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Liposan Production in the Self-Cycling Fermentor

by

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Abstract

Bioemulsifiers are biologically produced surfactants; of interest because of their ability to stabilize emulsions. Preference for microbial emulsifiers, over their synthetic counterparts, has been steadily increasing in recent years due to their environmentally friendly nature. Liposan is a polymeric bioemulsifier produced by the yeast *Yarrowia lipolytica* (ATCC 8662) when grown on hexadecane.

The industrial production of bioemulsifiers is difficult because of low product concentrations and contamination by residual hydrocarbon, which is often used as a substrate to promote the production of the emulsifier. Self-Cycling Fermentor (SCF) has been shown to increase the yield of many biosurfactants, and improve the degradation of insoluble substrates. Fed-batch fermentation, with a hydrocarbon substrate, has been shown to result in better substrate utilization when applied to standard batch growth. In this work, the effects of continuous hydrocarbon addition on growth, liposan production, and residual hydrocarbon in a Self-Cycling fermentor are studied. Continuous hydrocarbon addition to the SCF is compared to the standard method of initial hydrocarbon addition. The continuous addition method is shown to increase the final concentration of liposan, and reduce the concentration of residual hydrocarbon over the initial addition method.

Résumé

Les bio-emulsifiants sont des surfacatntes produits biologiquement et retinnent notre attention grâce à leur habileté de stabiliser les émulsions. Depuis qulques années, une préférence accrue pour les émulsifiants microbiologiques plutôt que pour leurs homologues synthétiques a été observée. Liposan est un bio-emulsifiant polymérique, produit par la levure *Yarrowia lipolytica* (ATCC 8662), dont la culture est stimulée par hexadecane.

La production industrielle des bio-émulsifiants est difficile à casue de ses basses concentrations et à cause de la contamination de l'hydrocarbure résiduel qui est souvent utilisé comme substrat pour promouvoir la production de l'émulsifiant. Par le passé, il a été démontré que le Self-Cycling Fermentor accroît la production de plusieurs biosurfactants et améliore la déchéance des substrats insolubles. La fermentation fed-batch avec un substrat d'hydrocarbure permettrait une meilleure utilisation du substrat quand la fermentation fed-batch est appliquée à une culture de lots standards. Ce présent travail étudie l'ajout contnuel d'hydrocarbure au SCF comme méthode pour accroître la production de liopsan et réduire la concentration d'hydrocarbure résiduel. Nous avons aussi acquis des connaissances sur le mécanisme de la production bio-émulsifiante et de la culture microbiologique au hexadecane.

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1 Introduction

1.1 Biosurfactants and Bioemulsifiers

Surfactants are amphiphilic molecules that partition preferentially at the interface between phases of different degrees of polarity and hydrogen bonding, such as oil and water or air and water. The surfactants form an ordered molecular film at the interface that lowers the interfacial energy between the two phases[1,2,3]. By affecting the interfacial properties, surfactants can stabilize emulsions of one phase held as droplets within the other. Emulsifiers also exhibit emulsification properties, and are often categorized with surfactants, although emulsifiers do not necessarily lower surface tension[4].

The properties of surfactants and emulsifiers make them potentially useful in any industry dealing with multiphase systems. The total sales of specialty synthetic surfactants in 1992, was estimated at 1.7 billion dollars in the USA[5], and 9.4 billion dollars worldwide[6]. Most of these synthetic surfactants are derived from petroleum[3].

A variety of organisms, including humans, synthesize chemicals with surface-active properties[7]. The physiochemical properties of these biological surfactants, such as their effects on interfacial tension, heat, and pH stability, have been shown to be comparable to their synthetic counterparts[8]. Properties such as higher biodegradability, better environmental compatibility, higher selectivity, and specific activity at extreme temperatures, pH, and salinity have been demonstrated in some cases.[7,9,10]

Interest in microbial emulsifiers has been steadily increasing in recent years due to their diversity and environmentally friendly nature[1,3,17]. Microbial emulsifiers are of

specific interest to the petroleum industry to aid in enhanced oil recovery, and to stabilize emulsions in the cosmetics and food industries[1,3,7,11,12,13,14,15,16,17]. There is also a growing interest in the isolation and characterization of bioemulsifiers for use in the bioremediation of hydrocarbon contaminated soils [1,7,11,13,14].

1.2 Liposan Production by *Yarrowia lipolytica*

Many new microbial emulsifiers have been isolated and characterized for their potential uses as gelling agents, stabilizers, flocculants and lubricants[17,18,19]. Liposan is a bioemulsifier produced by the organism *Yarrowia lipolytica*[3,4,12,17,22]. It was first isolated by Cirigliano for its potential use in the food industry[12,22]. It is water-soluble, and has been shown to be most effective under neutral to low pH, with maximum activity between pH 2 and 5. In this work, the organism was listed as being in the genus *Candida* but has now been moved to the genus of *Yarrowia*.

Bioemulsifiers are often divided into two groups[2,20,21]: low-molecular-weight compounds and polymers. The first group includes compounds such as glycolipids and phospholipids. These compounds lower the interfacial tension between hydrophobic liquids and water thereby reducing the energy required to form emulsions. The second group of bioemulsifiers stabilize emulsions by coating and interfering with the coalescence of the suspended phase. This group of compounds tends to not have a strong effect on surface tension.

Liposan is a polymeric emulsifier, which has been shown to stabilize oil in water emulsions[12,22]. It is similar to other polysaccharide thickeners such as gum Arabic and Xanthan gum, both used in the food industry[23]. The emulsan group of compounds,

produced by *Acinetobacter sp.*, and many of the cell wall components of *S. cerevisiae* (Baker's Yeast) are other, well studied, polymeric bioemulsifier[16,24].

1.3 Bioemulsifier Production

The commercial application of bioemulsifiers will be determined by their cost and properties in relation to competing synthetic compounds[1,2]. At present, the use of bioemulsifiers in the food and cosmetics industries is not generally competitive because of their higher production costs[1,25] (2 to 3 times more expensive)[3]. The petrochemicals industry is one area that has been successfully penetrated by biologically produced emulsifiers[1,7,20]. The production of the bioemulsifier emulsan for the oil industry has been recently estimated to be 400-500 tons annually[7]. As environmental factors become increasingly important, the push for biologically based chemicals will become stronger.

As with any chemical process, the goal in the production of a bioemulsifier is to maximize the productivity (g/l/h), to increase the yield of the product from the carbon source and to achieve a high final concentration. In addition, it is important to reduce the accumulation of other metabolic waste-products that may interfere with the recovery of the desired product[1,7,20,26].

The recovery of the biosurfactant product can account for a large proportion of the production cost[1] (60 % of total production cost)[3]. The low concentrations in which most bioemulsifiers are produced adds to the difficulty and to the cost of production and separation[1,27].

1.4 Residual Hydrocarbon Contamination

Liposan, along with many other biosurfactants, is produced by an organism growing on hydrocarbon[27,28]. One problem associated with the biological production of emulsifiers from a hydrocarbon substrate is that the substrate is not fully utilized at the end of the fermentation. The residual hydrocarbon may contaminate the spent fermentation broth. This can interfere with the properties of the emulsifier and can further complicate the separation procedure[1].

The problem of residual hydrocarbon has not been fully examined in the literature and few published works make note of it[1]. Studies are needed which quantify the residual hydrocarbon concentration in microbial fermentation and investigate methods for its reduction.

1.5 Self-Cycling Fermentation

Self-cycling fermentation (SCF) is a technique in which sequential batch fermentations are performed using a computerized feedback control scheme. For this work, the dissolved oxygen in the fermentor was used as the control parameter. As the concentration of cells in the fermentor increases, the dissolved oxygen decreases. When cell growth stops, the concentration of dissolved oxygen increases. The resulting minimum in dissolved oxygen is detected by the control computer and the fermentor is cycled; half of the reactor's contents are removed and replaced by fresh medium. The process then begins again. The period between consecutive minimums in dissolved oxygen is a cycle; the duration of a cycle is the cycle time.

Previous work with the SCF has shown its potential for complete utilization of the limiting nutrient, and a greater degree of degradation of toxic compounds[29,30]. This will help in reducing the concentration of residual hydrocarbon. The SCF has also been shown to increase the production of compounds similar to liposan[31,32,33]. The most important feature of the SCF is that it has been shown to give very repeatable results, which is difficult to achieve with many biological systems [30,32,33].

1.6 Continuous Hydrocarbon Addition

Most studies on fed-batch fermentation with hydrocarbon substrates have been done with compounds known to be toxic. Fed-batch strategies are employed in an attempt to reduce the toxic effects of these substrate[34,35].

The standard method of running the SCF, with an insoluble substrate, is to add it at the beginning of each cycle. An alternative to this would be to add the substrate continuously throughout the cycle. Continuous hydrocarbon addition to the SCF would be similar to the fed-batch fermentation of a hydrocarbon substrate. Earlier work on fed-batch fermentation with hydrocarbons focused on kinetics and degradation mechanisms, but did show the technique's potential for increasing the production of metabolites produced on hydrocarbon media[36].

2 Objectives

The main objective of this work was to investigate the effects of continuous hydrocarbon addition to the SCF on liposan production by *Yarrowia lipolytica.* To achieve this it was necessary to first find a simple medium on which the organism produced the desired product. A method of feeding the hydrocarbon to the reactor and an assay for measuring the concentration of liposan also needed to be found.

3 Materials and Methods

3.1 Culture Maintenance

Yarrowia lipolytica ATCC 8662 was maintained on Bacto YM Broth (Difco) slants at 4°. The cultures were transferred to new slants monthly. Samples of the organism were also kept frozen at -70° C.

3.2 Inoculum

Inocula for all experiments were grown on YM broth. Erlenmeyer flasks (500 ml) containing 100ml of YM broth were inoculated from slant cultures and grown for 2 days in an incubator shaker at 30°C (New Brunswick Scientific Co., model G25).

3.3 Medium and Growth Conditions

Initial shake flask experiments used the medium of Moo-Young[37]. The composition of this medium is shown in Table 3-1. This medium was eventually replaced by a modification of the Difco Bacto Yeast Nitrogen Base[38] originally used by Cirigliano[12,22]. The composition of the modified YNB medium is shown in Table 3-2. It contains all of the inorganic components contained in the original YNB medium, but does not contain any of the organic additives, except for thiamine hydrochloride.

Cultures in shake flasks were grown in an incubator shaker at 30°C. The heating water, used to control the temperature in the SCF, was maintained at 32 C.

For shake flask experiments, media were steam sterilized at 121°C and 1.2 bar for 30 min. Self-cycling fermentor experiments used larger 1L medium bottles. The SCF medium bottles were steam sterilized at 121°C and 1.2 bar for 3.5 hrs.

The sole carbon source used for all experiments was n-hexadecane, which was added to the media separately after it had been sterilized.

Table 3-1: Medium from Literature[37]

Water	1 L
Yeast Extract	0.1 g
KH2PO4	7.0 g
Na2HPO4	0.5 g
(NH4)2So4	4.0 g
CaCl2	38.2 g
NaCl	0.05 g
Trace elements solution	0.5 ml

Trace Elements Solution	
Water	1 L
CuSO4-5H2O	160 mg
KI	400 mg
FeCl3-6H2O	2000 mg
MnSO4-H2O	27 mg
Na2MoO4-2H20	11 mg
ZnSO4	160 mg
Boric Acid	400 mg

Table 3-2: Modified YNB Medium

Water	1 L
Ammonium Sulfate	5 g
Potassium Phosphate Monobasic	1 g
Magnesium Sulfate	0.5 g
Sodium Chloride	0.1 g
Calcium Chloride	0.1 g
Boric Acid	500 µg
Copper sulfate	40 µg
Potassium iodide	100 µg
Ferric Chloride	200 µg
Manganese Sulfate	400 µg
Sodium Molybdate	200 µg
Zinc Sulfate	400 µg
Thiamine Hydrochloride	400 µg

3.4 Emulsifier isolation

A crude isolation of the bio-emulsifier was done using the procedure of Cirigliano[22]. Shake flasks were inoculated with the organism and allowed to grow for 7 days. The final broth was distributed from the flasks into 30 ml centrifuge tubes and centrifuged for 30 min. at 8000 g and 4°C (Dupont Instruments RC-5 Superspeed Refrigerated centrifuge). After centrifugation, frozen hexadecane was removed from the centrifuge tubes using tweezers and the supernatant was vacuum filtered through 0.22 μm Milipore filter paper.

The cell free filtrate was poured into dialysis tubing (MW cutoff 3500). The tubing was dialyzed in distilled water for 2 hrs to remove any dissolved salts. The distilled water was replaced twice over the two hours of dialysis. The filtrate was then concentrated by pervaporation. Polypropylene glycol (average MW 8000) was used to draw the water out of the tubing. The pervaporation step was done at 4°C and required 3-4 days depending on the amount of polypropylene glycol used. The final volume of the solution was approximately 50 ml.

The concentrated solution was mixed with 450 ml of a chloroform-methanol solution (2:1) in a 1L separatory funnel. The aqueous phase was removed and the organic phase was discarded. The washing procedure was done three times. On the third washing, only one phase was present in the separatory funnel and a white precipitate had formed.

The precipitate was collected on Whatman 1Ps silicone coated filter paper and allowed to dry overnight in a glass vial. After the mass of the precipitate was determined

it was resuspended in distilled water. The solution was then tested for emulsion activity, protein, carbohydrate and fluorescence.

3.5 Biomass Measurements

Biomass measurements were made using a standard dry-weight analysis. 20 ml of broth was centrifuged for 30 min. at 8000•g. Centrifugation was carried out at 4°C to freeze any hydrocarbon. After centrifugation, any hydrocarbon frozen on the surface was removed using tweezers. The supernatant was collected for further tests and the pellet was resuspended in distilled water and centrifuged again (Fisher Vortex Genie 2). Following the second centrifugation, the supernatant was discarded and the solid pellet was washed out of the centrifuge tube, using distilled water, into a small aluminum pan. The pellet was allowed to dry for 48hrs at 105° (Fisher Isotemp Oven 100 series Model 126G). The final weight of the dried biomass was then measured.

3.6 Removal of cells from samples

Samples to be analyzed for extra-cellular components were first centrifuged for 30 min. at 8000g and 4°C. The resulting supernatant was then filtered through 0.22 μm filter paper (Osmonics Inc. Magna, nylon supported, plain, 25mm) using a 10-ml glass syringe and stainless steel filter support. Removing the biomass from samples by filtration, without first centrifuging, was difficult because the filter would become clogged with cells. It was also determined that centrifugation alone did not remove all of the cells from the samples.

3.7 Total carbohydrate

The total carbohydrate of the cell free broth was measured using the phenol reaction method[39]. 1 ml of cell free sample was mixed with 1 ml of a 50 g/L phenol solution and vortexed on maximum for 5 seconds (Fisher Vortex Genie 2). 5 ml of sulfuric acid was then added and the sample was quickly vortexed again. The treated samples were allowed to develop for 30 min. The absorbance of the developed samples was measured at 488 nm using distilled water as the reference (Varian DMS 200 UV visible spectrophotometer). Samples with an absorbance greater than 1.0 were diluted, to bring them into range, and then retested.

The assay was calibrated using a standard glucose solution diluted with distilled water. The calibration curve for the total carbohydrate assay is shown in Figure 3-1. Carbohydrate values are quoted as equivalent g/L of glucose.

3.8 Total Protein

The total protein of the cell free samples was measured using the Biorad DC Protein Assay. The procedure follows that of Biorad. The technique was calibrated using the Biorad Assay Standard II-Lypophilized Bovine Serum Albumin. The calibration curve is shown in Figure 3-2.

