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# Utilization of Organic Co-Solvents to Enhance Bacterial Conversion of Poorly Water-Soluble Compounds

Miguel Rodríguez Jr.
*University of Tennessee - Knoxville*

To the Graduate Council:

I am submitting herewith a thesis written by Miguel Rodríguez Jr. entitled "Utilization of Organic Co-Solvents to Enhance Bacterial Conversion of Poorly Water-Soluble Compounds." I have examined the final electronic copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Life Sciences.

Robert N. Moore, Major Professor

We have read this thesis and recommend its acceptance:

Brian H. Davison, Paul D. Frymier

Accepted for the Council:
Carolyn R. Hodges

Vice Provost and Dean of the Graduate School

(Original signatures are on file with official student records.)

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Brian H. Davison

Committee Member

Paul D. Frymier

Committee Member

Accepted for the Council:

Anne Mayhew

Vice Provost and
Dean of Graduate Studies

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# UTILIZATION OF ORGANIC CO-SOLVENTS TO ENHANCE BACTERIAL CONVERSION OF POORLY WATER-SOLUBLE COMPOUNDS

A Thesis

Presented for the

Master of Science Degree

The University of Tennessee, Knoxville

Miguel Rodríguez, Jr.

December, 2003

## Dedication

This thesis is dedicated to the loving memory of my brother, Rafael (Rafy) Rodríguez-Vélez, whose faith in God and excellence in humanity will always be an inspiration to me, and to my parents, Ms. María C. Vélez-Santiago and Mr. Miguel A. Rodríguez-Rodríguez, for their unconditional love and everlasting support.

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## Abstract

Mass transfer of a poorly water-soluble gas in fermentations can reduce the performance of bioreactors. In aerobic fermentations, oxygen often becomes a limiting factor. In the biodegradation of volatile organic contaminants, their poor water solubilities often regulate the process. In fermentations of synthesis gas to fuels and chemicals, the issue of mass transfer is apparent. Since the mass transfer rate of the gaseous substrate to the aqueous phase is often the rate-limiting step in the bacterial conversion, many different reactor configurations have been suggested. An alternative approach could be to incorporate a co-solvent, which has properties that will increase the interfacial mass-transfer area and thereby improve the overall conversion rate. In this thesis work, two bacterial systems were used to illustrate enhancement of the conversion of poorly water-soluble substrates by the addition of co-solvents. The first tested system was anaerobic conversion of synthesis gas by *Clostridium ljungdahlii* and the second was aerobic conversion of toluene by *Pseudomonas putida* F1.

*Clostridium ljungdahlii* (ATCC 55383) converts coal synthesis gas (carbon monoxide, hydrogen, and carbon dioxide) into ethanol and acetic acid. A limiting factor in this process is the low solubility of synthesis gas in aqueous media. Different co-solvent systems were evaluated to increase the effective conversion rate of carbon monoxide. The Ostwald coefficient and the octanol-water partition coefficient values were considered when selecting co-solvent systems, and serum bottle experiments were performed to test solvent systems for biocompatibility. Hexadecane proved to the best solvent tested in terms of both gas conversion rate and biocompatibility. Serum bottles

with 10% hexadecane (v/v) converted 100% of the available carbon monoxide to products, while in the control serum bottles, only 30% was converted during the same time.

*Pseudomonas putida* F1 (ATCC 700007) was used as a model organism to study the conversion enhancement of toluene in stirred tank reactors. Silicone oil was used as a co-solvent with and without rhamnolipids (biosurfactants) to enhance the mass transfer rate. Batch experiments were conducted in two side-by-side fermentation vessels; one with silicone oil and one without it. Silicone oil was tested at three different concentrations: 10%, 30%, and 50% (v/v). Results showed that the presence of 30% silicone oil resulted in a 20% higher conversion of toluene when compared to the control. Rhamnolipids were tested at two different concentrations: 0.025% (no silicone oil) and 0.0025% (with 30% silicone oil) (w/v). No significant enhancement in conversion was observed when rhamnolipids were only added by themselves or when they were added in conjunction with 30% silicone oil. In subsequent experiments, results showed that increasing the silicone oil from 20% to 35% in a continuous stirred tank reactor increased the conversion rate by 10%.

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# Chapter 1

## 1. Introduction

### 1.1. Research Objectives

The research objective of this thesis was to study the utilization of organic co-solvents to enhance bacterial conversion of poorly water-soluble compounds. This was performed using two model microorganisms in two different systems:

1. *Clostridium ljungdahlii* – Anaerobic conversion of synthesis gas (syngas)
2. *Pseudomonas putida* F1 – Aerobic conversion of toluene

Results from these studies provide new insights in the development of bioreactor design for the bioconversion of these compounds in these or similar biocatalytic processes. In the last two decades, there has been a growing interest in the development of a reactor design for the bioconversion of poorly water-soluble compounds. Some of these compounds, like synthesis gas, are used for the production of chemicals and fuels. Other poorly water-soluble compounds, like benzene, toluene, xylene, trichloroethylene, and phenols, are environmental contaminants of serious concern in air, soil, and water. Bioreactor technologies currently used to address this purpose include two-liquid phase systems: biofiltration and surfactant enhanced systems. Colleagues at the Daugulis laboratory have reported positive results for the application of two-phase bioreactors containing oleyl alcohol as a co-solvent for the degradation of benzene, toluene, xylene, and phenols by the *Pseudomonas* species (Collins and Daugulis, 1999; Vrionis, et al., 2002). Positive results have also been reported for the utilization of silicone oil in two-

phase bioreactors for biodegradation of polycyclic aromatic hydrocarbons like pyrene, chrysene, benzo[*a*]pyrene and perylene (Marcoux, et al., 2000).

## 1.2. Synthesis Gas (Syngas) Fermentation

Synthesis gas, also known as syngas, is a mixture of primarily carbon monoxide, hydrogen, and carbon dioxide. Syngas represents an easily obtainable, generic chemical feedstock for the production of numerous fuels and chemicals. It may be obtained directly as a waste gas from many manufacturing processes, or it may be obtained through gasification of any carbonaceous material such as agricultural, municipal, and paper wastes, coal, or natural gas. Traditional chemical catalytic processes at high temperatures and high pressures are being used for the conversion of synthesis gas into different fuels and chemicals, such as methanol and acetic acid (Rath and Longanbach, 1991; Wender, 1996). Figures 1.1 and 1.2 illustrate the potential chemicals and fuels that were projected to be produced from syngas as of 1994 (Wender, 1996). The four largest commercial uses of syngas are: 1) the manufacture of hydrogen gas (more than 50% is used for the synthesis of ammonia), 2) synthesis of methanol, 3) its conversion to paraffins, olefins and oxygenates via the Fischer-Tropsch reactions, and 4) conversion of olefins plus syngas to aldehydes and alcohols via the hydroformylation (oxo) reaction. The manufacture of hydrogen gas is very important. Due to continued environmental regulations, hydrogen is being promoted as the clean energy source for the 21$^{st}$ Century. Methanol is one of the top ten organic chemicals manufactured in the world. It is the source material for a large number of fuels and chemicals, mostly oxygenated compounds.

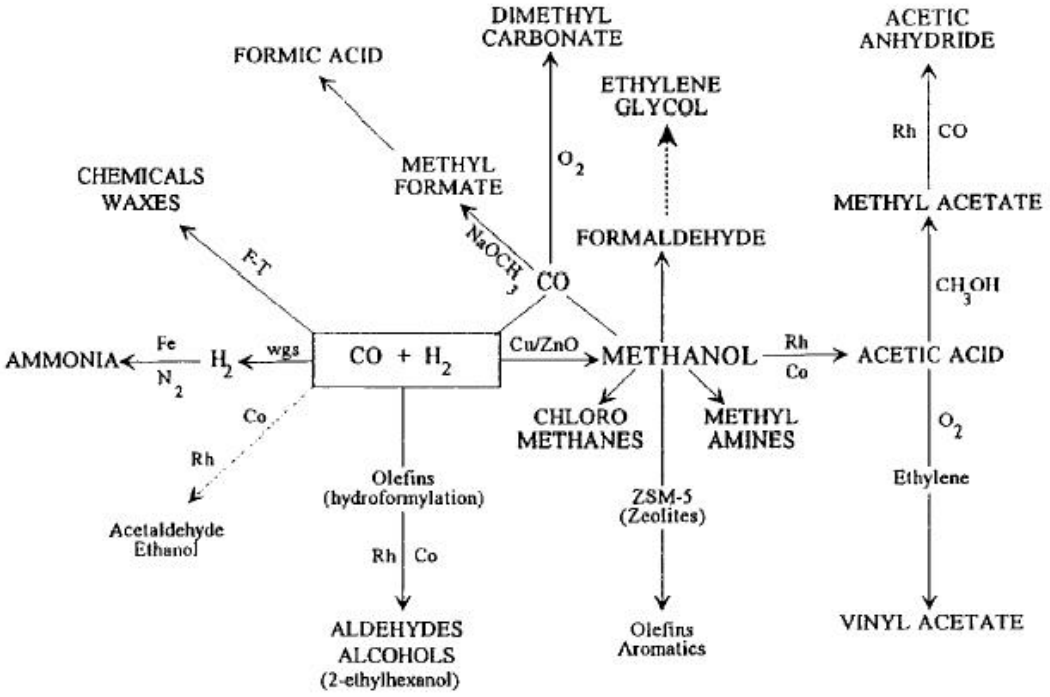



**Figure 1.1.** Potential chemicals from synthesis gas as of 1994 (Adapted from Wender, 1996)

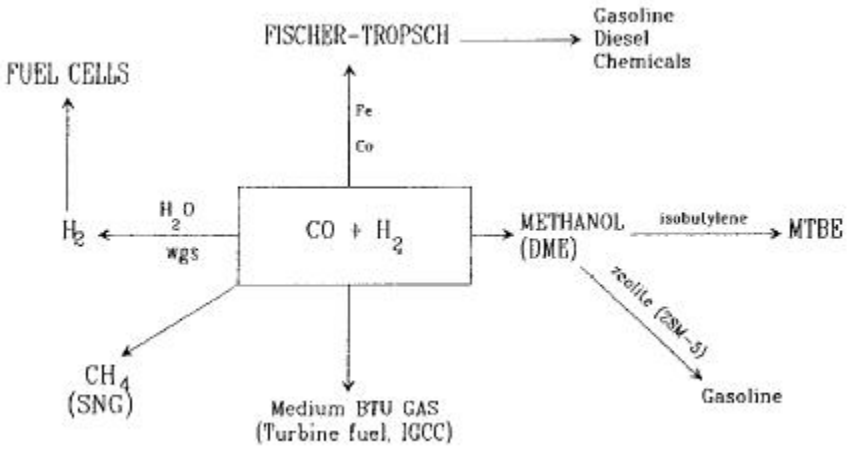



**Figure 1.2.** Potential fuels from synthesis gas as of 1994 (Adapted from Wender, 1996)

While conventional catalytic technology exists for the conversion of synthesis gas into fuels and chemicals, developing biological processes offer the potential to synthesize more specific product streams with high savings in capital and operating expenses at a variety of plant scales. Biological processes have the potential to offer several advantages over chemical synthesis, but further research and development is needed to fully realize this potential. Although biological processes are usually less rapid than chemical reactions, they occur at near ambient temperatures and pressures, significantly decreasing capital and operating costs. Enzyme-catalyzed biological reactions are also generally irreversible and quite specific, resulting in higher yields and product specificity.

Microbiological research has shown that different microorganisms can be used to convert synthesis gas into liquid and gaseous products (Madhukar, et al., 1996; Grethlein and Jain, 1992; Worden, et al., 1991). Microorganisms have been used for the production of ethanol from synthesis gas (Barik, et al., 1990; Vega, et al., 1989; Phillips, et al., 1993; Elmore, 1990; Gaddy and Clausen, 1992; Klasson, et al., 1990; 1992; 1993). Phillips, et al. (1994) reported the production of acetic acid and ethanol by *Clostridium ljungdahlii* grown on carbon monoxide or hydrogen. Vega, et al. (1990) studied the production of acetate by *Peptostreptococcus productus* from synthesis gas in different bioreactor configurations. Other researchers described the production of methane from synthesis gas by a mixed culture of *Rhodospirillum rubrum*, *Methanosarcina barkeri*, and *Methanobacterium formicicum* (Kimmel, et al., 1991; Klasson, et al., 1990). Madhukar, et al. (1996) reported the production of acetate, ethanol, and methanol by three rod

shaped, Gram-positive cultures isolated from petroleum-contaminated soil, a cow manure-soil mixture, and sheep rumen fluid.

Microorganisms that synthesize acetate from CO are called acetogens. Most acetogens can produce more than acetate as their reduced end product (Drake, et al., 2002). *Clostridium ljungdahlii* is a Gram-positive, motile, rod-shaped anaerobic acetogenic bacterium that was isolated from chicken waste. It occurs most often in single cells that rarely sporulate, and its optimal temperature for growth is 37°C. The pH range for growth is from 4.0 to 7.0 with an optimum at 6.0. The type strain was designated PETC (Pittsburgh Energy Technology Laboratory) and deposited in the American Type Culture Collection as strain ATCC 49587. This organism can grow on arabinose, xylose, glucose and fructose. It is capable of producing ethanol and acetate from CO and $H_2O$ and/or $H_2$ in synthesis gas (Tanner, et al., 1993; Elmore, 1990).

Ethanol is produced by this acetogen according to the following stoichiometry:

$$6\ CO + 3\ H_2O \rightarrow CH_3CH_2OH + 4\ CO_2 \quad (1)$$

$$2\ CO_2 + 6\ H_2 \rightarrow CH_3CH_2OH + 3\ H_2O \quad (2)$$

Acetic acid is also produced as a by-product by the equations:

$$4\ CO + 2\ H_2O \rightarrow CH_3COOH + 2\ CO_2 \quad (3)$$

$$2\ CO_2 + 4\ H_2 \rightarrow CH_3COOH + 2\ H_2O \quad (4)$$

A recently discovered clostridial bacterium from an agricultural lagoon, catalogued as P7 at the University of Oklahoma, was found to be able to produce butanol in addition to ethanol and acetic acid from synthesis gas (Rajagopalan, et al., 2002). Also, a research group from Belgium has described an acetogen with a fermentation

pattern similar to *C. ljungdahlii*. It was isolated from rabbit waste and was named *Clostridium autoethanogenum* (Abrini, et al., 1994).

A limiting factor in syngas biological processes is the low solubility of the individual gases in aqueous media. Syngas fermentations are generally mass-transfer limited in practical applications (Bredwell, et al., 1999; Klasson, et al., 1992; Vega, et al., 1990). When the cell concentration reaches a value at which mass transfer controls the bioconversion process, the concentration of carbon monoxide and hydrogen in the liquid becomes zero and the uptake rate is controlled by the rate of transport of the gaseous substrate into the liquid phase. Bioreactors that achieve high mass transfer rates and high cell concentrations are desirable for synthesis gas fermentations. Bredwell and Worden (1998) demonstrated an increase in mass transfer in synthesis gas fermentations by using microbubble sparging. This method requires a surfactant in order to generate bubbles in a spinning-disk apparatus from which they are pumped to the culture media in the bioreactor. Another approach to overcome this limitation is to use a second liquid phase with high solubility for the gaseous substrates in syngas and to increase the gases contact with the microorganisms. Separately, Breman, et al. (1994) reported hexadecane as a good reacor solvent for carbon monoxide and hydrogen based on its respective gas-liquid solubilities.

## 1.3. VOC Degradation and Biofiltration

Although atmospheric emissions of gaseous effluents containing volatile organic compounds (VOCs) have decreased 37% from 1970 to 1997, industries continue their efforts to comply with the regulations set forth by the Environmental Protection Agency.

The 1990 Clean Air Act Amendments alone are expected to increase the compliance cost to several billions of dollars (Chilton, 2000). Traditional chemical control technologies to decrease emissions are very expensive. They generally require processes that are performed at high temperatures and high pressures, and generate a significant amount and number of waste products. As a result, industries have been forced to look for alternative ways of dealing with these effluent streams. Biological technologies have become attractive, inexpensive solutions for the treatment of VOCs (Khan and Ghoshal, 2000). Microorganisms can be used as biocatalysts offering higher specificity and minimal hazardous waste products. This has generated an increased interest in the design and development of biofilters (Delhomenie, et al., 2002).

One VOC, toluene, is very volatile and is considered to be a priority hazardous air pollutant in United States and Canada (Harding, et al., 2003). Toluene is an aromatic hydrocarbon that forms a clear, colorless liquid and is obtained primarily from petroleum. It is used to make several commodity products including benzene, urethane foams, pharmaceuticals, dyes and cosmetic products (Harding, et al., 2003). Since the amount of toluene generally present in waste gases produced in many industrial processes is low, traditional chemical methods to remove toluene from the waste are not economically feasible. This leaves biological treatment as a viable option (Daugulis, 2001).

Biofiltration has been the most common technology used to remove low concentrations of poorly water-soluble VOCs from waste gases (see Figure 1.3). Two of the advantages of biofiltration are that it is performed at room temperature (15-30°C) and it does not produce toxic by-products. Examples of operating units include biofilters,

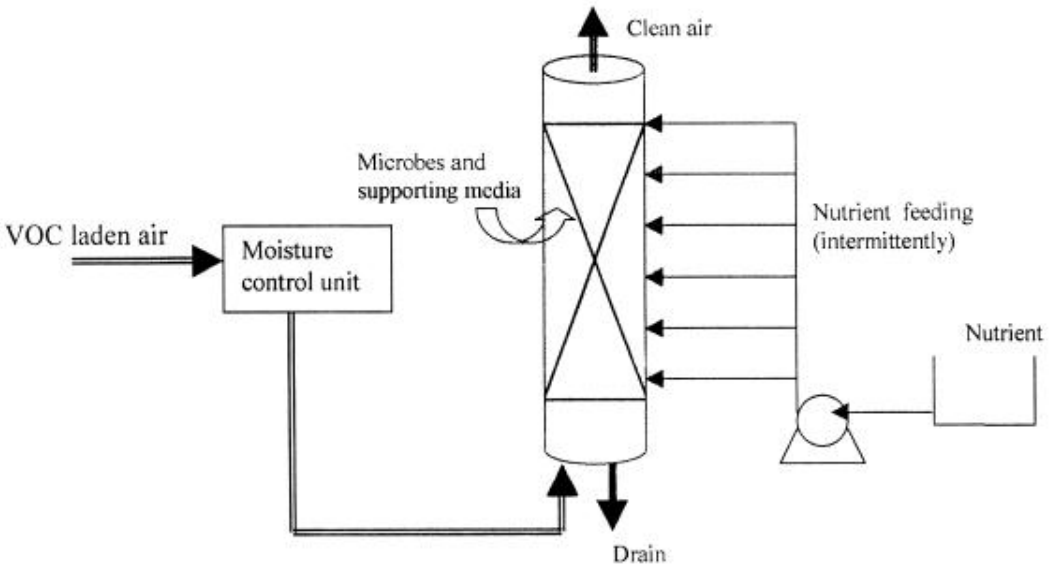



**Figure 1.3.** General schematic diagram of a biofiltration system (Adapted from Khan and Ghoshal, 2000)

biotrickling filters, and bioscrubbers. In a biofilter, microorganisms are retained on a solid support. This support can be composed of compost, soil, peat, wood chips, or polystyrene spheres. VOCs are used to provide the main source of carbon and energy for the aerobic microorganisms. They are metabolized to produce carbon dioxide or to be incorporated into biomass. An aqueous nutrient solution is periodically added to the filter-bed, which allows the microorganisms to grow and form a biofilm on the surface of the bed-forming particles (Delhomenie, et al., 2002; McNevin and Barford, 2000). Microorganisms can be self-attached to the filter-bed material or be artificially immobilized to or on it. Methods of artificial immobilization include: membrane separation, covalent bonding, entrapment within polymer beads, microencapsulation, and support-free immobilization (Cohen, 2001). For detailed information about biofiltration operating requirements, see Swanson and Loehr (1997).

