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Department of Chemical Engineering

Master's Thesis

The Adaptation of Self-Cycling Fermentation to a Continuously-Stirred Tank Reactor for Xenobiotic Degradation

By

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Abstract

The technique of self-cycling fermentation (SCF) was adapted to a continuously-stirred tank reactor (CSTR) using the production of CO_2 as the monitored parameter in the feedback control loop, mechanical level switches to determine the volumes removed and replaced during reactor cycling and a non-50% harvest fraction. Previous work with the technique indicated the potential for improvement of SCF by applying these changes. The work was performed to develop a system that can be used to study the degradation of xenobiotic compounds when exposed to microbial cultures. The development of the experimental apparatus was done using the degradation of the plasticizer di-(2-ethylhexyl) adipate by *Rhodococcus rhodochrous*.

The experimental results demonstrated that CO_2 production could be used in the feedback control loop to successfully trigger cycling of the reactor. The use of a 65% harvest fraction demonstrated that a 50% harvest fraction was not necessary to operate SCF.

Sommaire

La technique de fermentation auto-cyclique (FAC) a été adaptée à un réacteur biologique à agitation continue utilisant la concentration en CO_2 dans les effluents du réacteur comme paramètre mesuré dans la boucle de commande à rétroaction. Des contacteurs de niveau ont été ajoutés au système afin de permettre la récolte des produits traités à des volumes de liquide arbitraires (c.-à-d., différents de 50 %). En fait, des études antérieures ont indiqué que la technique FAC pouvait être améliorée avec de tels changements. L'objectif principal de ce projet de recherche était donc de mettre au point un système permettant l'étude de la dégradation par exposition à des cultures microbiennes de composés chimiques xénobiotiques. Le système modèle utilisé était constitué d'un matériau plastifiant, l'adipate de bis(2-éthylhexyle), et le microbe *Rhodococcus rhodochrous*.

Les résultats expérimentaux ont montré que la mesure du taux de production de CO_2 peut être utilisée pour enclencher le processus de récolte cyclique. Le bon fonctionnement de la technique FAC avec une récolte de 65 % du volume au lieu du 50 % habituel a été démontré.

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I really do hope that those people that I acknowledge for their help and support in this section already know how much I appreciate what they've done for me. Otherwise, I've clearly not done a good job of expressing myself during these past two years.

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Table of Contents

List of Figures

List of Tables

1. Introduction

General public awareness of the effects of xenobiotic compounds in the environment first gained prominence in the early 1960s with the effects of pesticides and other pollutants on wildlife [1]. Cultural and scientific awareness of this issue in the west has increased and substantial amounts of resources have been devoted to investigating the consequences of human- and environmental-exposure to xenobiotic compounds. The technology and techniques used for these studies have developed in tandem with improvements to the understanding of the degradation of these compounds. However, the sheer number of possible interactions that xenobiotics can have with the environment and microorganisms, and the development of new xenobiotics require the constant development of new microbiological techniques to assess the consequences of their presence in the environment.

Plasticizers are a group of xenobiotic chemical additives commonly found in a wide-range of commercial and industrial plastics, including those used in food-packaging film, medical equipment, upholstery, flooring, mouldings, gaskets, piping, clothing, electrical wire insulation, pond- and swimming pool-liners and roofing systems [2]. Since most polymers in their pure forms exhibit properties which make them ill-suited to their required applications, plasticizers are added to obtain usable end-products. As their name implies, these compounds usually impart functionality to the pure polymer by increasing its flexibility, although many other physical and chemical properties of the polymer can be altered depending on the types and amounts of plasticizers used [3]. The plasticizer di-

(2-ethylhexyl) adipate (DEHA) (shown in Figure 1-1) is primarily found in poly(vinyl chloride) (PVC) products such as food packaging films, cables and garden hose, where flexibility is required at low or room temperatures [4, 5].

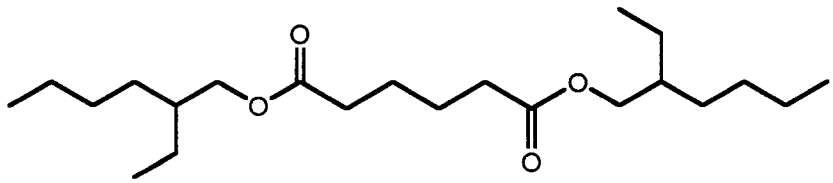

Figure 1-1 The chemical structure of DEHA.

As a consequence of their prevalent use, plasticizers have become an example of a widespread xenobiotic environmental contaminant. The global production of plasticizers in 2005 was estimated at 5.5 million tonnes annually, with DEHA accounting for approximately 5% of the plasticizer market [6, 7]. Various studies have documented the ability of plasticizers to leach from their polymer matrices under conditions similar to those in landfills [8-10]. Accordingly, the extent of plasticizer contamination in the environment is such that these compounds are considered ubiquitous contaminants [2, 8, 10-12].

The breakdown of DEHA by *Rhodococcus rhodochrous* has been shown to produce metabolites that are significantly more toxic than DEHA itself [2, 13-15]. However, the consequence of hazardous microbial metabolites is by no means limited to plasticizers. To better understand plasticizer degradation and, more broadly, the degradation of xenobiotics, the application of the semi-continuous cell-culture technique of self-cycling fermentation (SCF) was of interest.

SCF has been used to study the degradation of some xenobiotics [16-19] and has a number of characteristics that make it particularly well-suited to study the degradation of xenobiotics by microorganisms growing on complex substrates.

The technique requires a fermentation parameter that can be easily monitored and that directly reflects growth conditions in the culture. This monitored parameter is used as a signal for feedback control. When the parameter is observed by the control system to exhibit behaviour indicative of a desired state in the culture, usually the consumption of a primary substrate, 50% of the fermentation broth is harvested and replaced by an equivalent volume of sterile nutrient broth. Thus the growth state of the culture itself determines when each cycle is to be initiated.

The advantages of the repeatability and stability of this technique have been somewhat limited by restrictions due to the monitored parameter used and the reactor configuration. As well, previous work has only used 50% as the harvest fraction for cycling. In order to adapt SCF to study xenobiotic degradation, three characteristics of the technique were considered in this work. Firstly, a non-50% harvest fraction was used to demonstrate that a harvest fraction of 50% was not necessary for SCF. Secondly, CO_2 evolution was used as the monitored parameter, instead of the customarily-used dissolved oxygen (DO) or oxidation reduction potential (ORP) probes. This avoided problems associated with these probes being immersed in the liquid media, such as fouling from the accumulation of biomass. Lastly, the SCF technique was adapted to a continuously-stirred tank-reactor (CSTR), instead of the conventionally-used cyclone reactor. This application of SCF using these three modifications was developed using the degradation of DEHA by *R. rhodochrous* as a system to evaluate the effectiveness of this technique.

2. Literature Review

2.1. Prevalence and Effects of Plasticizer Contamination

The body of work concerning plasticizer contamination in the environment focuses on four areas: the extent of plasticizer contamination, the means of release into the environment, the measured and possible effects on human health and interactions with the environment.

Estimates of the amount of plasticizer released into the environment are varied and often concern either an individual chemical or a particular class of plasticizers. For example, estimates of 7.7×10^7 kg/a for all plasticizers released in Western Europe [20], 9.4×10^7 kg/a worldwide for di-(ethylhexyl) phthalate (DEHP) [21], 2.8×10^5 kg/a in the United States for DEHP, di-n-butyl phthalate and dimethyl phthalate [3], and 2.3×10^7 kg/a in North America for phthalic acid esters [22] have been reported. More commonly, contamination levels of different plasticizers are reported in various media, such as soil, fresh water and river sediments for specific geographic areas [2, 9, 12, 23, 24]. As a result of the areas and levels of contamination reported, plasticizers are considered ubiquitous contaminants [8-12]

Studies into the sources of plasticizer contamination have largely attributed the ubiquitous contamination in the environment to leaching from landfills and commercial products [8-10]. The leaching of plasticizers from manufactured goods such as food packaging [25-27], gloves used in meal preparation [28], and medical devices [29] is of particular concern as these items generally expose

humans directly to the leached plasticizers. From exposure to food products alone, an estimated rate of human ingestion of plasticizers has been made of 8.2 mg/person/day [30].

Adding to the concern over the issue of human exposure to plasticizers are chemical effects. Certain phthalate-ester plasticizers have been linked with the induction of early puberty in human females [31, 32], while animal models have linked the metabolism of plasticizers to tumour growth and cancer [33, 34].

The extent of plasticizer contamination has led to investigations of interactions in the environment, specifically those with microorganisms. The literature suggests that the microbial degradation of plasticizers is dependent upon the combination of plasticizer, microorganism, system studied and substrate. Certain studies have reported plasticizer degradation in the presence of another carbon substrate and have investigated the extent to which they were degraded [2, 8, 14, 15, 35]. There are also reports of complete plasticizer mineralization [36-38]. No general statements as to the consequences of plasticizer interaction with the environment can be made as plasticizer degradation research has been conducted in both pure- [13, 35, 37] and mixed-culture systems [39-41].

2.2. DEHA Degradation Studies

An early report of DEHA degradation in an activated sludge system recorded the incomplete degradation of DEHA when compared to the degradation of di-*n*-hexyl adipate under identical conditions [40]. More recent work has demonstrated that the incomplete degradation of DEHA by a variety of microorganisms in pure culture results in the accumulation of the metabolites 2-

ethylhexanol and 2-ethylhexanoic acid and that these metabolites cause an increase in the toxicity of the aqueous media [2, 35]. The degradation of DEHA by *R. rhodochrous* has been investigated, resulting in a proposed pathway for degradation, shown in Figure 2-1 [13-15].

It should be noted that the degradation of DEHA by *R. rhodochrous* was shown to be co-metabolic; the degradation of DEHA was not observed in the absence of a primary carbon and energy source [13]. Furthermore, the adipic acid released during degradation (Figure 2-1) can be utilized by the organism as a carbon and energy source.

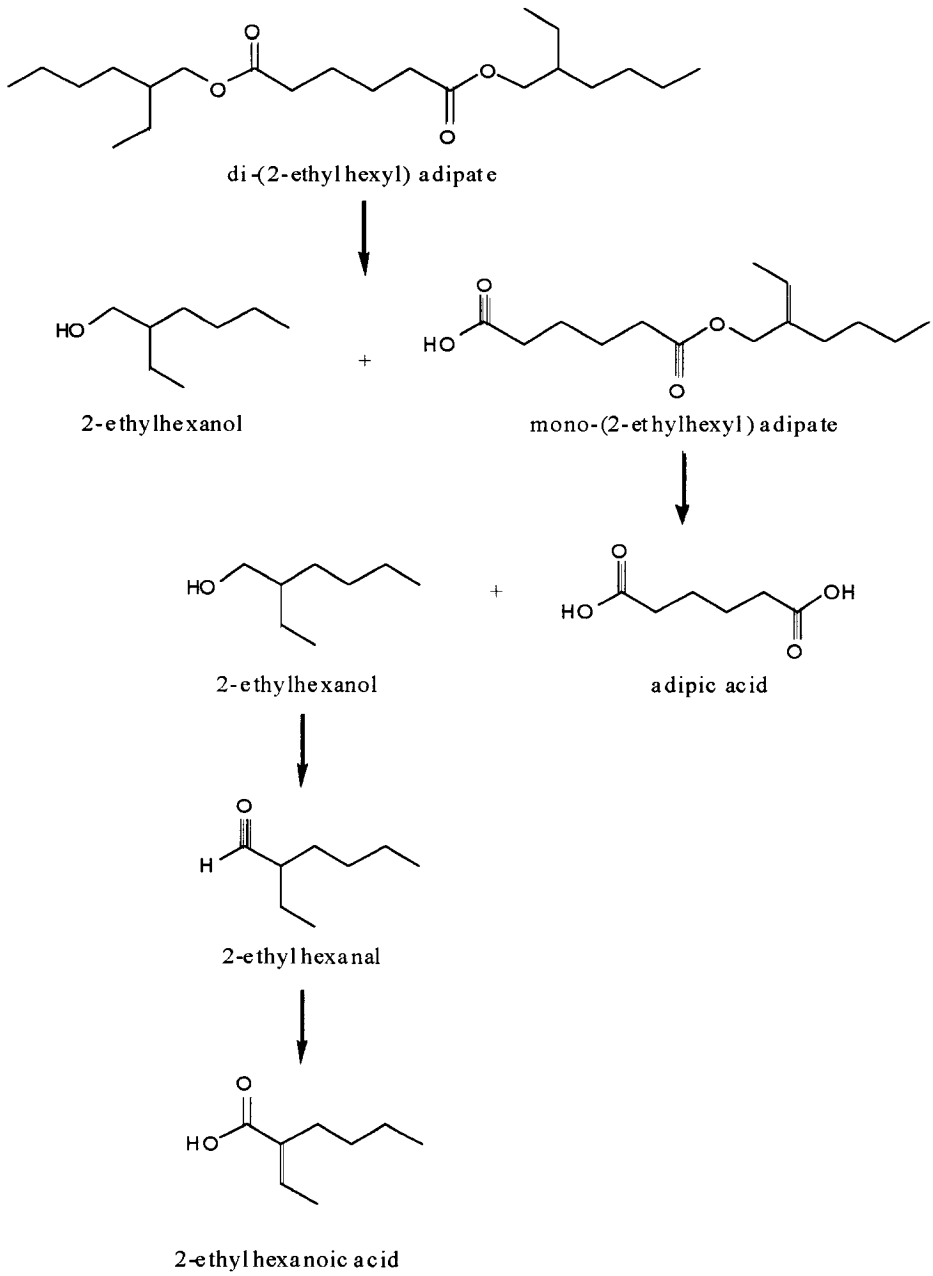

Figure 2-1 The pathway for the incomplete degradation of di-(2-ethylhexyl) adipate by *R. rhodochrous* [13].

