to toxic effects 2-HBP has an inhibitory effect on one or more of the desulfurization enzymes in the dsz
pathway (Hopkins 1991). The obvious result of this inhibitory effect is a direct limitation of the maximum desulfurization capacity for continuously operating systems due to the build up of HBP to levels above the inhibitory concentration (~ 0.5 g HBP/L, (Hopkins 1991)).

The deleterious effects of the end product of this pathway have created difficulties in the large scale application of whole respiring cells as biocatalysts for desulfurization of petroleum. Efforts at overcoming the effect of the end-product on catalyst stability and activity have been addressed by deleting the gene coding for the last enzyme in the pathway, dszB (2-(2’-hydroxyphenyl)benzene sulphinate desulfinase {HBPSi-desulfinase}). Mutant strains that are missing the dszB enzyme exhibit significantly higher stability and activity at the expense of not completely removing sulfur from the carbon skeleton.

The most evident drawback is that the sulfur is not removed from the carbon structure as originally intended. The advantages afforded by the oxidative pathway are no longer applicable if the pathway is cut short of the dszB enzyme. The net effect of resulting the pathway is that DBT is converted into a water soluble sulfinic acid. Modification of the pathway in this manner to overcome the toxic effects of 2-HPB carries several costs that cannot be justified by the value of the end product, HBPSi-.

The first cost associated with the clipped pathway is the loss of the heating value of the fuel as a result of the retention of HBPSi- in the aqueous phase. In high sulfur fuels (1.7 wt% sulfur) this can account for more than 10% of the heating value of the fuel, while in the more common fuels (0.3 wt% sulfur) this will account for more than 1.7% of the heating value of the total fuel stream.

The second cost associated with the modified pathway is the incomplete elimination of sulfur from the carbon skeleton. There are three scenarios for what can be done with the HBPSi- produced: further downstream remediation, specialty or bulk chemicals, or environmentally compliant disposal. All of these scenarios add to the total cost of processing fuel streams and would unlikely be justified, unless the resulting product were of much higher value than the original substrate.
There exist several process-driven strategies that can adequately address the shortcomings of the clipped pathway. One involves the augmentation of the existing DBT degradation pathway with elements from the polychlorinated biphenyl (PCB) degradation pathway.

In this case the merging of two pathways is carried out with the purpose of removing toxic end products from the native pathway, namely HBP and its substituted analogues. Figure 2 illustrates a proposed pathway which would accomplish the task of effectively removing toxic end products.

The pathway shown in Figure 2 was constructed using metabolic database information found in various databases accessible through the world wide web. The primary sites containing information used in constructing this pathway were The University of Minnesota Biocatalysis/Biodegradation Database[^1] and ExPASy Enzyme Database[^2].


nomenculture database. Construction of the pathway was accomplished by searching the appropriate databases for enzymes which use the end products of the original pathway as substrates. The resulting pathway can be extended indefinitely in this manner provided there are documented enzymes that act on the end products of the proposed pathway.

The extent of the pathway in Figure 2 was determined in part by the availability of published information on recombinant E. coli strains which had been successfully transformed with the appropriate genes. In this instance the availability of published enzymes would have permitted extension of the pathway to the full combustion of both benzoic acid and 2-hydroxypenta-2,4-dienoate to carbon dioxide. It is important to remember that the pathways presented here represent the combination of enzymes from different pathways and different organisms.

The non-dsz enzymes underlined in Figure 2 were isolate from two separate organisms unrelated to R. rhodochrous. 2-hydroxybiphenyl-3-monooxygenase (hbpA) was isolated from Pseudomonas azelaica by Suske et al. (Suske 1997). Both biphenyl-2,3-diol 1,2-dioxygenase (bphC) and HOPDA hydrolase (bhpD) were isolated from Pseudomonas pseudoalcaligenes KF707 by Furukawa et al. (Hayase 1990). All three genes for these enzymes have been successfully transferred to E. coli and have been functionally expressed. (Hyase 1990 and Suske 1997).