Successful removal of toluene by biofiltration has been reported in several studies. Delhomenie, et al. (2002) demonstrated an approximate 95% removal efficiency by using a compost-based bed. Garcia-Peña, et al. (2001) reported removal efficiencies of 98% by the fungus *Scedosporium apiospermum* using a vermiculite-based support. Moe and Irvine (2001) measured over 99% removal using a polyurethane foam support. Deshusses and colleagues have tested the performance of biotrickling filters (Cox and Deshusses, 1999; 2002; Cox, et al., 2000). Recently, silicone membrane bioreactors have been developed for toluene biofiltration (Xiao, et al., 2000; Brown, et al., 2000; Attaway, et al., 2001). These systems take advantage of the selective permeability for VOCs by silicone rubber.

Initially, *Pseudomonas putida* F1 (*Pp*F1) was best known for its ability to utilize toluene as a sole source of carbon and energy for growth (Zylstra and Gibson, 1989). However, this fluorescent soil bacterium can also do the same with ethylbenzene and benzene. Although the molecular basis is not known, *Pp*F1 is chemotactic toward toluene in an inducible manner (Phoenix, et al., 2003). The *Pp*F1 toluene degradation (*tod*) pathway consists of seven enzymatic reactions (Cho, et al., 2000) encoded by the genes *todFC1C2BADEGIH* (9.5 kb total). The sequence of these reactions is shown in Figure 1.4. Toluene dioxygenase (TDO; EC 1.14.12), encoded by the *tod C1C2BA* genes, catalyzes the first reaction of the pathway. TDO is a multicomponent enzyme system that oxidizes toluene to (+)-*cis*-(1*S*,2*R*)-dihydroxy-3-methylcyclohexa-3,5-diene (*cis*-toluene dihydrodiol) by adding two hydroxyl groups at the 2,3 position of toluene. The organization of the TDO system is shown in Figure 1.5. Electrons are transferred

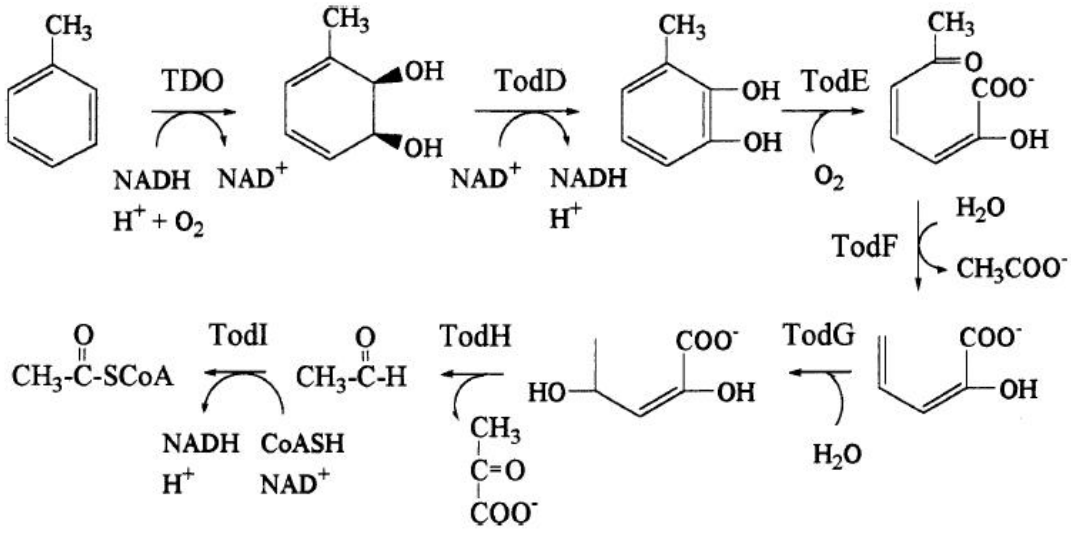



**Figure 1.4.** Toluene degradation (*tod*) pathway in *Pseudomonas putida* F1 (Adapted from Cho, et al., 2000)

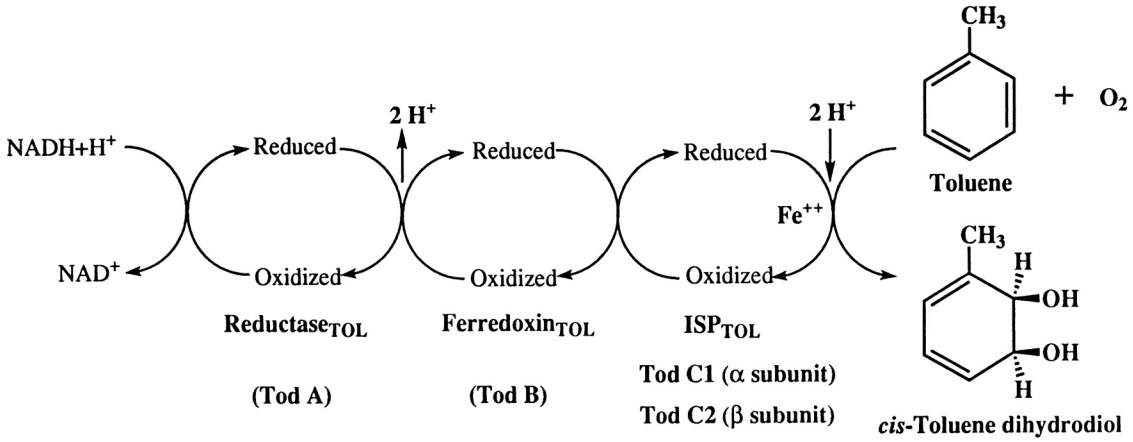



**Figure 1.5.** Electron flow in the toluene dioxygenase (TDO) system (Adapted from Jiang, et al., 1999)

from NADH through a flavoprotein reductase ($Reductase_{TOL}$) to a Rieske [2Fe-2S] protein ($Ferredoxin_{TOL}$). The latter reduces the oxygenase component, an iron-sulfur protein ($ISP_{TOL}$) which, in the presence of exogenous ferrous iron, catalyzes the stereospecific addition of dioxygen to the aromatic nucleus. $ISP_{TOL}$ has a $\alpha_2\beta_2$ subunit composition, and the α and β subunits are encoded by the *todC1* and *todC2* genes, respectively (Jiang, et al., 1999).

In the second reaction, the *cis*-toluene dihydrodiol is dehydrogenated to 3-methylcatechol by a $NAD^+$ - dependent *cis*-toluene dihydrodiol dehydrogenase (TodD). In the third reaction, 3-methylcatechol undergoes *meta* cleavage by 3-methylcatechol-2,3-dioxygenase (TodE) to form 2-hydroxy-6-oxo-6-methylhexa-2,4-dienoate (6-methyl-HOHD). In the fourth reaction, 6-methyl-HOHD is hydrolyzed to acetic acid and 2-hydroxypenta-2,4-dienoate by 6-methyl-HOHD hydrolase (TodF). Next, 2-hydroxypenta-2,4-dienoate is transformed in the last three reactions of the pathway by the enzymes 2-hydroxypenta-2,4-dienoate hydratase (TodG), 4-hydroxy-2-oxovalerate aldolase (TodH), and acylating aldehyde dehydrogenase (TodI), to produce pyruvate and acetyl-CoA.

The toluene dioxygenase pathway in *Pp*F1 is also capable of degrading trichloroethylene (TCE). The bacterium does not use TCE as an energy source, but the compound is fortuitously degraded when the organism utilizes toluene as a carbon and energy source. This phenomenon offers an advantage for monitoring microbial degradation of TCE, and a bioluminescence reporter gene system has been developed in *Pseudomonas putida* for this purpose. A linear relationship between the total amount of

toluene degraded and total bioluminescence has been reported (Cheng, 1998; Robinson, et al., 1998; Applegate, et al., 1997).

## 1.4. Fermentation with Co-solvents and Surfactants

Co-solvent use in fermentation increases the surface area between the aqueous liquid phase and the microbial substrate that is often present in a gas or solid phase. The substrate dissolves into the co-solvent, which is evenly dispersed in the aqueous phase. Co-solvents are often used in large quantities and exist as a separate phase in the fermentation. Surfactants are amphipatic (part hydrophilic and part hydrophobic) molecules that partition preferentially at the interface between fluid phases such as organic-aqueous or gas-aqueous interfaces. Their structural properties allow them to reduce surface and interfacial tension, which facilitate creation of new surface areas (e.g., bubbles or drops) or allow the aqueous phase to penetrate pores more efficiently. Surfactants are typically used in low concentrations and are usually soluble in water.

The best known application of two-liquid-phase bioreactors is the addition of a co-solvent to enhance the conversion of a poorly water-soluble substrate by increasing its availability to the cells in the aqueous phase (Figure 1.6). Here the substrate is almost insoluble in water and the solvent phase acts as a reservoir for the substrate and provides a larger interfacial area for substrate transfer. When compared to biofilters in general operating terms, Daugulis (2001) presented three main advantages for using co-solvents in two-liquid-phase bioreactors. First, two-liquid-phase bioreactors require less time to reach the desired biomass level at start up and in case of system failure. Second, biofilters are generally restricted to treatment of low gas concentrations of VOCs, less

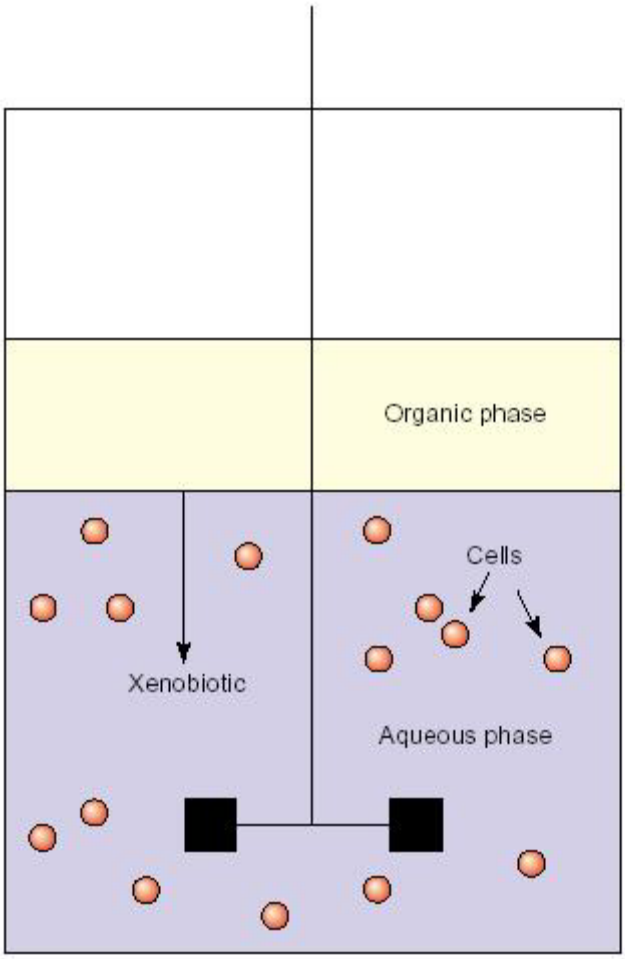



**Figure 1.6.** Schematic diagram of a two-phase-liquid bioreactor (Adapted from Daugulis, 2001)

than 5 mg/L. Many VOCs are hydrophobic and only partially dissolve during the contact time for them to flow through the biofilter. Also, if the incoming VOC levels fluctuate, the co-solvent phase in the liquid bioreactor acts as a "buffer" protecting the microorganisms from potential inhibitory substrate levels. Third, it is difficult to maintain a consistent performance in biofilters due to overgrowth, dehumidification, or deactivation; it can also be a challenge to maintain uniform temperatures and pH. Growth distribution of microorganisms in the filter bed is often not homogeneous and excess growth in certain areas can cause pressure increases and clogging. Two-liquid-phase bioreactors do not have these limitations, making them more suitable for most applications.

Three microbial uptake mechanisms for poorly water-soluble substrates in co-solvent systems are generally considered (Déziel, et al., 1999). In the first mechanism, only the substrate dissolved in the aqueous phase is available and the degradation rate depends on the mass-transfer rate from the organic to the aqueous phase. In the second mechanism, the cells obtain their substrate by contact of their membrane and the co-solvent (interface uptake). In a study conducted using a biphasic aqueous-silicone oil system for the degradation of 2,4,6-trichlorophenol, Ascon-Cabrera and Lebeault (1995) reported a high concentration of viable cells at the liquid-liquid interface in the reactor, suggesting interface uptake. Finally, in the third mechanism, cells produce surfactants causing the formation of small droplets, or micelles, which are directly assimilated. This last mechanism will be discussed later when biosurfactants are reviewed.

The following properties should be considered for selection of a co-solvent: biocompatibility, solubility for the substrate, immiscibility, and non-biodegradability (Déziel, et al., 1999). The Ostwald coefficient is one of the parameters that can be used to measure gas solubility of a substrate in a co-solvent (Battino, 1984; Wilhelm, et al., 1977). The Ostwald coefficient $L_v^0$ is defined as (Serra, et al., 1998):

$$L_v^0 = (V_g/V_l^0)_{\text{equil}} \tag{5}$$

where $V_g$ is the volume of the dissolved gas and $V_l^0$ is the total volume of the pure liquid after the equilibrium is reached, at 101.3 kPa, or 1 atm, total pressure. In the case of synthesis gas fermentations, the Ostwald coefficient for hydrogen and carbon monoxide illustrate that they are more soluble in organic co-solvents than water (see Table 1.1).

The logarithm of the partition coefficient of an organic co-solvent in a standard octanol-water two-phase system (log $P_{OW}$) is a useful empirically calculated parameter used to determine the best co-solvent to use in a particular bioconversion (Inoue and Horikoshi, 1991; Laane, et al., 1987). At ambient temperature and pressure, water and octanol are partially miscible, forming an octanol-rich phase (72.5 mol% 1-octanol), denoted as OR here, and a water-rich phase (99.99 mol% water), indicated by the superscript WR. The octanol-water partition coefficient of a solute $i$ (log $P_{OW,i}$) is defined as the equilibrium ratio of concentrations of this solute between the two liquid phases when a very small amount is added to an equilibrated water and 1-octanol mixture (Equation 6),

$$\log P_{OW,i} = \log[C_i^{OR}/C_i^{WR}]. \tag{6}$$

**Table 1.1.** Ostwald coefficient values for selected gases and solvents at 298.15K and 1 atm (Young, 1981; Cargill, 1990; IEEE, 1991 for silicone oil values)

| Co-solvent | Gas | | |
|---|---|---|---|
| | **Hydrogen** | **Carbon Monoxide** | **Oxygen** |
| Water | 0.02 | 0.02 | 0.03 |
| Ethanol | 0.08 | 0.19 | 0.21 |
| 1-Heptanol | 0.06 | 0.12 | 0.19 |
| 1-Octanol | 0.05 | 0.12 | 0.18 |
| 1-Decanol | 0.05 | 0.11 | 0.12 |
| 1-Dodecanol | 0.04 | 0.10 | 0.12 |
| Hexane | 0.12 | 0.33 | 0.37 |
| Heptane | 0.11 | 0.28 | 0.32 |
| Octane | 0.10 | 0.23 | 0.28 |
| Decane | 0.08 | 0.19 | 0.23 |
| Dodecane | 0.08 | 0.17 | 0.20 |
| Hexadecane | 0.06 | 0.12 | 0.15 |
| Silicone Oil | 0.06 | 0.10 | 0.18 |

This coefficient is a measure of the hydrophobicity of the solute. A hydrophobic chemical preferentially partitions into the octanol-rich phase, resulting in a large value of log $P_{OW}$, while a hydrophilic chemical will have a small log $P_{OW}$ value (Lin and Sandler, 1999).

The partition coefficients for some common organic co-solvents are listed in Table 1.2. The relationship between log $P_{OW}$ and biocompatibility is based on the assumption that the octanol-water system provides a sufficient description of hydrophobic and transport interactions when a co-solvent is introduced into a biological system (Aono and Inoue, 1998). Co-solvents with a log $P_{OW}$ value between -2.5 and 0 are water miscible. Co-solvents with a log $P_{OW}$ value above 0 are water immiscible. In general, organic co-solvents with a log $P_{OW}$ value between 1 and 5 are toxic to microorganisms, but co-solvents with a log $P_{OW}$ value over 5 are generally not toxic to microorganisms (Laane and Tramper, 1990; Isken, 1998).