2.3. Self-Cycling Fermentation

Compared to the traditional cell-culture techniques of batch and continuous (i.e. chemostat) culture, SCF is relatively new. The defining feature of the technique is the use of feedback control to determine when the contents of the

reactor are cycled (the "time-to-cycle"). SCF was created by Sheppard and Cooper, who applied feedback control to the technique of continuous phasing to develop SCF in 1990 [42]. Since then, the technique has been used to study the degradation of aromatic compounds, hydrocarbons, and nitrogen removal [16-19], although other investigations have focused on its use for the production of compounds [43-46] and mathematical modelling [47-49].

Despite limited reports of its use in studying the degradation of xenobiotics, the characteristics of SCF make it very useful for degradation studies. Firstly, results obtained from cycles following the initial cycles are highly-repeatable when compared to results from series of batch experiments [50, 51]. Secondly, the system has been demonstrated to be stable and robust, with operation upwards of 100 cycles having been reported [18, 19, 42]. Thirdly, cells cultured via SCF grow at their maximum growth rates and exhibit high levels of synchronicity as a result of the periodic reactor volume cycling with fresh media [45, 50]. Culture synchronicity is of interest from a research standpoint, because as synchronicity increases, results obtained from that culture will increasingly represent that organism's performance during a given growth phase, rather than be averaged over a distribution of cells in varying stages of growth [50]. Given these benefits with regards to repeatability and the reporting of results, SCF is an ideal tool for work in xenobiotic degradation.

Experiments with SCF have historically been conducted in cyclone reactors [42, 46, 52] and CSTR systems [17, 18, 53, 54]. The use of cyclone reactors provided excellent mixing of the reactor contents. However, these reactors required gravimetric measurements to determine the volumes to be added to and

removed from the reactor, as their geometries were not conducive to the use of level sensors or mechanical level switches. This was problematic as technically-complex solutions were required to cycle the reactor contents, yet variations in the volumes transferred were still observed [55]. Studies using SCF in CSTR systems also utilized gravimetric measurements as the use of level sensors could not be used as a result of foaming in the reactor [56]. To date, mechanical level switches have not been used for SCF in a CSTR system.

All SCF studies have utilized harvest fractions of 50% [50]. Questions have been raised as to determining the optimum harvest fraction for SCF and model-based solutions have been proposed [57]. However, no experimental results for this modification have been reported, despite the potential for improvements such as superior reactor throughputs.

The monitored parameter used to determine the time-to-cycle in SCF has usually been dissolved oxygen (DO) [18, 19, 44, 45, 52, 54, 56]. The cultures were grown under a slight oxygen limitation that did not significantly impede growth. The oxygen demand was calculated from the rate of change in the DO measurements or alternatively, a "critical value" in the DO level was determined empirically that indicated the time-to-cycle, usually when the primary carbon and energy source in the system had been consumed. Logically, DO can only be used for aerobic systems that use oxygen as the terminal electron acceptor [50], thus other work with anoxic systems has utilized an oxidation reduction potential (ORP) probe and calculations of the first- and second-derivatives of its signal to facilitate reactor cycling [51, 53].

Problematically, both DO and ORP probes require direct contact with the liquid reactor media to obtain readings. Consequently, fouling of the probes in extended fermentations can occur as a result of biomass accumulation on the sensing element. This results in inaccurate measurements from the probe that cannot be used to determine the time-to-cycle of the system.

The monitoring of CO_2 production in SCF systems to determine the time-to-cycle has been suggested, as the patterns of CO_2 production have shown behaviour analogous to those of DO with respect to the consumption of primary substrates [18, 51, 54]. The use of CO_2 production is inherently advantageous over DO or ORP probes as these sensors do not need to be in direct contact with the liquid media and may be placed in the non-sterile gas-outlet stream of reactors, thereby removing their need for sterilization. Despite these benefits, CO_2 production has only been used once as the monitored parameter in the SCF control loop [51].

3. Objectives

The presence of xenobiotic contamination in the environment is a current and increasing problem. While contaminants may or may not be chemically- and biologically-inert, the metabolites produced by their exposure to microorganisms can have unforeseen consequences in the local ecosystem. Given the advantages of SCF for studying cell populations and their metabolic products, and the potential improvements to the technique that could be realized by utilizing a CO_2 sensor, mechanical level switches and a non-50% harvest fraction, it was of interest to develop this system further for current work with plasticizers and other xenobiotic compounds. Consequently, the objectives of this research were to:

1. Adapt the SCF technique to a CSTR as a technique for determining the degradation products of xenobiotic compounds when exposed to aqueous microbial cultures.
2. Evaluate the effectiveness of this application of SCF by monitoring the degradation of DEHA by *Rhodococcus rhodochrous*.

4. Materials and Methods

4.1. Storage and Maintenance of *Rhodococcus rhodochrous*

All experiments were conducted using *R. rhodochrous*, ATCC 13808 from the American Type Culture Collection (ATCC, United States). The strain used was stored at -70 °C in a freezer (REVCO, model ULT1386, United States) in a 20% glycerol (Sigma-Aldrich, Canada), 20% NB broth (DIFCO Brand, Fisher Scientific, Montreal, QC) in a sealed 1.5 mL plastic vial (Fisher Scientific, Montreal, QC).

This culture was revived as follows. The culture was thawed at -4 °C, re-suspended by shaking the vial by hand, and transferred into a 500 mL Erlenmeyer flask containing 100 mL of Brain Heart Infusion (BHI) at a concentration of 30 g/L (DIFCO Brand, Fisher Scientific, Montreal, QC). This culture was capped with a foam plug and then incubated at room temperature in a rotary incubator shaker operated at 200 RPM (New Brunswick Scientific, model G-25, United States) for 30 h.

The contents of the Erlenmeyer flask were then divided into 24 1.5 mL plastic vials in 1 mL volumes and the contents of the Erlenmeyer flask checked for contamination by plating on a prepared BHI-agar plate [58]. The vials were centrifuged at 10 000 RPM for 10 min (International Equipment Company, model Micromax, United States). The supernatant of each vial was discarded and replaced by BHI at a concentration of 30 g/L containing 10% (vol/vol) glycerol

(Fisher Scientific, Montreal, QC). The pellet of each vial was re-suspended by shaking the vial by hand and then the sealed vials were stored at -70 °C.

A culture of *R. rhodochrous* was maintained by reviving the culture as above, and incubating the culture in a rotary shaker at room temperature in 100 mL of 30 g/L of sterile BHI media in a 500 mL Erlenmeyer flask, capped with a foam plug, for one week. The following week, 10 mL of the culture was transferred to 100 mL of sterile BHI media and the maintained culture was plated on BHI-agar plates to check for contamination by visual inspection. This process was repeated for a period not exceeding one month, when another frozen culture was revived and maintained in the same manner.

The BHI-Agar plates were prepared using 30 g/L BHI and 20 g/L technical agar (DIFCO brand, Fisher Scientific, Montreal, QC) in distilled water. This was sterilized for 30 min using saturated steam at 121 °C. The media was allowed to cool slightly before being poured into 100 mm x 25 mm sterile Petri dishes (Fisher Scientific, Montreal, QC). The dishes were then left to solidify and stored in sealed plastic bags.

All inocula were prepared by transferring 10 mL of the maintained culture of *R. rhodochrous* to 100 mL of sterile 30 g/L BHI media in a 500 mL Erlenmeyer flask. The flask was capped with a foam plug and was incubated for 30 h at room temperature in a rotary shaker at 200 RPM. 10 mL of the flask contents were then withdrawn into a 10 mL sterile syringe and used as the inoculum for all experiments.

The methods described were performed using sterile techniques inside a laminar fumehood (Baker Company, model VBM600, United States). All media

was sterilized for 30 min using an autoclave (AMSCO, model SG-116 and model 3021-S, United States) using saturated steam at 121 °C.

4.2. Experimental Apparatus

4.2.1. CSTR Equipment

Figure 4-1 is a schematic of the CSTR vessel and the modifications used for batch and SCF experiments. The vessel was part of a BioFlo® 3000 unit (New Brunswick Scientific, NJ, USA) and was connected to its control unit, which monitored and controlled the agitation rate, air inlet flowrate and reactor temperature. The media reservoirs connected to the reservoir inlet were three 20 L Nalgene autoclavable carboys (Fisher Scientific, Model 02-690-23, Montreal, QC) connected in series using PharMed® BPT tubing (Cole-Parmer, Anjou, QC). Each reservoir was vented using a HEPA-Vent 0.2 μm inline filter (Fisher Scientific, Montreal QC) to allow media flow by gravity into the CSTR and maintain sterile conditions.

All solenoid valves used in the apparatus were fail-closed and constructed of stainless steel (Parker, Model 71215SN1MN00, Skinner Valve Division, CT, USA). The CO_2 sensor (Vernier Instruments, Model CO2-DIN, OR, USA) was mounted in a small polypropylene bottle connected to the air outlet stream of the reactor. The flowrate past the CO_2 sensor was measured manually using a calibrated rotameter (Brooks Instrument, Model Sho-Rate, Markham, ON). In order to prevent saturation of the CO_2 sensor, the flow past the sensor was diluted with a constant flow of air using a similar rotameter. The level switches and

magnetic floats used in the apparatus were constructed of stainless steel (Davis Controls, Model LS-1750, Oakville, ON). During reactor cycling, media was removed using a peristaltic pump (Masterflex® Model 7520-20, Cole-Parmer, Anjou, QC). Sterility of the air flowrates through the reactor was maintained using HEPA-Vent 0.2 μm inline filters. All sterile tubing connections in the experimental were made using PharMed® BPT tubing; non-sterile connections were made using MasterFlex® C-Flex® silicon tubing (Cole-Parmer, Anjou, QC).