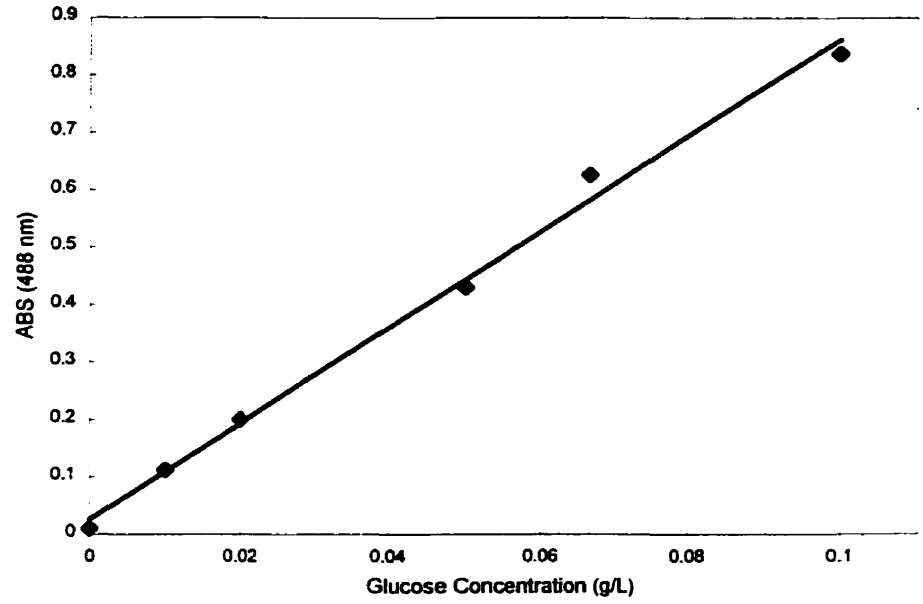

Figure 3-1: Total carbohydrate calibration curve

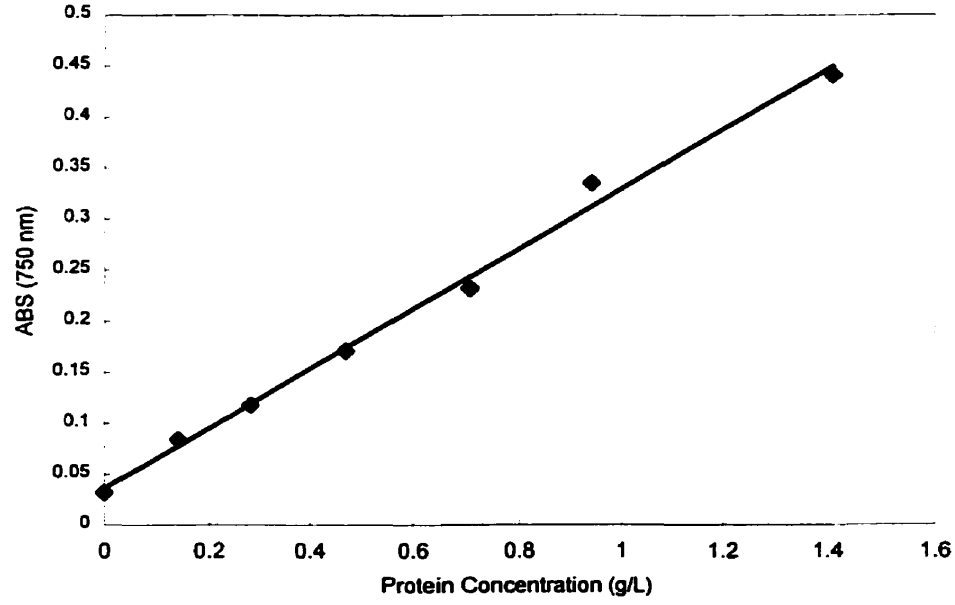

Figure 3-2: Total protein calibration curve

3.9 Fluorescence

The fluorescence of the cell free samples was measured using a Shimadzu RF-1501 spectrofluorophotometer with xenon arc light bulb (Ushio Inc.). Samples were excited at 265 nm, and fluorescence was measured at 340 nm. All samples were diluted ten fold with distilled water to bring their fluorescence intensities into the range of the machine.

3.10 Emulsion Activity

The emulsion activity test was used to measure the activity of the bioemulsifier The test was done by mixing 2ml of cell free filtrate with 2 ml of a sodium-acetate buffer (pH 3.0) and 4 ml of n-hexadecane in a test tube. The mixture was vortexed for 2 minutes and allowed to settle for 6 minutes.

Upon settling, the mixture separated into an upper organic phase, a middle emulsion phase and a lower aqueous phase. The aqueous phase was removed from the test tube using a Pasteur pipette and the remaining emulsion phase was poured into a cuvette. The volume of the organic phase, usually small, would be poured into the cuvette along with the emulsion. If the organic phase was too large, it was removed. Once in the cuvette the optical density of the emulsion was measured at 600nm. If no emulsion was present after settling, the emulsion activity was taken to be zero.

The sodium acetate buffer was made by mixing 3 parts of a 58.14 molal glacial acetic acid solution with 1 part of an 8.203 molal sodium acetate solution.

3.11 Measurement of pH

pH was measured using a Fisher pH electrode (Model 13-620-252) connected to an Orion Research analog pH meter (model 301). pH measurements were taken of the whole reactor broth.

3.12 Toxicity Test

The toxicity of hexadecane was measured using the Microtox toxicity assay. The procedure follows that of Microtox.

3.13 Hydrocarbon

The hexadecane concentration was measured using the technique of Marino[40]. 2 ml of broth were mixed with a 0.01% solution of pentadecane in chloroform and vortexed for 2 min. The organic phase was then analyzed using a gas chromatograph (Hewlett Packard 8950 Series II GC with flame ionization detector; Supelco 2-5361 SPB-1 colum). Operating conditions for the gas chromatograph are summarized in the following table (Table 3-1). Peaks from the gas chromatograph were sent to a personal computer to be integrated (Varian Star Chromatography Workstation software). The ratio of the peak area for hexadecane to the peak area for pentadecane was then calculated.

Table 3-1: Gas chromatograph operating conditions for hexadecane analysis

Initial Temperature	65°C
Initial Time	2.5 min
Rate	10.00 °C/min
Final Temperature	200 °C
Final Time	0.00
Injector Temperature	225 °C
Detector Temperature	250 °C
Carrier Flow Rate	15 ml/min
Head Pressure	50 KPa

The technique was calibrated using mixtures of distilled water and hexadecane. The hexadecane in water mixtures were quickly swirled in 500 ml Erlenmeyer flask and sampled using a Pipetman micropipette. The samples were then tested for hexadecane. The area ratio of hexadecane to pentadecane was plotted against the original concentration of hexadecane. This calibration curve is shown in Figure 3-1.

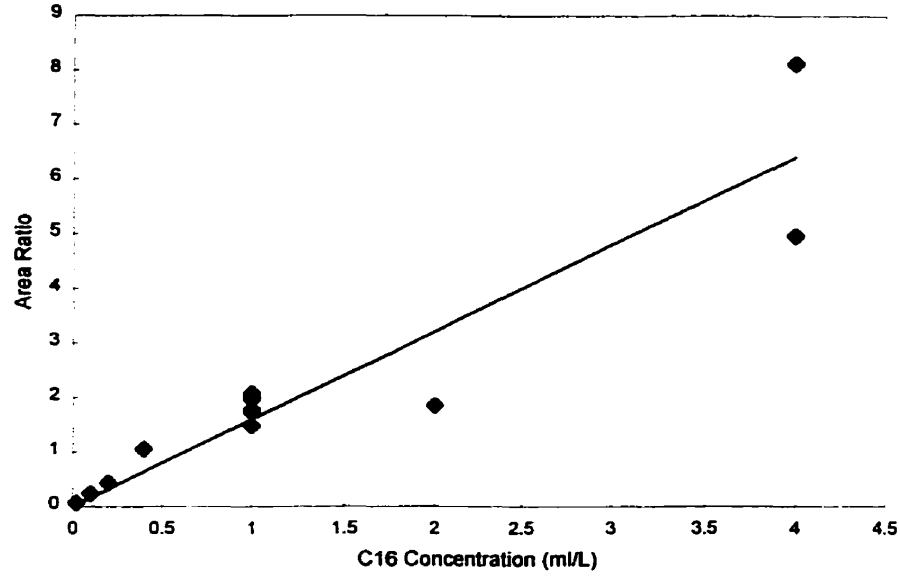

Figure 3-1: Hexadecane calibration curve

3.14 Experimental Apparatus

The experimental apparatus used for the self-cycling fermentation of *Yarrowia lipolytica* was similar to that described by Marino[40]. Changes were made to the location of the hydrocarbon feed line for experiments using continuous hydrocarbon addition. A schematic of the reactor setup is shown in Figure 3-1.

The reactor was set up as a flow loop in which the liquid was circulated through the main cyclone reactor, dissolved oxygen measuring point and water jacket heat exchanger by a centrifugal pump (0.2 hp, March Model MDX). The pump circulated the reactor contents at a rate of 185 ml/sec. The heating water temperature in the heat exchanger was maintained by a recirculating water bath (Haake, model FE2). Air was bubbled into the cyclone portion of the reactor, midway between the liquid level and the bottom of the reactor. The air flow-rate into the reactor was 180 ml/min and was monitored using a gas rotameter (Gilmont 250). A Friedrich's condenser was used at the exhaust point of the reactor to prevent evaporation of the liquid medium. Nalgene air filters were used to isolate the reactor at all points open to the atmosphere including the air inlet line.

Hydrocarbon was added to the reactor using a syringe pump, (Orion Model 341B) and either a 30 cm^3, or 10 cm^3 syringe (Becton Dickinson & Co) connected to the main reactor with Masteflex Viton tubing. The syringe pump calibration curve is shown in Figure 3-2.

For experiments using initial addition, the hydrocarbon inlet was located at the top of the cyclone reactor just below the condenser (Hydrocarbon Feed A on Figure 3-1). For experiments using continuous addition, the hydrocarbon inlet was located between the dissolved oxygen measuring point and the suction side of the recirculating pump (Hydrocarbon Feed B on Figure 3-1).

The dissolved oxygen concentration was monitored using an Ingold polargraphic oxygen sensor (model IL 531). The signal from the probe was fed through a Cole-Parmer amplifier (model 01971-00) and then to a data acquisition board and strip chart recorder (Linear 1200).

It was necessary to periodically remove the dissolved oxygen probe, to clean off biomass that had accumulated over the membrane. The probe was cleaned with a 70% ethanol solution and passed through a flame before being reinserted into the reactor.

The reactor was controlled using an IBM compatible 8088 PC interfaced with a data acquisition board (Data translation Model DT-2801). The control program was similar to that used by Marino except for modifications made to the cycling algorithm, and the additional syringe pump control required for experiment using continuos hydrocarbon addition.

A balance (Precisa 6000D) was used to monitor the liquid level in the cyclone portion of the reactor. The cyclone was held with clamps, and supported by a stand placed on the balance. The stand was stabilized by rings attached to another stand anchored to the workbench. The other components of the reactor were attached to a wood board, also on the workbench.

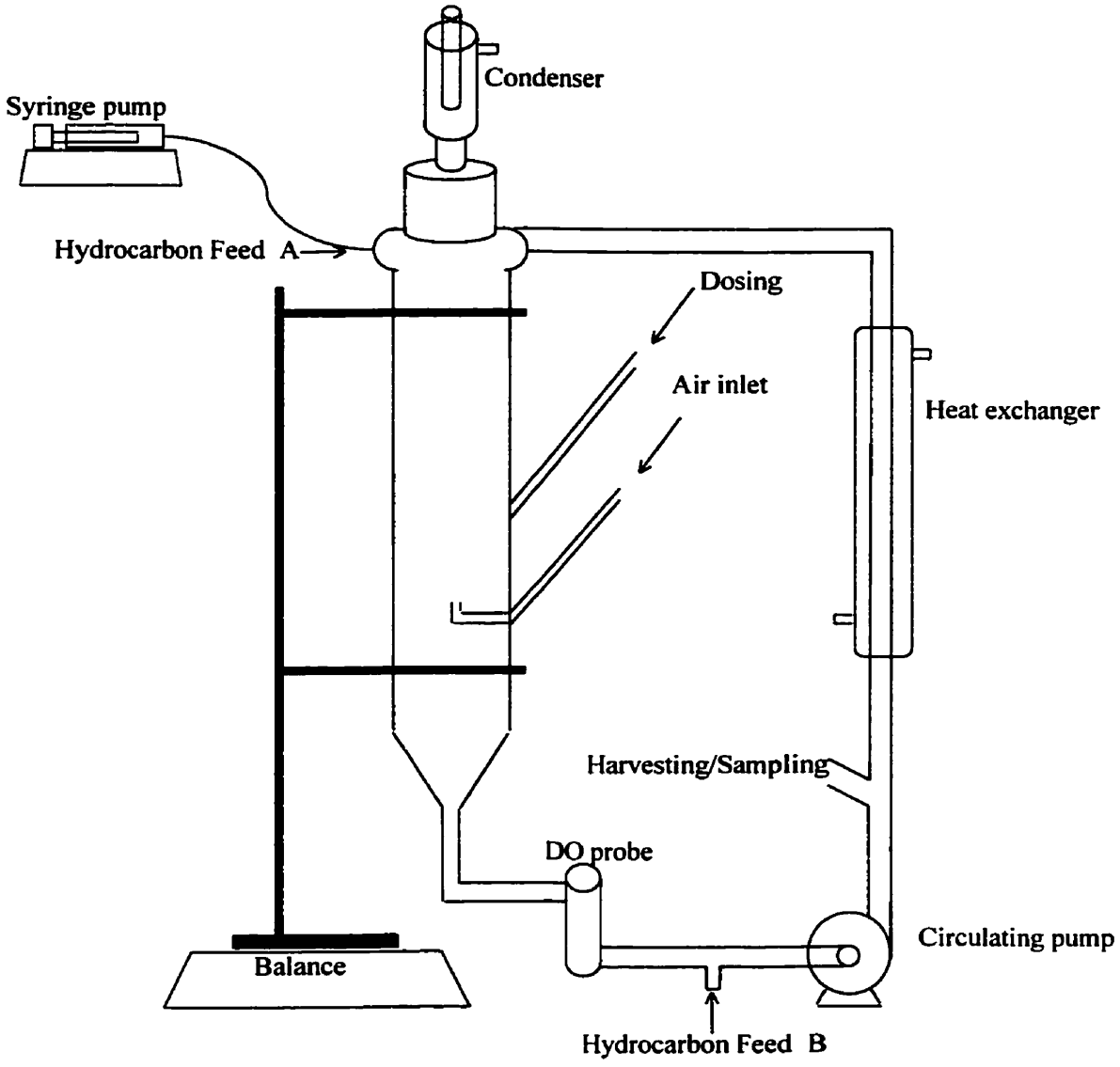

Figure 3-1: Schematic of the Self- Cycling Fermentor

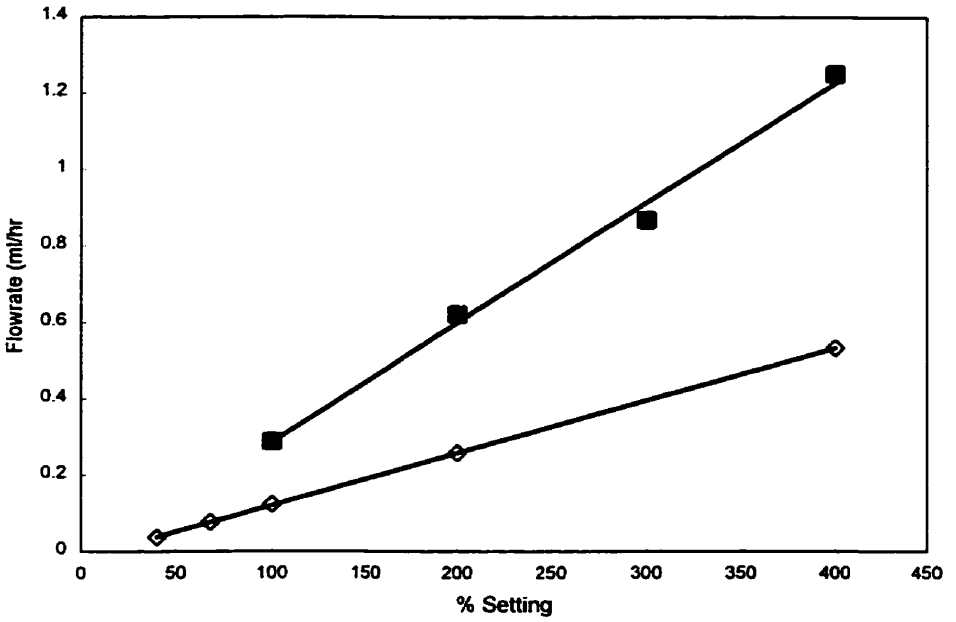

Figure 3-2: Syringe pump calibration curve: 30 ml syringe, 10 ml syringe

4 Results

4.1 Medium Formulation

Yeast Nitrogen Base (YNB), used by Cirigliano *et al.* for the isolation of liposan, is a complex medium consisting of a standard mixture of salts and a large number of additional amino acids and vitamins[12,22]. When *Y. lipolytica* was grown on just the inorganic components of the YNB medium no emulsifier was produced.

Nine of the amino acid and vitamin additives of the YNB medium were tested for their effects on the growth of *Y. lipolytica* and the production of liposan. Nine shake flasks, containing 100 ml of the YNB inorganic base and one of the nine additives, at the same concentration as in YNB medium, were inoculated with *Y. lipolytica*. Samples were taken at three and five days to test for emulsion activity. Growth of the organism was judged by visual inspection of the turbidity of the shake flasks. Table 4-1 summarizes the results of this preliminary screening. No effect on growth, but dramatic effects on emulsifier production were observed. The flask containing thiamine was the only one that, when tested, gave strong emulsions. The flasks containing biotin and riboflavin showed weak emulsion activity. The remaining additives did not have a measurable effect on the emulsion activity.

Table 4-1: Effects of Organic Additives on Growth and Emulsifier Production

Biotin	+	+
Folic Acid	+	-
Methionine	+	-
Tryptophane	+	-
Thiamine	+	++
Riboflavin	+	+
Inositol	+	-
Aminobenzoic	+	-
Pantothenate	+	-
Legend: - Negative	+ Positive	++ Strong

To confirm the effect of thiamine on the production of liposan, a shake flask, containing only the YNB inorganic base, was inoculated from a flask supplemented with thiamine and showing positive emulsion activity. On the first transfer of the growing culture into the new medium emulsification activity dropped significantly. A second transfer into a thiamine free medium resulted in no emulsifier activity. Based on these results, thiamine was included in the growth medium for all subsequent experiments.