**Table 1.2.** Log $P_{OW}$ values for selected solvents (Laane, et al., 1987; Watanabe, et al., 1984 for silicone oil value)

| **Co-solvent** | **log $P_{OW}$** | **Co-solvent** | **log $P_{OW}$** |
|---|---|---|---|
| Ethanol | -0.24 | Heptane | 4.0 |
| Acetic Acid | -0.23 | 1-Decanol | 4.0 |
| 1-Heptanol | 2.4 | Dodecanol | 5.0 |
| Toluene | 2.5 | Decane | 5.6 |
| 1-Octanol | 2.9 | Dodecane | 6.6 |
| Silicone Oil | 2.9 | Oleyl Alcohol | 7.5 |
| Hexane | 3.5 | Hexadecane | 8.8 |

In general, Gram-negative bacteria appear to have a higher solvent tolerance than Gram-positive bacteria, and species within a genus sometimes show a range of tolerances (Harrop, et al., 1989; Rajagopal, 1996; Vermue, et al., 1993; Weber and deBont, 1996). It has been suggested that the difference in solvent tolerance is caused by the presence of the outer membrane in Gram-negative bacteria containing lipopolysaccharides, which protects the cells against hydrophobic compounds (Ramos, et al., 2002). The most resistant Gram-negative species have been reported in the genus *Pseudomonas* (Cruden, et al., 1992; Harbron, et al, 1986; Inoue and Horikoshi, 1989). One of the key processes in the adaptation of some *Pseudomonas* strains enabling them to tolerate organic solvents appears to be the isomerization of *cis*- into *trans*-unsaturated fatty acids (Weber, et al., 1994; Huertas, et al., 2000; von Wallbrunn, et al., 2003).

Structural properties of surfactants allow them to reduce surface and interfacial tension. Biosurfactants are surfactants synthesized by microorganisms (Desai and Banat, 1997), and most known biosurfactants are glycolipids; part carbohydrate and part lipid. The best studied glycolipids are rhamnolipids. Rhamnolipids consist of one or two molecules of rhamnose linked to one or two molecules of β-hydroxydecanoic acid. Strains of *Pseudomonas aeruginosa* have been found to produce rhamnolipid biosurfactants, which enhance hexadecane degradation (Zhang and Miller, 1995; Noordman, et al., 2002). L-Rhamnosyl-L-rhamnosyl-b-hydroxydecanoyl-b-hydroxy-decanoate (dirhamnolipid) and L-rhamnosyl-b-hydroxydecanoyl-b-hydroxydecanoate (monorhamnolipid), are the principal glycolipids produced by *P. aeruginosa.* The release

of lipopolysaccharides (LPSs) induced by these agents has been considered to be the mechanism for enhanced cell surface hydrophobicity (Al-Tahhan, et al., 2000).

Biosurfactants can act in two different ways. First, they can solubilize hydrophobic compounds within micelle structures. This will efficiently increase the aqueous solubility of the insoluble substrate from the co-solvent and its availability for uptake by the cells. Second, they can cause the cell surface to become more hydrophobic, thereby increasing the direct physical contact between the cells and the insoluble substrate (Al-Tahhan, et al., 2000).

# Chapter 2

## 2. Synthesis Gas Fermentation – *Clostridium ljungdahlii*

### 2.1. Introduction

As previously mentioned, *Clostridium ljungdahlii* is an anaerobic Gram positive bacterium able to produce ethanol and acetic acid from the carbon monoxide and hydrogen in synthesis gas. At the time this organism was isolated by researchers at the University of Arkansas, synthesis gas was primarily produced from gasified coal (Gaddy and Clausen, 1992) and for the last few decades, coal utilization research has been a high priority to the U.S. Department of Energy due to its potential as a national energy source. Thus, much of the work with *C. ljungdahlii* targeted coal synthesis gas as substrate. At the present time, synthesis gas has become an even more attractive feedstock for fuels and chemicals since it can be produced from many more sources, including gasified biomass. Recently, production of synthesis gas has been reported from the steam gasification of biomass-derived oil (Panigrahi, et al, 2003). This fuel is renewable and is free of sulfur dioxide emissions and less nitrogen oxide emissions when compared to fossil fuels.

This part of the thesis consists of experiments conducted in serum bottles to study the effect of co-solvents in synthesis gas fermentations by *C. ljungdahlii*. The goal was to enhance the conversion of synthesis gas to improve the production of ethanol and acetic acid. The following issues were studied: transfer of synthesis gas components (carbon monoxide and hydrogen) from the head space, biocompatibility of the co-solvent in terms of growth in dry cell weight, uptake of synthesis gas components (carbon

monoxide and hydrogen) by the biocatalyst, carbon dioxide production, fermentation products, and ethanol extraction by co-solvents.

## 2.2. Materials and Methods

### 2.2.1. Microbial Culture and Media

*Clostridium ljungdahlii* strain 55383 from the American Type Culture Collection (Manassas, VA) and medium recipe was kindly provided by Dr. Marshall D. Bredwell from Michigan State University. Cells were grown anaerobically in serum bottles in a horizontal position in an orbital shaker at 160 RPM and 37$^{o}$C in a phosphate-buffered basal (PBB) sulfide-reduced medium with 0.1% (w/v) yeast extract (DIFCO) on synthesis gas (ordered as 36% $H_2$, 47% CO, 10% $CO_2$, 5% $CH_4$ and balance in $N_2$ from Air Liquide, LaPorte, TX). Resazurin (DIFCO; 0.1%, w/v) was used as oxygen indicator in maintaining stock cultures. The medium was distributed into 125 ml serum bottles (Wheaton, Millville, NJ) at 100 ml/bottle, and sealed with butyl rubber stoppers and aluminum crimp seals (Wheaton, Millville, NJ). After sterilization for 20 minutes at 121°C and 15 psig (while medium was still hot), the reducing agent [2.5% (w/v) $Na_2S{\cdot}9H_2O$] was added to each serum bottle. For complete medium composition, see Tables 2.1 to 2.4. Before inoculation, the headspace of each bottle was sparged with nitrogen filter-sterilized through a 0.2-µm-pore-size Acrodisc filter (Pall, Ann Arbor, MI) for two minutes. After inoculation, the headspace of each bottle was sparged with filter-sterilized synthesis gas for one minute. A 5% (v/v) inoculum from a 72-hour-seed-culture grown in, and using synthesis gas, was used to start experiments.

**Table 2.1.** Phosphate buffered basal medium (PBB)

| Ingredient | Concentration (per liter) |
|---|---|
| NaCl – EM Science | 0.8 g |
| $MgSO_4 \cdot 7H_2O$ – EM Science | 0.2 g |
| $CaCl_2 \cdot 2H_2O$ – JT Baker | 0.1 g |
| $NH_4Cl$ – Mallinckrodt | 1.0 g |
| KCl – JT Baker | 0.1 g |
| Yeast Extract – DIFCO | 1.0 g |
| Wolfe's Vitamins – see Table 2.2 | 1.0 ml |
| Trace Elements – see Table 2.3 | 1.0 ml |
| 0.1% Resazurin - DIFCO | 2.0 ml |
| Phosphate buffer – see Table 2.4 | 25 ml |
| 2.5% $Na_2S \cdot 9H_2O$ – JT Baker | 25 ml |

**Table 2.2.** Composition of Wolfe's vitamins solution

| Ingredient | Concentration (mg per liter) |
|---|---|
| Biotin – Sigma | 20 |
| Folic acid – Sigma | 20 |
| Pyridoxine HCl – Sigma | 100 |
| Thiamine HCl – Sigma | 50 |
| Riboflavin – Sigma | 50 |
| Nicotinic acid – Sigma | 50 |
| Calcium D-(+)-pantothenate – Sigma | 50 |
| Cyanocobalamine – Sigma | 1 |
| *p*-aminobenzoic acid -Sigma | 50 |
| Thioctic acid - Sigma | 50 |

**Table 2.3.** Composition of trace elements solution*

| Ingredient | Concentration (per liter) |
|---|---|
| Nitrilotriacetic acid – JT Baker | 20 g |
| $MnSO_4 \cdot H_2O$ – JT Baker | 10 g |
| $Fe(SO_4)_2(NH_4)_2 \cdot 6H_2O$ – Sigma | 8 g |
| $CoCl_2 \cdot 6H_2O$ – Matheson, Coleman + Bell | 2 g |
| $ZnSO_4 \cdot 7H_2O$ – JT Baker | 2 mg |
| $CuCl_2 \cdot 2H_2O$ – Fisher Scientific | 200 mg |
| $NiCl_2 \cdot 6H_2O$ – EM Science | 200 mg |
| $Na_2MoO_4 \cdot 2H_2O$ – Fisher Scientific | 200 mg |
| $Na_2SeO_4$ – JT Baker | 200 mg |
| $Na_2WO_4$ – EM Science | 200 mg |

*Add the nitrilotriacetic to water and adjust the pH to 6.0 with KOH and then add the remainder of the ingredients.

**Table 2.4.** Composition of phosphate buffer

| Ingredient | Concentration (g per liter) |
|---|---|
| $KH_2PO_4$ – EM Science | 369.6 |
| $K_2HPO_4$ – EM Science | 11.4 |

### 2.2.2. Experimental Techniques

To study the effect of co-solvents on the 'absorption' of carbon monoxide and hydrogen in the absence of bacteria, 40 ml of the following co-solvents were used in 60 ml serum bottles: oleyl alcohol (Aldrich), hexadecane (Aldrich), heptane (Mallinckrodt), decane (Eastman), dodecane (Matheson, Coleman and Bell), and 2,6-dimethyl-4-heptanol (Aldrich). Bottles were sterilized for 20 minutes at 121°C and 15 psig. The headspace of each bottle was sparged with nitrogen filter-sterilized through a 0.2-µm-pore-size Acrodisc filter (Pall, Ann Arbor, MI) for two minutes. Then, 30 ml of nitrogen were removed from the headspace and immediately 30 ml of filter-sterilized synthesis gas were added. Bottles were incubated in a horizontal position in an orbital shaker at 160 RPM and 37°C. The decrease of gaseous substrates was observed for up to 19 hours.

Since ethanol was produced by the biocatalyst, it was desired to track its fate in the two liquid phases without microorganisms. In these studies, a total concentration (w/v) of ethanol (2 g/L; AAPER Alcohol and Chemical Company) was added to the aqueous phase (75%, v/v) of a serum bottle previously sterilized for 20 minutes at 121°C and 15 psig containing 25% (v/v) of hexadecane (Aldrich). The bottles were placed in a horizontal position in an orbital shaker at 160 RPM and 37°C. Samples were taken at intervals from both the organic phase and the aqueous phase of each bottle. The ethanol concentration in each phase was measured by gas chromatography.

Studies were also conducted to assess the biocompatibility of different co-solvents with *C. ljungdahlii*. Cell growth was assessed by measuring the increase in optical

density at 660 nm in bottles containing solvents, which was compared with those that did not contain solvents.

### 2.2.3. Analytical Techniques

To determine cell concentration, 1-ml liquid samples were centrifuged for 10 minutes at 14,000 RPM and the pellet resuspended in 1 ml phosphate buffer (Table 2.4). This was done to remove any residual amount of co-solvent from the aqueous phase before performing the measurements. Then the optical density at 660 nm was measured in a Bausch and Lomb (Milton Roy Company, Rochester, NY) Spectronic 21 spectrophotometer and the absorbance was compared to a standard calibration curve, which correlated dry cell weight (DCW) and optical density (OD). For this purpose, the following equation was used:

$$DCW\ (g/L) = (0.742 \times OD_{660} + 0.013)\ . \qquad (7)$$

Equation 7 was developed by diluting a concentrated cell broth and measuring the optical density of each dilution. At the same time, a portion of the concentrated cell broth was filtered on a pre-weighed filtered paper (0.45-μm-pore-size, Whatman, Clifton, NY) and the filter was dried at 100°C. This method allowed the dry cell weight to be calculated for each dilution.

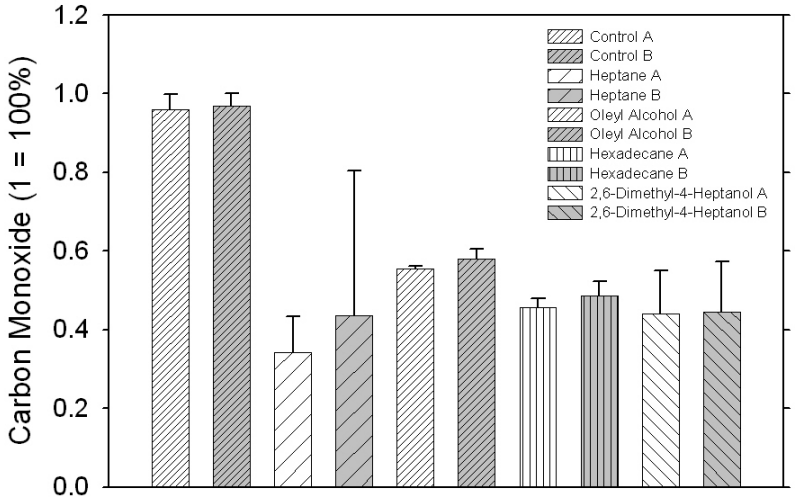
Carbon dioxide in the bottle headspace was analyzed using a gas chromatograph (Hewlett Packard HP 5890 Series II) equipped with a Teflon column (0.76 m × 3.2 mm) packed with Super Q (80/100 mesh) (Alltech, Waukeegen, WI). Temperatures of the column, injection port, and thermal conductivity detector were 70$^{o}$C, 125$^{o}$C, and 125$^{o}$C respectively. The carrier gas was helium at 25 ml/min. Carbon monoxide, hydrogen,

methane, and nitrogen were measured using a gas chromatograph (Hewlett Packard HP 5890 Series II) equipped with an HP PLOT molecular sieve 5A capillary column (30 m × 0.32 mm) with a 12-μm film thickness. Temperatures on the column, injection port, and thermal conductivity detector were 55°C, 100°C, and 240°C respectively.

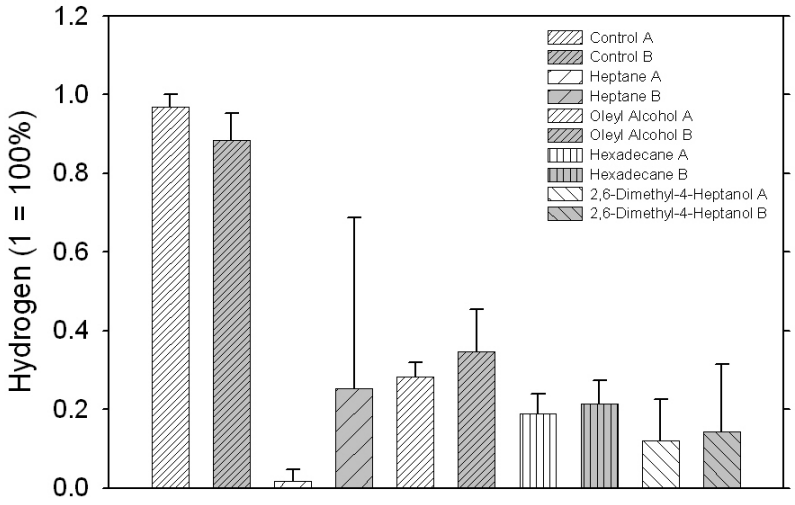
Liquid samples were acidified with 0.1 N phosphoric acid (JT Baker; 0.1 ml per 1 ml of sample) and centrifuged for 10 minutes at 14,000 RPM before chromatographic analysis for fermentation products. The acidified liquid samples were analyzed by gas chromatography using a HP 5890 Series II with an HP WAT (crosslinked PEG) capillary column (30 m × 0.53 mm) with a 1.0-μm film thickness. The column temperature program was initially 70°C followed by ramping to 200°C at 25°C/min with a 1.2 min hold then followed by ramping to 225°C at 25°C/min with a 3.0 min hold. The injection port temperature was 245°C while the flame ionization detector was 265°C. The following volatile fatty acids (VFAs) were measured: propionic acid, isobutyric acid, butyric acid, isovaleric acid, valeric acid, and caproic acid.

## 2.3. Results and Discussion

Values of the Ostwald coefficient (see Table 1.1) and log $P_{OW}$ (see Table 1.2) were used to select a number of potential organic co-solvents for synthesis gas fermentations in serum bottles. Based on these values (it is desired for both of these values to be high), the solvents selected for testing were heptane, decane, dodecane, 2,6-dimethyl-4-heptanol, hexadecane, and oleyl alcohol. Control experiments were conducted to study the transfer of the main components of synthesis gas, carbon monoxide and hydrogen, in serum bottles with sterile co-solvents (see Figure 2.1). These




(a)



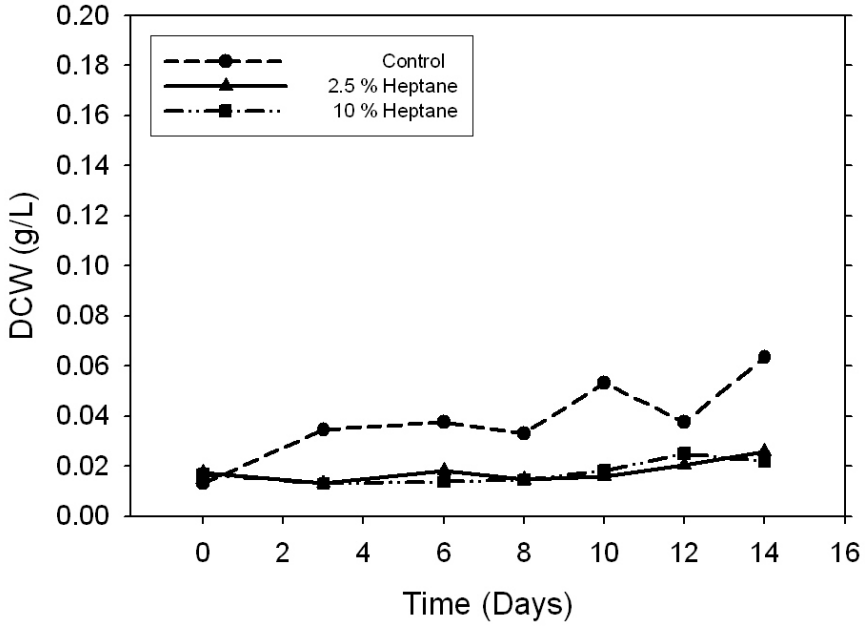

(b)

**Figure 2.1.** Gas transfer into solvents in sterile serum bottles. Fraction of gas remaining in headspace after 5 hours of incubation. Error bars reflect one standard deviation among triplicate experiments. A gas level of 1 (100%) indicates the initial value at the beginning of the experiment. (a) Carbon monoxide transfer. (b) Hydrogen transfer.