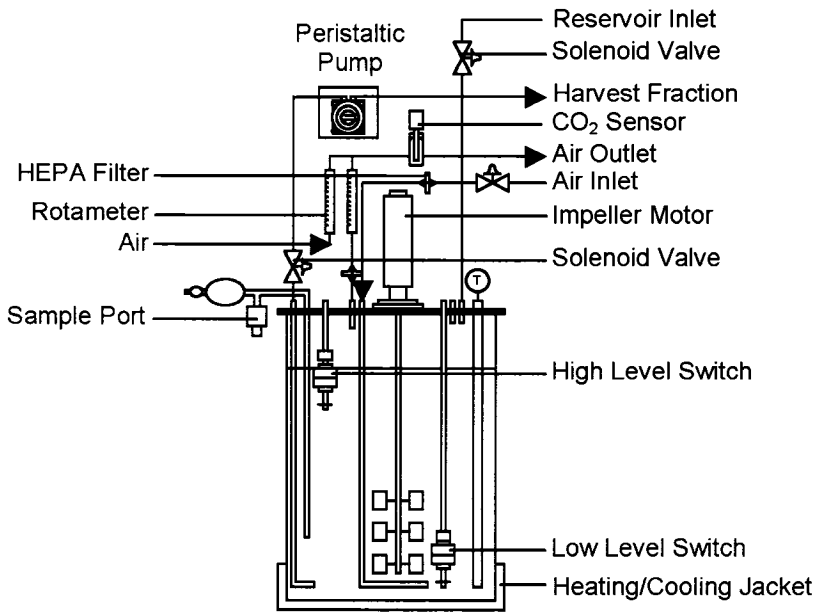

Figure 4-1 Diagram of the modified BioFlo® 3000 CSTR and selected connections.

4.2.2. Electronic Equipment and Computer Interface

For the purposes of data acquisition and equipment control, all signals were acquired and sent using a miniLAB data acquisition card (DAQ) (Measurement Computing, Model 1008, MA, USA), connected to the control computer via a USB 1.1 connection. 5 V_{DC} power was supplied to the CO_2 sensor and level

switches directly from the DAQ. The signal from the CO_2 sensor was measured in differential mode with a resolution of 1.22×10^{-3} $V_{measured}$/bit and calibrated in the experimental apparatus using varied flowrates of CO_2 diluted by air (both MEGS, Montreal, QC), each connected to the rotameters in Figure 4-1. The state of each level switch was determined using a TTL signal input to two digital in ports on the DAQ.

Operation of the peristaltic pump and solenoid valves required mains voltage, which was supplied through a series of optical relays (Opto22, Model OAC5, CA, USA) installed on a mounting rack (Opto 22, Model PB16A, CA, USA). This provided electrical isolation between the mains power and the DC control signals sent from the DAQ.

Control signals from the DAQ were sent using TTL signals, routed through two Darlington transistor array integrated circuits (Motorola, ULN2003A) to supply sufficient current to operate the optical relays. An external power supply of 5 V_{DC} was used to supply power to the relay mounting rack and the transistor arrays.

4.2.3. Self-Cycling Fermentation Control Program

The control program that determined the time-to-cycle and automated the cycling routine was written using LabVIEW 7.1 (National Instruments, TX, USA). The time-to-cycle was determined by examining the first derivative of the CO_2 concentration in the air outlet stream of the reactor in a manner similar to those previously described [51, 54]. In this work, this was calculated by sampling

the CO_2 concentration every 30 s and fitting a linear regression to the most recent 120 CO_2 data points (i.e. all CO_2 data points within the previous hour).

The time-to-cycle was detected when two conditions were met. Firstly, the first-derivative of the CO_2 concentration in the air outlet stream had to be less than -100 ppm/h. This ensured that cycling took place just after the maximum CO_2 production had occurred and not during a stationary phase. Secondly, the time elapsed in the current cycle had to be greater than a threshold value of 7 h. This prevented cycling as a result of decreasing CO_2 concentrations immediately after reactor cycling caused by the removal of 65% of the reactor culture.

Cycling in the reactor was accomplished as follows. The control program closed the solenoid valve in the air inlet stream and opened the solenoid valve in the harvest fraction stream and activated the peristaltic pump. The pump ran until enough media in the reactor had been removed such that the low level switch was closed. At this point, the solenoid valve was closed again, and the solenoid valve in the reservoir inlet stream was opened to allow fresh media to drain into the reactor by gravity. The valve remained open until the high level switch was opened, at which point the valve was closed and the air inlet stream solenoid valve opened again, thereby completing cycling of the reactor. At no point in the cycle was it necessary to stop agitation of the reactor.

4.3. Batch Reactor Experiments

Three batch reactor experiments were conducted in order to assess the production of CO_2 by *R. rhodochrous* as an appropriate variable to be monitored

on-line to determine the point at which a self-cycling fermentation system would cycle. An overview of the batch experimental conditions is listed in Table 4-1.

Table 4-1 Overview of the experimental conditions in batch reactor experiments.

Experiment	Substrate(s) Present	Sample Volume (mL)	Data Obtained
Simple Batch	Adipic Acid	5	CO_2 OD_{500}
Complex Batch A	Adipic Acid DEHA	15	CO_2 OD_{500} Chemical Concentrations
Complex Batch B	Adipic Acid DEHA	15	CO_2 OD_{500} Chemical Concentrations

In the Simple Batch experiment, the reactor system was filled with 4 L of mineral salt medium (MSM) containing 10 mmol/L adipic acid (Fisher Scientific, Montreal, QC) and 0.1 g/L yeast extract (DIFCO, United States). The composition of MSM is listed in Table 4-2. The reactor and its contents were sterilized using saturated steam at 121 °C for 2 h and allowed to cool to room temperature before it was connected to the control unit. The reactor was operated at 30 °C and agitated at 200 RPM with an air flowrate of 4 L/min through the reactor. 0.4 mL of sterile 2,2,4,4,6,8,8-heptamethylnonane (Sigma-Aldrich, Montreal, QC) was added as an anti-foam agent via the injection port of the reactor using a 1 mL sterile syringe. The reactor was then inoculated using 10 mL of *R. rhodochrous* via the injection port. 5 mL samples were taken periodically using the sample port of the reactor and analyzed for optical density at 500 nm.

Table 4-2 Composition of mineral salt medium (MSM), prepared in distilled water.

Component	Concentration (g/L)
NH_4NO_3	4.0
KH_2PO_4	4.0
Na_2HPO_4	6.0
$MgSO_4 \cdot 7H_2O$	0.2
$CaCl_2 \cdot 2H_2O$	0.010
$FeSO_4 \cdot 7H_2O$	0.010
EDTA (di-sodium salt)	0.014

The experiments Complex Batch A and B were identical to the Simple Batch experiment with the following modifications. Prior to inoculation, 13 mL of sterile DEHA was added via the injection port of the reactor using two 10 mL sterile syringes. 15 mL samples were taken periodically via the sample port of the reactor and were analyzed for OD_{500}, DEHA and DEHA metabolites using chloroform extraction and gas chromatography.

4.4. Self Cycling Fermentation Experiments

Three SCF experiments were conducted; an overview of the experimental conditions is listed in Table 4-3. In the Simple SCF A experiment, the reactor was filled with 4 L of MSM containing 10 mmol/L adipic acid (Fisher Scientific, Montreal, QC), 0.1 g/L yeast extract (DIFCO, United States), 5 mmol/L NaOH (added to neutralise the acidity of adipic acid) and 10 mL of silicon oil as an anti-foam agent (Fisher Scientific, Montreal, QC). The media reservoirs contained 10 mmol/L adipic acid and 5 mmol/L NaOH in MSM. The reservoirs, reactor and their contents were sterilized using saturated steam at 121 °C for 2 h and allowed to cool to room temperature before being connected to the control unit. The reactor was operated at 30 °C and agitated at 200 RPM with an air flowrate of 2 L/min through the reactor. The reactor was then inoculated using 10 mL of *R.*

rhodochrous via the injection port and the equipment was operated and cycled automatically by the operating program.

Table 4-3 Overview of the experimental conditions in SCF experiments.

Experiment	Substrate(s) Present	Sample Volume (mL)	Data Obtained
Simple SCF A	Adipic Acid	0	CO_2
Simple SCF B	Adipic Acid	0	CO_2
Complex SCF	Adipic Acid	30	CO_2
	DEHA		OD_{500}
			Chemical Concentrations

The Simple SCF B and Complex SCF experiments were operated identically to the Simple SCF A experiment except for the following modifications. The media reservoirs contained 0.1 g/L yeast extract (DIFCO, United States) in addition to 10 mmol/L adipic acid and 5 mmol/L NaOH. During SCF, silicon oil was added in 1 mL increments when foaming became pronounced in the reactor. This was done no more than 4 times during each experiment. In the Complex SCF experiment at the beginning of the final cycle, 9 mL of sterile DEHA was added to the reactor via the injection port using a 10 mL sterile syringe. After the addition of DEHA, 30 mL samples were taken periodically and were analyzed for OD_{500}, DEHA, DEHA metabolites and adipic acid using chloroform- and ethyl-acetate extraction and gas chromatography.

4.5. Analytical Procedures

4.5.1. UV-Visible Measurements

Given its widespread acceptance as an indicator of bacterial cell concentrations [59], optical density measurements at 500 nm (OD_{500}) were made

to obtain an indication of the growth of *R. rhodochrous* in the liquid culture media for all experiments. 1 mL of the liquid culture was transferred from the sample volume to a 3 mL glass optical cell (Fisher Scientific, Montreal QC) using a 1000 µL pipette (Gilson Pipetman, Model P1000). To this, 2 mL of MSM was added and the absorbance at 500 nm was measured using a UV-visible spectrophotometer (Varian, Model Cary 50 Bio). A total of six measurements were made and the average calculated as the OD_{500} for that sample. MSM was used as the zero reference for all samples.

4.5.2. Sample Preparation Procedures

4.5.2.1. Chloroform Extraction

10 mL of the liquid culture was transferred to a 38 mL glass screw-cap vial (Fisher Scientific, Model 03-338K) using a 10 mL disposable pipette (Fisher Scientific, Model 13-678-27F). The liquid sample was acidified using 3 drops of 98% H_2SO_4 (Fisher Scientific, Montreal, QC). To this, 1 mL of reagent-grade chloroform (Fisher Scientific, Montreal QC) containing n-pentadecane (A&C American Chemicals, Montreal, QC) as an internal standard was added and the vial was covered with aluminium foil and capped. The vial was vortex-mixed (Scientific Industries, Bohemia, NY, USA, Model G-560) for 30 s and the organic layer removed using a 1 mL syringe (Hamilton, Reno, NV, USA, Model Gastight #1001). The chloroform extract was transferred to a 6 mL screw-cap vial (Fisher Scientific, Model 03-339-21C) and stored at -4 °C until it was analyzed by gas chromatography.

4.5.2.2. Ethyl Acetate Extraction

10 mL of the liquid culture was transferred to a 38 mL glass screw-cap vial (Fisher Scientific, Model 03-338K) using a 10 mL disposable pipette (Fisher Scientific, Model 13-678-27F). The liquid sample was acidified using 3 drops of 98% H_2SO_4 (Fisher Scientific, Montreal, QC). To this, 2 mL of reagent-grade ethyl-acetate (Fisher Scientific, Montreal QC) containing myristic acid (Fisher Scientific, Montreal QC) as an internal standard was added and the vial was covered with aluminium foil and capped. The vial was vortex-mixed (Scientific Industries, Bohemia, NY, USA, Model G-560) for 30 s and the organic layer removed using a 1 mL syringe (Hamilton, Ren, NV, USA, Model Gastight #1001). The liquid sample was extracted as above two more times, each with 2 mL of ethyl-acetate containing myristic acid. All the ethyl-acetate extract was transferred to a 6 mL screw-cap vial (Fisher Scientific, Model 03-339-21C) and evaporated under a stream of dry nitrogen gas before being derivatized and subsequently analyzed by gas chromatography.

4.5.2.3. Derivatization Procedure

In order to increase the volatility of adipic acid so that it could be analyzed using gas chromatography, the adipic acid in the evaporated ethyl-acetate extraction sample was methylated using reagent grade tetramethylammonium hydroxide (TMAH) in a variation of the procedure described ASTM International designation D 5974-96 [60].

The evaporated sample was re-suspended in 1 mL of a 50/50 (v/v) ethyl-ether/methanol (Fisher Scientific, Montreal, QC) mixture and 2 drops of a 1% (w/v) phenolphthalein in methanol solution (Fisher Scientific, Montreal, QC) were added. This was derivatized as described in the cited work; however a 1.25% (v/v) TMAH in methanol solution (Fisher Scientific, Montreal, QC) was used to compensate for the low concentrations of adipic acid present in the sample. The derivatized samples were injected into the gas chromatograph immediately after derivatization.