7.2 Experimental Protocol for Future Experiments

Two separate transformed E. coli strains containing the individual enzymes hbpA and bphCD, respectively, were obtained from the aforementioned groups. Coincidentally physical separation of the two sets of enzymes allowed for greater control in the extension of the native pathway. The individual strains could be used as metabolic building blocks allowing the native pathway to be extended either one or three steps beyond HBP, as desired. Table (1) lists the specific strains used in the pathway extension experiments and details about the specific expression systems used.

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33 http://www.expasy.ch/sprot/enzyme.html
Experimental implementation of the strategy outlined above was not possible in the time given; however with the information given in Table 1 on the expression systems for each of these enzymes one can devise a series of experiments to gauge the impact of introducing these enzymes into systems where HBP or one of its substituted analogues is being produced.

### 7.3 DNA Shuffling

Recent work in the area of DNA shuffling and chimera formation has given rise to the development of accelerated genetic evolution. In its most basic form DNA or "gene shuffling", as it is known, consists of taking genes which code for enzymes with identical or nearly identical biological activity/function from two or more distinct species and recombining fragments of appropriate lengths of these genes in a random manner. The end result in many cases is a gene with enhanced activity and broadened specificity for substrate.

The most obvious application of this technique is in broadening the enzyme specificity of the dsz enzymes to by shuffling genetic elements from the dsz pathway with those from other organisms which have similar activity. McFarland et al (1998) provide a fairly comprehensive review of prokaryotes that exhibit desulfurizing activity (ref. ppg. 110). Candidates from among these organisms would be chose based specific knowledge of the mechanisms of enzyme activity. Divergent pathways (i.e. in this case non-oxidative pathways) or enzymes with substrate specificity that are radically different would not be good candidates. An excellent example of experimental implementation of gene shuffling is given by Kumamaru et al. (1998) (coincidently this group also provided the bphCD genes shown in Table 7.0).

The problem of low glucose utilization efficiency could also be addressed using the techniques described above. The creation of knockout mutants of one or more

<table>
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<tr>
<th>Strain</th>
<th>Enzyme(s)</th>
<th>Antibiotic Resistance</th>
<th>Promoter</th>
<th>Origin</th>
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<tbody>
<tr>
<td>phbpA</td>
<td>hbpA</td>
<td>Amp</td>
<td>lacZ</td>
<td>P. azelaica</td>
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<tr>
<td>pbphCD</td>
<td>bphC and bphD</td>
<td>Amp</td>
<td>lacZ</td>
<td>P. pseudoalcaligenes</td>
</tr>
</tbody>
</table>
secondary metabolic enzymes may address the problem along with DNA shuffling of enzymes for desulfurization from other organisms where glucose utilization efficiency is higher. Likely candidates for knock-out (or deletion) would be enzymes that could drain intermediate glucose metabolites from glycolysis and the TCA cycle. Among the enzymes responsible for diverting glucose away from these pathways are lactate dehydrogenase, glucose oxidase, citrate lyase, lactate 2- monooxygenase, and glutamate dehydrogenase.

Another area where glucose utilization could be improved is in the activity of frdA (the flavin reductase associated with the dsz pathway). It is possible that the activity of frdA is not closely coupled with the monooxygenation carried out by dszC and dszA. A decoupling of the activity of frdA from that of dszC and dszA would result in a futile cycle. Because frdA is not in the same operon as the dsz enzymes, a lack of physical as well as regulatory proximity of these sets of enzymes might be one cause of the decoupling. The regrouping of the dsz and frdA enzymes under a single promoter or the design of a fusion protein which contains all enzymes needed would address this problem. Studies into the design of a fusion protein for desulfurization would likely borrow from the structure and function of pyruvate dehydrogenase (PDH).
Conclusions

The following conclusions can be drawn based on the work presented in this study:

1.) A volumetric productivity of 0.36 g/L-h of *R. rhodochrous* biomass was achieved by optimization of fed-batch fermentation under sulfur controlled conditions.