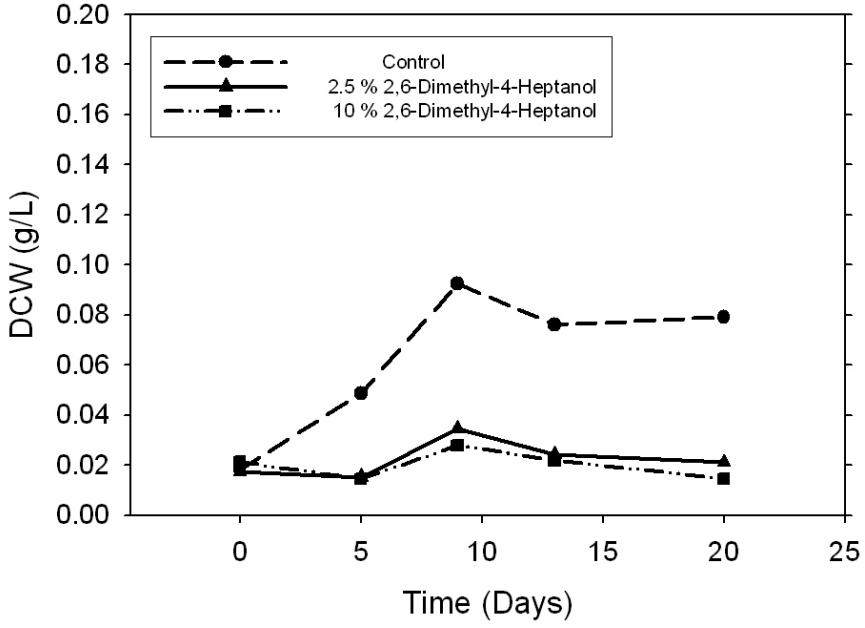
experiments were performed twice (denoted “A” and “B” in the figure legends); each of these experiments was performed in triplicate. As is noted in Figure 2.1, the levels of carbon monoxide and hydrogen in bottles containing any of the tested solvents were significantly lower than in the control bottles, which contained no solvent. From these results, it was concluded that heptane was slightly better than hexadecane, 2-6-dimethyl-4-heptanol, and oleyl alcohol at transferring carbon monoxide and hydrogen from the gas phase.

Although it appeared that the removal of carbon monoxide and hydrogen was enhanced by the presence of any tested co-solvent, biocompatibility studies with heptane, 2,6-dimethyl-4-heptanol, decane, and dodecane showed that these co-solvents were inhibitory to the growth of the biocatalyst [see Figures 2.2(a) to 2.2(d) respectively]. There was a very small increase in dry cell weight in the serum bottles containing these co-solvents when compared to the control bottles. On the other hand, biocompatibility studies with hexadecane as a co-solvent showed promising results for growth of the biocatalyst [see Figure 2.2(e)].

A final screening study, measuring the transfer of carbon monoxide in the gas phase with respect to time in the presence of bacteria, was performed over three days in the following co-solvents: 2,6-dimethyl-4-heptanol, decane, dodecane, hexadecane, and oleyl alcohol (see Figure 2.3). The three-day experiment clearly showed that the biocatalyst consumed carbon monoxide faster in the presence of hexadecane as a co-solvent. The other solvents did not show better consumption than the control experiment with no co-solvent. Oleyl alcohol showed slightly less carbon monoxide removal than

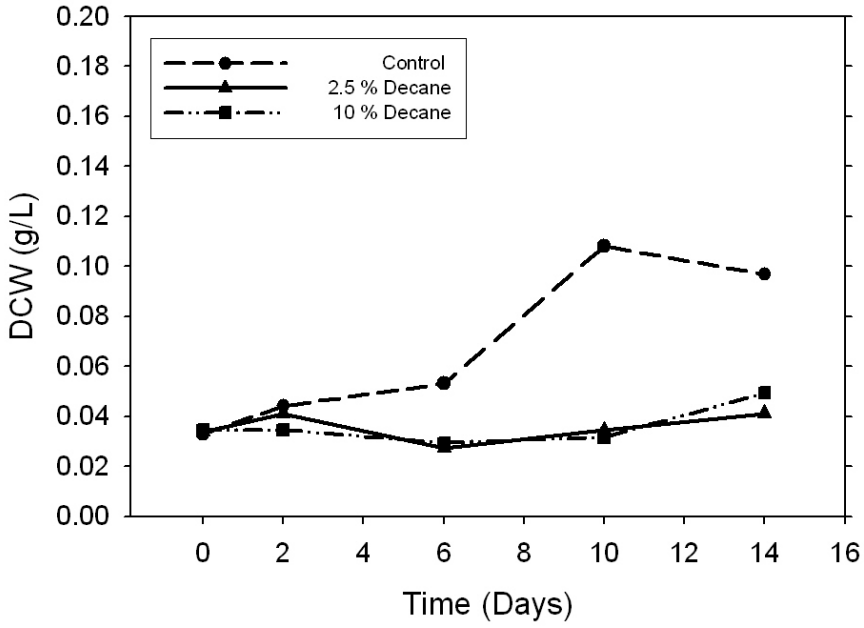



(a)

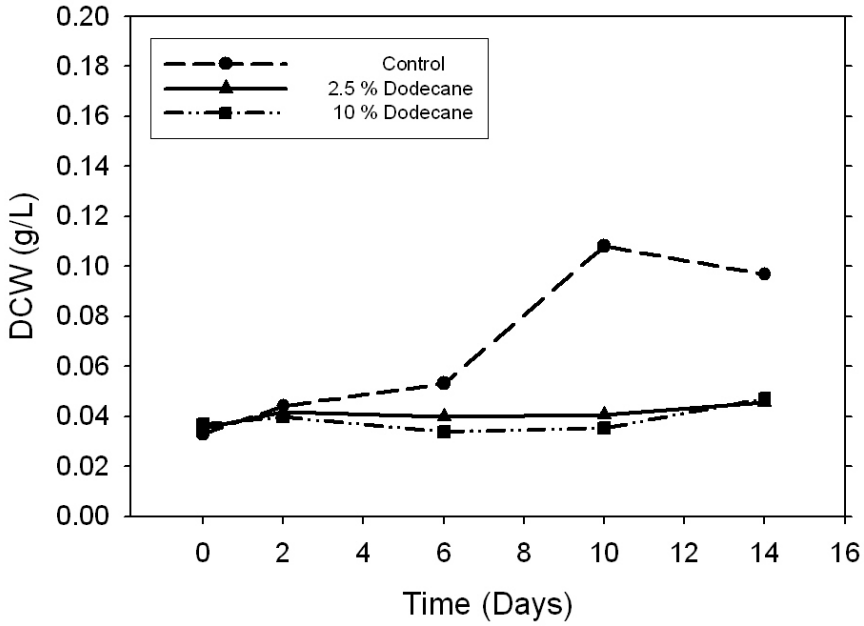



(b)

**Figure 2.2.** Growth of *C. ljungdahlii* on syngas in the presence of co-solvent. (a) heptane. (b) 2,6-dimethyl-4-heptanol.

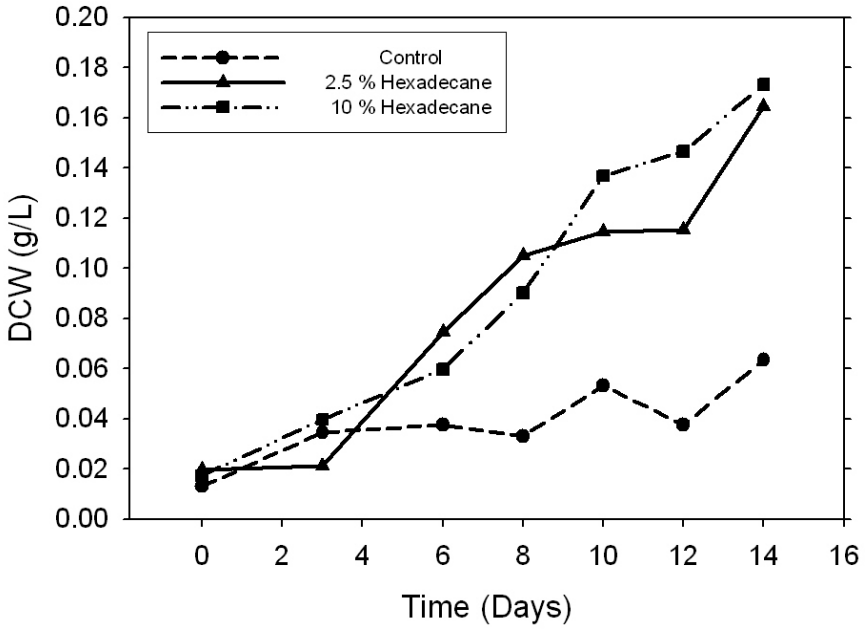



(c)




(d)

**Figure 2.2.** Continued. (c) decane. (d) dodecane.




(e)

**Figure 2.2**. Continued. (e) Hexadecane.

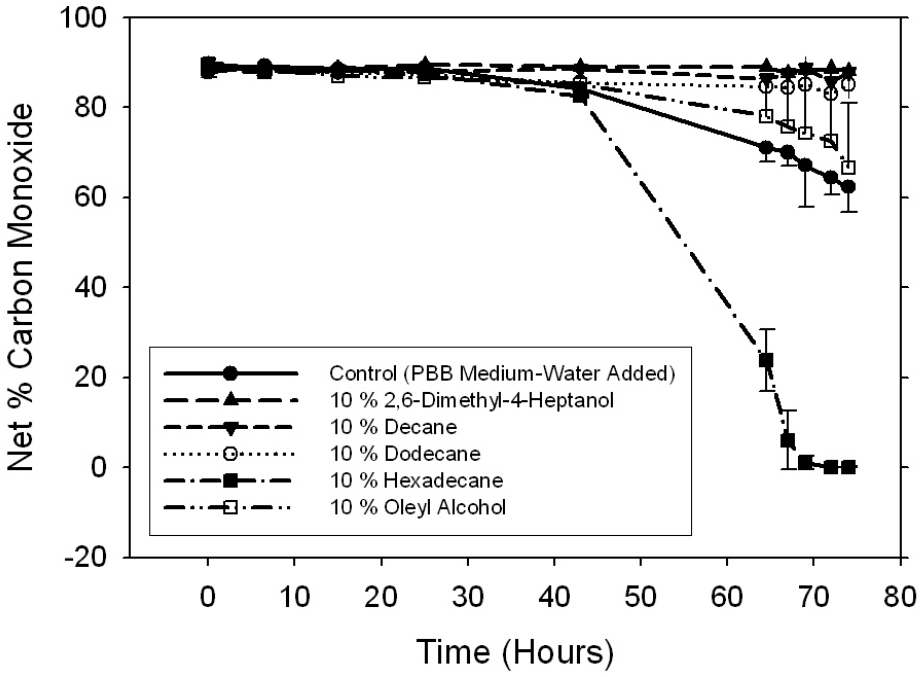



**Figure 2.3.** Consumption of carbon monoxide by *C. ljungdahlii* over several days of incubation. Fraction of original carbon monoxide remaining in headspace. Error bars reflecting one standard deviation are shown for the control (upper half), oleyl alcohol (lower half), and hexadecane (both) from experiments performed in duplicate.

the control, but within the statistical error. In conjunction with these experiments, a few control experiments with hexadecane in the presence or absence of synthesis gas and/or biocatalyst were conducted. These control experiments were designed to investigate the possibility of hexadecane serving as a carbon source (and producing carbon dioxide in the process). As expected from the previous experiments, cells growing in the presence of hexadecane and synthesis gas showed the highest concentration of carbon dioxide in the headspace (see Figure 2.4). After a few hours, it generally remained constant throughout the experiment. The apparent jump in carbon dioxide level between 0 and 6.5 hours may be attributed to establishing equilibrium between the gas and liquid phases following inoculation. It should be noted that the inoculum may contain dissolved carbon

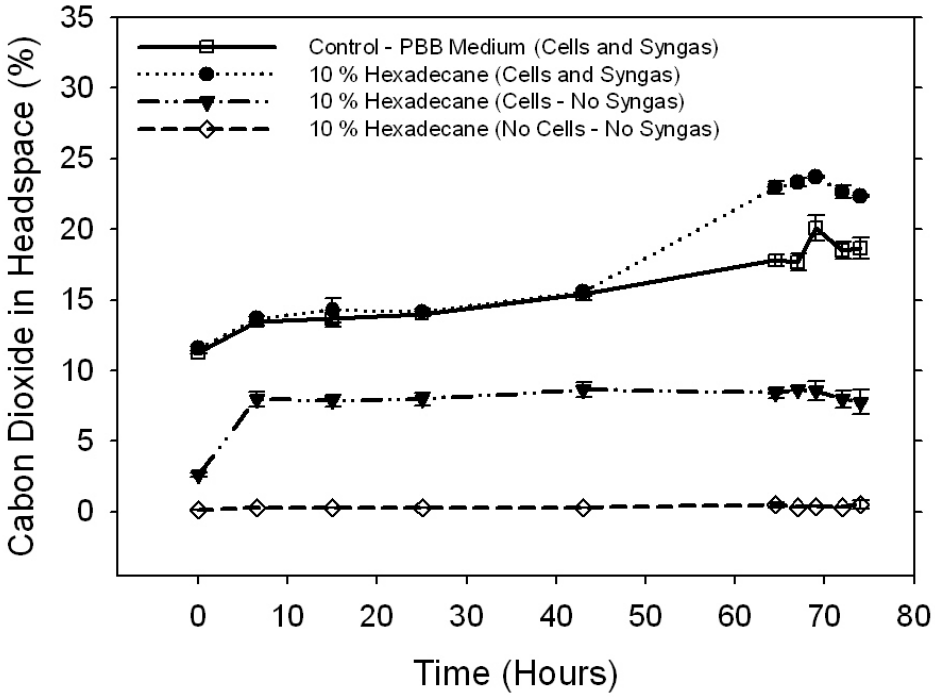


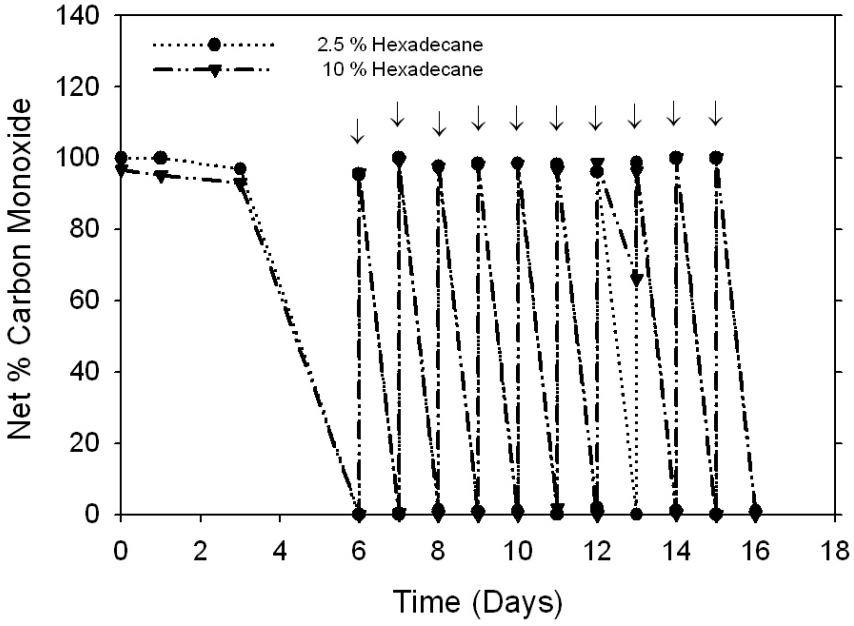

**Figure 2.4.** Production of carbon dioxide by *C. ljungdahlii*. Fraction $CO_2$ of total headspace gases is shown. Error bars reflecting one standard deviation are shown from

experiments performed in duplicate. Changes in pressure or other gas components are not reported.

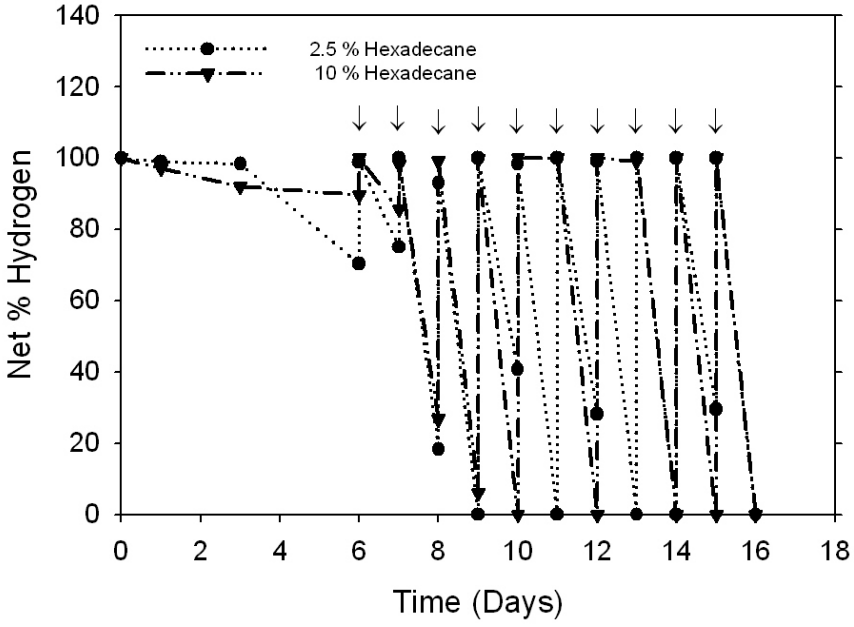
dioxide from the seed culture. Overall, the data suggested that the biocatalyst was not using hexadecane as a carbon source. The amount of carbon dioxide produced in serum bottles without syngas and cells was almost zero, as expected. To study the effect of solvent quantity, experiments were conducted with 2.5 and 10% (v/v) of co-solvents. In these experiments, the consumption of carbon monoxide by *C. ljungdahlii* was monitored for over two weeks in the presence of hexadecane, heptane, 2,6-dimethyl-4-heptanol, decane, and dodecane. In these experiments, the headspace of the bottles was re-sparged periodically with fresh synthesis gas, when most of the gas had been consumed.

The results from these experiments did not yield much additional information. The consumption of carbon monoxide and hydrogen in the presence of hexadecane is shown in Figures 2.5(a) and 2.5(b), respectively. After an initial lag phase of 3 to 5 days, consumption of gas was noted regardless of the level of hexadecane present. After that point, gas consumption was rapid and the headspace was re-sparged with new synthesis gas each day over a period of 2 weeks. Overall, there was no significant difference between gas consumption in bottles with 2.5 and 10% hexadecane. This suggests that a lesser quantity of co-solvent may be used to achieve the desired results.

Results of similar experiments with different co-solvents are shown in Figures 2.6 to 2.8. However, due to the lack of syngas consumption these bottles and their controls were not re-sparged as frequently. The results from studies with heptane (Figure 2.6), 2,6-dimethyl-4-heptanol (Figure 2.7), decane (Figure 2.8), and dodecane (Figure 2.9) did not show any benefits to co-solvent addition at the two levels investigated.