4.5.3. Gas Chromatography

All samples analyzed by gas chromatography were injected into a Varian CP-3800 gas chromatograph equipped with a flame-ionization detector (FID) and SPB-5 fused silica column (Supelco, Model 24048). The injection port used was the supplied Varian injection port (Model 1177) using Thermogreen™ 9.5 mm septa (Supelco, Model 20652) and a splitless, single-taper injection sleeve with the deactivated glass wool packing removed (Supleco, Model 2-0478,05). The operating parameters of the gas chromatograph are listed in Table 4-4. DEHA, DEHA metabolites and adipic acid were quantified using calibration curves prepared from pure samples of the compounds of interest.

Table 4-4 Gas chromatograph operating parameters for DEHA, DEHA metabolites and adipic acid quantification.

Parameter	Value	
Injection Temperature	300	°C
Initial Oven Temperature	60	°C
Hold Time	2.00	min
Temperature Ramp	10	°C/min
Final Column Temperature	300	°C
Hold Time	2.50	min
FID Temperature	300	°C
Column Flowrate	1.0	mL/min (constant)

5. Results

5.1. CO_2 Monitored as a Variable During Fermentation

Figure 5-1 shows the CO_2 evolution by *R. rhodochrous* in the Simple Batch experiment, where adipic acid was the only substrate available to the organism. The amount of CO_2 evolved by the organism increased with time and paralleled the OD_{500} measurements. The concentration of CO_2 in the air outlet stream of the reactor increased in a pattern consistent with an organism passing through a lag phase between 0 h and 5 h, then an exponential growth phase between 5 h and 22 h. Neither the CO_2 production, nor the OD_{500} measurements exhibited a stationary phase. The error bars on the OD_{500} measurements indicate one standard deviation of six measurements on one sample.

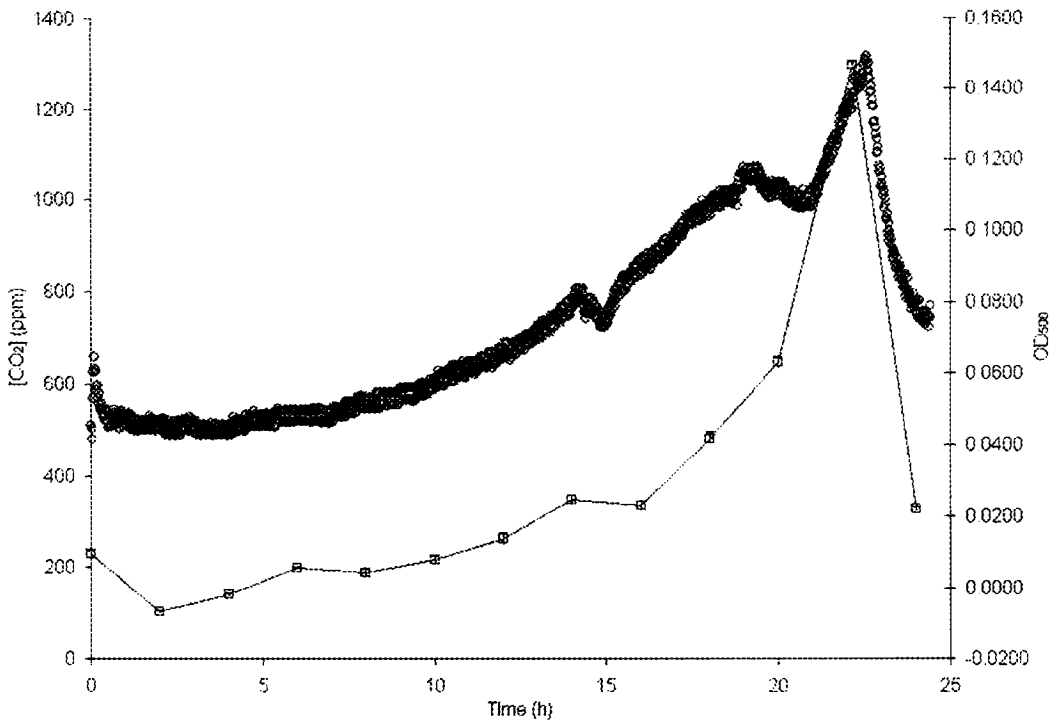

Figure 5-1 Generation of CO_2 (○) by *R. rhodochrous* and media OD_{500} (□) measurements for the Simple Batch experiment, which had an initial substrate concentration of 10 mmol/L adipic acid.

Figure 5-2 is a plot of the CO_2 measurements from Figure 5-1 against the OD_{500} measurements. The linear trend observed indicated a direct correlation between these two measurements during the growth of *R. rhodochrous*. The error bars on the OD_{500} measurements indicate one standard deviation of six measurements on one sample.

In the absence of DEHA, the results in Figure 5-1 and Figure 5-2 indicated that the CO_2 production by *R. rhodochrous* was a good indication of the growth state of the culture.

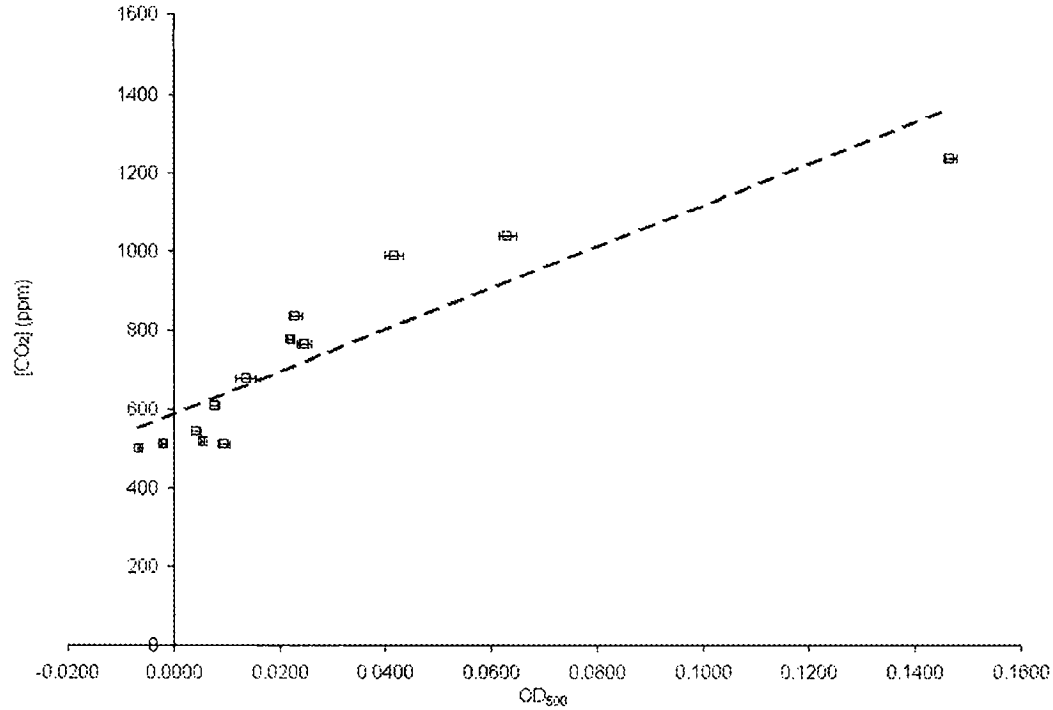

Figure 5-2 Correlation between CO_2 evolution by *R. rhodochrous* and media OD_{500} measurements for the Simple Batch experiment.

5.2. Biodegradation of Di-2-Ethyl-Hexyl Adipate (DEHA), in the Presence of Adipic Acid, by *Rhodococcus rhodochrous*

Figure 5-3 shows the CO_2 evolution during the Complex Batch A experiment where both adipic acid and DEHA were present in the system. OD_{500} was used as a measurement of growth, with vertical error bars in the figure indicating one standard deviation of six measurements on one sample.

Both the CO_2 measurements and, to a lesser extent, the OD_{500} measurements suggested two phases of exponential growth (having started at 6 h and 19 h), with each followed by a stationary phase. At 27 h, a sharp decrease in the CO_2 evolution was observed.

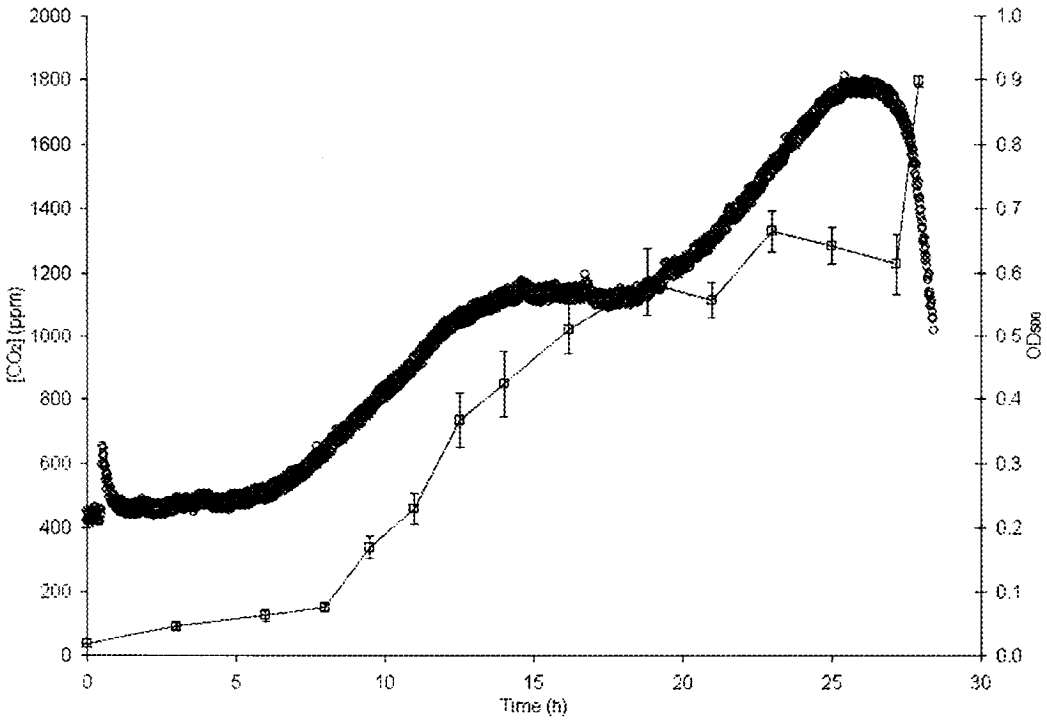

Figure 5-3 Generation of CO_2 (○) by *R. rhodochrous* and media OD_{500} (□) measurements for the Complex Batch A experiment, which had an initial substrate concentrations of 10 mmol/L adipic acid and 8.5 mmol/L DEHA.

Figure 5-4 is a plot of the CO_2 measurements from Figure 5-3 against the OD_{500} measurements. A linear trend was observed, indicating a direct correlation between CO_2 evolution and growth of the culture.