4.2 Properties of the Crude Isolate

A crude purification of liposan was done, based on the procedure of Cirigliano *et al.*, so that a method for measuring its concentration could be determined. Diluting a sample of the crude isolate with distilled water showed the effectiveness of the emulsification activity assay and its correlation with the concentration of liposan. The results of this test are shown in Figure 4-1.

Analysis of the crude isolate showed it to be composed of 14.7% protein and 33% carbohydrate. Based on this, protein and carbohydrate assays were tested as alternative methods of measuring the concentration of liposan. Protein and carbohydrate

concentrations were compared to emulsification activity for a variety of different growth conditions. The concentration of carbohydrate correlated well with emulsion activity. This comparison is shown in Figure 4-2. The protein concentration was found to correlate with emulsification activity for individual experiments, but not when data from different experiments were combined. The partially purified liposan fluoresced at 340 nm when excited at 265 nm. This property was later shown not to be a direct result of the emulsifier, but most likely caused by another microbial product found in the crude isolate.

Figure 4-3 shows the effect of the age of refrigerated samples on emulsion activity. The observed decrease in emulsification activity indicated that long periods of storage were undesirable. All subsequent samples were tested for emulsification activity within two days of having been collected.

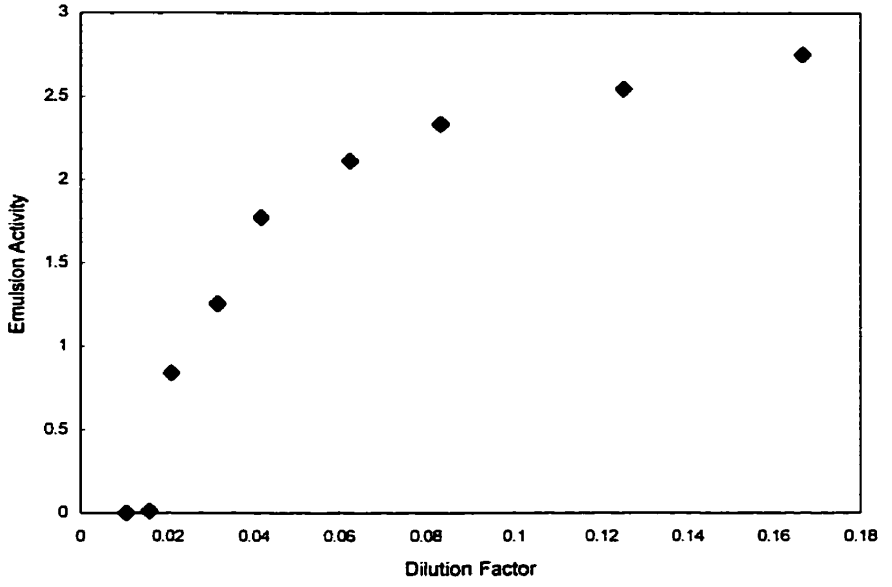

Figure 4-1: Emulsion activity (EA) of diluted crude-isolate

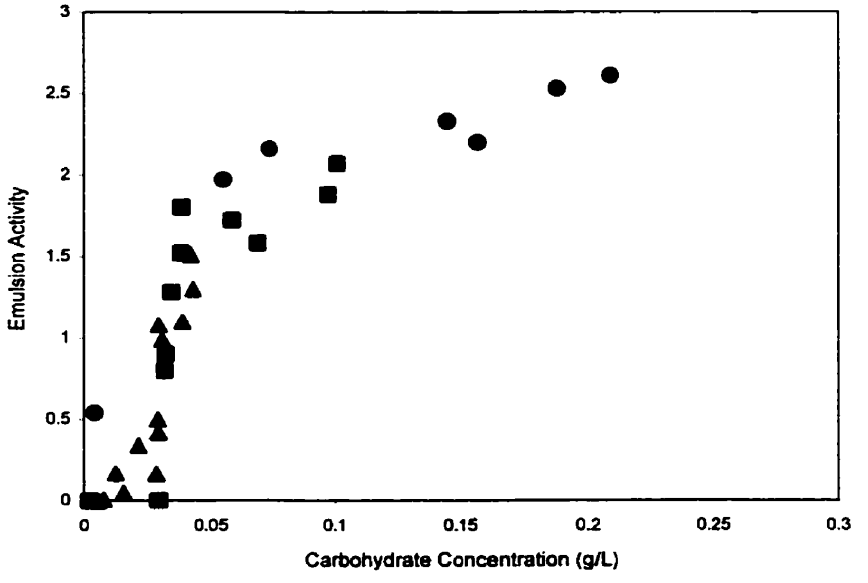

Figure 4-2: Correlation between carbohydrate concentration and emulsion activity. ■ End of cycle, ▲ Batch, ● Two-Stage Reactor

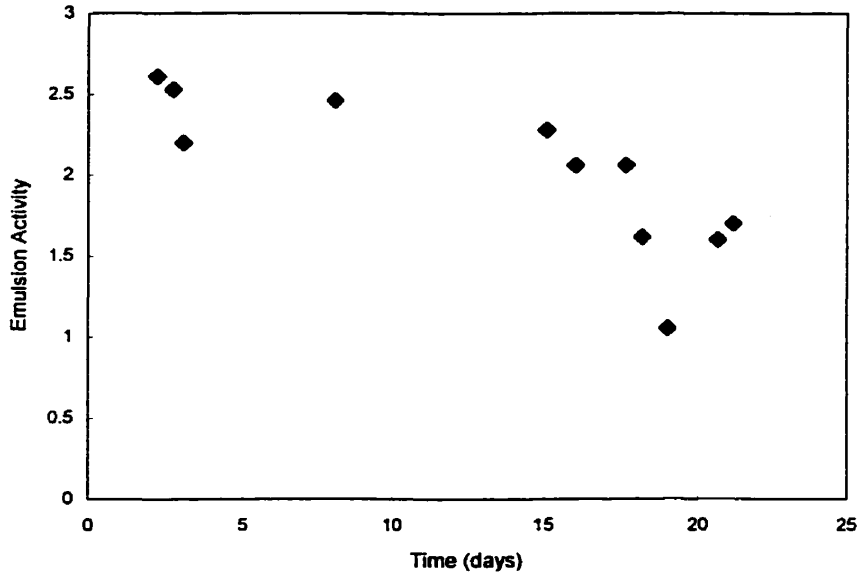

Figure 4-3: Effect of refrigeration on emulsion activity

4.3 Pretreatment of samples

To properly measure the extra-cellular carbohydrate concentration, and to eliminate any emulsifying properties of the biomass, it was necessary to remove the cells in a two step process of centrifugation and filtration. Centrifugation alone did not remove a sufficient number of cells to prevent interference with the measurements. Figure 4-1 compares the measured carbohydrate concentration of filtered and unfiltered samples. Without filtering, the trend of increasing carbohydrate was obscured by the interference of the cells still in the sample.

4.4 Batch Growth

In its first cycle, the Self-Cycling Fermentor is equivalent to a batch reactor. The batch production of liposan was studied in the first cycle of the Self-Cycling Fermentor with an initial hydrocarbon concentration of 5 ml/L. Figure 4-2 shows the carbohydrate concentration and emulsion activity for the batch growth of *Y. lipolytica*.

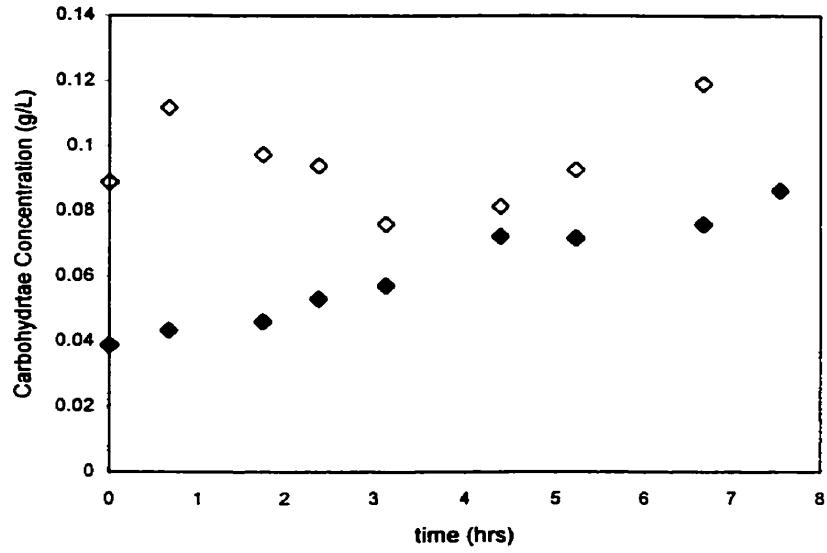

Figure 4-1: Comparison of measured carbohydrate concentrations for filtered and non-filtered samples: ◊ Unfiltered, ♦ Filtered

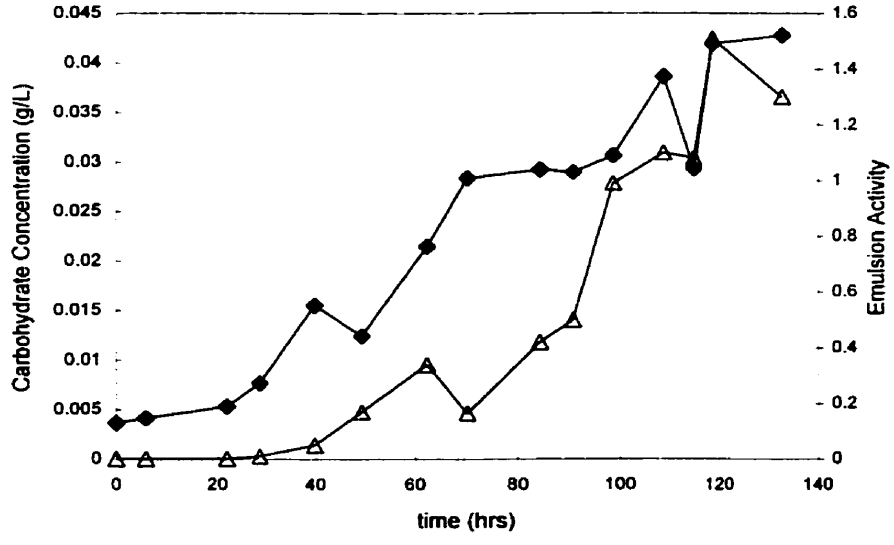

Figure 4-2: Emulsifier production during batch growth: ♦ Carbohydrate, Δ Emulsification Activity

4.5 Method of Initial Hydrocarbon Addition

A water insoluble substrate must be added to the SCF separately from the aqueous growth medium. The standard method for adding a water insoluble substrate to the SCF is by injecting it, at the beginning of each cycle, using a syringe pump. [30,31,33,40]. Throughout this thesis, this method is referred to as initial addition. To establish a baseline by which to compare future work, preliminary experiments were done with the initial addition of 5ml of hexadecane to the reactor.

The dissolved oxygen for two consecutive cycles in the SCF, using initial addition, is shown in Figure 4-1. The SCF is cycled when a minimum in dissolved oxygen is detected by the control computer. The minimum in dissolved oxygen was detected by comparing the concentration of dissolved oxygen in the reactor to the lowest concentration measured in the current cycle. When the current concentration was greater than the lowest concentration by 2% of the saturated value, the reactor was cycled. This method caused a delay in the recognition of the minimum in dissolved oxygen by the control computer. This time delay can be seen in Figure 4-1.

To obtain values for the various parameters that represent the end of cycle conditions, the reactor was run until the duration of the individual cycles stabilized. End of cycle samples were then collected and for five to eleven cycles and averaged. This procedure was repeated for each new set of experimental conditions. Figure 4-2 shows the cycle time and end of cycle carbohydrate for a series of consecutive cycles using the initial addition of 5 ml/cycle. As the reactor stabilizes, the cycle time decreases from

1000 minutes to 400 minutes. After the cycle time had stabilized, sample collection began. For this experiment, the end of cycle carbohydrate concentrations stabilized at the same cycle as the cycle time. For all subsequent experiments samples were not taken until the cycle time was judged to be stable for three consecutive cycles. Table 5-2 summarizes the end of cycle values for the initial addition of 5 milliliters of hexadecane per cycle.

Table 4-1: Summary of end of cycle values for initial hydrocarbon addition of 5 ml/cycle

Cycle Time	6.7 hrs
Carbohydrate	0.046 g/L
Dry-weight	1.1 g/L
Emulsion Activity	1.4
Residual Hydrocarbon	3.77 ml/L
pH	2.9

Samples of the reactor contents could also be taken during a cycle. Figure 4-3 shows the inter-cycle concentrations of hexadecane and carbohydrate for the initial addition of 5 ml/cycle of hexadecane. The initial, low hydrocarbon concentrations were caused by a delay before the system became well mixed. The inter-cycle carbohydrate profile showed two periods of increased production, one beginning at 50 minutes and the second beginning at 200 minutes. The rapid drop in dissolved oxygen, seen in Figure 4-1, corresponded with the second, larger, increase in carbohydrate production. It was not possible to measure the concentration of biomass during a cycle because the dry-weight assay required too large a sample to be taken without disturbing the reactor.

To extend the period of liposan production after the normal end of a cycle, two different techniques were employed. The first method involved taking a large sample, at the end of a cycle, and transferring it to a second reactor where growth could continue.

This was referred to as a two-stage reactor. The second reactor, in the two-stage method, was a shake flask incubated at the same temperature as the reactor. Figure 4-4 shows the carbohydrate concentration and emulsion activity in the second reactor with the initial addition of 5 ml/cycle of hydrocarbon. After 70 hours in the second reactor, the carbohydrate concentration was 0.22 g/L. This was more than four times the final carbohydrate concentration in the primary reactor. The drop in emulsion activity seen towards the end of the experiment was due to the age of the samples and was the reason that the effect of sample age was investigated (Figure 4-3).

The second method of extending the period of liposan production was to delay cycling the reactor after the minimum in dissolved oxygen was detected. This method is presented in section 4.9.

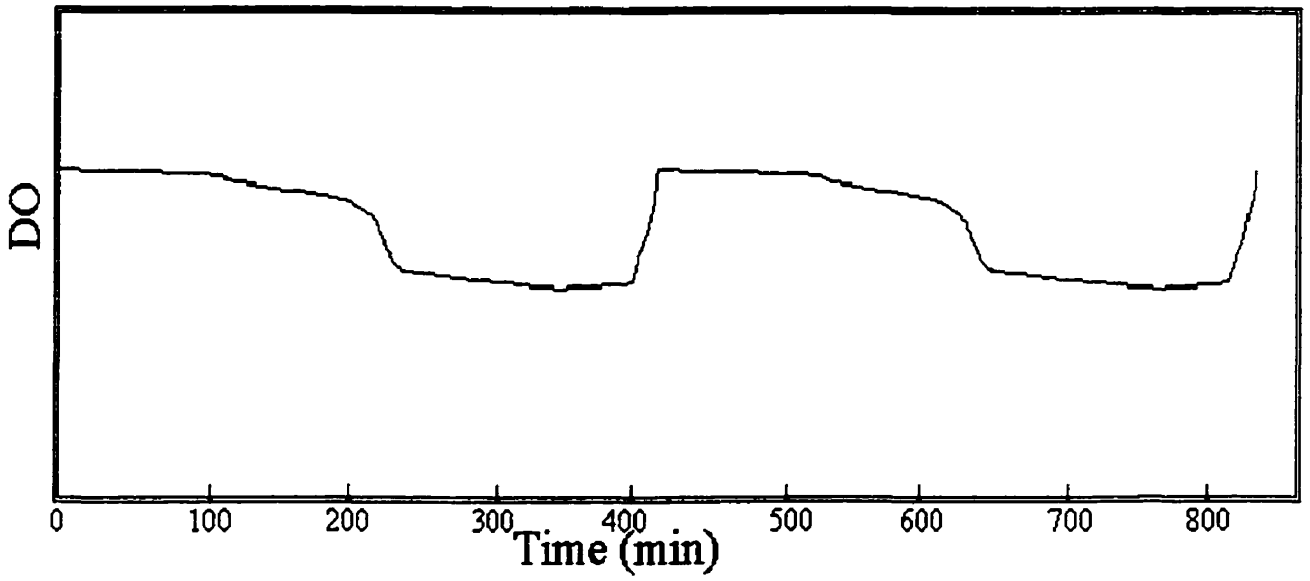

Figure 4-1: Dissolved Oxygen for two consecutive cycles using initial addition of 5 ml/cycle