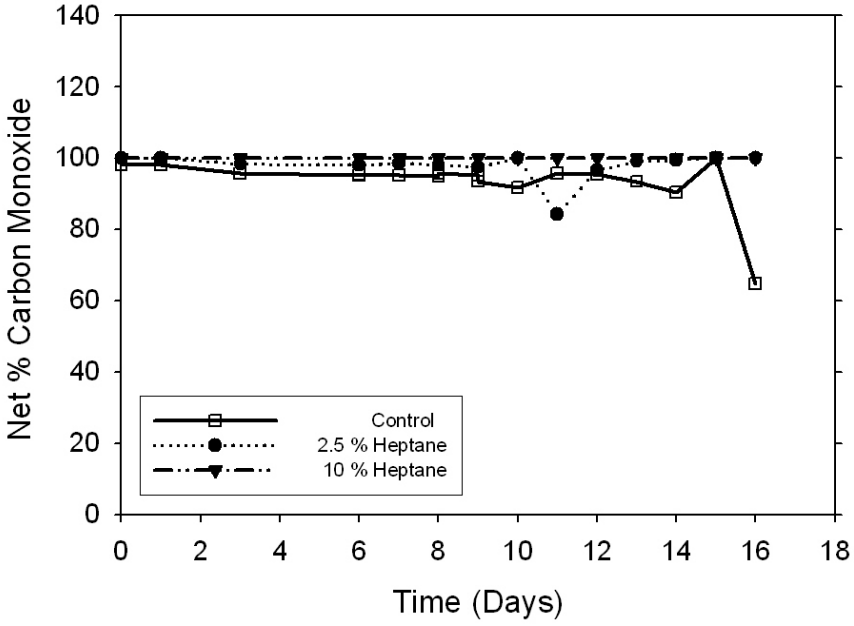



(a)

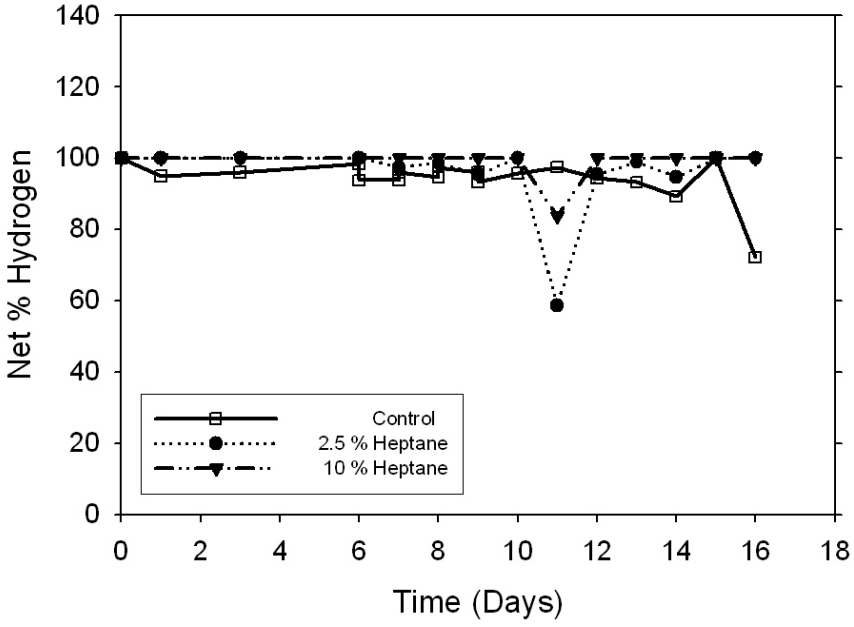



(b)

**Figure 2.5.** Gas uptake in serum bottles with hexadecane. Arrows indicate re-sparging with fresh syngas. (a) Carbon monoxide uptake. (b) Hydrogen uptake.

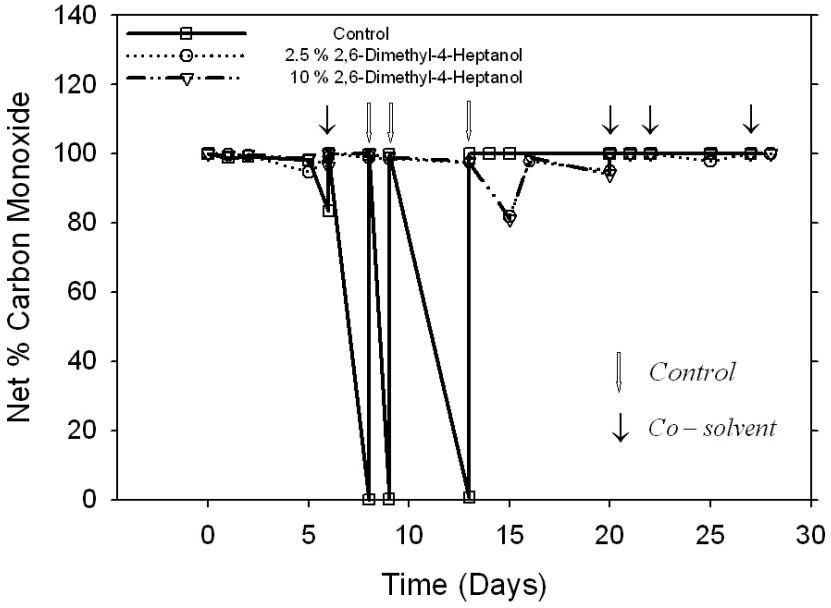



(a)

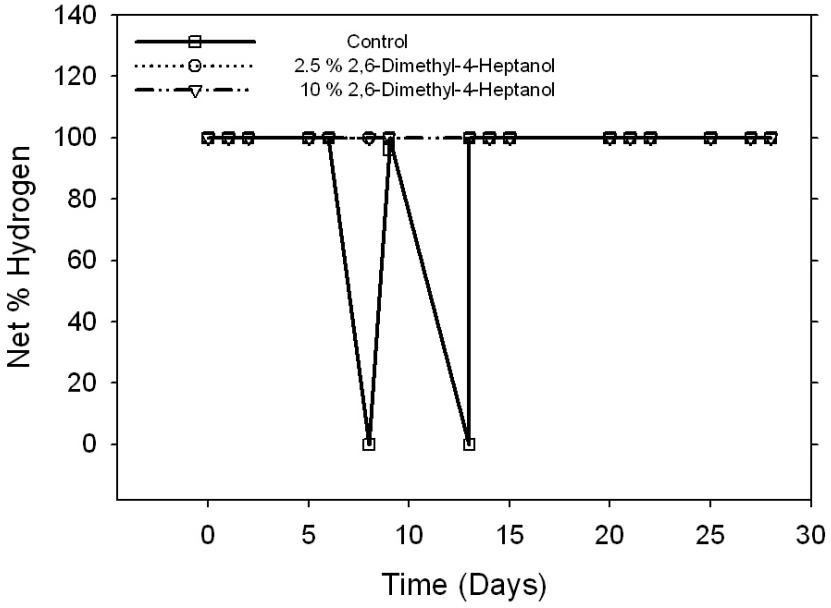



(b)

**Figure 2.6.** Gas uptake in serum bottles with heptane. (The apparent decrease at 11 h probably reflects an analytical error.) (a) Carbon monoxide uptake. (b) Hydrogen uptake.

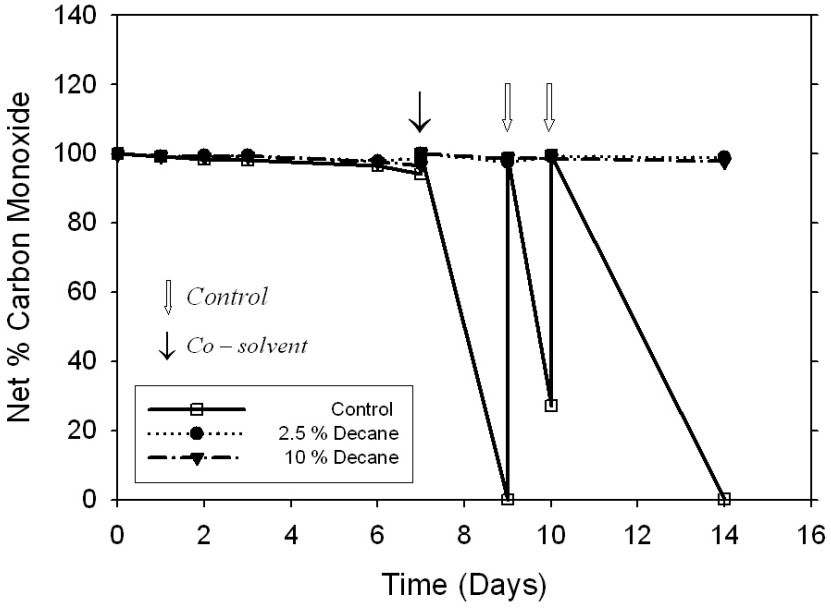



(a)

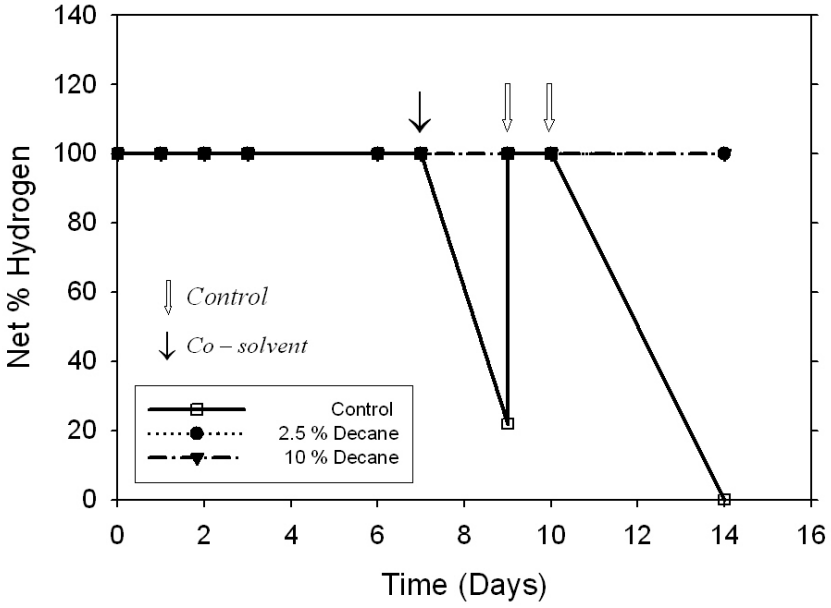



(b)

**Figure 2.7.** Gas uptake in serum bottles with 2,6-dimethyl-4-heptanol. Open and solid arrows indicate re-sparging with fresh syngas of the control and co-solvent bottles, respectively. (a) Carbon monoxide uptake. (b) Hydrogen uptake.

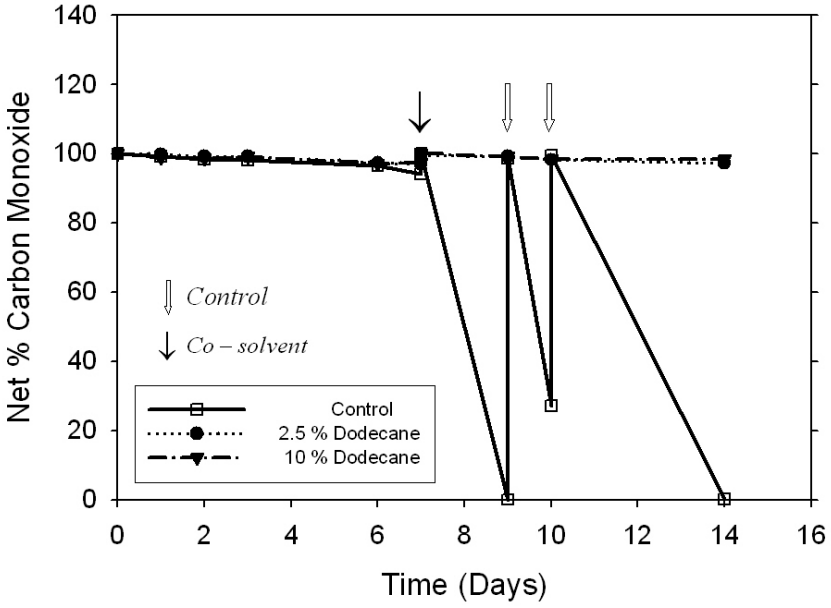



(a)

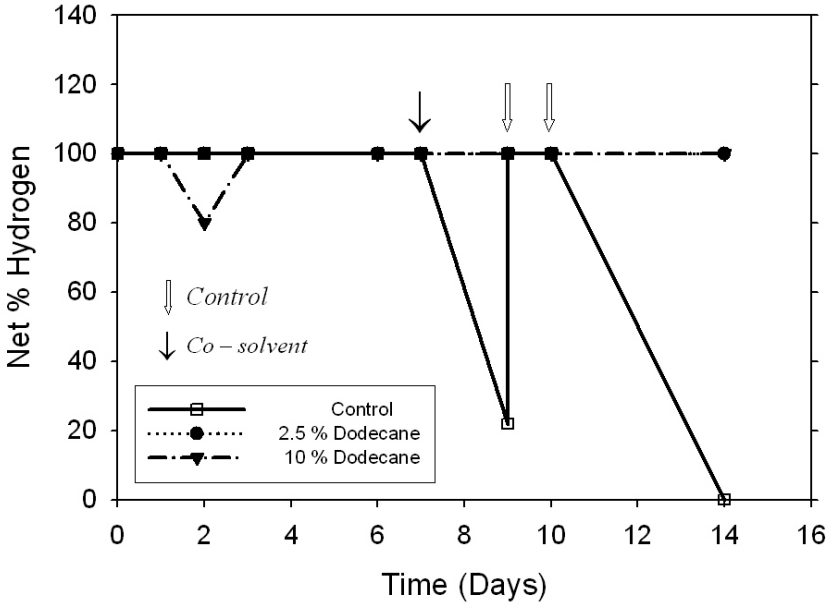



(b)

**Figure 2.8.** Gas uptake in serum bottles with decane. Open and solid arrows indicate re-sparging with fresh syngas of the control and co-solvent bottles, respectively. (a) Carbon monoxide uptake. (b) Hydrogen uptake.




(a)




(b)

**Figure 2.9.** Gas uptake in serum bottles with dodecane. Open and solid arrows indicate re-sparging with fresh syngas of the control and co-solvent bottles, respectively. (a) Carbon monoxide uptake. (b) Hydrogen uptake.

The production of total fermentation products (ethanol, acetic acid, butanol, propionic acid, isobutyric acid, butyric acid, isovaleric acid, valeric acid, and caproic acid) for the two best-performing co-solvents (hexadecane and heptane) were measured in the previously described experiments. The results showed that liquid products accumulated over time (Figure 2.10). The highest product concentrations were noted in the experiment with 2.5% hexadecane, where 1.4 g/L products were achieved.

It was assumed that ethanol would be the most likely product extracted by hexadecane and may be the reason lower product concentrations were noted in the experiment with 10% hexadecane compared with 2.5% hexadecane (Figure 2.10), even though similar gas consumptions were noted (see Figure 2.5). For that reason, an experiment was conducted to determine the ethanol extraction efficiency of hexadecane.

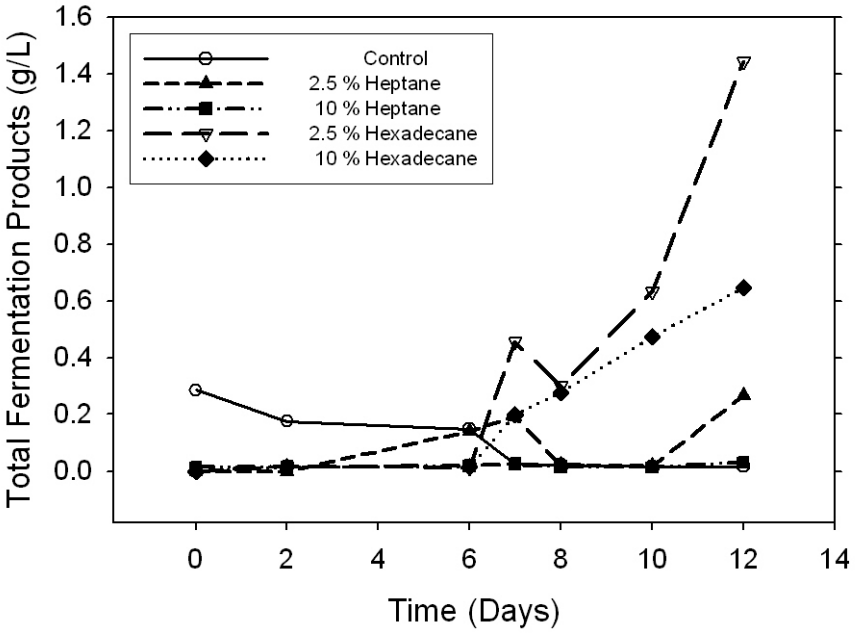



**Figure 2.10.** Total fermentation products in the aqueous phase with heptane and hexadecane as co-solvents

No change was observed in the ethanol concentration in the co-solvent for a period of 6 days (see Table 2.5). Synthesis gas was not added to bottles, but the bottles were inoculated with cells. No increase in optical density or fermentation products was observed. Based on these results, it was concluded that ethanol was not significantly extracted by hexadecane in the experiments.

## 2.4. Conclusions – The Use of Co-solvents in Synthesis Gas Fermentations

Several studies were performed in serum bottles with different co-solvents to study the effect of cell growth and gas consumption by *Clostridium ljungdahlii* in the presence of the co-solvents. Some of the co-solvents were inhibitory to growth. These included heptane, 2,6-dimethyl-4-heptanol, decane, and dodecane. Some of these same co-solvents (most notably heptane) appeared to have a positive effect on the consumption of the main components of synthesis gas; carbon monoxide and hydrogen. However, the only co-solvent that consistently out-performed the other co-solvents and control, both for cell growth, carbon monoxide consumption, and hydrogen consumption, was hexadecane. When two levels of hexadecane (2.5% and 10%, v/v) were studied, carbon

**Table 2.5.** Results from ethanol extraction by hexadecane

| Time (Days) | Ethanol (g per liter) | |
|---|---|---|
| | Aqueous Phase (Growth Medium and Cells) | Organic Phase (Hexadecane) |
| 0 | 2.615 | 0.006 |
| 1 | 2.752 | 0.006 |
| 2 | 3.466 | 0.005 |
| 3 | 3.673 | 0.005 |
| 6 | 2.699 | 0.004 |

monoxide and hydrogen consumption was almost identical in each case. However, higher fermentation products concentrations were observed with 2.5% hexadecane. Extraction experiments with growth medium, ethanol, and hexadecane showed that ethanol loss due to hexadecane extraction was not significant.