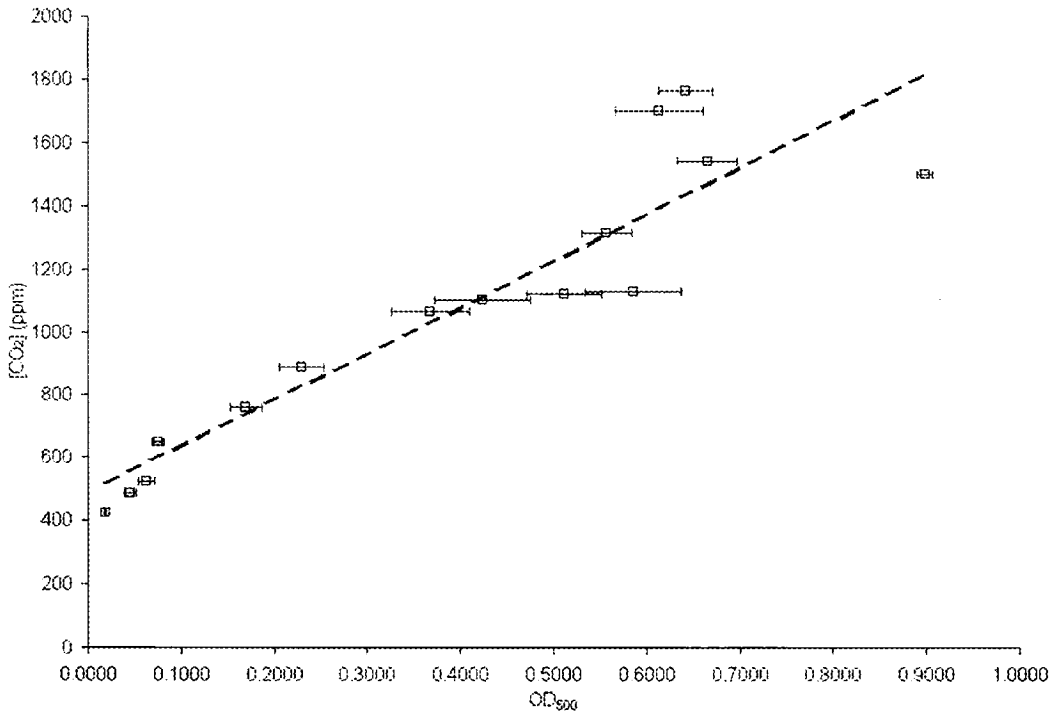

Figure 5-4 Correlation between CO_2 evolution by *R. rhodochrous* and media OD_{500} measurements for the Complex Batch A experiment.

Figure 5-5 is a plot of the concentration of the degradation products 2-ethyl-hexanol and 2-ethylhexanoic acid as well as that of DEHA during the Complex Batch A experiment. Neither of the two DEHA degradation products were observed before 15 h.

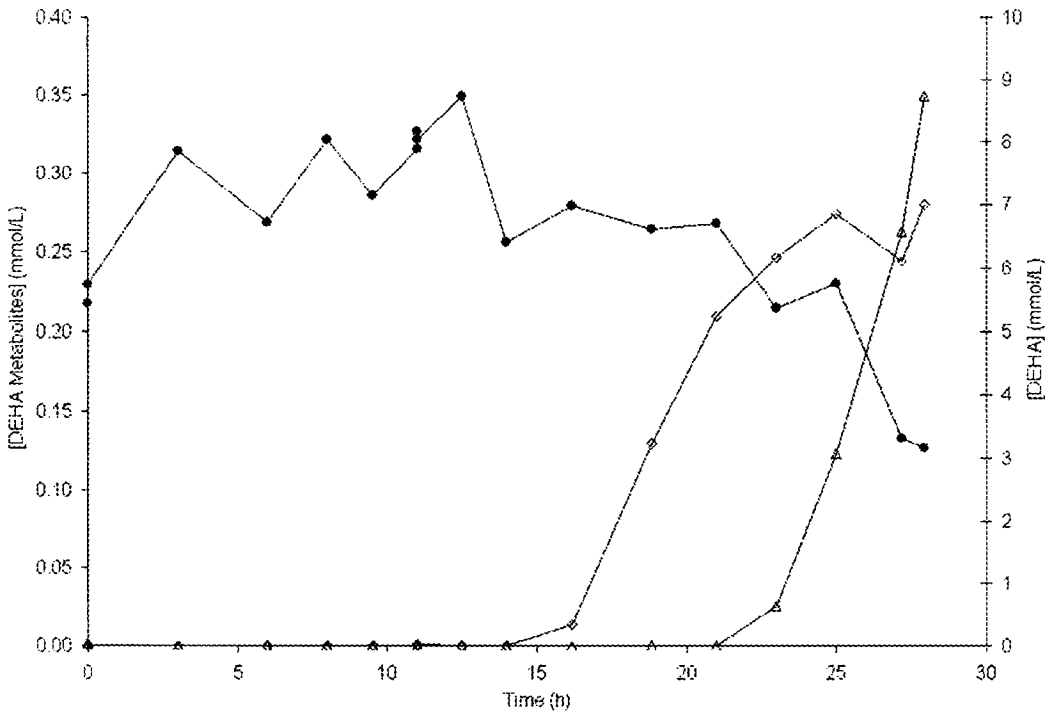

Figure 5-5 Production of 2-ethyl-hexanol (◇) and 2-ethyl-hexanoic acid (△) and the degradation of DEHA (●) in the Complex Batch A experiment, which had an initial substrate concentrations of 10 mmol/L adipic acid and 8.5 mmol/L DEHA.

Figure 5-6, Figure 5-7 and Figure 5-8 contain data for the Complex Batch B experiment. A number of sharp peaks were observed in the CO_2 evolution (Figure 5-6) and the concentrations of CO_2 observed in the air outlet stream of the fermentor were attenuated, as compared to those of Figure 5-1 and Figure 5-3.

The CO_2 measurements in Figure 5-6 indicated that two phases of growth, with each followed by a stationary phase that started at 7 h and 26 h. However, this pattern of growth was not observed in the OD_{500} measurements. It is likely that the interruptions in the air supply disturbed the growth of *R. rhodochrous* in the system and are responsible for discrepancies between the Complex Batch A and Complex Batch B experiments.

The interruption in the air flowrate through the reactor caused stagnation of the air flowing past the CO_2 sensor, which resulted in sharp increases in the

concentration of CO_2 observed. To address this issue, adjustments were made to the power supply of the unit to increase its tolerance to voltage fluctuations in the power line, thereby increasing its tolerance to power-supply failures. Additionally, a line-conditioning unit (TrippLite, Chicago IL, USA, Model 60610-4188) was installed to reduce the voltage fluctuations experienced by the experimental apparatus.

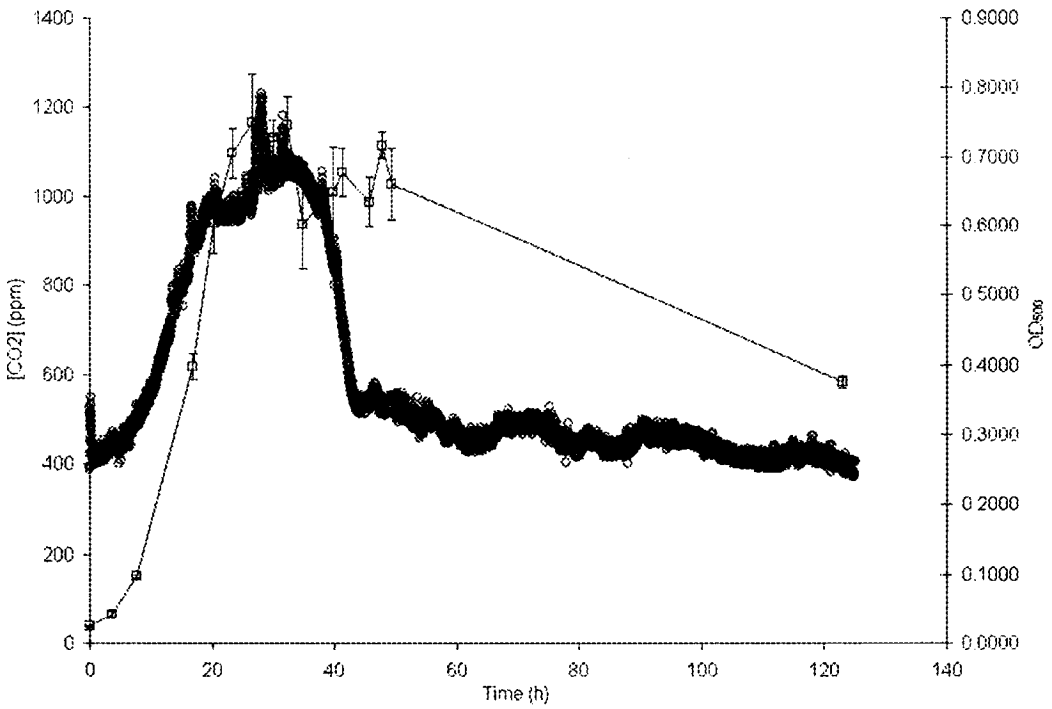

Figure 5-6 Generation of CO_2 (○) by *R. rhodochrous* and media OD_{500} (□) measurements for the Complex Batch B experiment, which was operated for an extended period and had an initial substrate concentrations of 10 mmol/L adipic acid and 8.5 mmol/L DEHA.

Figure 5-7 is a plot of the correlation between the CO_2 and OD_{500} measurements from the Complex Batch B experiment. A general trend of increasing CO_2 evolution with increased bacterial growth was observed, however, a range of CO_2 concentrations were measured for relatively similar values of OD_{500}, notably at OD_{500} values of approximately 0.4000 and 0.7000.

Consequently this trend was not as distinct as observed in Figure 5-2 and Figure 5-4.

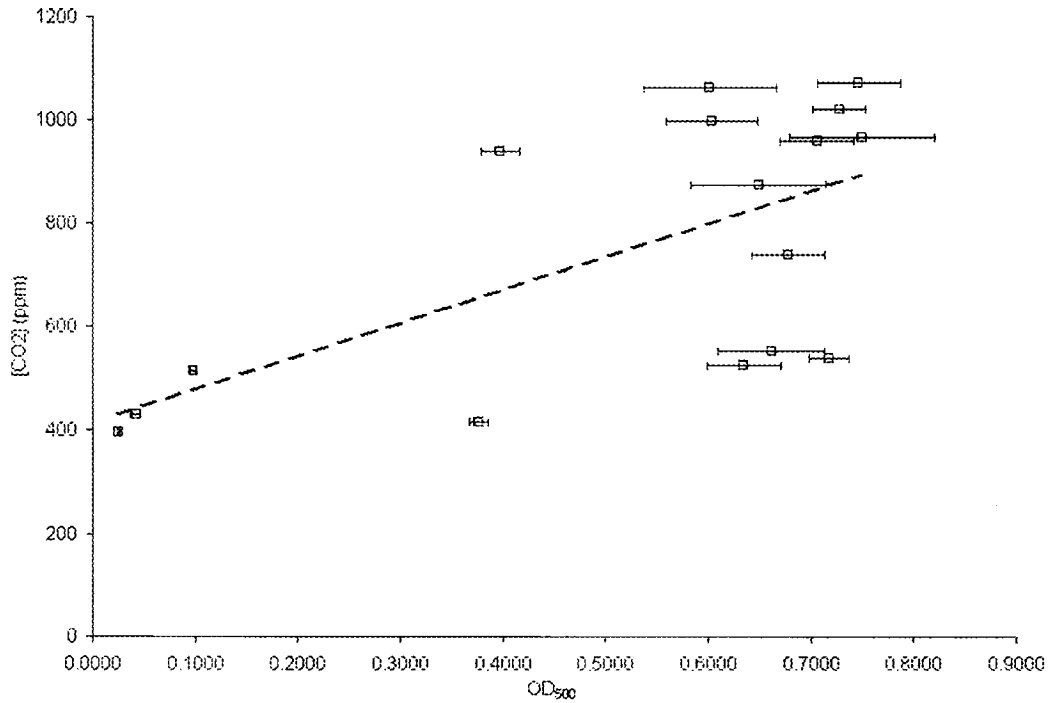

Figure 5-7 Correlation between CO_2 evolution by *R. rhodochrous* and media OD_{500} measurements for the Complex Batch B experiment.

The metabolite concentrations shown in Figure 5-8 exhibited behaviour similar to that in Figure 5-5 and showed that DEHA was degraded in the system, despite disturbances in the air flowrate and growth of the organism.