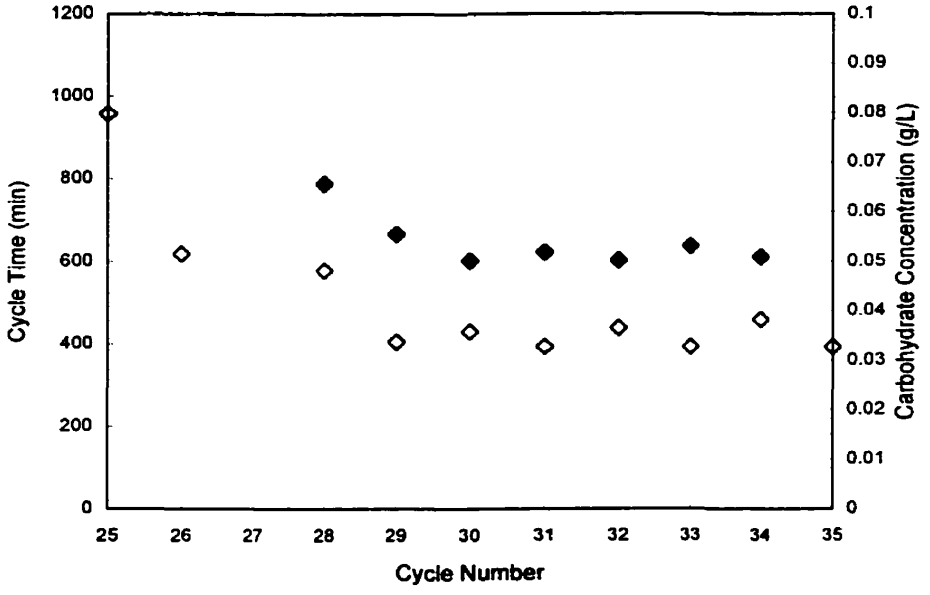

Figure 4-2: Cycle time and end of cycle carbohydrate for a series of consecutive cycles using initial addition of 5 ml/cycle: ◊ Cycle Time, ♦ Carbohydrate

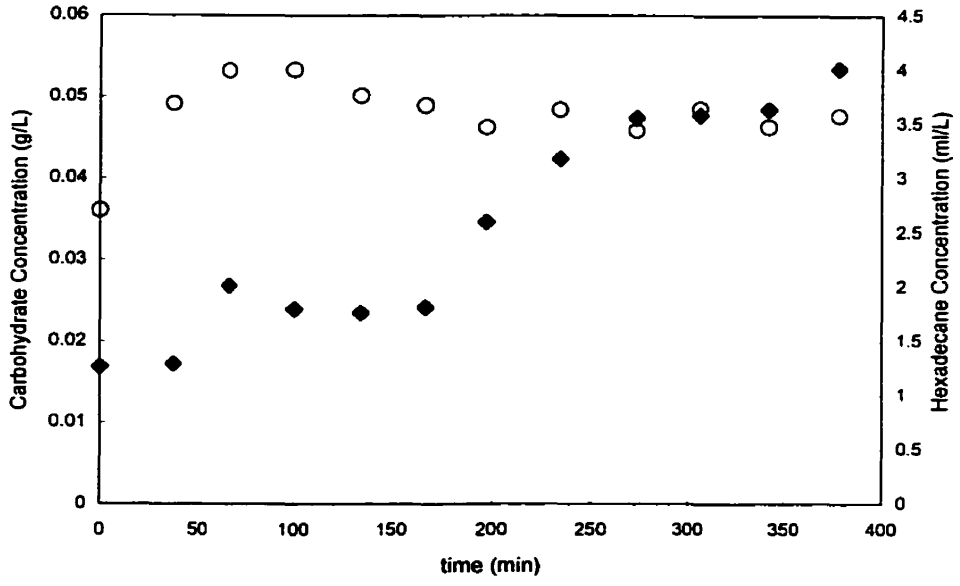

Figure 4-3: Inter-cycle carbohydrate and hexadecane for the initial addition of 5 ml/cycle: ♦ Carbohydrate, O Hexadecane

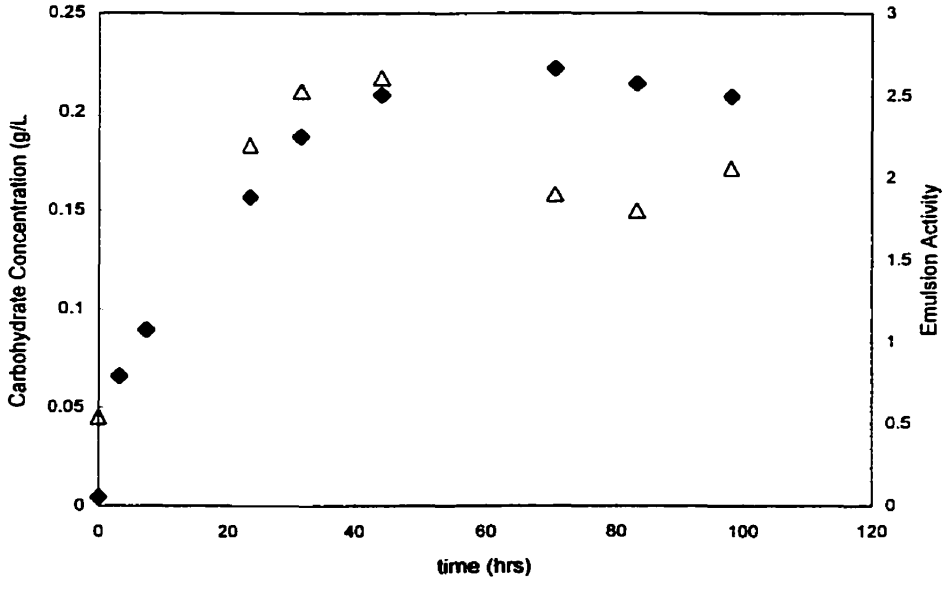

Figure 4-4: Emulsifier production in two-stage reactor following the initial addition of 5 ml/cycle: ♦ Carbohydrate, Δ Emulsion Activity

4.6 Method of Continuous Addition of Hydrocarbon

An alternative method of adding a water insoluble substrate to the Self-Cycling Fermentor was investigated for its potential to increase liposan production and reduce the end of cycle hydrocarbon residue. Using this technique, the hydrocarbon was added to the reactor continuously throughout the cycle. As mentioned previously, all end of cycle data presented are an average of five to eight stable, consecutive cycles. Figure 4-1 shows the end of cycle residual hydrocarbon and cycle time for the continuous addition of hydrocarbon at different flow-rates. Cycle time increased rapidly as the flow rate of hydrocarbon into the reactor was reduced. The cycle time for the lowest flow-rate was 28 hrs, and was left off the figure. Residual hydrocarbon concentrations for the lower addition rates were well below 1 ml/L. This is much lower than the residual hydrocarbon concentration for the preliminary work, done with the initial addition of 5ml/cycle.

End of cycle dry-weight and carbohydrate concentrations for continuous addition are shown in Figure 4-2 and Figure 4-3. Both parameters increased as the hydrocarbon flow-rate was decreased. The lowest values for both parameters are similar to those of the preliminary case of 5 ml/cycle initial addition.

The total hydrocarbon added to each cycle, based on flow-rate and cycle times, is plotted in Figure 4-4. This figure shows that the total hydrocarbon added to each cycle, using continuous addition, is much less than 5 ml/cycle.

The biomass and liposan yields were calculated and are shown in Figure 4-5 and Figure 4-6. Maximum in both yield coefficients occurred at a feed rate of 0.2 ml/hr of hexadecane.

To improve the quality of the end of cycle product, it was desirable to increase the amount of liposan produced relative to the amount of biomass. The ratio of these two parameters is shown in Figure 4-7 and was constant for all addition rates. It would be advantageous if these two fermentation products could be decoupled, and the ratio shifted in favor of the emulsifier.

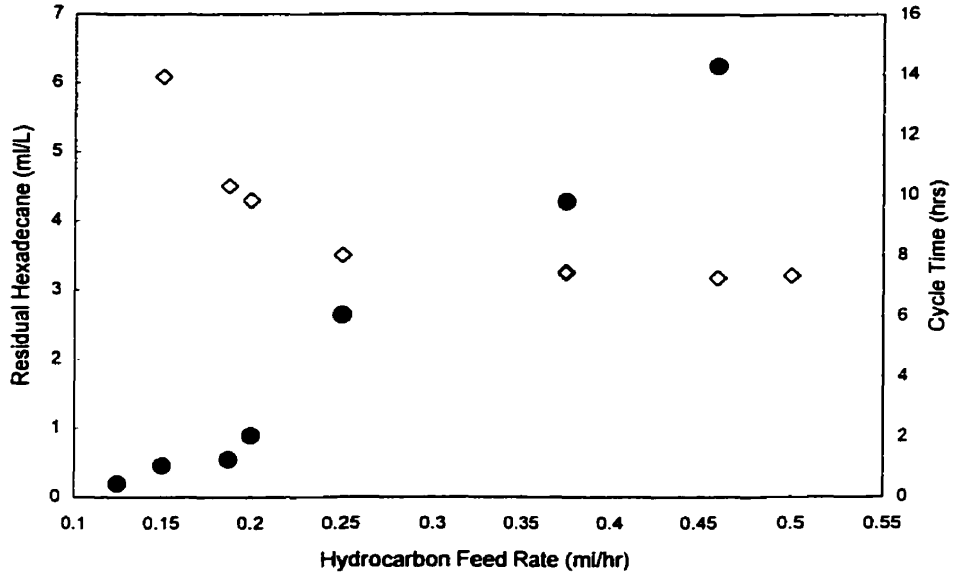

Figure 4-1: Cycle time and residual hexadecane for continuous addition at varying feed rates: ◊ Cycle Time, • Residual Hexadecane

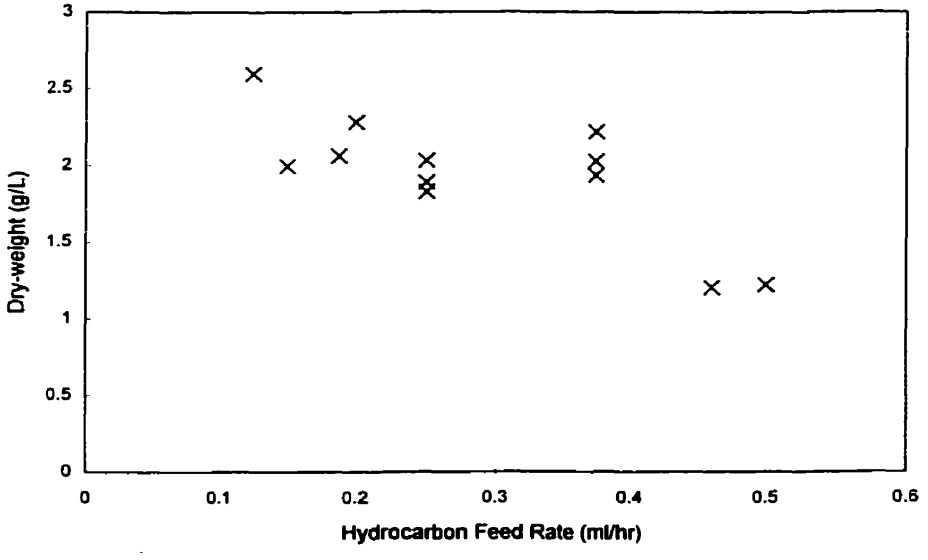

Figure 4-2: End of cycle dry-weight for continuous addition at varying feed rates

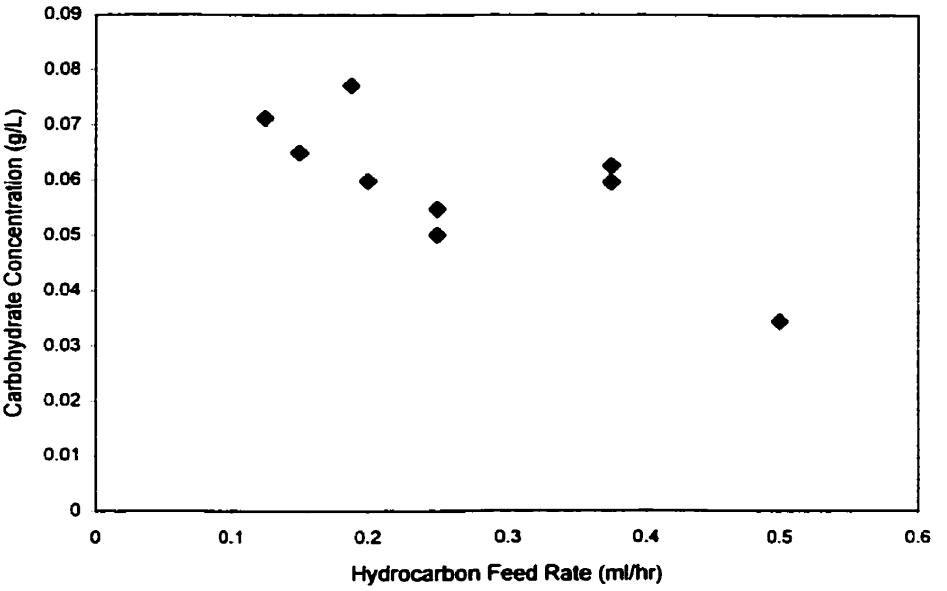

Figure 4-3: End of cycle carbohydrate for continuous addition at varying feed rates

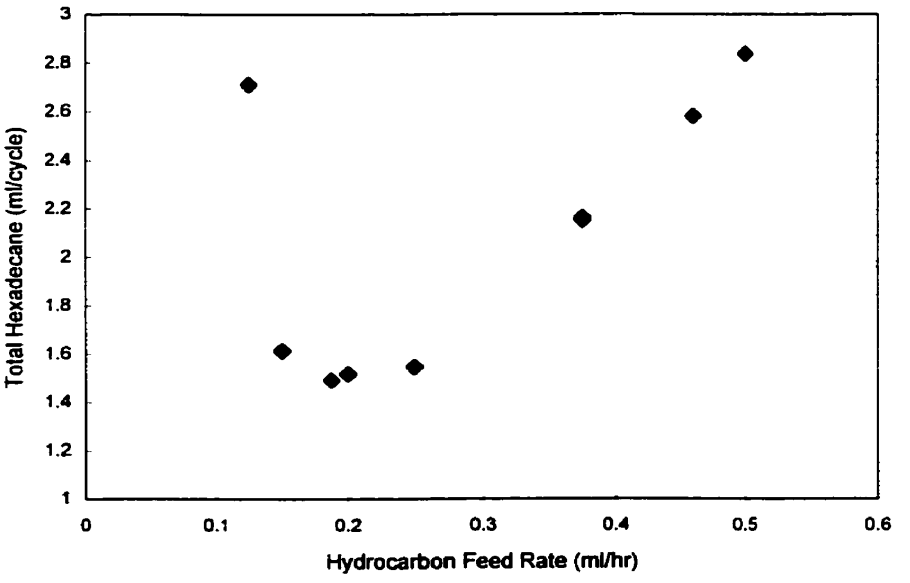

Figure 4-4: Total hexadecane added per cycle for continuous addition at varying feed rates