The studies with synthesis gas consumption by *C. ljungdahlii* were successful overall, showing that hexadecane have the potential to improve the rate of synthesis gas consumption. However, detailed experiments in controlled stirred batch systems are needed to elucidate the mechanisms of the observed improvements (for example, improvements in mass transfer). It is difficult to do this in smaller-scale experiments. Although these additional types of experiments were planned, new funding directions of the Fossil Fuel Energy Program of the U. S. Department of Energy did not allow the research with *C. ljungdahlii* to continue. Experiments in stirred tank systems with addition of a different co-solvent (silicone oil) are discussed in Chapter 3.

# Chapter 3

## 3. Toluene Fermentation – *Pseudomonas putida* F1

### 3.1. Introduction

The previous chapter was devoted to synthesis gas fermentations as a model system for the enhancement of the anaerobic conversion of gaseous substrates using organic co-solvents. As mentioned in the conclusions for Chapter 2, the funding directions changed. As a result, the research efforts in synthesis gas fermentations for the production of ethanol and acetic acid were discontinued. However, new funding directions included enhancement of the biological conversion of gaseous contaminants present in the atmosphere as a result of many industrial processes. After performing a literature search on co-solvent systems used in the bioconversions of volatile organic compounds, the aerobic conversion of toluene by *Pseudomonas putida* F1 was chosen as a new model system. Silicone oil was selected as a co-solvent to enhance the conversion of toluene gas by the biocatalyst.

Experiments were conducted in stirred batch reactors and in a continuous stirred tank reactor. Silicone oil was tested in stirred batch reactors in three different concentrations (v/v): 10%, 30% and 50%. Next, the effect of biosurfactants was tested in two different concentrations: 0.025% and 0.0025% (w/v). Finally, the effect of the increase in silicone oil from 15% to 35% was studied in continuous reactors. This chapter of the thesis will consist of, a published scientific article in a peer-reviewed journal, additional materials and methods, supplemental results and discussion, and conclusions.

## 3.2. Published Scientific Article in a Peer-Reviewed Journal

The article in the Appendix was published in *Applied Biochemistry and Biotechnology*, Volume 91-93, pages 195-204, 2001 (Figure 3.1). The article reported on many of the experiments conducted on the model system of gaseous toluene conversion in stirred reactors by the bacterium *Pseudomonas putida* F1.

## 3.3. Additional Materials and Methods

### 3.3.1. Microbial Culture and Media

*Pseudomonas putida* F1 strain 700007 was obtained from the American Type Culture Collection (Manassas, VA). Seed cultures were grown aerobically at 30$^{o}$C in a mineral salts medium (SM + $KNO_3$ medium, see Tables 3.1 and 3.2). The medium was distributed in 50 ml aliquots in 125 ml serum bottles (Wheaton, Millville, NJ), and sealed with butyl rubber stoppers and aluminum crimps (Wheaton, Millville, NJ). Toluene (HPLC grade, JT Baker) was added (20 μL) to each culture as a carbon source.

### 3.3.2. Reactor Configuration

Experiments were performed in 1-liter nominal volume, stirred batch reactors (SBRs) and in a continuous stirred tank reactor (CSTR) from VirTis (Gardiner, NY). Silicone oil (DC 200, ~50 mPa.s, polydimethylsiloxane, Sigma/Fluka) was selected as an organic co-solvent to increase the aerobic conversion of toluene. Figures 3.2 and 3.3 show pictures of the stirred batch reactors. For side-by-side fermentations, the two reactors (each containing 1 liter of the mineral salts medium) were sterilized. Sterilization was performed for 45 minutes at 121°C and 15 psig. (When one reactor was used, sterilization was performed for 20 minutes at 121°C and 15 psig.)

# Enhancement of the Conversion of Toluene by *Pseudomonas putida* F-1 Using Organic Cosolvents

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## Abstract

*Pseudomonas putida* F-1 (ATCC 700007) was used as a model organism in stirred tank reactors to study conversion enhancement of poorly soluble substrates by organic cosolvents. After a literature study, silicone oil was used as a solvent system to enhance the mass transfer rate. To study the benefits of the organic solvent addition, batch experiments were conducted in two side-by-side fermentation vessels (experimental and control) at three different levels of silicone oil (10, 30, and 50%). Results showed that the presence of silicone oil resulted in a 100% increase in the toluene mass transfer compared to the control. Experiments in continuous stirred-tank reactors showed that improved conversion could be obtained at higher agitation rates.

**Index Entries:** *Pseudomonas putida* F-1; toluene; organic solvents; silicone oil.

**Figure 3.1.** Abstract of peer-reviewed manuscript. See Appendix for complete text.

**Table 3.1.** Composition of SM + $KNO_3$ medium (Rodríguez, et al., 2001)

| Ingredient | Concentration (per liter) |
|---|---|
| $KH_2PO_4$ – EM Science | 0.4 g |
| $K_2HPO_4$ – EM Science | 0.5 g |
| $MgSO_4 \cdot 7H_2O$ – EM Science | 0.5 g |
| $CaCl_2$ - JT Baker | 0.04 g |
| $NH_4Cl$ - Mallinckrodt | 0.5 g |
| $KNO_3$ – JT Baker | 0.5 g |
| Pfennig's Trace Metals – see Table 3.2 | 1 ml |

**Table 3.2.** Composition of Pfennig's trace metals

| Ingredient | Concentration (g per liter) |
|---|---|
| $ZnSO_4 \cdot 7H_2O$ – JT Baker | 0.1 |
| $MnCl_2 \cdot 4H_2O$ – JT Baker | 0.03 |
| $H_3BO_3$ – JT Baker | 0.3 |
| $CoCl_2 \cdot 6H_2O$ – Matheson, Coleman + Bell | 0.2 |
| $CuCl_2 \cdot H_2O$ – Fisher Scientific | 0.01 |
| $NiCl_2 \cdot 6H_2O$ – EM Science | 0.02 |
| $Na_2MoO_4 \cdot 2H_2O$ – Fisher Scientific | 0.03 |
| $FeCl_2 \cdot 4H_2O$ – JT Baker | 1.5 |
| $Na_2SeO_3$ – JT Baker | 0.01 |

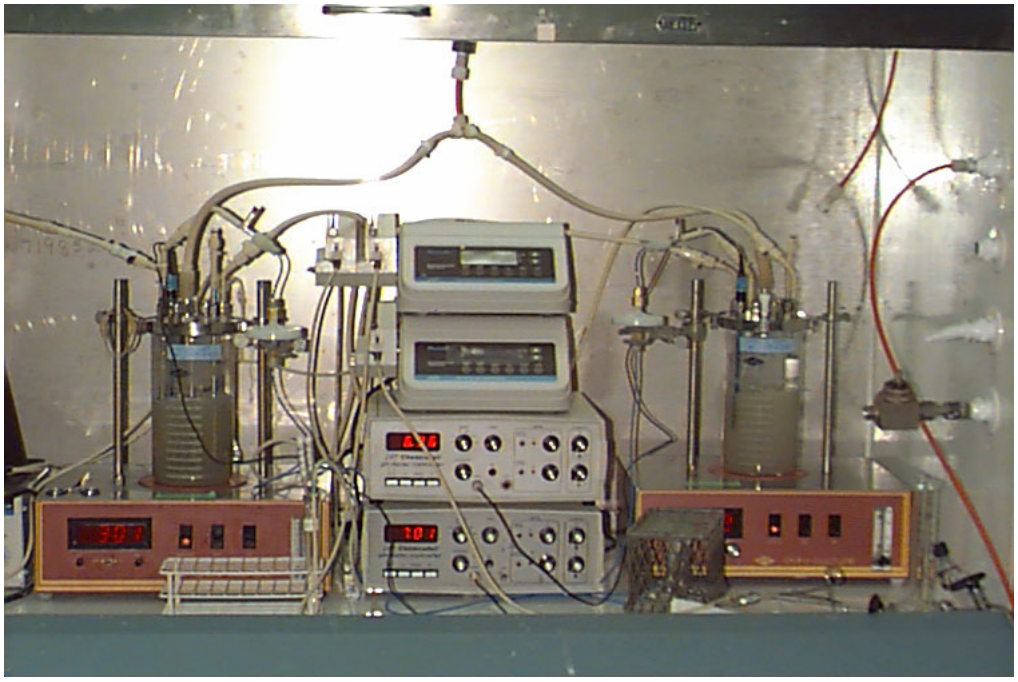

**Figure 3.2.** Experimental configuration for dual stirred batch reactors. The consoles are pH-controllers and peristaltic pumps.

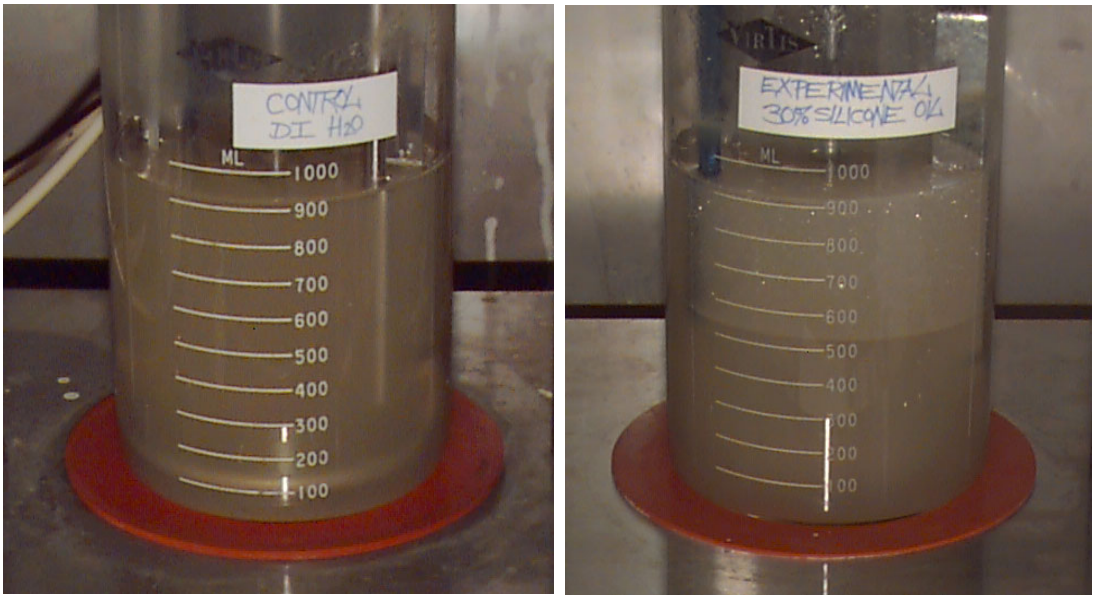


**Figure 3.3.** Control reactor versus 30% silicone oil reactor during operation. The agitation was turned off before this photo was taken.

### 3.3.3. Experiments in Stirred Batch Reactors (SBR)

Batch experiments were performed in order to study the effect of silicone oil on the degradation of toluene in the gas-phase. Prior to the addition of silicone oil, the reactors were not sampled; however qualitative observations of microbial growth were made in terms of turbidity. The bacteria were grown in the reactors for one or two days before the contents of both reactors were mixed together in a sterile 2-L bottle. After mixing, part of the broth was returned into the reactors and either silicone oil or sterile, distilled water (in the case of the control) was added to reactors. This mixing procedure was performed to ensure identical cell quantities in both reactors.

Experiments were also performed in stirred batch reactors to study the effect of addition of biosurfactant. The biosurfactant used was a concentrated rhamnolipid biosurfactant (product number JBR-425, Jeneil Biosurfactant Company, Saukville, WI).

To prevent foaming, antifoam (Polyglycol P-2000, Dow Chemical Company, Midland, MI) was added to each SBR at a concentration of 0.4 ml/liter.

### 3.3.4. Experiments in the Continuous Stirred Tank Reactor (CSTR)

In addition to the experiment discussed in the manuscript in which the effect of agitation in a CSTR with 15% silicone oil (v/v) was studied, other experiments were performed in the CSTR to study the reactor silicone oil fraction and how it affected the conversion of toluene by the biocatalyst. In this experiment, the reactor was operated with a continuous liquid feed of medium without the addition of silicone oil. Then, the silicone oil feed was initiated and the medium feed reduced to maintain an aqueous phase residence time equal to the one before oil addition. The gas-phase toluene conversion was monitored as alterations in the silicone oil flow rates were performed (see Table 3.3).

### 3.3.5. Analytical Techniques

Both liquid and gaseous samples were taken during the fermentations. The increase in cell density was measured by optical density (OD) at 600 nm in a Bausch and Lomb (Milton Roy Company, Rochester, NY) Spectronic 21 spectrophotometer with a standard calibration curve using the equation:

$$DCW\ (mg/L) = (176.91 \times OD_{600}^{2}) + (710.21 \times OD_{600})\ . \quad (8)$$

**Table 3.3.** Feed rates for continuous experiments with silicone oil addition

| Silicone Oil Addition | Medium Feed Rate (ml/min) | Silicone Oil Feed Rate (ml/min) |
|---|---|---|
| 0% | 0.45 | 0 |
| 20% | 0.35 | 0.1 |
| 35% | 0.3 | 0.15 |

Equation 8 was developed by diluting a concentrated cell broth and measuring the optical density of each dilution. At the same time, a portion of the concentrated cell broth was filtered on a pre-weighed filtered paper (0.45-μm-pore-size, Whatman, Clifton, NY) and the filter was dried at 100°C. This method allowed the dry cell weight (DCW) to be calculated for each dilution. Hexane (Burdick and Jackson) was used to extract the silicone oil present in each sample by adding two milliliters of sample and one milliliter of hexane to a cuvette. The cuvette was shaken and the phases were allowed to separate before reading the optical density of the aqueous phase. The optical density of control samples with and without hexane was compared and a conversion factor was developed to compensate for the presence of hexane. Gas samples of 100 μL from the inlet and the exhaust were analyzed using a gas chromatograph (Hewlett Packard HP 5890 Series II) equipped with an HP PLOT molecular sieve 5A capillary column (30 m × 0.32 mm) with a 12-μm film thickness. Temperatures of the column, injection port, and thermal conductivity detector were 70$^{o}$C, 175$^{o}$C, and 200$^{o}$C respectively. The carrier gas was helium at a flow rate of 25 ml/min. To measure toluene in the liquid, the samples were centrifuged for 5 minutes at 14,000 RPM and analyzed by gas chromatography using a HP 5890 Series II with an HP WAT (cross-linked PEG) capillary column (30 m × 0.53 mm) with a 1.0-μm film thickness. The column temperature program was initially 70$^{o}$C followed by a 25$^{o}$C/min ramp to 200$^{o}$C, then held for 1.2 min, followed by a second 25$^{o}$C/min ramp to 225$^{o}$C and held there for 3.0 min. The injection port temperature was 1$^{o}$C while the flame ionization detector temperature was 265$^{o}$C. Calibration of the

methods for toluene analysis was performed by injecting 8.66 and 86.6 ng toluene (dissolved in hexane).

## 3.4. Supplemental Results and Discussion

The bulk of the results from the experiments with *Pseudomonas putida* F1 where incorporated in a journal article (see Appendix). However, additional experiments were performed as part of this thesis and these are reported here. These experiments included the addition of a biosurfactant to stirred batch reactors with or without silicone oil.

The effect of biosurfactant addition at a level of 0.0025% (w/v) was negligible when used in batch fermentations as shown in Figures 3.4 and 3.5., where several experimental conditions are displayed. Figure 3.4 clearly shows that the biosurfactant did

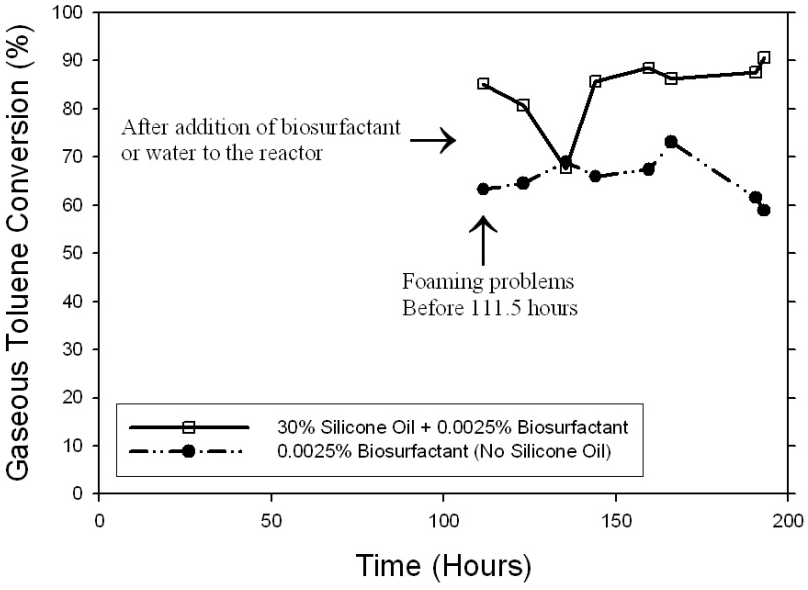


**Figure 3.4.** Effect of 0.0025% (w/v) biosurfactant (rhamnolipids) on the fermentation of gaseous toluene

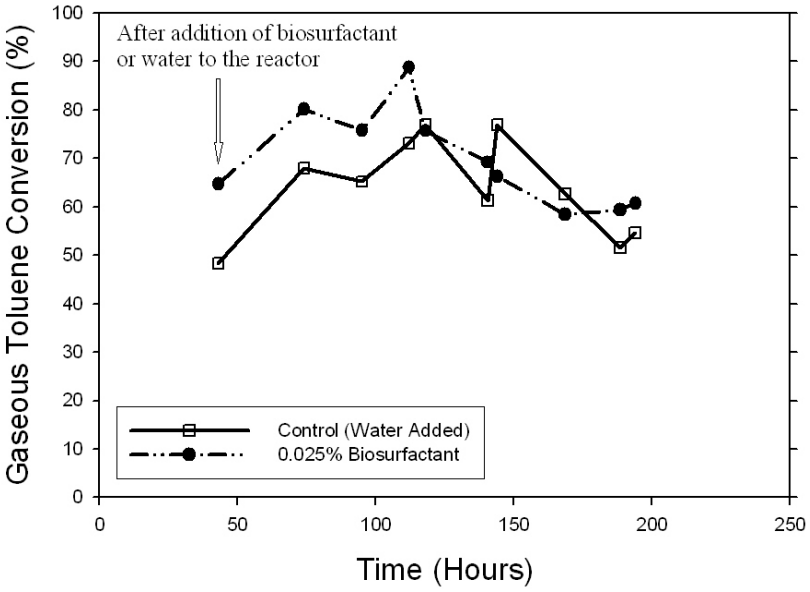


**Figure 3.5.** Effect of 0.025% (w/v) biosurfactant (rhamnolipids) in SBR with antifoam added to the medium to control foaming

not have an effect on the conversion of toluene by the biocatalyst whether silicone oil was present or not. The conversion of gas-phase toluene remained the same with or without the surfactant. Data before 115.5 hours in the experiment with biosurfactant but without silicone oil have been excluded from the graph – results were highly variable due to foaming problems and foam carry-over into the gas phase exit lines. Antifoam was added to alleviate the problem. In Figure 3.5, another experiment is shown using a higher concentration of biosurfactant (0.025%, w/v). Even at the higher level, the addition of the biosurfactant did not have an effect on the conversion of toluene by the biocatalyst.