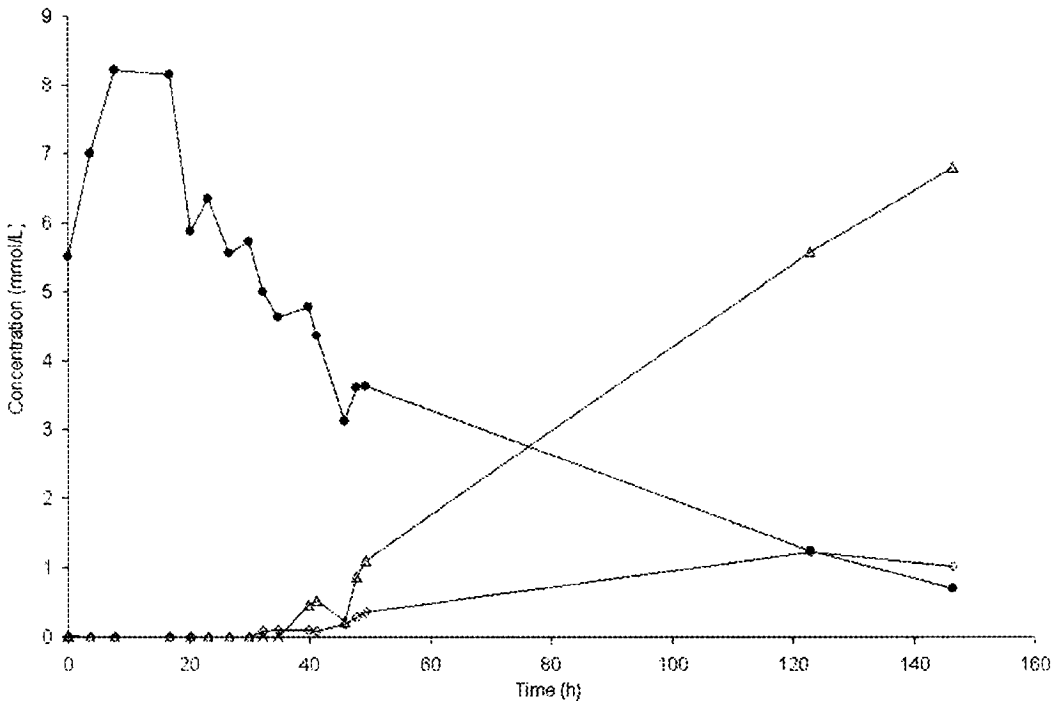

Figure 5-8 Production of 2-ethyl-hexanol (◇) and 2-ethyl-hexanoic acid (△) and the degradation of DEHA (●) for the Complex Batch B experiment, which was operated for an extended period and had an initial substrate concentrations of 10 mmol/L adipic acid and 8.5 mmol/L DEHA.

5.3. Self-Cycling Fermentation

Figure 5-9 is a plot of the CO_2 evolution in the Simple SCF A experiment. The lack of data after 1 d was caused by a blockage in the feed to the fermentor that occurred during cycling of the reactor. Consequently, the fraction of *R. rhodochrous* remaining in the fermentor had no new substrate upon which to grow during this period. Upon removal of the blockage on day 2, media from the reservoir drained into the fermentor, thus completing the cycle. Subsequent cycles exhibited an unexpected pattern of CO_2 production; up until day 7, the culture of *R. rhodochrous* produced decreasing amounts of CO_2 in a trend towards producing almost-constant concentrations of CO_2 despite the addition of fresh media containing adipic acid at a concentration of 10 mmol/L during each cycle.

On day 7, yeast extract was added to the reservoir media at a concentration of 0.1 g/L and when a cycle was initiated, the culture of *R. rhodochrous* began producing CO_2 in a pattern consistent with growth on one substrate, as seen in Figure 5-1. On day 10, biomass partially-blocked the air flow through the reactor, which caused saturation of the CO_2 sensor. Removal of the blockage was attempted but was unsuccessful as the remaining biomass obstruction caused fluctuations in the air flowrate. Consequently, subsequent CO_2 measurements did not accurately represent the growth conditions of the culture and the experiment was terminated on day 11.

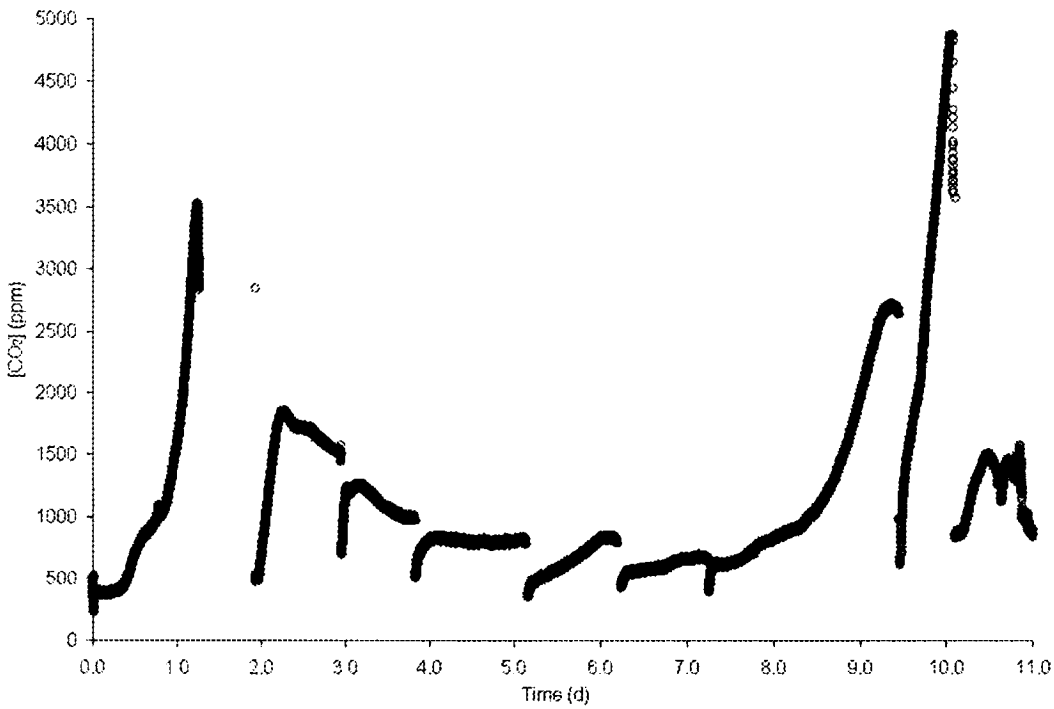

Figure 5-9 CO_2 generation by *R. rhodochrous* in the Simple SCF A experiment, which had an initial substrate concentration and reservoir media substrate concentration of 10 mmol/L adipic acid.

Figure 5-10 shows the pattern of CO_2 generation in the Simple SCF B experiment. A pattern of repeatable cycles was observed between day 7 and day 12 (cycles 6 and 15, respectively), however the reactor became contaminated after

day 12. As seen in Figure 5-10 and Figure 5-11, the length of each cycle began to increase after day 12. Contamination of the culture was confirmed at day 15 via agar plate.

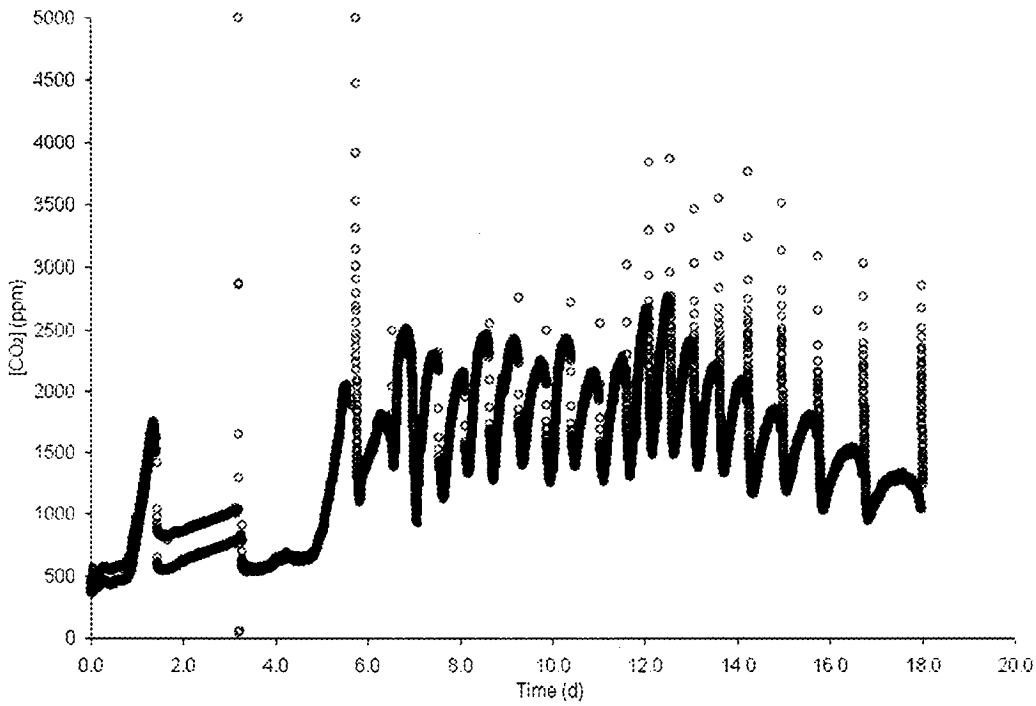

Figure 5-10 CO_2 generation by *R. rhodochrous* in the Simple SCF B experiment, which had an initial substrate concentration and reservoir media substrate concentration of 10 mmol/L adipic acid.

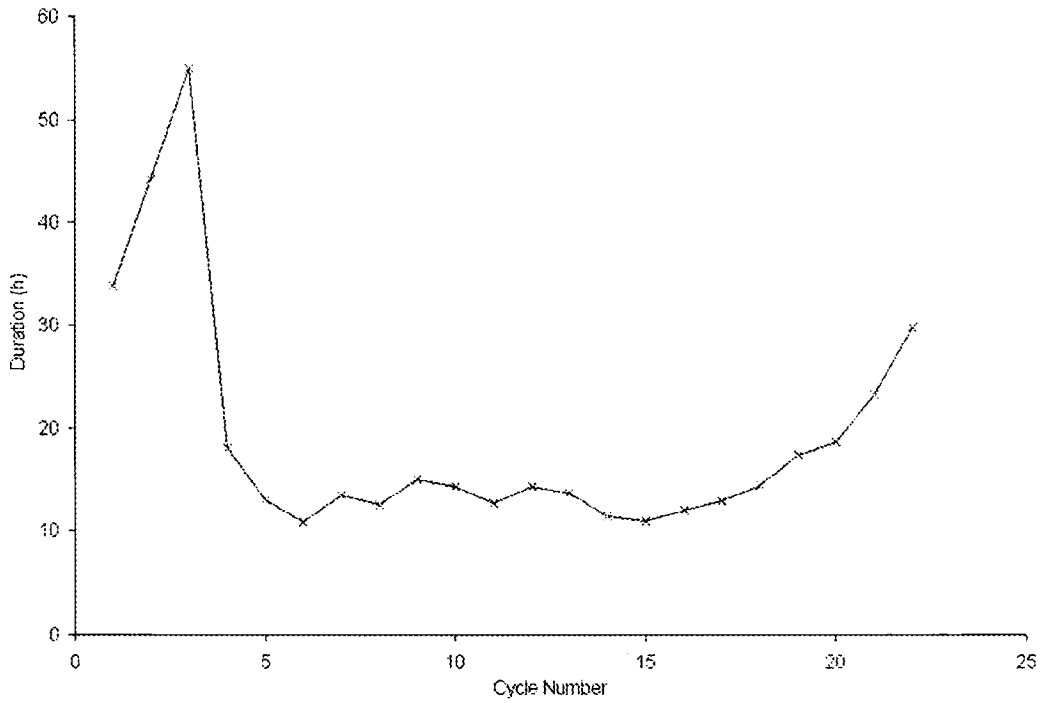

Figure 5-11 Cycle durations in the Simple SCF B experiment.

The Simple SCF B experiment produced relatively stable cycles until contamination was observed (Figure 5-11). During the period of stable cycles between day 7 and day 12 (cycles 6 and 15, respectively), the duration of these stable cycles exhibited variation not previously reported [42].