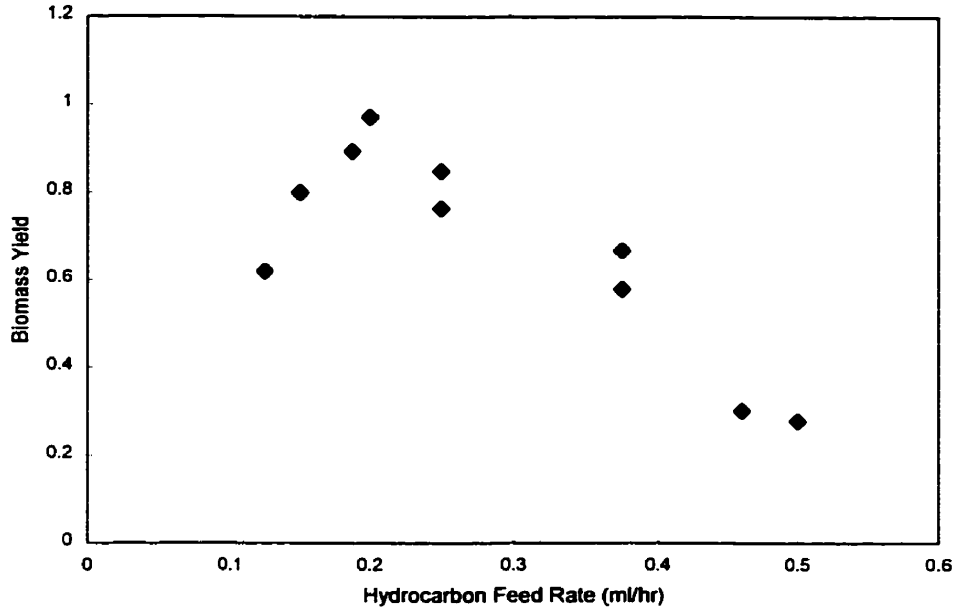

Figure 4-5: Biomass yield for continuous addition at varying feed rates

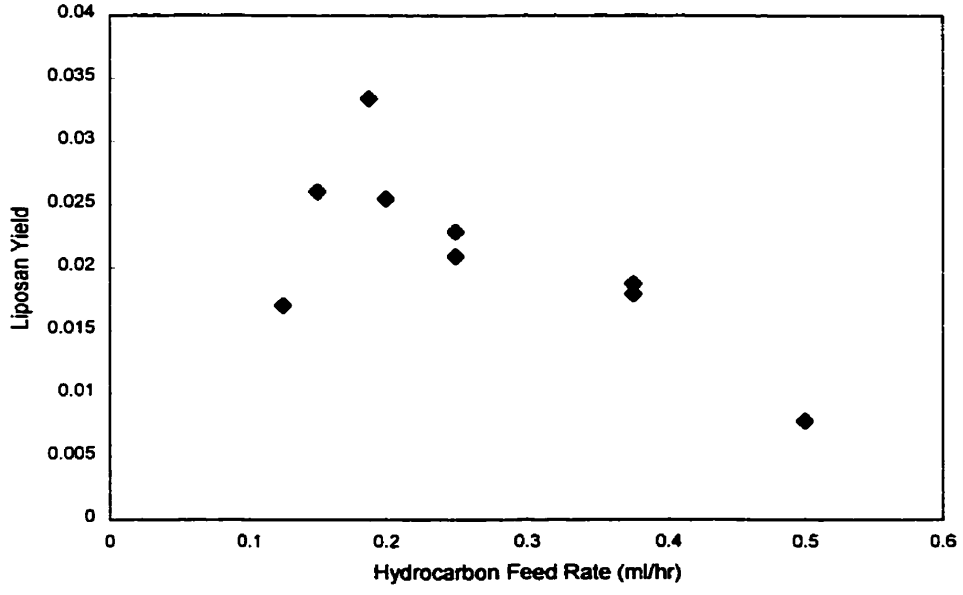

Figure 4-6: Liposan yield for continuous addition at varying feed rates

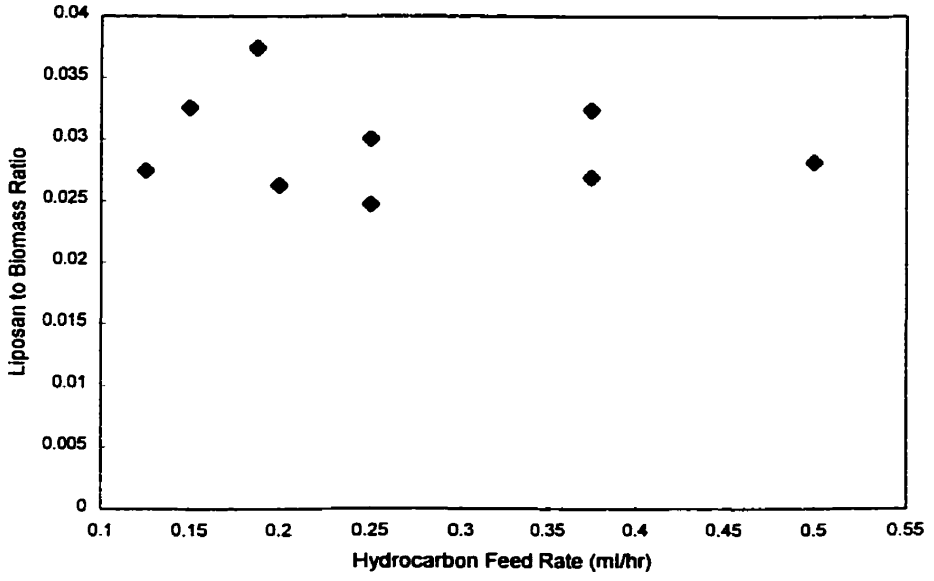

Figure 4-7: Liposan to biomass ratio for continuous addition at varying feed rates

4.7 Initial Addition of Varying Amounts of Hydrocarbon

The total hydrocarbon added to each cycle during continuous addition was much less than the preliminary work done with initial addition. To show that the effects of continuous addition on growth and liposan production were a result of the addition method and not the decreased volume of hydrocarbon added to the reactor, experiments were done with initial addition using less hydrocarbon.

Figure 4-1 shows the cycle time and residual hydrocarbon concentration for the initial addition of varying volumes of hydrocarbon. Figure 4-2 shows the end of cycle biomass and carbohydrate concentrations for this set of experiments. The biomass and liposan yields are shown in Figure 4-3 and the product to biomass ratio is shown in Figure 4-4.

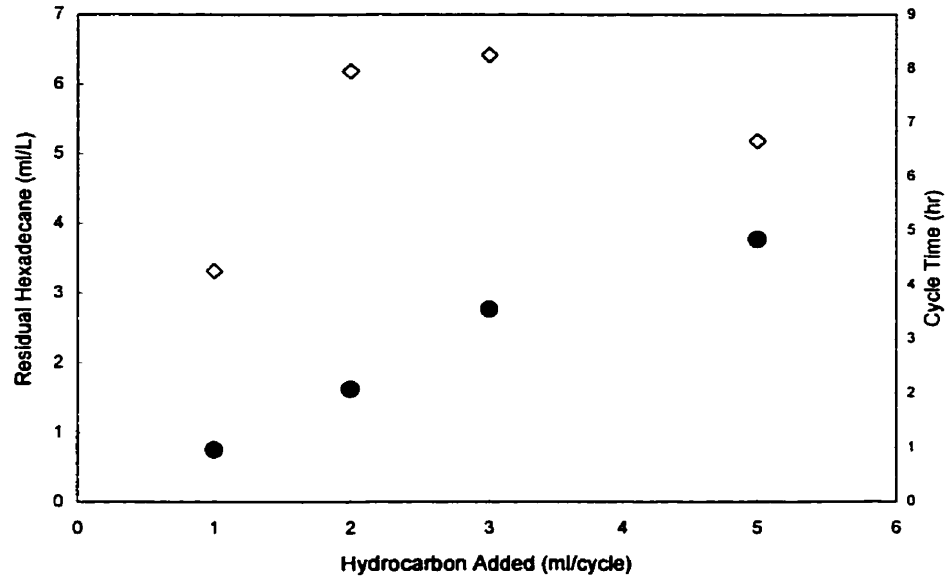

Figure 4-1: Cycle time and residual hexadecane for the initial addition of varying amounts of hydrocarbon: ◊ Cycle time, • Residual hexadecane

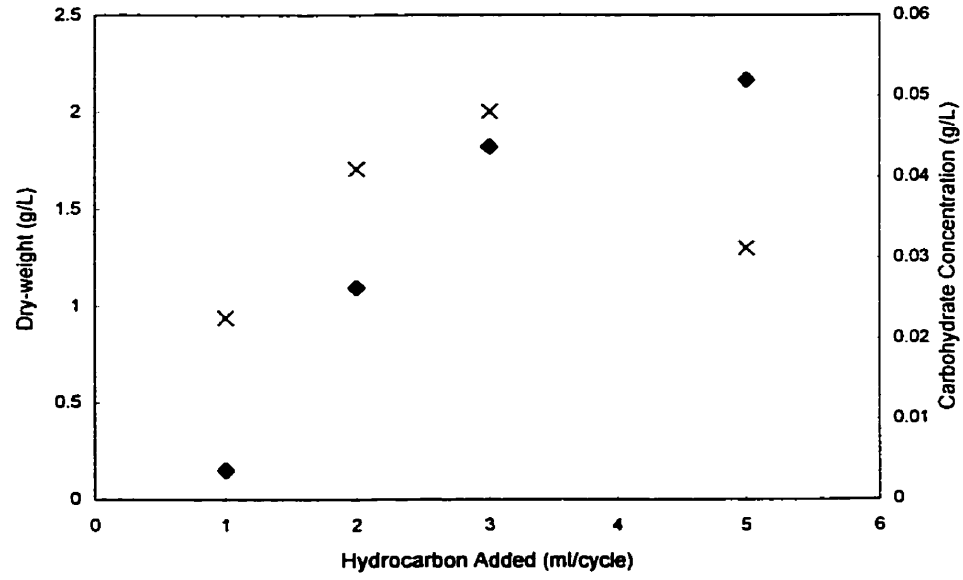

Figure 4-2: End of cycle dry-weight and carbohydrate for the initial addition of varying amounts of hydrocarbon: X Dry-weight, ♦ Carbohydrate

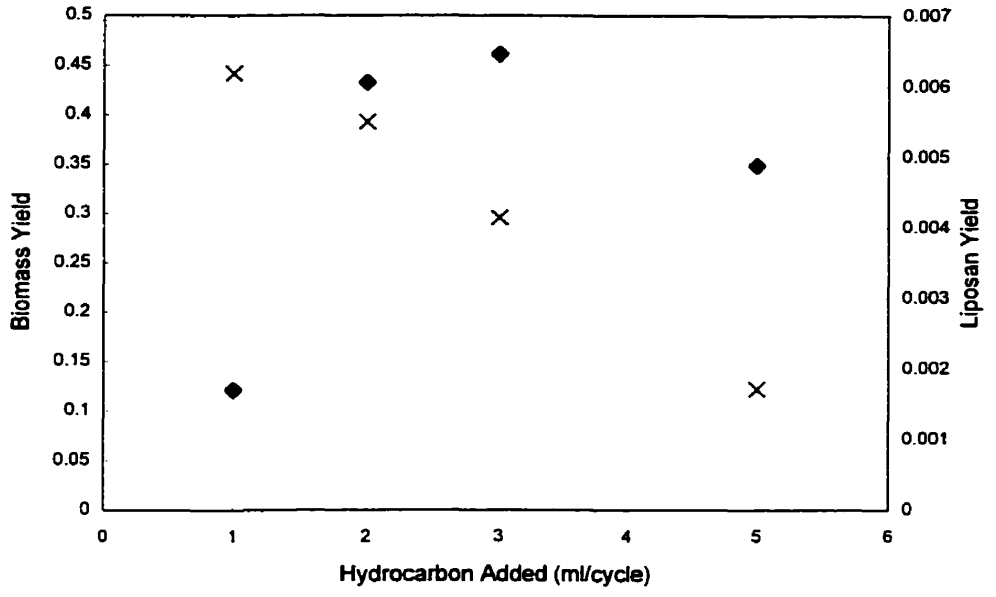

Figure 4-3: Biomass and liposan yield for the initial addition of varying amounts of hydrocarbon: X Biomass yield, ♦ Liposan yield

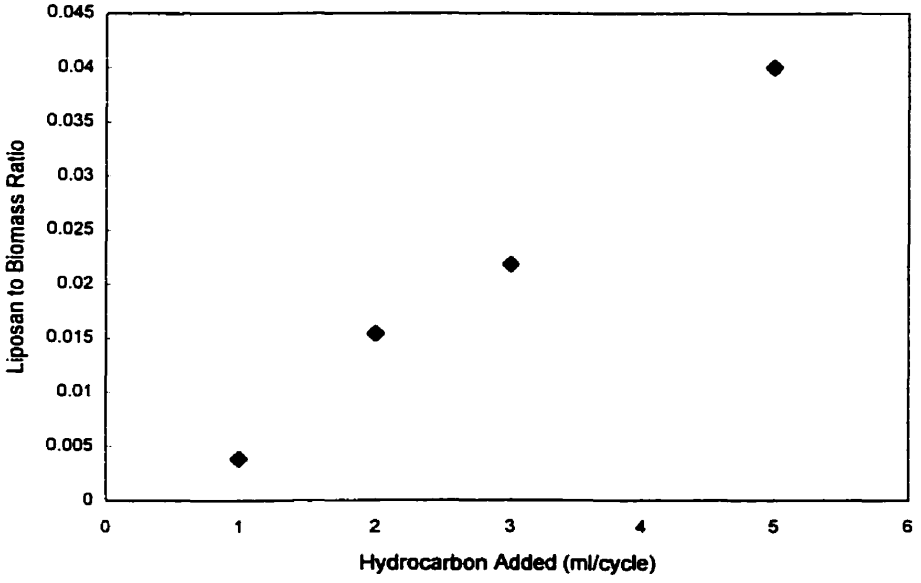

Figure 4-4: Liposan to biomass ratio for the initial addition of varying amounts of hydrocarbon

4.8 Comparison Between Initial and Continuous Methods of Hydrocarbon Addition

Figure 4-1 compares the final carbohydrate concentrations for the two methods of hydrocarbon addition against the total amount of hydrocarbon added per cycle.

The average biomass production rate was calculated from the cycle time and the final biomass concentration. The biomass production rate is plotted against the average hexadecane concentration in Figure 4-2. The figure shows a sudden drop in biomass production at 3 ml/L of hexadecane.

To investigate the potential toxicity of hexadecane, the average specific growth rate of *Y. lipolytica* was calculated from the cycle time. This is plotted against the average hexadecane concentration in Figure 4-3. The specific growth rate was estimated by assuming a constant rate of change in biomass at an average biomass concentration. If the substrate were inhibiting, the figure would have shown a maximum in the specific growth rate[41]. The inhibitory effect of hexadecane was also tested using the Microtox toxicity assay[42]. The assay showed that hexadecane was not toxic.

The productivity of the organism can be compared using the specific liposan production rate. The average specific production rates are compared to the average hexadecane concentration in Figure 4-5.

The final comparison between addition methods is between the residual hydrocarbon concentration and the liposan production rate (Figure 4-6).

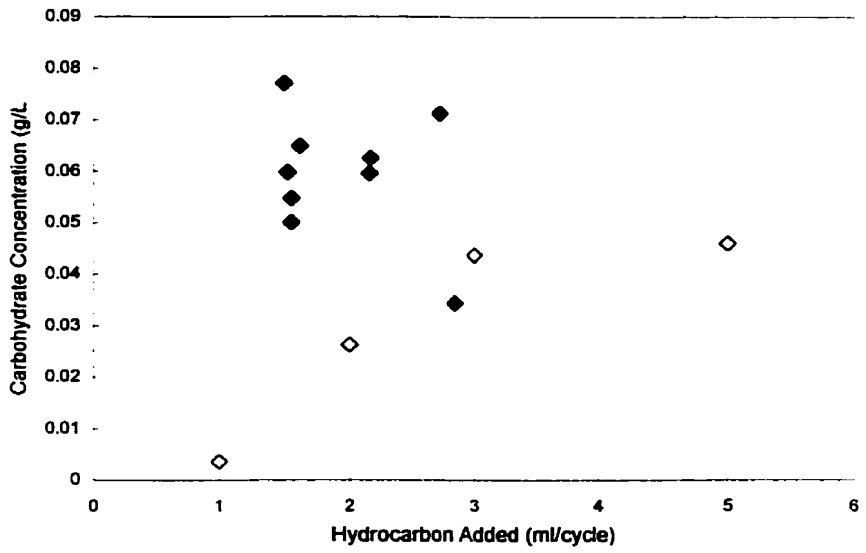

Figure 4-1: Comparison of end of cycle carbohydrate concentrations for continuous and initial addition: ◊ initial addition, ♦ continuous addition

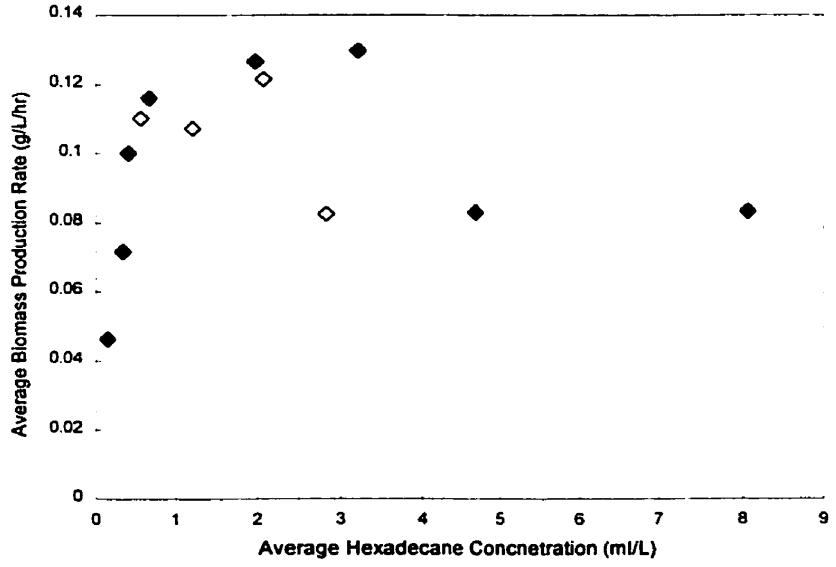

Figure 4-2: Average biomass production rate for continuous and initial addition: ◊ initial addition, ♦ continuous addition

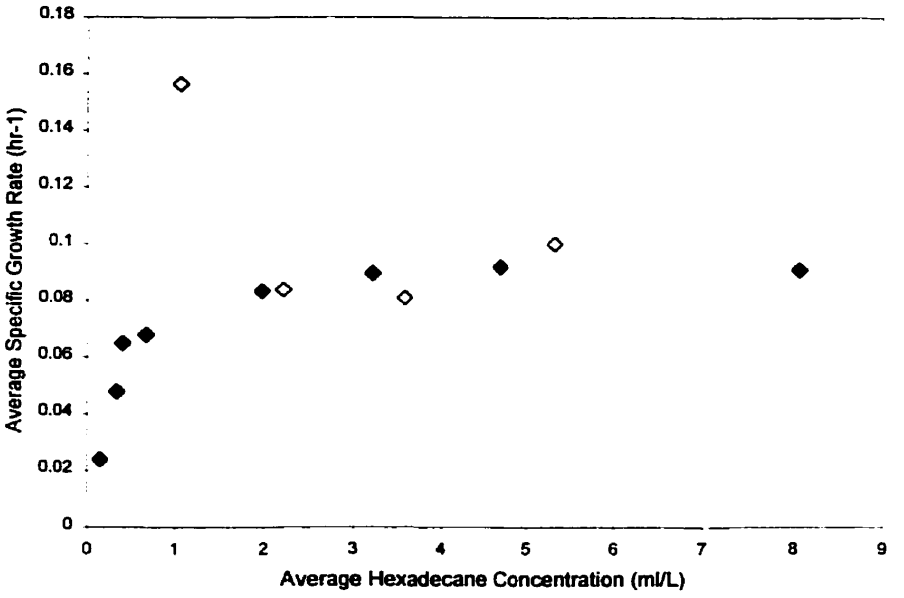

Figure 4-3: Average specific growth rate versus average substrate concentration for continuous and initial addition: ◊ initial addition, ◆ continuous addition