2.) The volumetric productivity obtained is higher than the highest values reported in the literature to date.

3.) An efficient and rapid method for medium optimization using techniques borrowed from high throughput screening and Response Surface Methodology (RSM) was developed.

4.) The technique for medium optimization mentioned in 3.) resulted in higher model significance, higher $R^2$, $R^2_{adj}$, and $R^2_{prediction}$ than values reported in the literature for comparable RSM studies in medium optimization using conventional techniques.

5.) The use of DMSO as a sole sulfur source resulted in cells of higher specific activity than those grown in LB or with sulfate salts. This result confirms work carried out by Hopkins (1992) and Gray (1998).

6.) A model which explains the decrease in enzyme activity with time was developed and agrees well with experimental results.

7.) A model of enzyme activity in a continuous biodesulfurization process was developed which incorporates the model developed in 6.) and which agreed well with experiment.

8.) The model developed in 7.) was used to obtain theoretical optimal operating ranges for process parameters such as dilution rate and enzyme concentrations. Experiments confirmed theoretical predictions thus validating the model.

9.) Sustained desulfurization in a laboratory scale continuous process was successfully demonstrated for several residence times.

10.) Three fold increases in specific activity (over that reported in the literature) were obtained as a result of applying the theoretical modeling results described in 8.).

11.) A model of glucose uptake in desulfurizing systems was developed based on first principles. Preliminary experimental results agreed well with predictions of glucose uptake rates based on the model.
12.) Evidence of intermediate buildup and inhibition of certain dsz was observed based on deviations from mass balance and on differential activities based on DBT consumption versus HBP production. These results are in agreement with observations made by Hopkins (1993).

13.) A method of determining glucose and oxygen channeling ratios by measuring glucose uptake rate and oxygen uptake rates was derived theoretically. Based on these results it is possible to measure the extent to which glucose (in the form of reducing equivalents) and oxygen are being used by desulfurization and cellular respiration respectively.

14.) Enzymes from the polychlorinated biphenyl degradation pathway (from Pseudomonas azelaica and Pseudomonas pseudoalcaligenes KF707) were identified as potential candidates for metabolic reconstruction of an augmented pathway which would combine elements from the dsz, pcb and hbp operons.

15.) All three genes for these enzymes have been successfully transferred to E. coli and have been functionally expressed (Hyase 1990 and Suske 1997) and are available for use in a form which would permit 14.) to be carried out.
References


APPENDIX A1

The calculation of the oxygen uptake rate (OUR), the carbon dioxide evolution rate (CER) and the respiratory quotient (RQ) were carried out by applying the equations shown below to the data obtained from the mass spectrometer.

\[
OUR = \frac{F_s}{22.4} \left( \frac{\%N_2}{100} \right)_{\text{ref}} \left[ \left( \frac{\%O_2}{\%N_2} \right)_{\text{ref}} - \left( \frac{\%O_2}{\%N_2} \right)_{\text{sample}} \right] \quad \text{in mol/min}
\]

\[
CER = \frac{F_s}{22.4} \left( \frac{\%N_2}{100} \right)_{\text{ref}} \left[ \left( \frac{\%CO_2}{\%N_2} \right)_{\text{sample}} - \left( \frac{\%CO_2}{\%N_2} \right)_{\text{ref}} \right]
\]

\[
RQ = \frac{CER}{OUR}
\]

where \(\%N_2\), \(\%O_2\), and \(\%H_2O\) are the percentages of the off gas that are nitrogen, oxygen and water vapor for each stream, respectively. For significant water vapor generation during the fermentation or bioconversion the sample off gas percentages can be corrected by renormalizing with respect to a stream containing no water vapor.

\[
(\%G_i)_{\text{renormalized-sample}} = \frac{(\%G_i)_{\text{original-sample}}}{\sum_{i=1}^{n-1} (\%G_i)_{\text{original-sample}}}
\]

where \(n\) is \(\%H_2O\).