In another experiment, using a continuous stirred tank reactor (CSTR), data were collected over 1100 hours of experimentation (Figure 3.6). The conversion of gas-phase toluene in the CSTR, when initially operated without silicone oil, was between 50 and

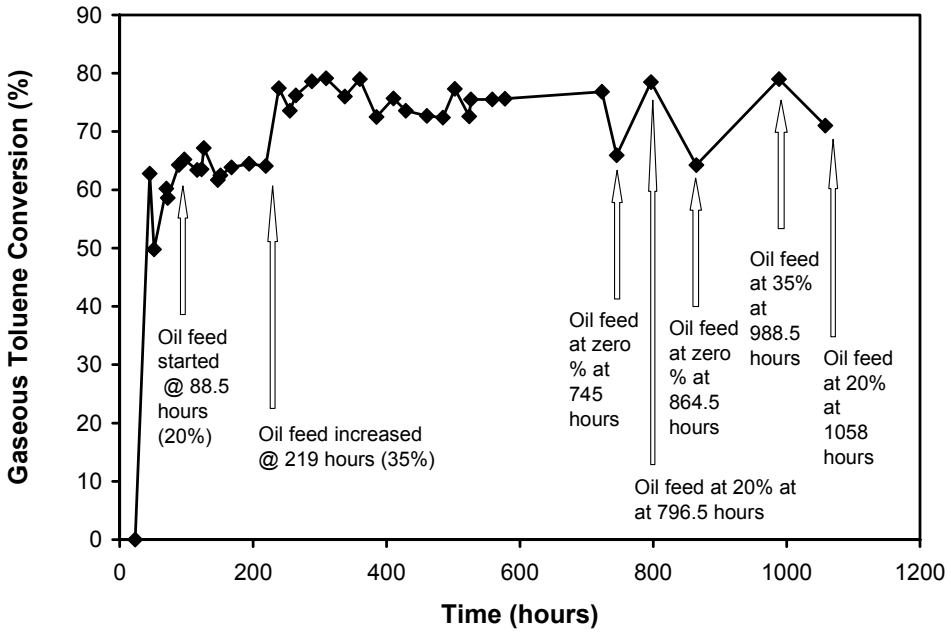


**Figure 3.6.** Effect of silicone oil concentration (v/v) on toluene conversion in a CSTR. Arrows indicate changes in silicone oil concentration.

63% and did not change significantly when operation was changed to a 20% silicone oil feed. However, when a 35% silicone oil feed was provided, a clear improvement was observed as the conversion of toluene increased (almost instantaneously) from 65 to 75%. When the silicone oil was again reduced (or omitted), the toluene conversion changed as expected. Part of the data presented in Figure 3.6 is arguably qualitative as steady state values may not have been reached in the beginning and also toward the end of the fermentation when the silicone oil feed rates were changed in a rapid fashion. The total liquid flow rate to the reactor was 0.45 ml/min (see Table 3.3). This corresponds to a residence time of 37 hours. Three constant data points collected after three residence times are commonly used to confirm a steady state. Regardless of the non-steady state data, the results in Figure 3.6 clearly showed that silicone oil addition improved the

conversion of toluene in the CSTR; at least when the feed consisted of 35% silicone oil. It is difficult to draw the same conclusion when 20% silicone oil was added. Another continuous stirred tank reactor experiment with over 1500 hours of operation is shown in Figure 3.7. This is a partial experiment (no data are reported before 307.5 hours); however, there are enough data points to show the effect of silicone concentration in the conversion of gas toluene by the biocatalyst. The data shown in the figure start at a silicone oil feed of 35%. When the feed was changed from 35 to 20%, the conversion decreased. The same effect was observed when the oil feed was decreased from 20 to 0%. Both CSTR fermentations were performed using an agitation of 300 RPM.

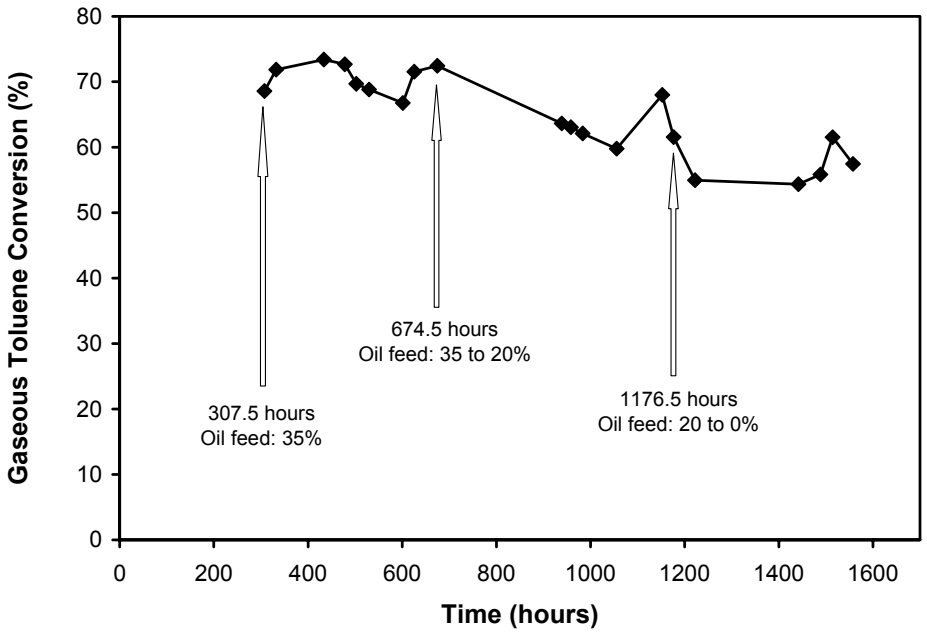


**Figure 3.7.** Effect of reduction in silicone oil concentration (v/v) on toluene conversion in a CSTR. Arrows indicate changes in silicone oil concentration.

## 3.5. Conclusions – The Use of Co-solvent in Toluene Fermentations

Both results presented in the peer-reviewed article (see Appendix) and in the supplemental experiments (Section 3.4) support the conclusion that the addition of silicone oil improved the conversion of gaseous toluene by *Pseudomonas putida* F1. The apparent mass transfer coefficient values were 2-3 times higher in the presence of silicone oil (10-50%) and the majority of data suggested that 30-35% silicone oil may have been optimal in the stirred reactors investigated in this work. The results presented in this thesis suggest that performance improvement may be realized if poorly water-soluble substrates are converted in fermenters with co-solvents.

# Chapter 4

## 4. Overall Conclusions

Biological systems can be developed for the treatment of volatile organic compounds present in industrial gaseous wastes and for the conversion of gases into more valuable chemicals or fuels. Microbiological methods are specific, generate a minimum amount of byproducts, and substantially less expensive when compared to traditional chemical methods performed at high temperatures and pressures. The challenge is to develop systems that can efficiently degrade these poorly water-soluble pollutants. As shown in this thesis, two-phase-liquid systems offer an effective way of addressing this challenge in bacterial fermentations. An additional organic phase (co-solvent) can be used acting as a carrier of the poorly water-soluble substrates to the microorganisms present in the aqueous phase, thus enhancing their conversion. The co-solvent enhanced conversion by providing increased mass transfer and surface area from a second gaseous substrate rich phase.

Observed results demonstrated that co-solvents may be added in bacterial fermentations to increase the metabolic performance of microorganisms. Positive results were obtained when hexadecane was used as a co-solvent in serum bottles experiments to enhance the production of ethanol and acetic acid by *Clostridium ljungdahlii* in synthesis gas fermentations. In batch tests, syngas consumption was increased from 30% in controls with no hexadecane to 100% with the co-solvent present. However, many potentially thermodynamically attractive co-solvents were eliminated from consideration due to bioincompatibility. Synthesis gas has become a very important inexpensive

feedstock for the production of fuels and chemicals. Bioreactor systems that can be developed for the use of co-solvents in synthesis gas fermentations should be considered in the future; since they may provide valuable ways of producing chemicals from synthesis gas with considerable cost savings when compared to traditional chemical methods mostly used today.

As found in the literature, several biofiltration systems have been developed for the treatment of poorly water-soluble gaseous contaminants while several two-liquid-phase systems have been reported for the degradation of poorly-soluble liquid contaminants. In this thesis work, the application of a two-liquid-phase system with silicone oil showed positive results for the degradation of toluene; a model poorly water-soluble gaseous contaminant. An increase in the conversion of toluene gas by *Pseudomonas putida* F1 was observed in stirred batch reactors and in a continuous stirred tank reactor in the presence of silicone oil. Continuous flow conversion increased from about 50% to near 80%.

It is difficult to compare results from the two models systems studied and to conclude which system benefited more from co-solvent addition. There are multiple differences between these systems, including reactor configuration and substrate solubility. In the case of synthesis gas, the solubility of the gases is poor in both the aqueous and organic phases. In the case of toluene, the solute is completely soluble in the organic phase and slightly soluble in the aqueous phase. However, results from this thesis work can be useful in addressing many challenges in two-liquid-phase systems gas fermentations.

Different co-solvents were used for each system. It would have been interesting to see if results could have been repeated if the co-solvents used in one system were applied to the other system. In the case of the studies presented in this thesis, silicone oil with a log $P_{OW}$ value of 3.9 was successfully used with *Pseudomonas putida* F1 but may have been toxic to *Clostridium ljungdahlii*. The latter organism is Gram-positive and generally cannot tolerate co-solvents with log $P_{OW}$ values less than 4.0, while some Gram-negative organisms (e.g, *Pseudomonas* sp.) can tolerate co-solvents with lower log $P_{OW}$ values. Nevertheless, it would have been interesting to test silicone oil with the clostridial system.

In general terms, the microorganism or microbial consortium used in a two-liquid-phase system should be able to tolerate the presence of a co-solvent. On the other hand, the co-solvent's "biocompatibility" should not be so high that will allow the microorganism to use the co-solvent as a carbon source. In the case of the studies presented in this thesis, *P. putida* may have been able to use hexadecane as a carbon source, even if *C. ljungdahlii* did not. In summary, optimization experiments should be conducted in two-liquid-phase fermentations to find the optimum co-solvent type, concentration, biocompatibility, gaseous substrate solubility, and optimal metabolic performance (e.g., product formation rate).

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# Appendix

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# Enhancement of the Conversion of Toluene by *Pseudomonas putida* F-1 Using Organic Cosolvents

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## Abstract

*Pseudomonas putida* F-1 (ATCC 700007) was used as a model organism in stirred tank reactors to study conversion enhancement of poorly soluble substrates by organic cosolvents. After a literature study, silicone oil was used as a solvent system to enhance the mass transfer rate. To study the benefits of the organic solvent addition, batch experiments were conducted in two side-by-side fermentation vessels (experimental and control) at three different levels of silicone oil (10, 30, and 50%). Results showed that the presence of silicone oil resulted in a 100% increase in the toluene mass transfer compared to the control. Experiments in continuous stirred-tank reactors showed that improved conversion could be obtained at higher agitation rates.

**Index Entries:** *Pseudomonas putida* F-1; toluene; organic solvents; silicone oil.

## Introduction

To promote higher bioconversion of poorly water-soluble components, cosolvents and surfactants are often added to the fermentation broth. The logarithm of the partition coefficient (log $P$ or log $P_{ow}$) of an organic solvent in a standard octanol-water two-phase system is a useful parameter to predict what solvent would be most suitable for a bioconversion *(1,2)*. The partition coefficients for some common organic solvents are listed in Table 1. The relationship between log $P$ and bioactivity is based on the assumption that the octanol-water system provides a sufficient description of hydrophobic and transport interactions when it is introduced into a biologic system *(3,4)*. In general, organic solvents with a log $P$ value between 1 and 5 are toxic to microorganisms *(5)*.

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Table 1
Log *P* Values for Selected Solvents *(2)*

| Solvent | Log *P* | Solvent | Log *P* |
|---|---|---|---|
| Ethanol | –0.24 | 1-Decanol | 4.0 |
| 1-Heptanol | 2.4 | Dodecanol | 5.0 |
| Toluene | 2.5 | Decane | 5.6 |
| 1-Octanol | 2.9 | Dodecane | 6.6 |
| Hexane | 3.5 | Oleyl alcohol | 7.5 |
| Heptane | 4.0 | Hexadecane | 8.8 |

In general, Gram-negative bacteria appear to have a higher solvent tolerance than Gram-positive bacteria, and species within a genus sometimes show a range of tolerances *(6–9)*. It has been suggested that the difference in solvent tolerance is caused by the presence of the outer membrane in Gram-negative bacteria containing lipopolysaccharides, which protect the cells against hydrophobic compounds. The most resistant Gram-negative species have been reported in the genus *Pseudomonas (10–12)*. One of the key processes in the adaptation of some *Pseudomonas* strains enabling them to tolerate organic solvents appears to be the isomerization of *cis*- into *trans*-unsaturated fatty acids *(13)*.

Silicone oil has been used for the biological elimination of alkanes from gases using biotrickling filters. One study reported the use of silicone oil with an aqueous medium in a 1:1 ratio being recirculated in a biotrickling filter to remove hexane *(14)*. An 89% elimination efficiency of hexane was achieved. The oil was reused after separation by natural gravity or centrifugation. Column experiments were performed with intermittent replacement of nutrients. In these studies, a control column was not used, making it difficult to positively prove the benefit of the oil addition.

In a study by Budwill and Coleman *(15)*, silicone oil was used as an additive in peat-based biofilters for the removal of hexane. Peat was coated with 20% (v/v) silicone oil and loaded into the biofilter columns. An average 60%, or 16 g/($m^3$·h), hexane removal was reported in the column containing silicone oil compared to 24%, or 8.2 g/($m^3$·h), in the untreated control. These investigators speculated that the presence of silicone oil increased the mass transfer of hexane from the gas to the liquid phase by increasing the contact of microorganisms with the dissolved gas at the water–silicone oil interface.

A group of French researchers have been investigating the applications of silicone oil as an organic solvent for the degradation of poorly water-soluble xenobiotic compounds such as xylene, butyl acetate, 2,4,6-trichlorophenol, and styrene *(16–19)*. One of their studies reported that microorganisms were able to grow more in a two-phase system (70% medium and 30% silicone oil [v/v]) on xylene and butyl acetate (70%/30% [w/w]) than in a one-phase system *(16)*. The two-phase system resulted in an

increase in optical density at 540 nm for several substrate concentrations. No appreciable growth was noted in the one-phase system.

Other studies performed by the French group have also used a biphasic aqueous-silicone oil system with 20% oil (v/v) in a continuous stirred-tank reactor (CSTR). One of these studies demonstrated a more efficient degradation of 2,4,6-trichlorophenol in the biphasic system when compared to a monophasic aqueous system *(17)*. As the dilution rate was changed from 0.033 to 0.22/h, the volumetric conversion rate increased from 21.3 to 85.8 g/($m^3$·h) in the biphasic system compared with an increase from 13.9 to 40.2 g/($m^3$·h) in the monophasic system. Ascon-Cabrera and Lebeault *(18)* demonstrated the effect of silicone oil in the degradation of chlorinated and nonchlorinated mixed compounds. They found that the specific growth rate of the microorganisms used in the study was about two times higher in the biphasic system (0.48/h) than in the monophasic system (0.27/h). Statistical analysis showed that the biphasic system was more efficient in the degradation process when compared to the monophasic system. Finally, the French group showed in another study that this type of biphasic system was effective in the degradation of styrene by *Pseudomonas aeruginosa (19)*. Without silicone oil, the microorganisms were unable to oxidize styrene, but in the presence of silicone oil, the lethal dose of styrene in aqueous medium (70 mg/L) was avoided.

The French studies were performed by mixing an aqueous phase and silicone oil laden with organic contaminants, and they were able to demonstrate the benefit of silicone oil as a "reservoir" for the contaminant. By doing this, the toxic levels of some of the compounds in the aqueous phase could be lowered and toxicity avoided. By contrast, our scope was to study the benefit of using silicone oil for enhancement of mass transfer of dilute gaseous organics (not necessarily toxic) to microorganisms in the aqueous phase when conducting batch or continuous fermentations.

## Materials and Methods

### *Microbial Culture and Media*

*Pseudomonas putida* F-1 strain ATCC 700007 was obtained from the American Type Culture Collection (Manassas, VA). Seed cultures were grown aerobically at 30°C in a mineral salts medium consisting of the following ingredients: 0.4 g/L of $KH_2PO_4$, 0.5 g/L of $K_2HPO_4$, 0.5 g/L of $MgSO_4 \cdot 7H_2O$, 0.04 g/L of $CaCl_2$, 0.5 g/L of $NH_4Cl$, 0.5 g/L of $KNO_3$, and 1 mL of Pfennig trace metals *(20)*. The medium was distributed in 50-mL aliquots in 125-mL serum bottles and sealed with butyl rubber stoppers and aluminum crimps (Wheaton, Millville, NJ). Toluene was added (20 µL) as a liquid to each culture as a carbon source.

### *Reactor Experiments*

Experiments were conducted in 1-L nominal volume, stirred batch reactors (SBRs) and in a CSTR (Virtis, Gardiner, NY). Silicone oil (DC 200,

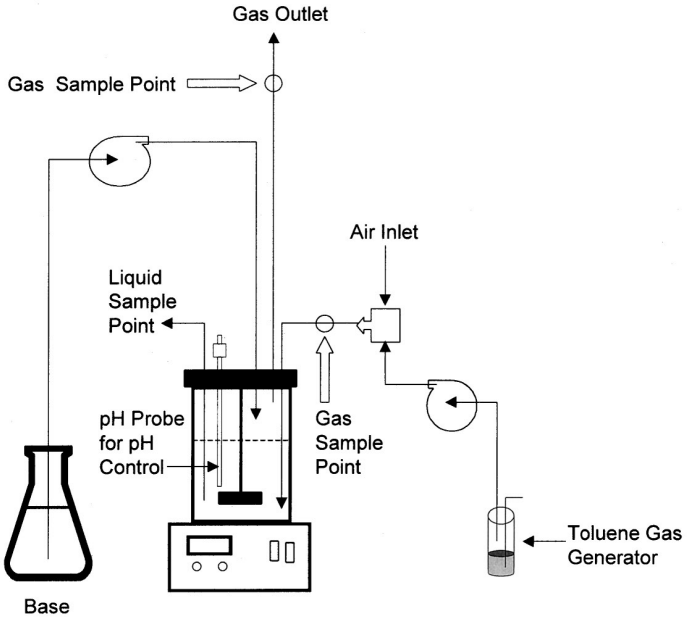


Fig. 1. Schematic of experimental setup for SBRs.