5.4. Biodegradation of Di-2-Ethyl-Hexyl Adipate (DEHA), in the Presence of Adipic Acid, by *Rhodococcus rhodochrous* Using Self Cycling Fermentation

Figure 5-12 shows the CO_2 data obtained from the Complex SCF experiment and Figure 5-13 shows the cycle durations. It appeared on day 6 (cycle 5) that the system would begin producing a series of repeatable cycles similar to that seen in Figure 5-10, however this was not observed beyond day 7 (cycle 8). On day 12, 9

mL of DEHA was injected into the system during the cycling of the reactor. The CO_2 and OD_{500} data for the cycle following the injection of DEHA are plotted against time in Figure 5-14 and against each other in Figure 5-15.

Figure 5-13 indicated that while there were periods during which the cycle durations were relatively stable, the variation in the patterns of CO_2 evolution seen in Figure 5-12 was not representative of the reproducibility previously observed [18, 51, 54].

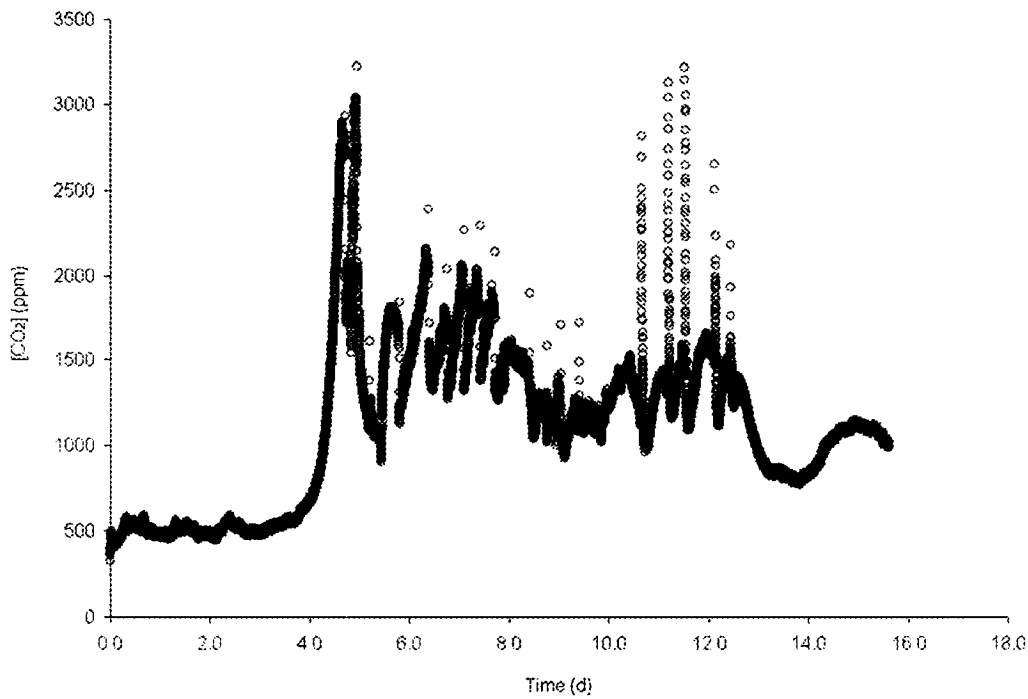

Figure 5-12 CO_2 generation by *R. rhodochrous* in the Complex SCF experiment, which had an initial substrate concentration and reservoir media substrate concentration of 10 mmol/L adipic acid. At the beginning of the last cycle of the experiment, 9 mL of DEHA was injected into the reactor.

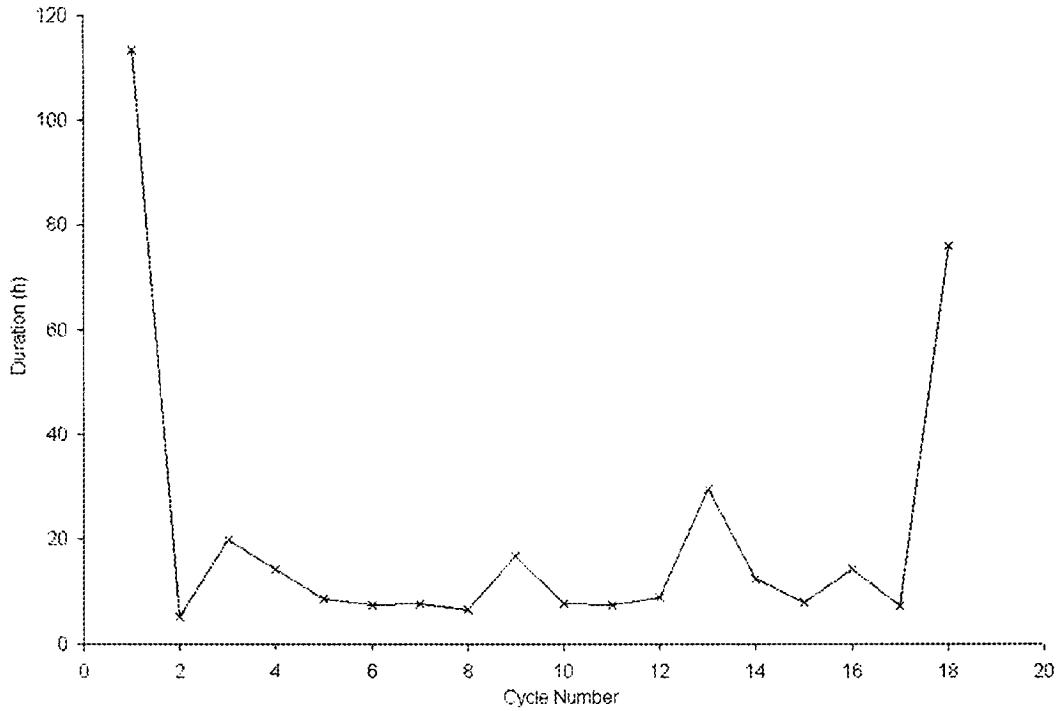

Figure 5-13 Cycle durations in the Complex SCF experiment.

Following the injection of DEHA, the CO_2 data in Figure 5-14 showed the expected increase in CO_2 production following the beginning of a cycle; however this increase in CO_2 production reached a maximum at 4.5 h into the cycle and began to decrease. This peak occurred before the threshold time for cycling and the subsequent decrease in CO_2 production was less than 100 ppm/h. The rate of CO_2 production remained relatively constant between 13 d and 14 d, after which the CO_2 production began to increase again, though no distinct maximum was observed between 14 d and the end of the experiment. This combination of factors resulted in the extended cycle observed.

The OD_{500} measurements somewhat followed the CO_2 measurements, although the pattern was not as clear as with the batch experiments. Additionally, there were discrepancies between the CO_2 and OD_{500} data. There was a distinct increase in the OD_{500} measurements between 12.5 d and 13.5 d when the CO_2

measurements were decreasing. More generally, the OD_{500} measurements were at their lowest in the first 10 h of the cycle when the CO_2 readings were at their highest, while the OD_{500} measurements were at their highest at 14.5 d and 15.5 d when the CO_2 measurements were at a relatively constant, mid-range level compared to the other CO_2 readings taken during the experiment (see Figure 5-12). As a consequence of this pattern of CO_2 production, the correlation in Figure 5-15 was not nearly as clear as those seen in Figure 5-2 and Figure 5-4 and it appeared to have a general trend of the CO_2 evolution decreasing with increasing microbial growth, as indicated by OD_{500} measurements.

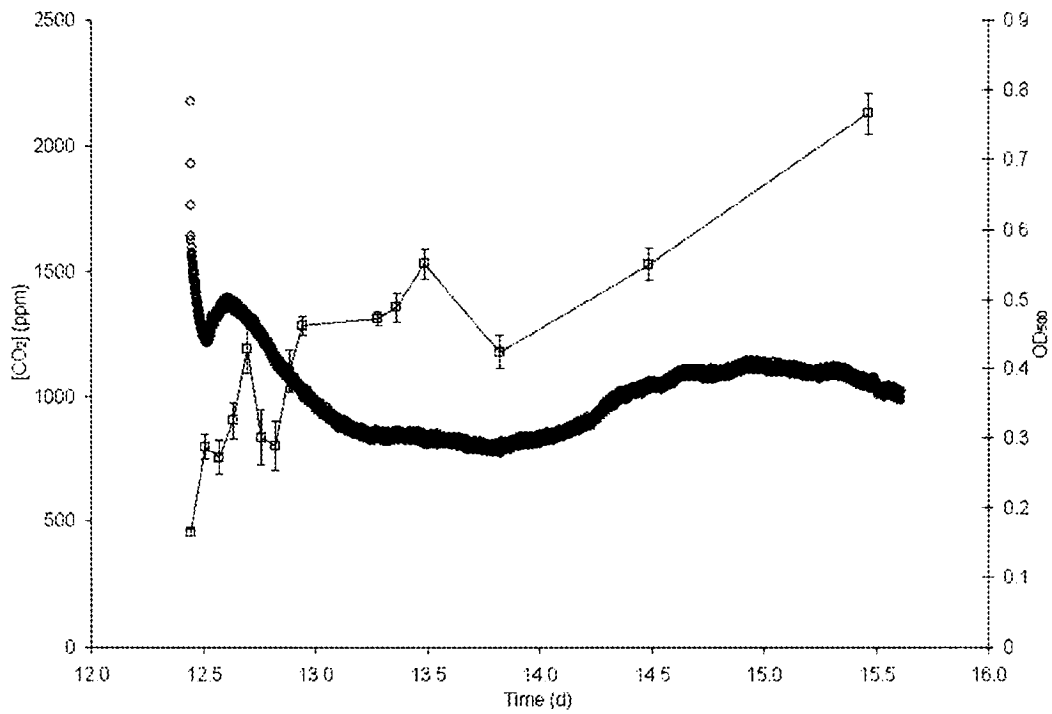

Figure 5-14 Generation of CO_2 (○) by *R. rhodochrous* and media OD_{500} (□) measurements in the final cycle of the Complex SCF experiment.

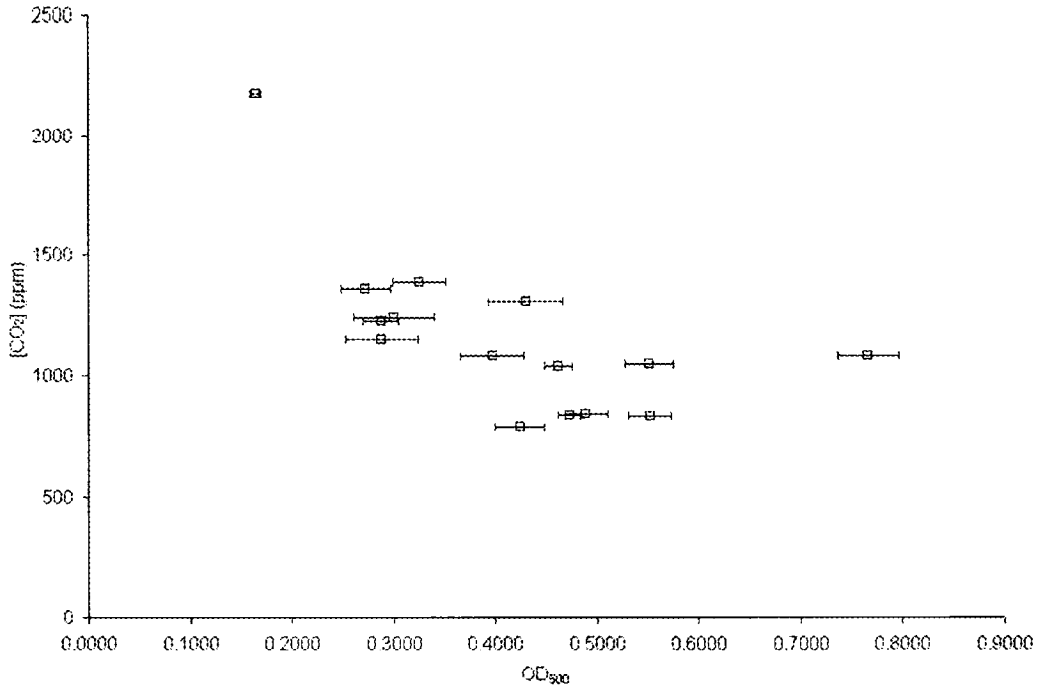

Figure 5-15 Correlation between CO_2 evolution by *R. rhodochrous* and media OD_{500} measurements in the final cycle of the Complex SCF experiment.