An example is given for renormalization of \(\%CO_2\). In this example we assume that there are only 4 gases that contribute significantly to the overall percentages outside of water vapor.

\[
(\%CO_2)_{\text{renormalized-sample}} = \frac{(\%CO_2)_{\text{original-sample}}}{(\%CO_2 + \%O_2 + \%N_2 + \%Ar)}
\]
APPENDIX A5

Proof that $D_r = D_{r,aq}$

The following is a proof that the total BDS unit dilution rate is equal to the BDS unit aqueous fraction dilution rate, regardless of oil/aqueous volume ratio, provided that $(R_{o/w})_{inlet} = (R_{o/w})_{BDS\ unit}$.

The proof begins with a definition of the respective dilution rates.

$$D_{aq} = \frac{F_{aq}}{V_{aq}} \quad (A5-1)$$

$$D_r = \frac{F_r}{V_r} = \frac{F_{oil} + F_{aq}}{V_{oil} + V_{aq}} \quad (A5-2)$$

with the following definition of oil/water ratios,

$$F_{oil} = R_{o/w} F_{aq} \quad (A5-3)$$

$$V_{oil} = R_{o/w} V_{aq} \quad (A5-4)$$

If the assumption $\left(\frac{F_{oil}}{F_{aq}}\right)_{t=0} = \left(\frac{V_{oil}}{V_{aq}}\right)_{t=0}$ holds then substituting Eqs. (A5-3) and (A5-4) into Eq. (A5-2) yields,

$$D_r = \frac{F_r}{V_r} = \frac{R_{o/w} F_{aq} + F_{aq}}{R_{o/w} V_{aq} + V_{aq}} = \frac{F_{aq}}{V_{aq}} \left(\frac{R_{o/w} + 1}{R_{o/w} + 1}\right) = D_{aq} \quad (A5-5)$$

$$D_r = D_{aq}, \; Q.E.D.$$}

It is possible to apply this result to the derivation of optimal dilution rate given by Eq. (5-15). This yields $D_{r}^{opt} = D_{aq}^{opt}$. 

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A5.1 Derivation of Enzyme Activity Relation for a Continuous System with Recycle

The following is a derivation of the enzyme activity model carried out in Chapter 5 for a continuous system with recycle. The behavior of such a system would be expected to differ from that of purely continuous systems. Predictions concerning desulfurization performance can be made before carrying out any experimental work. A mathematical derivation of system performance based on knowledge of enzyme decay and reactor flow conditions allows one to explore the performance of various process designs.

The derivation of an enzyme activity model for a continuous system with recycle is analogous to the derivation given in Chapter 5. The material balance begins with the same form of differential equation.

\[ \frac{dE(t)}{dt} = \frac{FE_{in}}{V_r} - \frac{FE(t)}{V_r} - kE(t) \]  

(5.1)

In this case the inlet enzyme concentration is not \( E_0 \), but a combination of the fresh inlet and the recycled outlet thus,

\[ E_{in} = \frac{E_o F + E(t)F_R}{F + F_R} \]  

(A5-1)

The recycle ratio can be defined as follows,
\[ R = \frac{F_R}{F} \quad \text{(A5-2)} \]

Solving Eq. A5-2 for \( F_R \) and substituting the result into Eq. A5-1 gives,

\[ E_{in} = \frac{E_o + E(t)R}{1 + R} \quad \text{(A5-3)} \]

Substituting Eq. A5-3 into Eq. 5-1 and defining the dilution rate as \( D = F/V \) gives,

\[ \frac{dE(t)}{dt} = \frac{D_r E_o}{(1 + R)} - E(t) \left[ \frac{D_r R}{(1 + R)} + k + D_r \right] \quad \text{(A5-4)} \]