~53 mPa·s, polydimethylsiloxane) (Sigma/Fluka) was selected as an organic solvent to increase the conversion of toluene. A schematic of the batch reactor configuration is shown in Fig. 1. Modifications to the reactor for continuous feed included the addition of two feed pumps (for medium and oil). Both gas and liquid exited the continuous reactor through a tube through the top of the reactor positioned at the interface (Fig. 2).

The batch experiments were conducted in two side-by-side fermentation vessels (experimental and control) with a total liquid volume in each reactor of 1 L. The SBRs were operated at 30°C and an agitation of 300 rpm. The pH was controlled at 7.0. The aeration was set at 1 L/min, and the air was prefiltered using a Gelman Sciences Acrodisk (ACRO 50 APT, 0.2 µm polytetrafluoroethylene). Toluene was pumped as a gas into the inlet air at a flow rate of 10 mL/min, resulting in concentration of approx 35 ppmv ($1.4 \times 10^{-6}$ mol/L). After an initial growth phase without silicone oil, the contents of both reactors were mixed and a portion of the broth was returned to the fermentation vessels. Then, either silicone oil or water was added to a total volume of 1 L. Thus, the same population of viable cells was present in each reactor when measurements began. Silicone oil was tested at three different concentrations: 10, 30, and 50% (v/v).

The continuous experiments were conducted with a total liquid volume in the reactor of 1 L. The CSTR was operated at 30°C and an agitation of 300 rpm. The pH, aeration, and toluene addition was the same as in the SBRs. After an initial growth phase without silicone oil, the oil and medium feed streams were started at a total flow rate of 0.45 mL/min (15% silicone oil).

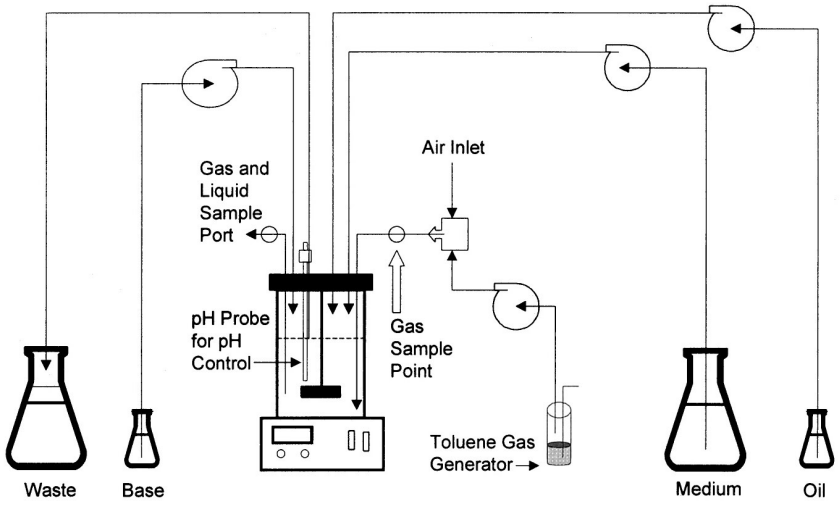


Fig. 2. Schematic of experimental setup for CSTR.

### *Analytical Techniques*

Gas samples were collected from the inlet gas and the reactor headspace in gastight syringes, and 100 µL was injected into a gas chromatograph (Hewlett Packard HP 5890 Series II) equipped with an HP WAT (cross-linked polyethylene glycol) capillary column (30 m × 0.53 mm) with a 1.0-µm film thickness. Temperatures of the column, injection port, and flame ionization detector were 40, 175, and 200°C, respectively. Helium was used as a carrier gas. The calibration was based on 8.66 and 86.6 µg/L of toluene standards in hexane. Liquid samples were collected from the aqueous phase, which was allowed to settle by temporarily turning off the agitation. To measure toluene in the aqueous phase, samples were centrifuged for 5 min at 14,200*g* and 2 µL of the aqueous phase was injected into the gas chromatograph. The column temperature program was initially 35°C followed by ramping to 50°C at 25°C/min with a 2.0-min hold, then followed by ramping to 150°C at 20°C/min with a 0-min hold. Temperatures of the injection port and the flame ionization detector were 245 and 265°C, respectively.

The increase in dry cell weight (DCW) was measured by optical density (OD) at 600 nm after hexane had been used to extract the remaining silicone oil present in each sample. No emulsion was observed in the samples after extraction. The calibration curve was prepared from samples with known cell concentration. The potential interference of hexane in the procedure was determined by comparing OD measurements of hexane-extracted and nonhexane-extracted samples.

## Results and Discussion

The addition of silicone oil enhanced the conversion of gaseous toluene for all conditions studied in the batch experiments. In Fig. 3, the conver-

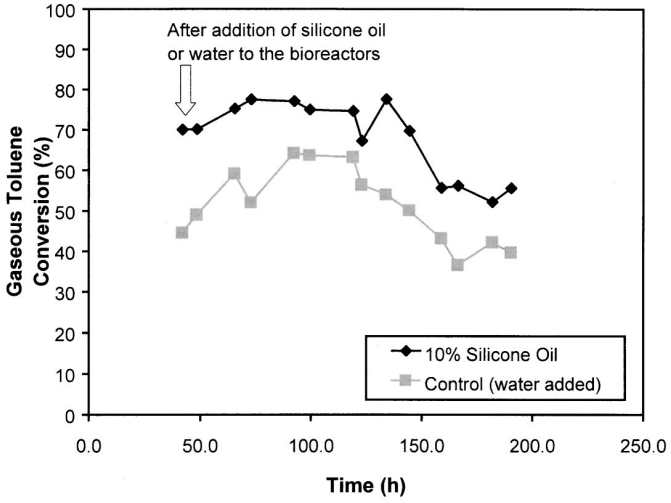


Fig. 3. Toluene conversion in SBRs with or without the addition of 10% (v/v) silicone oil.

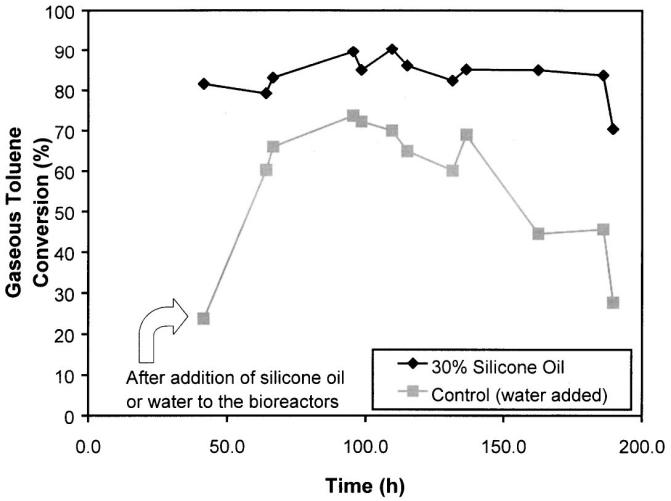


Fig. 4. Toluene conversion in SBRs with or without the addition of 30% (v/v) silicone oil.

sion of toluene has been plotted as a function of fermentation time when 10% (v/v) silicone oil was present in one of the reactors. Data prior to the addition of the oil has not been plotted, and it should be noted that the contents of the reactors were mixed just before the addition of the silicone oil. The toluene conversion was substantially higher when silicone oil was present. At the end of the batch fermentation, the conversion dropped

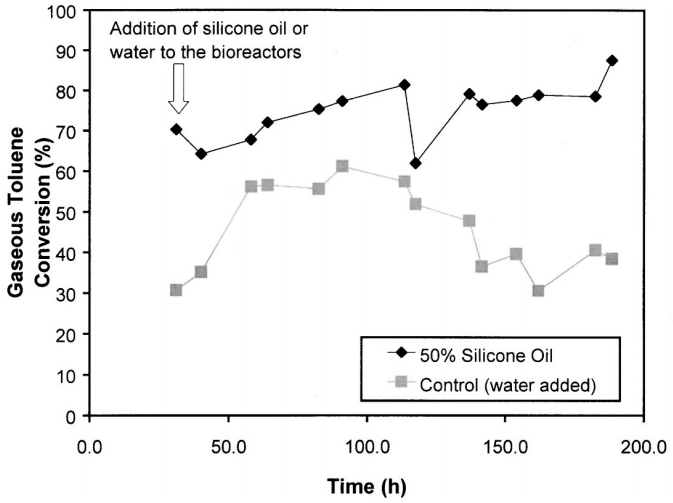


Fig. 5. Toluene conversion in SBRs with or without the addition of 50% (v/v) silicone oil.

in both cases, presumably because of nutrient limitations. A short lag phase was noted in most cases after the mixing/initiation of the experiment. Figures 4 and 5 show results from the addition of 30 and 50% silicone oil. In both cases, the conversion of toluene was higher in the reactor containing silicone oil. Toluene concentration in the aqueous phase remained below detection (data not shown) in these experiments, indicating gas mass transfer limiting conditions.

The cell growth in the study in which 30% silicone oil was used is shown in Fig. 6. The increase in cell concentration is dramatic in the case in which silicone oil was used. This, of course, can be attributed to the higher conversion obtained in this reactor by the apparent improved mass transfer rate of the toluene from the gas to the aqueous phase. It is clear from the results that the silicone oil was not toxic to the cells. The log $P$ value for silicone oil used in these experiments with a molecular mass of 3000 g/mol was estimated to be 2.93 *(21)*.

The mass transfer coefficient ($K_L a$) was calculated (Eq. 1) from the measured consumption of toluene, the composition of the headspace in the reactors, and the assumption that mass transfer–limited conditions were present.

$$\text{rate of toluene conversion} = (K_L a / H)(p_{\text{toluene}})(V_{\text{liq}}) \quad (1)$$

in which $p_{\text{toluene}}$ is the partial pressure of toluene in the headspace and $V_{\text{liq}}$ is the total liquid volume in the reactor. The results showed that the mass transfer of toluene from the gas increased by a factor of 2 in the presence of silicone oil (Table 2). No trend was found between the mass transfer rate and amount of silicone oil added to the reactor.

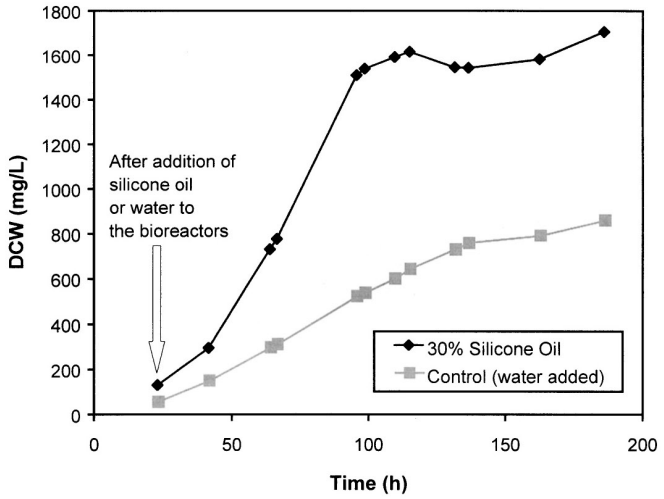


Fig. 6. Cell growth in SBRs with or without the addition of 30% (v/v) silicone oil.

Table 2
Average $K_La'$ for Conversion of Toluene
by *P. putida* F-1 at Different Concentrations of Silicone Oil[a]

| Silicone oil (%) | Experimental average $K_La'$ ±SD | Control average $K_La'$ ±SD |
|---|---|---|
| 10 | 7.9 ± 1.6 | 3.7 ± 1.1 |
| 30 | 16.7 ± 6.6 | 5.3 ± 2.1 |
| 50 | 8.2 ± 3.0 | 3.1 ± 1.0 |

[a]Average $K_La'$ was determined from samples taken in an interval of approx 40–140 h during the course of the experiment. The unit for the mass transfer coefficient ($K_La' = K_La/H$) is mol/(h·L·atm) and includes the Henry's law constant ($H$).

To confirm the enhancement of the mass transfer rate by the addition of silicone oil, the control and experimental reactors were switched so that the reactor normally used as control became the experimental reactor and vice versa. Within experimental error, the results were the same as before (data not shown).

Since this was a non-steady-state condition, it is important to estimate the amount of toluene that initially may accumulate in the silicone oil. An overestimated microbial uptake rate may be calculated if the accumulation is significant. Using a Henry's law constant of 0.071 atm/(L·mol) *(22)* for the toluene/silicone oil system, we can calculate that it would take 15–76 min to saturate the silicone oil if no microbial conversion existed. This time is considerably shorter than the fermentation time with silicone oil, which lasted approx 150 h.

The CSTR was operated for 35 d in a study conducted to investigate the effect of agitation rate. After an initial batch growth without silicone oil,

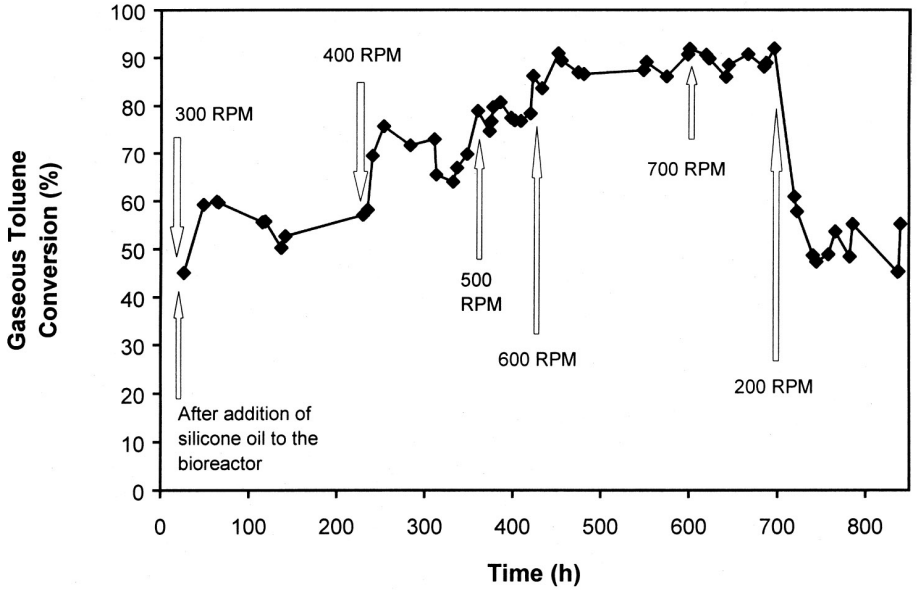


Fig. 7. Effect of agitation on toluene conversion in CSTR with 15% (v/v) silicone oil. Arrows indicate agitation changes (in revolutions per minute).

the liquid feeds were started and oil was added to the reactor. As expected, the conversion of toluene was higher at higher agitation rates, although the dependence was more apparent at a low agitation rate (Fig. 7). In these studies we also confirmed that it was possible to recycle the silicone oil after a gravity separation and filtration through a filter paper (Whatman, Clifton, NJ). Calculations using a simple mass balance over the CSTR showed that the maximum toluene loss in the silicone oil exiting the reactor was 3.5% of the gaseous toluene exiting the reactor.

## Conclusion

Silicone oil is an organic cosolvent that efficiently enhances the conversion of toluene by *P. putida* F-1. We speculate that the increases seen may be attributed to an increase in effective transfer area between the toluene-rich and toluene-poor phases. This is more apparent at lower agitation rates where the gas holdup in the liquid is low. Toluene is absorbed into the silicone oil, and the silicone oil disperses the toluene into the aqueous medium. Subsequently, the availability of toluene increases, thus increasing the consumption of the gas by the microorganism. In our studies with low levels of toluene (average of 15 ppmv) in the headspace air, the calculated concentration of oxygen in the aqueous phase ($2.5 \times 10^{-4}$ mol of $O_2$/L) is 70 times greater than the calculated toluene concentration ($3.5 \times 10^{-6}$ mol of toluene/L) under equilibrium conditions *(23,24)*. Thus, the concentration of $O_2$ is eight times higher than is needed to completely oxidize the

toluene, assuming a theoretic oxygen-to-toluene molar ratio of 9. It is therefore safe to assume that the limiting reactant is toluene.

This process would be applicable for biologic conversion of other poorly water-soluble gases such as nitric oxide or synthesis gases. Since the silicone oil can be reused, it minimizes the generation of waste and the capital cost. Further research needs to be conducted to expand this process for industrial applications.

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# Vita

Miguel Rodríguez, Jr. was born in Utuado and grew up in Caguana, Utuado, Puerto Rico. He received an Associate of Science Degree from the University of Puerto Rico, Arecibo Campus in June 1987. Later, he received a Bachelor of Science Degree in Industrial Microbiology from the Mayagüez Campus in June 1989. In the spring and summer of 1989, he was an intern in Oak Ridge National Laboratory (ORNL) working in the Environmental Sciences Division under the mentorship of Dr. Anthony V. Palumbo. From 1990 to 1994, he worked as a Microbiologist in the former Fermentation/Microbiology Group in Ricerca, LLC in Painesville, Ohio under the supervision of Drs. Joseph N. Silvernale and Suzan Woodhead. In 1994, he returned to ORNL to become a staff member of the Chemical Technology Division under the supervision of Dr. Brian H. Davison. He is now a staff member in the Biochemical Engineering Research Group of the Life Sciences Division at ORNL. During his career development in ORNL, he has received the following awards: 2002 Significant Event Award, UT-Battelle, LLC; 2000 Significant Event Award, UT-Battelle, LLC; 1999 Technical Support Award, Lockheed Martin Energy Research Corporation; and 1998 Significant Event Award, Lockheed Martin Energy Research Corporation. In January 2003, he was promoted to Research Associate. He completed his first semester at the University of Tennessee on a full-time basis in the Department of Botany. Since his second semester, he attended the university on a part-time basis to complete the requirements for the Master of Science Degree in Life Sciences in Biotechnology.