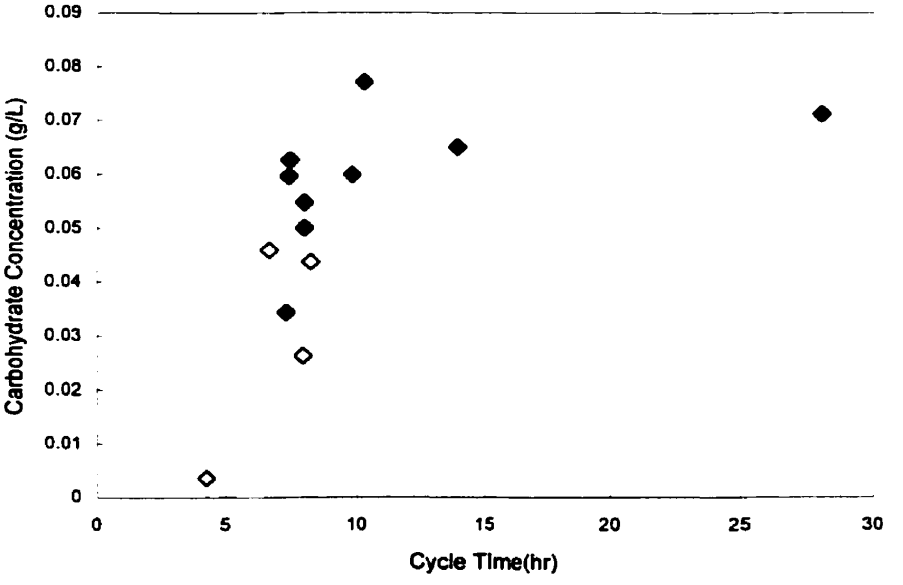

Figure 4-4: Comparison of end of cycle carbohydrate concentration and cycle time for continuous and initial addition: ◊ initial addition, ◆ continuous addition

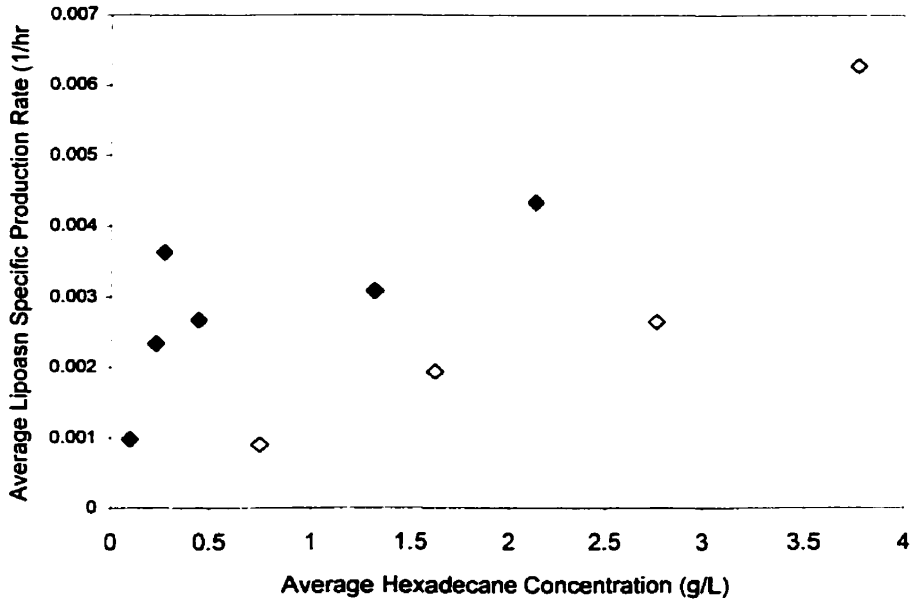

Figure 4-5: Comparison of average liposan specific production rates for continuous and initial addition: ◊ initial addition, ♦ continuous addition

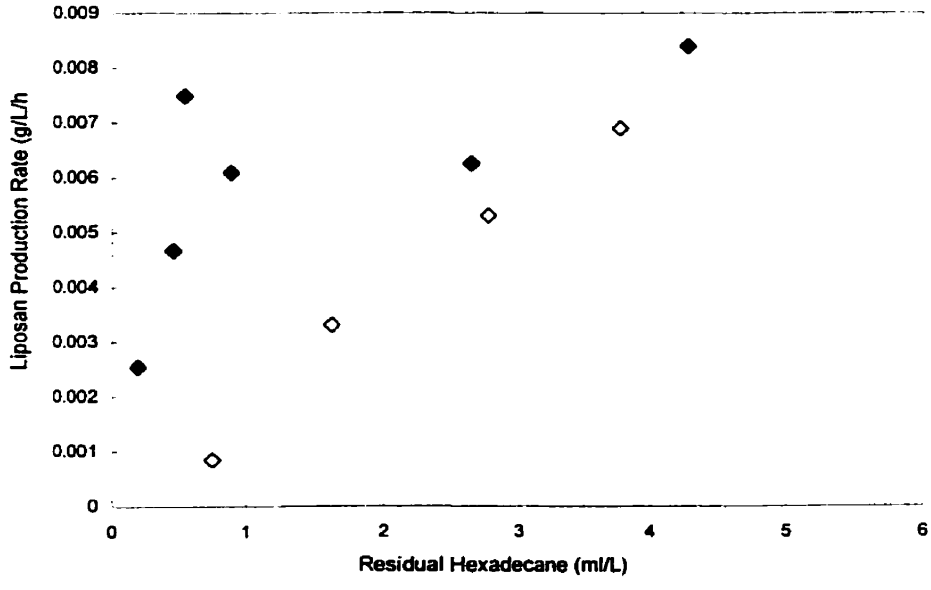

Figure 4-6: Comparison of liposan production rate and residual hexadecane for continuous and initial addition: ◊ initial addition, ♦ continuous addition

4.9 Time Delay Following Continuous Addition

Imposing a time delay upon detection of the minimum in dissolved oxygen and before cycling the reactor, extends the liposan production period and forces the organism to continue growing under stressful conditions. Experiments, where a time delay was imposed at the end of the cycle, were done with continuous addition flow rates of 0.25 ml/hr and 0.2 ml/hr. These two flow rates were used for delay experiments because they represented cases of high and low hydrocarbon concentration at the end of the cycle. At a continuous feed rate of 0.25 ml/hr, end of cycle residual hydrocarbon was significant (2.64 ml/L), while at a feed rate of 0.2 ml/hr the residual hydrocarbon concentration was much lower (0.89 ml/L). Hydrocarbon flow rates lower than 0.2 ml/hr were not studied because the resulting long cycle times were not practical.

Figure 4-1 shows the total cycle time and residual hydrocarbon concentration for a continuous hexadecane flow-rate of 0.2 ml/hr with an end of cycle time delay. Figure 4-2 shows the total cycle time and residual hydrocarbon for a hydrocarbon flow-rate of 0.25 ml/hr with an end of cycle time delay.

Figure 4-3 and Figure 4-4 show the end of cycle biomass and carbohydrate concentrations for the two hydrocarbon feed-rates studied with an end of cycle time delay. The carbohydrate concentration of 0.09 g/L for the 0.2 ml/hr addition rate and 2 hrs delay, was the highest end of cycle concentration obtained in the main reactor.

Figure 4-5 and Figure 4-6 show the product to biomass ratio for the two continuous hydrocarbon flow-rates studied with an end of cycle time delay.

Inter-cycle data was also collected for the 0.25 ml/hr hydrocarbon addition rate and a 2 hr end of cycle time delay. Figure 4-7 shows the inter-cycle profiles of hydrocarbon and carbohydrate concentration. The figure also shows the time when the minimum in dissolved oxygen was detected and the hydrocarbon feed to the reactor was stopped.

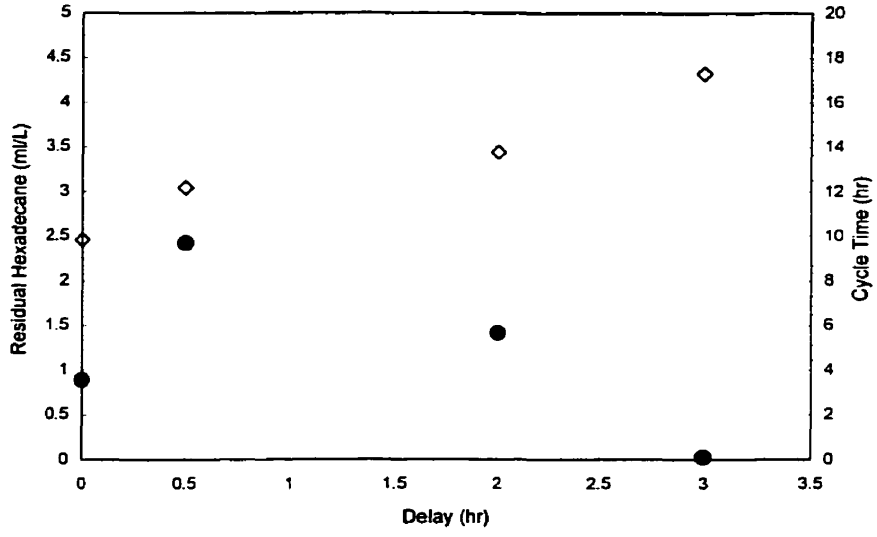

Figure 4-1: Cycle time and residual hexadecane for the continuous hydrocarbon addition of 0.2 ml/hr and end of cycle time delay: ◊ Cycle time, • Residual Hexadecane

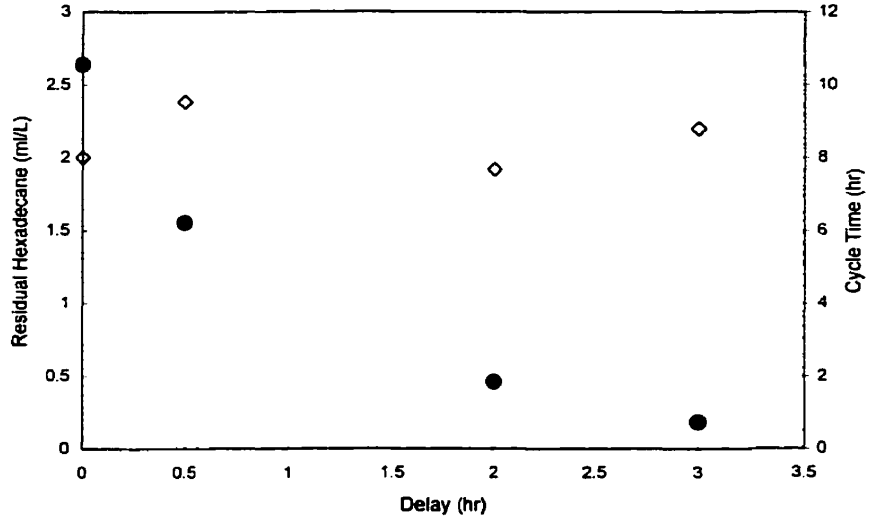

Figure 4-2: Cycle time and residual hexadecane for the continuous hydrocarbon addition of 0.25 ml/hr and end of cycle time delay: ◊ Cycle time, • Residual hexadecane

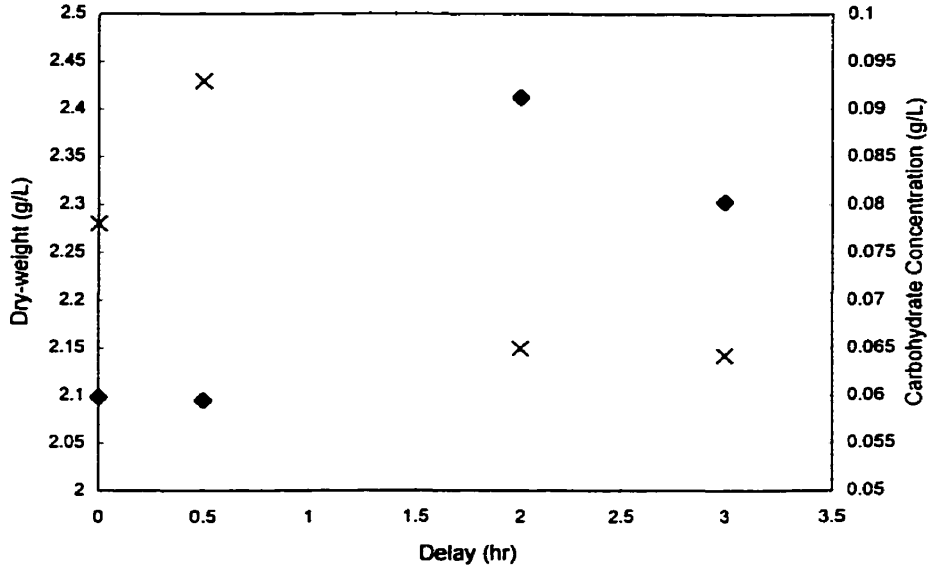

Figure 4-3: End of cycle dry-weight and carbohydrate for the continuous hydrocarbon addition of 0.2 ml/hr and end of cycle time delay: x Dry-weight, ◆ Carbohydrate

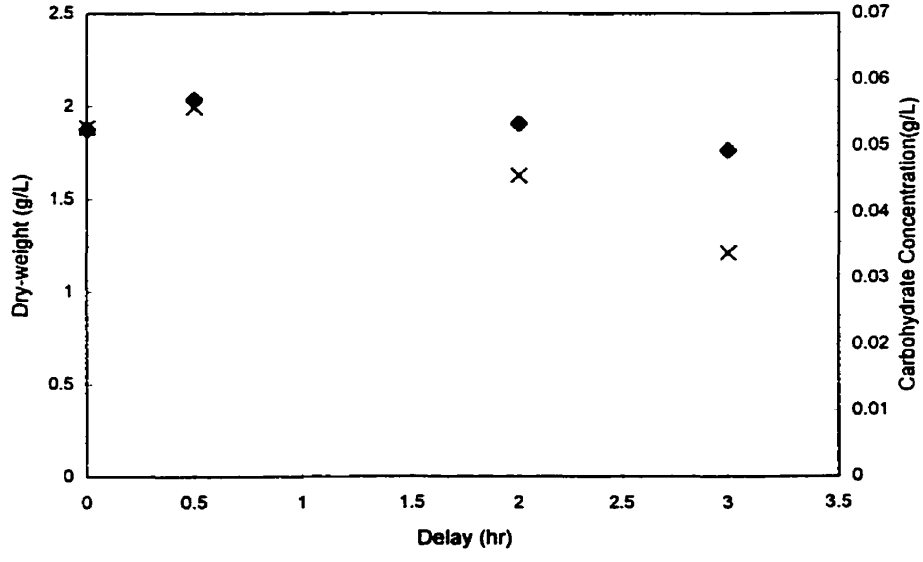

Figure 4-4: End of cycle dry-weight and carbohydrate for the continuous hydrocarbon addition of 0.25 ml/hr and end of cycle time delay: x Dry-weight, ◆ Carbohydrate

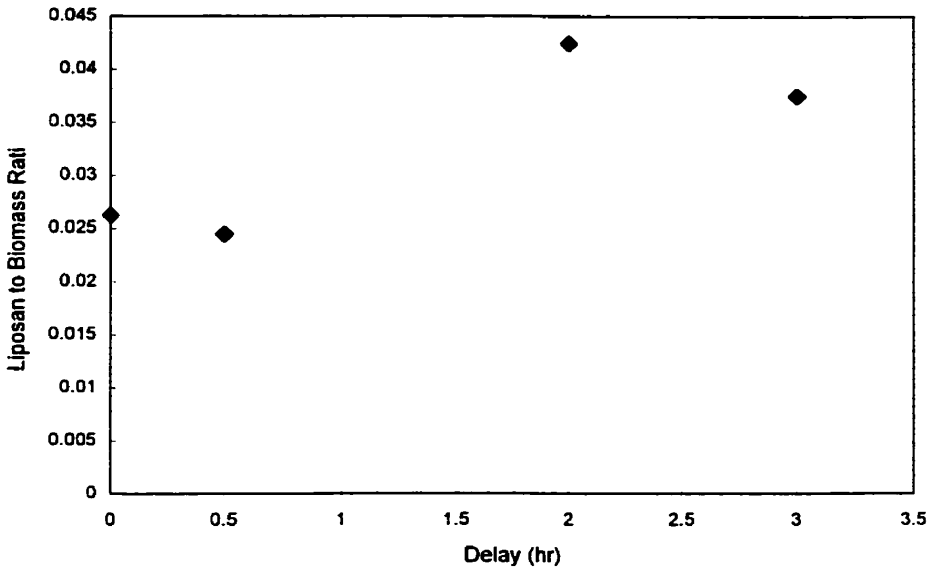

Figure 4-5: Liposan to biomass ratio for the continuous hydrocarbon addition of 0.2 ml/hr and end of cycle time delay