Defining the following quantity,

\[ \overline{D} = \left[ \frac{D_r R}{(1 + R)} + k + D_r \right] \quad \text{(A5-5)} \]

and solving Eq. A5-4 with the initial condition \( E(0) = E_i \) gives,

\[ E(t) = \left[ E_i - \frac{D_r E_o}{D(1 + R)} \right] e^{-\overline{D}t} + \frac{D_r E_o}{D(1 + R)} \quad \text{(A5-6)} \]

Setting the coefficient of the transient term equal to zero and solving for the critical enzyme concentration ratio (as in section 5.1) gives,

\[ \left( \frac{E_i}{E_o} \right) = \frac{D_r}{D(1 + R)} \quad \text{(A5-7)} \]

One can confirm by inspection that substitution of \( R = 0 \) into A5-7 returns Eq. 5.8, as it should.
Derivation with a Concentration Step in Addition to Recycle

If the outlet stream is not only recycled but concentrated, such as would be the case with a centrifugation of the outlet stream, then the analysis needs to be modified in order to take into account the concentration of cell mass (and consequently enzyme).

The analysis is identical to that given in the previous section with the exception of the definition of $E_{in}$. This quantity is defined as follows,

$$E_{in} = \frac{E_o F + E(t) F_R}{F + F_R} \quad (A5-1)$$

Because a concentration step is also included a concentration factor must be included in the definition of $E_{in}$. This factor is defined as follows,

$$\frac{E_R(t)}{E(t)} = Q \quad (A5-8)$$

The recycle ratio is defined as before. Solving Eq. A5-8 for $Q$ and substituting this result and the result from A5-2 into Eq. A5-1 gives,

$$E_{in} = \frac{E_o + E(t) R Q}{1 + R} \quad (A5-9)$$
Substituting A5-9 into 5.1 gives,

\[
\frac{dE(t)}{dt} = \frac{D, E_0}{(1 + R)} - E(t) \left[ \frac{D_r R Q}{(1 + R)} + k + D_r \right]
\]  

(A5-10)

Defining the following quantity,

\[
\bar{D} = \left[ \frac{D_r R Q}{(1 + R)} + k + D_r \right]
\]

(A5-11)

and solving Eq. A5-4 with the initial condition \( E(0) = E_i \) gives,

\[
E(t) = \left[ E_i - \frac{D_r E_0}{\bar{D}(1 + R)} \right] e^{-\bar{D}t} + \frac{D_r E_0}{\bar{D}(1 + R)}
\]

(A5-12)

Setting the coefficient of the transient term equal to zero and solving for the critical enzyme concentration ratio (as in section 5.1) gives,

\[
\left( \frac{E_i}{E_o} \right) = \frac{D_r}{\bar{D}(1 + R)}
\]

(A5-12)

This result is identical in form to that given by A5-7; however, note that \( \bar{D} \) takes into account recycle with concentration, whereas \( \bar{D} \) only takes into account recycle.
Dependence of Optimal Dilution Rate on $C_{DBT}^o$

The dependence of the optimal dilution rate on $C_{DBT}^o$ as given in Eq. (5.15) is illustrated in Figure (A5-1). The function $(X_r - X_d)$ is plotted for a series of $C_{DBT}^o$ concentration ranges (0.2, 1.0, 2.0, 3.0, and 4.0 g/L). Also plotted is the contour which contains the maxima for all curves in the range depicted. As expected the amount of time required to carry out desulfurization at optimal dilution increases (i.e. dilution rate decreases) as $C_{DBT}^o$ increases.

The shape of the maxima contour contains a great deal of information concerning the impact of increasing $C_{DBT}^o$ on the optimal dilution rate. It would appear that in the lower DBT concentration ranges the magnitude of the increase in throughput increases more rapidly per change in DBT concentration than in regions of high DBT concentration. This is an indication of the sensitivity of throughput to $C_{DBT}^o$. This fact should be kept in mind when throughput is fixed and other parameters are free to vary.