Figure 5-16 shows the consumption of the adipic acid and DEHA substrates and the production of metabolites in the cycle following the injection of DEHA into the system. In contrast to the batch experiments, the degradation of DEHA was observed immediately by the production of 2-ethyl-heaxnol.

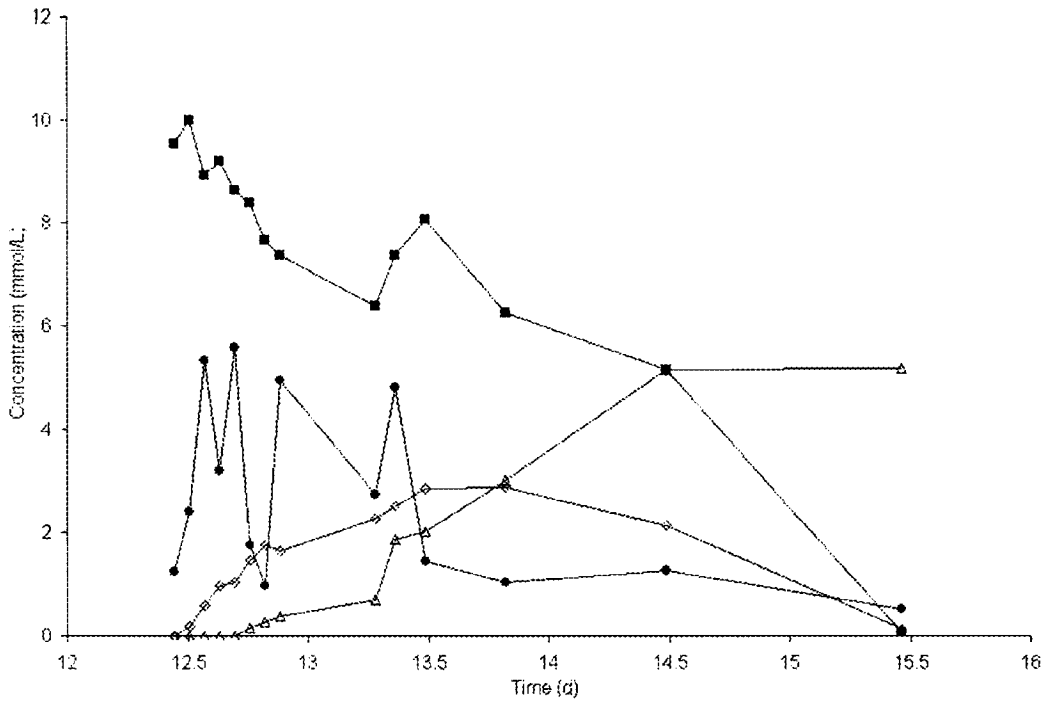

Figure 5-16 Concentrations of Adipic Acid (■), DEHA (●), 2-ethyl-hexanol (◇), and 2-ethyl-hexanoic acid (△) during the final cycle of the Complex SCF experiment.

6. Discussion

6.1. Adaptation of Self-Cycling Fermentation to a Continuously-Stirred Tank Reactor

The motivation for adapting self-cycling fermentation (SCF) to a continuously-stirred tank reactor (CSTR) was to develop a technique to study microbial growth in systems with complex substrates; in particular the degradation of recalcitrant xenobiotic compounds. The co-metabolic degradation of DEHA by *R. rhodochrous* is an example of such a system. DEHA is not easily biodegraded and significant degradation only takes place in the presence of a primary, more easily degraded substrate. As well, most of the biodegradation occurs after the primary substrate has been utilized [14].

There were several types of problems that needed to be overcome. Previous SCF studies used dissolved oxygen (DO) as a control parameter. This required the immersion of a "direct-contact" probe in the fermentation media and led to problems such as fouling of the sensor from biomass accumulation on the probe surface. This in turn resulted in inaccurate measurements in extended fermentations [51]. Another problem associated with DO is that many of these probes could not be autoclaved, which is necessary in these experiments to ensure that cultures did not become contaminated [50]. The solution used was to adapt the SCF system for control based on CO_2 production by *R. rhodochrous* to indicate the growth state. The CO_2 probe can be placed in the non-sterile air outlet stream of the reactor, which avoided problems with fouling from biofilm

formation and also made it much easier to ensure sterile conditions in the experiment.

The use of level switches in the experimental apparatus was necessary to cycle the stirred tank reactor. Previous SCF studies with cyclone reactors made use of gravimetric measurements to automate reactor cycling. However, this approach was prone to fluctuations in the volumes removed [42, 55] and this problem would only have been worse with the much heavier CSTR set-up.

Finally, with the complexities of monitoring the use and degradation of multiple substrates, it was desirable to have flexibility in the size of the harvest fractions. The earlier work with SCF had always used a 50% harvest fraction. It was important to show that this was not an essential factor for stable SCF operation. The work presented here used a harvest fraction of 65%.

6.2. CO_2 Evolution by *Rhodococcus rhodochrous* Resulting from the Co-Metabolic Degradation of DEHA

Batch experiments indicated that CO_2 production by *R. rhodochrous* could be used as the monitored variable for SCF with a simple system of growth on adipic acid as the sole carbon and energy source. There was the expected pattern of CO_2 production and a direct correlation between the number of *R. rhodochrous* cells present in the media and the concentration of CO_2 measured in the air outlet stream.

Experiments with a complex substrate of both adipic acid and DEHA were also encouraging in that there was a direct correlation between the number of *R.*

rhodochrous cells present in the media and the concentration of CO_2 measured in the air outlet stream. The fact that this correlation existed for growth on both the simple substrate of adipic acid and the complex substrate suggested that the concentrations of DEHA used in experimentation did not adversely affect the growth of the culture, in agreement with other investigations [13, 14]. However, the pattern of CO_2 production was a more complicated pattern and was consistent with two phases of growth.

The presence of what appeared to be two growth phases can be explained by the release of adipic acid during the co-metabolic degradation of DEHA [61]. Although *R. rhodochrous* cannot use DEHA as its sole carbon source [14, 61], the release of adipic acid during the degradation of DEHA effectively allowed DEHA to act as a secondary source of energy.

Other evidence of the CO_2 production representing the growth state of the culture was seen in the sharp decrease in CO_2 production, which corresponded to the accumulation of DEHA metabolites in the media. Increased concentrations of 2-ethyl-hexanol and 2-ethyl-hexanoic acid are associated with an increase in media toxicity [2, 13]. Also considering that the degradation of DEHA by *R. Rhodococcus* releases adipic acid as an additional source of energy and that DEHA was still present in the liquid media (Figure 5-5 and Figure 5-8), the sharpness of the decrease in CO_2 production following the secondary growth phase was consistent with a reduction in the metabolism of the culture caused by this increase in toxicity.

Despite the above complexity, there was still an obvious linear correlation between the CO_2 produced and the OD_{500} measurements. This correlation

indicated that the time of maximum CO_2 production corresponded to the time of maximum biomass in the culture. Thus the detection of the CO_2 maximum via computer monitoring could be used to determine the time-to-cycle for the SCF reactor as previously proposed [18, 54] and implemented [51], in other work.

The CO_2 data from SCF experiments demonstrated that computer-controlled cycling of the reactor was successful when using this control strategy as the culture exhibited growth during cycles and remained viable during extended operation or periods of nutrient limitation (Figure 5-9). Detection of the CO_2 maximum as the time-to-cycle the reactor in the feedback control loop was effective, since cycling of the reactor was unaffected by variations in the pattern of CO_2 production seen in different cycles.

6.3. The Use of Level Switches in Self-Cycling Fermentation

In earlier SCF experiments that used the weight of the whole apparatus to monitor the fluid levels during emptying and filling, it was necessary to stop the mixing process during cycling to minimize fluctuations in the measurements. Many recalcitrant xenobiotic compounds have limited aqueous solubility [12]. If agitation was stopped during cycling, these insoluble components in the media could accumulate in the reactor over time as a result of phase separation causing the removal of a greater proportion of one phase than the other. Level switches were used while adapting SCF to a CSTR system because this method for reactor cycling did not require stopping the reactor agitation.

The use of level switches worked well and resulted in a significant improvement to the SCF technique, as variations in the volumes removed and replaced during cycling were insignificant compared to those previously reported [42, 55]. As well, this method gives flexibility in setting the harvest ratio. By adjusting the lengths of the mounting stems of the level switches, the harvest fraction in the experimental apparatus used could be easily adjusted.

6.4. Self-Cycling Fermentation Using a 65% Harvest Fraction

Within the context of using SCF to study the degradation of xenobiotic compounds, there are number of situations where harvest fractions other than 50% are desirable. For example, the degradation of xenobiotic compounds by established cell cultures can be studied using small harvest fractions. After injection into the reactor, the degradation of a compound can be monitored, then cycling of the reactor could be used to re-establish the culture before being exposed to another sample. In other cases, studying degradation using a range of harvest fractions can be used to establish degradation kinetics and the degradation efficiencies for different systems. It was also of general interest to show that a 50% harvest fraction was not an essential condition to obtain stable SCF operation.

The use of a harvest fraction of 65% demonstrated that the cell-culture technique of SCF did not require a 50% harvest fraction in order to function. This was seen in the growth of *R. rhodochrous* on a simple substrate of adipic acid over a repeated number of automated cycles (Figure 5-10 and Figure 5-12).

Although more variation in the pattern of cycle periods was observed than in previous studies that utilized a harvest fraction of 50% [50, 51], this could be a consequence of the non-synchronous growth in the culture. It has been shown before that there is a link between synchronous growth and stability and reproducibility of SCF. It is not possible to achieve synchrony with a harvest fraction other than 50% [42].

6.5. Future Work

If SCF controlled by CO_2 production is to be used as a model system to study the degradation of xenobiotic compounds, the effect of culture acclimation on CO_2 evolution must be investigated. In this work, an extended cycle was caused by an altered pattern of CO_2 production following the injection of DEHA into a culture unacclimated to its presence (Figure 5-14). Although DEHA degradation was subsequently observed, the correlation between CO_2 and OD_{500} measurements (Figure 5-15) was not comparable to batch experiments. To study the effects of culture acclimation further, a strategy can be used where the substrate of interest is injected in one cycle and followed by a series of cycles without any addition, at which point the process is repeated. Alternatively, modifications could be made to the experimental apparatus such that small amounts of the substrate of interest are added at the beginning of each cycle. Either of these modifications should result in a culture acclimated to the presence of DEHA whose CO_2 production will trigger automatic cycling of the reactor.

As previously noted, much work has been done with SCF using harvest fractions of 50%. While this study demonstrated this is not necessary to maintain

cycling, a logical extension is the investigation of the effects of a range of harvest fractions upon this system.

Although the maximum CO_2 production was used to determine the time-to-cycle in this work, results from batch experiments suggested other points in the pattern of CO_2 production could be used to trigger cycling; for example, the detection of an increase in CO_2 production after a stationary phase or a return in CO_2 concentrations to a previous value could both be used in the feedback control loop. Moreover, a range of advanced control strategies for biological systems have been developed [62] and could be implemented in the SCF system to trigger cycling for a desired condition in a specific degradation system.

7. Conclusions

The cell-culture technique of SCF was adapted to a CSTR to study the microbial growth on complex substrates, specifically the degradation of xenobiotic compounds and their metabolites. The development of the experimental apparatus and its operation was facilitated by investigating the degradation of DEHA by *R. rhodochrous* in the presence of adipic acid. The degradation of DEHA and the appearance of its metabolites were monitored using gas chromatography.

Results from this study demonstrated that CO_2 production indicated the growth condition of the culture and could be used successfully as the monitored variable in the feedback control loop that trigged reactor cycling. Level switches were used to automate reactor cycling without stopping reactor agitation, which allowed for the presence of insoluble substrates in the media. The use of level switches also improved the repeatability with which volumes were removed and replaced in the system.

The use of a harvest fraction of 65% in experiments demonstrated that it was not necessary to use a harvest fraction of 50% in order to obtain stable SCF cycles.

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