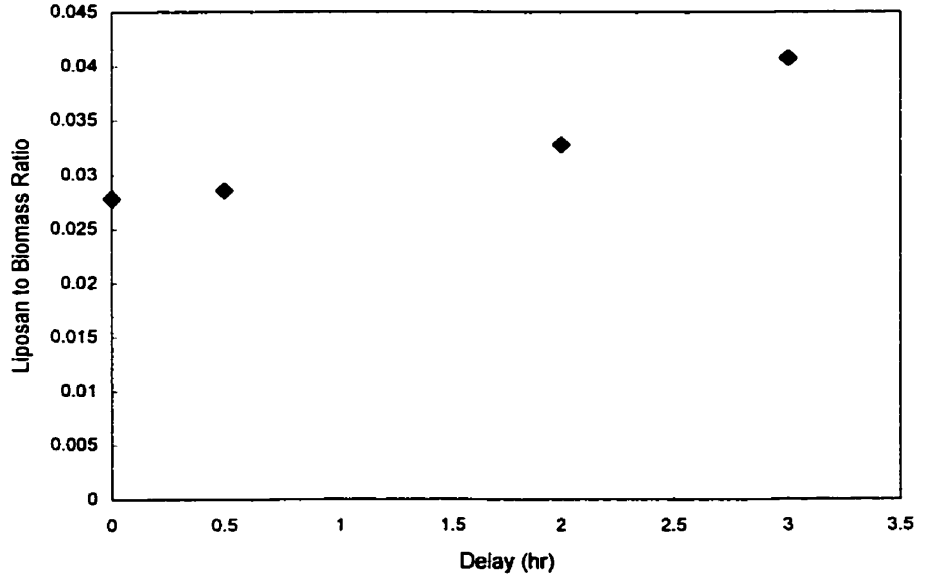

Figure 4-6: Liposan to biomass ratio for the continuous hydrocarbon addition of 0.25 ml/hr and end of cycle time delay

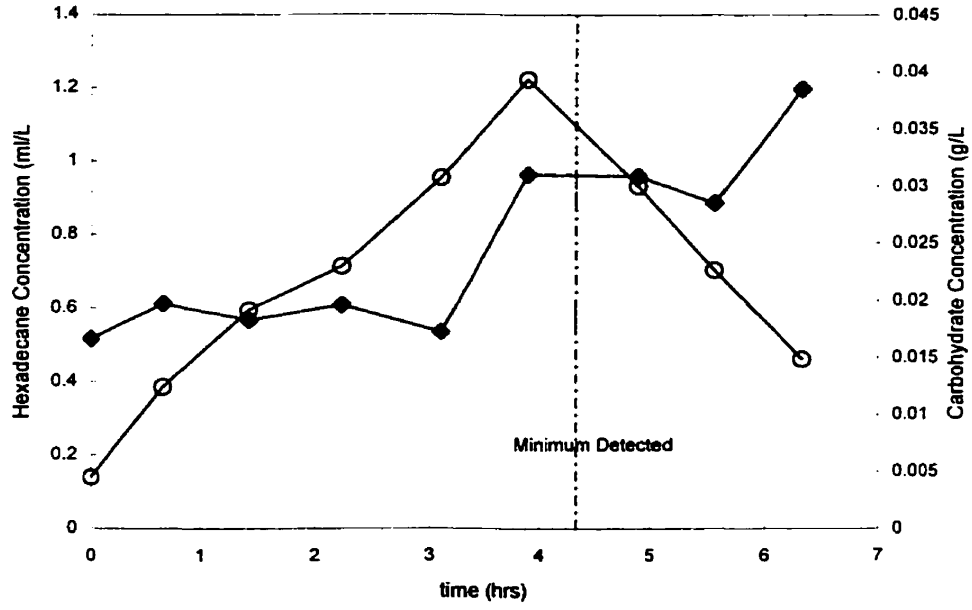

Figure 4-7: Inter-cycle carbohydrate and hexadecane for the continuous hydrocarbon addition of 0.25 ml/hr and end of cycle time delay: O Hexadecane, ♦ Carbohydrate

4.10 Method of Initial Hydrocarbon Addition With End of Cycle Time Delay

To further examine the effects of the end of cycle time delay and the hydrocarbon addition method, experiments were done with the initial addition of 2 ml/cycle with an end of cycle time delay. Figure 4-1 shows the cycle times and residual hydrocarbon concentration for these experiments. Figure 4-2 shows both the final biomass and final carbohydrate concentrations for this series of experiments. Both biomass and carbohydrate concentrations increased during the time delay. The relative increases in liposan and biomass are shown by the product to biomass ratio plotted in Figure 4-3.

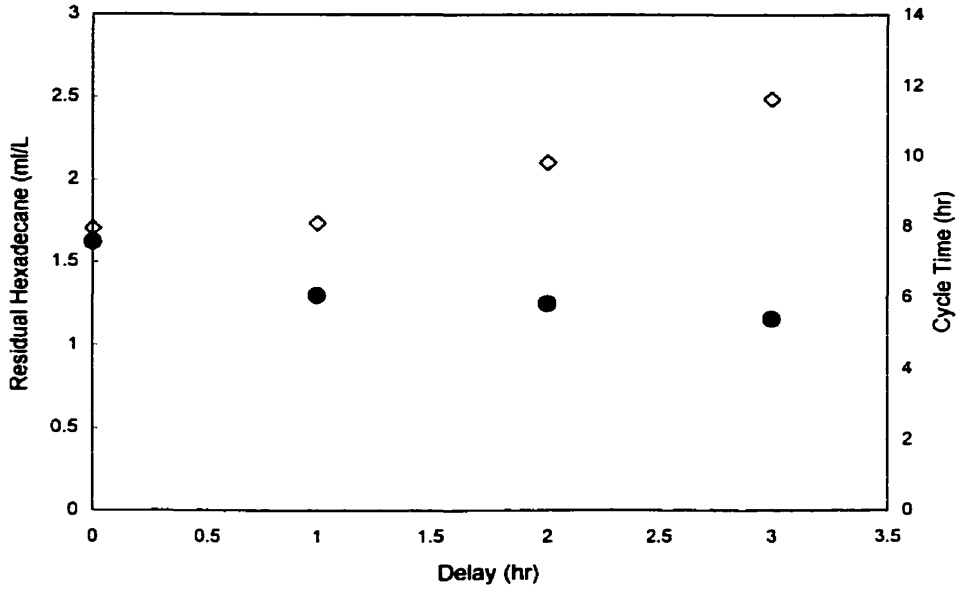

Figure 4-1: Cycle time and residual hexadecane for the initial addition of 2 ml/cycle with end of cycle time delay: ◊ Cycle time, • Residual hexadecane

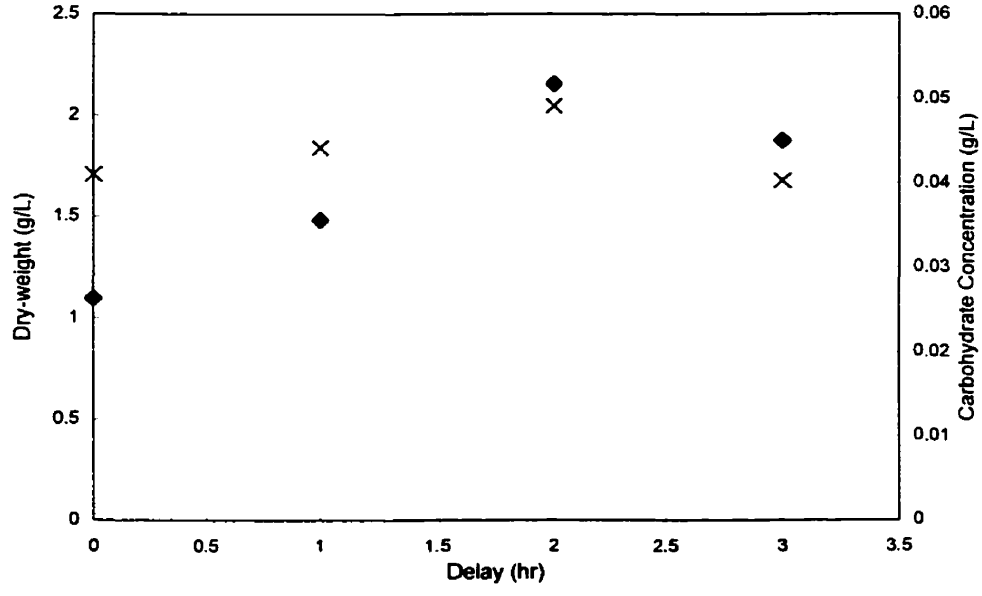

Figure 4-2: End of cycle dry-weight and carbohydrate for the initial additional of 2 ml/cycle with end of cycle time delay: x Dry-weight, ♦ Carbohydrate

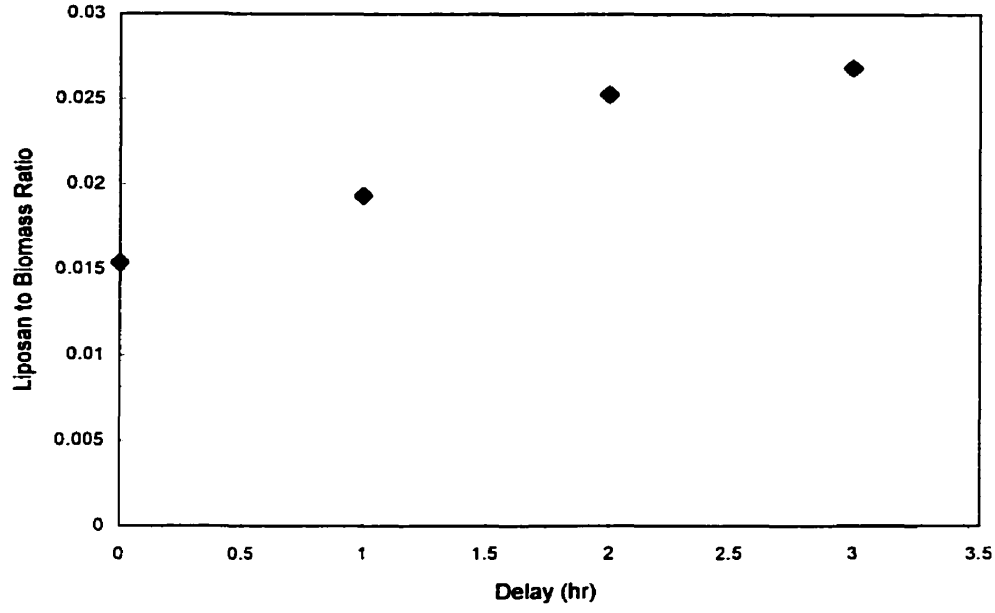

Figure 4-3: Liposan to biomass ratio for the initial additional of 2ml/cycle with end of cycle time delay

4.11 Comparison Between Continuous Addition With and Without Time Delay

Figure 4-1 compares the end of cycle liposan concentration and cycle time for the continuous addition of hydrocarbon with, and without, an end of cycle time delay. The second comparison is of the liposan production rate and the residual hydrocarbon concentration (Figure 4-2).

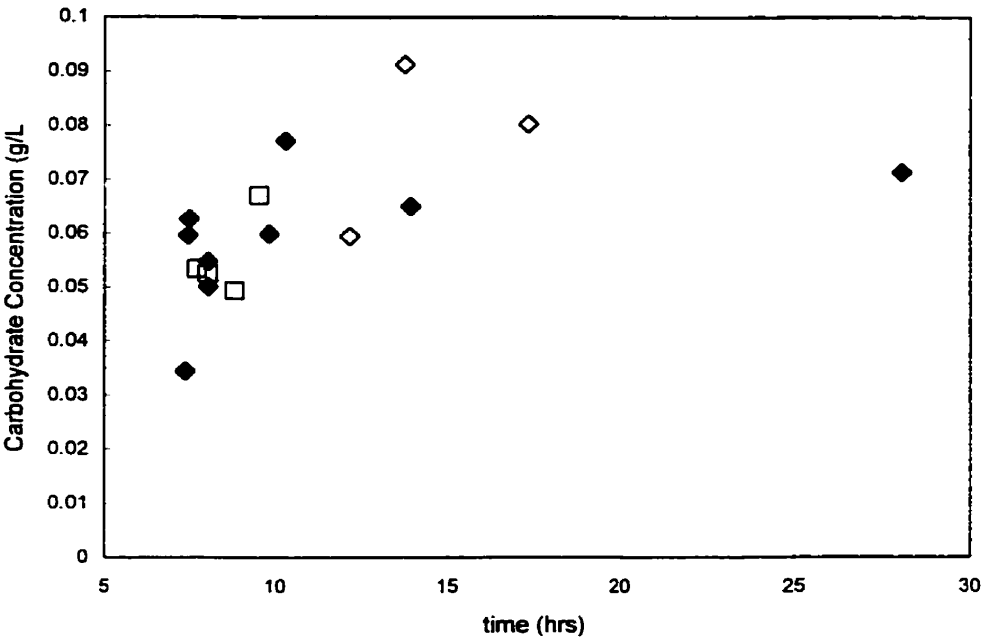

Figure 4-1: Comparison of end of cycle carbohydrate concentrations for continuous addition with and without time delay: ♦ continuous addition with no time delay, ◊ continuous addition of 0.2 ml/hr with time delay, c ontinuous addition of 0.25 ml/hr with time delay

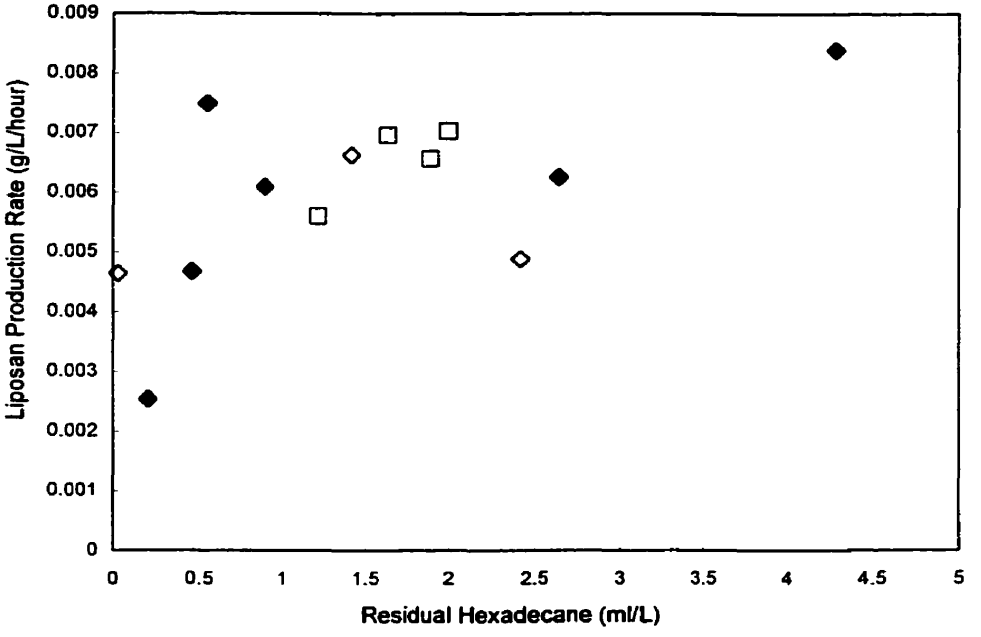

Figure 4-2: Comparison of liposan production rate and residual hydrocarbon for continuous addition with and without time delay: ♦ continuous addition with no time delay, ◊ continuous addition of 0.2 ml/hr with time delay, c ontinuous addition of 0.25 ml/hr with time delay

5 Discussion

5.1 Medium Formulation and Measurement of Liposan Concentration

Candida lipolytica, now in the genus *Yarrowia*, was shown to be able to degrade hydrocarbon in 1971[37]. No mention of emulsifier production by the organism was made in this initial work. In the current work, when *Yarrowia lipolytica* was grown in the medium the previous researchers had used, there was no measurable emulsification activity.

A study by Cirigliano *et al.* involved growing *Y. lipolytica* on a complex medium composed of mineral salts, a wide range of amino acids, and other growth factors[12,22]. Growth on this medium resulted in the production of an emulsifier, which they named liposan. This medium was not suitable for the present work, because its complexity made it expensive and resulted in interference with the measurement of growth parameters. When the medium was simplified, by eliminating the organic additives, it supported the growth of *Y. lipolytica* but the yeast did not produce liposan. This showed that some, or all, of the additives were required for emulsifier production.

Different media, each containing one of the organic additives, were screened to determine which were required for liposan production. Several were found to promote liposan production, but thiamine had, by far, the greatest effect.

Other work with this organism had shown the effects of thiamine on the cellular metabolism of citrate and iso-citrate[43]. The vitamin was included, with no explanation, in the growth media, of many studies on the production of citric acid by Y.

lipolytica[44,45,46,47]. One paper labeled the strain of Y. *lipolytica* as a thiamine auxotroph[48], meaning that the organism can not synthesize thiamine, but requires it for growth. This may have been confirmed by other work showing the strong effect of thiamine on the growth of *Y. lipolytica*[49].

The current research required a quick and reliable method for determining liposan concentration. The emulsification activity assay was not a reliable way of doing this. In particular, the technique was insensitive to changes in liposan concentration at higher values. At lower concentrations of liposan, weak emulsions were formed, which would break upon transfer to a cuvette.

Three assays were tested as measures of liposan concentration by comparing them with emulsification activity: fluorescence, total protein and total carbohydrate. All three assays showed initial promise, correlating well with emulsification activity during individual experiments. When data for multiple experiments were combined, only the total carbohydrate concentration exhibited a reasonable, overall, correlation with emulsification activity. Based on this correlation, the total carbohydrate assay was used, indirectly, to measure liposan concentration. Because the carbohydrate test was an indirect measure, it was necessary to periodically confirm its relationship with emulsification activity. This ensured that other components of the broth did not interfere with the measurement.

5.2 Growth of *Y. lipolytica* in the Self-Cycling Fermentor

It is inevitable, that using the mode of initial addition of the total amount of the carbon source required for the entire fermentation, will result in a large variation in

substrate concentration throughout each cycle of growth. Many studies have been done using gradual substrate feeds into a fermentation medium. The simplest of these strategies is to maintain a constant feed rate. Other, more complex strategies, try to achieve either a constant substrate concentration or a constant specific growth rate[35,51].

Many substrates, while supplying essential nutrients and energy, can also inhibit growth. The most dramatic effect is observed when one of these growth inhibiting substrates is added initially in one large amount[50,51].

As the continuous flow of hexadecane into the SCF was increased, biomass concentration, yield and cycle time all decreased. Based on the work of others, this result would seem to indicate that the hydrocarbon substrate was inhibiting[52]. Although a sudden drop in the rate of biomass production was observed at higher substrate concentrations, substrate inhibition was not demonstrated by the data collected.

The standard model of substrate inhibition predicts a maximum in the specific growth rate when it is plotted against the concentration of the growth limiting substrate. The specific growth rate, as estimated from the cycle time, did not demonstrate a clear maximum when plotted against hexadecane concentration. The change in the final biomass concentration and in the biomass yield, was not explained by the substrate inhibition model, which predicts only changes in the specific growth rate. Clearly, there is another mechanism, which is affecting both growth and substrate utilization in a manner which is deferent from the standard model of growth inhibition.

The specific growth rate showed different trends for continuous and initial addition when plotted against hydrocarbon concentration. Values of specific growth rate were similar for both addition methods at high substrate concentrations but differed

greatly at lower concentrations of hydrocarbon. The divergence was due to the drop in cycle time observed when the volume of hydrocarbon added initially was reduced. This is contrary to what would be expected if hexadecane were the growth limiting component in the medium. This may indicate significant differences in the growth of *Y. lipolytica* for the different addition methods. It may also show that the assumptions made to estimate the growth rate were not accurate for both hydrocarbon addition methods.

Hexadecane was shown to be non-toxic by the Microtox assay. Most studies of alkane inhibition have focused on the shorter, water-soluble homologues, which are by far the most toxic[53,54,55]. The longer alkanes have been shown to be less toxic, due to their low water solubility, but there is some evidence that the combination of emulsifier and long chain alkane is more toxic than either compound alone[56].

Continuous addition of hexadecane into the SCF is similar to hydrocarbon fed-batch fermentation. Work on hydrocarbon fed-batch showed similar results to those obtained in the SCF during continuous addition[36]. In both studies, increasing the hydrocarbon feed rate into the reactor resulted in increased biomass concentration and biomass yield.

Lee investigated the continuous addition of a p-xylene and toluene mixture to a reactor similar to the SCF, but control was based on monitoring the concentration of ammonium, the nitrogen source in the medium, and not dissolved oxygen[34]. As with the results of the current work, cycle time, biomass concentration and yield all decreased when the feed rate was increased. This was caused by increases in both cell growth and death rates at higher feed-rates of the hydrocarbon mixture. This resulted in a higher uptake rate, and quicker exhaustion of the nitrogen source whose concentration was used

to control the reactor. Nitrogen is used mostly for the synthesis of cellular components[41]. These components remain in the system and can be reincorporated into growing cells after being freed from dead cells. However, once the nitrogen is incorporated into a growing cell, it is no longer in the form of ammonia, which was monitored to control the reactor. A greater rate of ammonia uptake resulted in shorter cycle times and lower biomass concentrations.

Cycling in the SCF is controlled by dissolved oxygen, which is a measure of growth. The reactor cycles when the growth of the organism has stopped and the dissolved oxygen rises. This occurs when the limiting nutrient in the medium is exhausted. For the explanation of Lee to apply to the current work, the limiting nutrient in the medium must be something that, once incorporated into a growing cell and released upon the death of that cell, cannot be reused by other growing cells. This would not be the case if the liming nutrient were nitrogen. It is tempting to propose that thiamine is the limiting nutrient in the present work, since it has been shown to be required for growth.

When an end of cycle time delay was imposed on the reactor running with continuous hydrocarbon addition, biomass concentration decreased. This may have been caused by cell death during the extended portion of the cycle. This has been shown to occur for this organism in batch growth, but at a slower rate than observed here[37]. The apparent drop in biomass may also have been caused by the release of cell bound material into the medium. This often occurs during the transition of the fermentation from the growth to stationary phase[57].

When the fermentor was run using initial hydrocarbon addition, and an end of cycle time delay was imposed, the final biomass concentration increased. The different effect of the delay showed that conditions in the reactor were different for the different addition methods when the minimum in dissolved oxygen was detected.

5.3 Liposan Production in the Self-Cycling Fermentor

Preliminary experiments showed the advantage of using the SCF to improve liposan production. When *Y. lipolytica* was grown in a batch reactor, the carbohydrate concentration reached 0.044 g/L after 140 hr. The same concentration was obtained in the SCF after 6.7 hrs. Increased production of similar compounds in the SCF has been previously reported[31,32,33]. Residual hydrocarbon for this earlier work was very high, showing that a large excess of hydrocarbon was being fed into the reactor.

To further increase the production of liposan, a portion of the reactor contents, at the end of a cycle, were placed in a shake flask and incubated. This allowed growth to continue. This technique has been referred to as a two-stage reactor, and has been shown to increase the production of similar compounds, beyond what was possible in the SCF alone[31,32]. This technique takes advantage of the increased production in the SCF, but allows for the indefinite continuation of a cycle. This is not normally possible without upsetting the operation of the reactor. Using the two-stage reactor technique, carbohydrate concentrations of 0.2 g/l were obtained. This is more than twice the liposan concentration achieved using any other method. Unfortunately, this approach also took significantly longer to complete the fermentation.

The effect of continuous hydrocarbon addition on the growth of *Y. lipolytica* in the SCF discussed in the previous section is interesting, but the real objective of this work was to consider the effect of the addition method on the production of liposan. For all continuous flow-rates studied, the ratio of liposan to biomass was constant. This showed a coupling between the two fermentation end products: biomass and liposan. Because of this relationship, the increased biomass, resulting from the positive effects of continuous addition, also gave increased liposan concentrations. As observed with the biomass, lower flow-rates of hydrocarbon gave the highest product concentrations. Although this coupling between liposan and biomass resulted in higher liposan concentrations, it would be preferable to overcome this relationship, and increase liposan at the expense of the amount of biomass generated.

The total hydrocarbon being added to the SCF during continuous addition varied between 2.8 ml/cycle and 1.4 ml/cycle. This was much less than the 5 ml/cycle added during the preliminary work with initial addition. It was necessary to show that the increase in liposan, seen during continuous addition, was caused by the addition method and not a reduction in the total hydrocarbon being added to the reactor.

When the volume of hydrocarbon, being added to the reactor initially, was reduced, the final liposan concentration decreased. Although growth of the organism was similar for both addition methods, the effect on liposan was contrary to what had occurred during continuous addition. This is the most significant difference between the two addition methods. This is demonstrated by plotting the final carbohydrate concentration, for the two addition methods, against the total hydrocarbon added (Figure

4-1). Liposan concentration did not show a clear trend with total hydrocarbon added for continuous addition, but was greater than for initial addition.

The different effects on biomass and liposan production, in response to changing amounts of total hydrocarbon, for the different addition modes, were made apparent by the yield coefficients and the product to biomass ratio. For continuous addition, the product to biomass ratio remained constant. Lower hydrocarbon concentrations resulted in increased growth, and a proportional increase in liposan production. For initial addition, the same increase in growth was observed when the total amount of hydrocarbon was reduced, but liposan concentration was also reduced, and the product to biomass ratio was decreased.

Higher liposan yield coefficients for continuous addition relative to initial addition indicated an increase in production efficiency. This, in combination with the effects of the addition method on the product to biomass ratio, indicated a more productive biomass during continuous addition. This was further demonstrated by the average specific production rate of liposan. Unlike the specific growth rate, the average specific production rates, for each addition method, formed a distinct trend when plotted against the average hexadecane concentration. The larger specific liposan production rates, resulting from continuous addition, confirmed the positive effects of this addition method on the production of liposan, at low hydrocarbon concentrations.

An increase in the production of surfactants at low growth rates has been shown for some organisms[26]. A fed-batch strategy was used to increase the production of emulsan by controlling the growth rate of *Acinetobacter sp*[58]. The increase in liposan

production in the current work may be due to the lower specific growth rate that results from a low feed rate of the carbon source.

The two-stage reactor experiments showed that there was a potential for higher levels of liposan production with the system. Inter-cycle data showed that liposan production occurred towards the end of the cycle. To extend the production period, and increase the end of cycle liposan concentration, a time delay was placed between the detection of the minimum in dissolved oxygen and cycling the reactor. Extended growth, under nutrient limitation, has been shown to promote production of compounds similar to liposan [3,18,19,27,28]. End of cycle time delays stressed the organism by forcing it to grow under low concentrations of one or more nutrients.

Time delays of different lengths were placed at the end of cycle for the continuous addition of 0.2 ml/hr and 0.25 ml/hr of hexadecane. Minimum change in final carbohydrate concentration was seen when the delay was extended for the larger, 0.25 ml/hr addition rate. For the lower flow-rate of 0.2 ml/hr, carbohydrate concentrations increased rapidly after 0.5 hrs of delay, to a maximum at 2 hrs of delay. The maximum carbohydrate concentration of 0.091 g/L was the highest concentration obtained in the primary reactor, and was double that of the preliminary experiments. The additional liposan, seen during the delay, may have been the result of the biomass releasing cellular material during the transition from the exponential to stationary growth phase[57].

For continuous addition experiments without time delay, the product to biomass ratio was not affected by the flow-rate of hexadecane into the reactor. In order to improve product quality, it was desirable to decouple the two fermentation end products. The

opposite trends in biomass and liposan during the time delay resulted in an increasing product to biomass ratio.

Liposan concentration was also increased when an end of cycle time delay was imposed, following initial hydrocarbon addition. This increase was not as large as that observed for continuous addition with a time delay. As with continuous addition, the maximum in liposan occurred after 2-hrs of delay.

5.4 Elimination of the Residual Hydrocarbon

The presence of a hydrocarbon residue is undesirable because it will be associated with the emulsifier. This makes purification of the product difficult. As the continuous hexadecane feed rate was reduced, there was a decrease in the amount of residual hydrocarbon. By using the SCF with continuous hydrocarbon addition and optimizing the feed rate, it was possible to reduce the amount of residual hexadecane to less than 0.5 ml/L.

Similar results were achieved using the method of initial hydrocarbon addition. As would be expected, when less hydrocarbon was added initially the residual hydrocarbon was lower. For all initial addition experiments, the volume of hydrocarbon remaining at the end of the cycle was close to the volume of hydrocarbon added at the beginning. Since the initial concentration of hexadecane in the reactor, before additional hydrocarbon is injected, is half of the final concentration, the hydrocarbon degradation rate was approximately 60% for all initial addition experiments.

Attempts to further reduce the hydrocarbon residue, using the continuous addition method, resulted in very long cycle times. This made the reactor difficult to operate, and

significantly reduced its productivity. For many hydrocarbon fermentation studies, the residual concentration of hydrocarbon reported has been significant[31,32,37]. This residual hydrocarbon may play an important role in the growth of the organism. One study showed the requirement for a minimum amount of hydrocarbon before any growth would occur[36].

A different strategy was employed in trying to eliminate the hydrocarbon residue. A time delay was placed between the detection of the minimum in dissolved oxygen and cycling the reactor. Input of hexadecane was halted during the time delay so that the remaining hydrocarbon could be consumed by the organism.

Two continuous hydrocarbon flow-rates were used in combination with the time delay. The effect on residual hydrocarbon was different for the different hydrocarbon flow-rates. It was expected that cycle time would increase due to the time extension added to the end of the cycle. This occurred with the lower hydrocarbon flow-rate, but the increase in cycle time was more than the duration of the time delay. This indicated that the minimum in dissolved oxygen was occurring later, when a time delay was imposed. The time required to complete a cycle did not change for the higher hydrocarbon flow-rate when an end of cycle time delay was imposed. For this flow-rate, the minimum in dissolved oxygen occurred earlier as the time delay was extended. The effect of the time delay, on the period before the minimum in dissolved oxygen was observed, resulted in different volumes of hydrocarbon being added to the system, at different time delays.

For the lower hydrocarbon flow-rate, more hydrocarbon was fed to the reactor because the period before the minimum in dissolved oxygen became longer. For the

shortest delays, the residual hydrocarbon increased. As the delay became longer, the additional degradation during the delay overcame the extra hydrocarbon, and the residual hydrocarbon concentration was reduced below the detection limit.

The constant cycle time for the larger hydrocarbon flow-rate resulted in less hydrocarbon being fed to the system, because the minimum in dissolved oxygen occurred earlier. Less hydrocarbon was added to the system, and additional hydrocarbon degradation occurred during the delay. This allowed for further reduction of the residual hydrocarbon concentration, without the cycle increasing beyond a practical length of time. However, when the larger hydrocarbon flow-rate was used, the hydrocarbon residue could not be completely eliminated.

For experiments with a time delay and initial hydrocarbon addition, the total hydrocarbon fed to the reactor remained approximately constant, since the time to the minimum in dissolved oxygen did not change. This showed either a different form of growth or different conditions in the reactor, when the minimum was detected. Residual hydrocarbon concentrations decreased slightly during the delay following initial addition.

5.5 Productivity and Product Quality of Liposan in the Self-Cycling Fermentor

The variables describing the production of liposan in the SCF, as with any process, can be categorized as either product quality, or productivity. Both the concentration of liposan, and the concentration of any side products affect the quality of the product. The major contaminants in this system are the residue of hydrocarbon that was necessary for the production of liposan and the biomass. Production rate, yield, and specific productivity are parameters that deal with the productivity of the system. In

optimizing any system there is usually a trade-off between productivity and product quality.

As the continuous flow rate of hydrocarbon into the reactor was reduced, the duration of the cycles increased. This increase was minor for larger and intermediate rates of addition, but significant at low hydrocarbon feed rates. Cycle time increased much faster than liposan concentration, resulting in a drop in production rate. The maximum in production rate occurred at a continuous feed of 0.4 ml/hr of hexadecane. This is far from optimal for liposan and residual hydrocarbon concentrations, demonstrating the trade off among yield, productivity and product quality. Continuous addition allows for a higher liposan production rate than initial addition, at lower residual hydrocarbon concentrations. The highest liposan production rates, for both addition methods, occur when a large volume of hydrocarbon was added to the reactor, resulting in a high concentration of residual hydrocarbon.

When a time delay was imposed at the end of the cycle, the liposan concentration was further increased. The cycle time was also increased by the delay, reducing the rate of liposan production below its value with no delay. The time delay did allow for higher rates of liposan production at very low concentrations of residual hydrocarbon. In general, the parameters dealing with product quality were improved by the time delay, while parameters related to productivity were decreased.

For most biological systems, the most expensive step in production is purification of the desired product[1,3,27]. The alternative to separation is to use the whole growth broth for the desired application. Emulsan is used in this way by the petrochemicals industry. Instead of separating the emulsifier, the whole broth is used, reducing the cost. Since

emulsan is used mostly for cleaning tanks, the purity of the product is not important, as long as the final preparation exhibits the desired properties[1]. The final concentration of liposan for continuous addition with a time delay was high enough to be used as an emulsifier without separation. For either production scenario, the advantage of having increased product concentration and decreased waste product levels is more important than the small reduction in production rate.

6 Conclusions

A simple medium was found for the production of liposan from *Y. lipolytica*. It was determined that the growth of the organism was heavily dependent on thiamine, and that no liposan would be produced if thiamine were not included in the medium.

Using continuous hydrocarbon addition with the SCF gave a maximum liposan concetration of 0.08 g/L. This was double the maximum liposan concentration obtained with the standard initial addition method. The rate of hydrocarbon addition to the SCF was shown to have a strong effect on the growth of *Y. lipolytica* and the production of liposan.

By controlling the hydrocarbon addition rate, the residual hydrocarbon concentration was reduced below 0.1%, without reducing liposan production. The initial addition method did not allow for the residual hydrocarbon concentration to be reduced without also reducing the production of liposan.

The final concentration of liposan was further increased to 0.095 g/L by imposing an end of cycle time delay, during which continuous hydrocarbon addition was stopped. The end of cycle time delay eliminated any residual hydrocarbon, further increasing product quality. The end of cycle time delay was most effective when combined with a hydrocarbon addition rate that was optimal for liposan production and residual hydrocarbon concentration.

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