Citric acid production using the self-cycling fermentation technique

By

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Abstract

Self-cycling fermentation is a new technique used to cultivate synchronous populations of cells. This work used the technique in the production of citric acid from a strain of *Candida lipolytica*. Application of this technique resulted in extremely stable, reproducible patterns of growth and induced a high level of cell synchrony. Synchrony was maintained even throughout long periods of extended nutrient starvation. Thus, self-cycling fermentations can be operated with cycle times significantly longer than the doubling time of the microorganism. Use of this technique led to a full order of magnitude increase in the specific biomass production rate compared to literature values. This biomass was found to be suitable for the production of citric acid in a second stage reactor. Although this preliminary work with the second stage reactor was not optimized, the specific citric acid production rate obtained was comparable to rates found in the literature.

Résumé

La fermentation auto-cyclique est une nouvelle technique utilisée pour cultiver des populations de cellules synchronisées. Ce travail a utilisé cette technique dans la production d'acide citrique par une variété de *Candida lipolytica*. L'application de cette technique a eu pour résultat des profils de croissance stables et reproduisibles et a provoqué un grand degré de synchronisation des cellules. La synchronisation a même été maintenue à travers des périodes prolongées de privation de nutritifs. Ainsi, les fermentations auto-cycliques peuvent être opérées avec des durées de cycles beaucoup plus longues que le temps de doublage des microorganismes. L'utilisation de cette technique a mené à une augmentation d'un ordre de grandeur de la production spécifique de la biomasse comparé aux valeurs bibliographiques obtenues. Cette biomasse a été trouvée convenable pour la production d'acide citrique dans un deuxième réacteur. Quoique ce travail préliminaire avec ce deuxième réacteur n'a pas été optimisé, le taux de production spécifique d'acide citrique obtenue était comparable aux taux obtenus dans la bibliographie.

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1. Introduction

1.1 Cell Synchrony

Understanding the cell cycle has long been a goal of the microbiologist. For many years, microbiologists have used the microscope to observe the individual cell and its development during the cell cycle. On a macroscopic level, it is only possible to study the entire cell population. It has therefore become the basic unit of study in microbiology. Since cells in a population are present in a distribution of ages, it is obvious that studies of such populations will yield, at best, only average values of metabolic parameters.[17]

In recent years the development of cell synchrony has improved the study of microorganisms.[17] In these cultures, the cells in the population are all, or almost all, in the same stage of development, therefore the averaging factor accompanying a distribution of ages is reduced. Thus, the results obtained from such populations more closely reflect the processes of a single cell.[17,25] Various techniques to produce synchronous cultures exist and can be grouped into three categories: selection, culture fractionation and induction.

1.2 Techniques Used to Produce Synchronous Cultures

1.2.1 Selection Methods

Synchronous cultures are produced by passive techniques based on the assumption that a direct correlation exists

between cell size and cell age.[22,35] Methods established when selecting for synchronous populations are size selection by filtration and velocity sedimentation, and age selection by membrane elution.[22]

1.2.2. Culture Fractionation Methods

Culture fractionation procedures permit physical separation into sub-populations of organisms in an exponentially growing culture. This method provides a rapid classification of an asynchronous population into its various age classes. In this method, the exponentially growing culture is subjected to some treatment in which all cells receive an identical exposure before instantaneous arrest by rapid cooling. The cells are then separated into fractions of increasing size by centrifugation. A wide range of applications has been reported for the technique of culture fractionation.[22,35] This range includes the measurement of the rate of enzyme induction at successive stages of the cell cycle, the chemical analysis of different size classes and the measurement of the rate of induction of enzymes at successive stages of the cell cycle.

1.2.3 Induction Methods

Induction methods usually involve the perturbation of environmental variables and, as a result, provide important information on the control mechanisms involved in the cell

division process. Throughout the years, many methods have been used to induce cell synchrony, however, most have been found to be extremely organism-dependent.[22] Some of these include: cyclic changes of temperature or pressure, the use of DNA synthesis inhibitors, nutrient deprivation followed by refeeding, regimes of illumination followed by periods of darkness, pulse addition of the limiting nutrient at doubling time intervals in a chemostat and continuous phased growth with periodic feeding.

1.3 Induction of Synchronous Cultures by Nutrient Manipulation

Induction of synchronous cultures through essential nutrient manipulation is one of the few induction methods which show promise for large scale development.[17,22,35] The problem with selection methods and culture fractionation methods is that, generally, they are used in conjunction with batch growth and maintain synchrony for only a few generations.

A cell is thought to prepare for division along many more or less independent channels.[35] Synchronization is promoted by the establishment of conditions which will reduce the rate of progression towards cell division in one of these channels. The cells that are closest to division are most retarded by this reduced rate, whereas those farthest away from division are least affected. The cells are given the opportunity to mature along all other channels. The release of the blocking

mechanism of the specific channel is triggered, for example, by the depletion of a limiting nutrient, allowing synchronous division to take place.

Two continuous methods of induced synchronization have been developed: pulsed culture and phased culture. The pulsed culture involves pulsed additions of limiting nutrient at intervals equalling the doubling time of the organism, to a chemostat growing on a medium deficient in that nutrient. Instead of a continuous supply of fresh medium and pulsed additions of limiting nutrient to the fermenter, the phased culture involves a total addition of nutrient rations to the fermenter at cycle intervals of *chosen* doubling time.[14,15,16,17] Also, one half of the cell-laden broth is removed prior to each nutrient addition to avoid increase in the volume of the system. Both systems yield similar results, however, the phased culture method ensures an identical nutrient environment for each cycle. As a result of this uniform nutrient environment, the data collected from these phased fermentations is extremely repeatable.

1.4 Self-Cycling Fermentation Technique

In the phased culture method introduced by Dawson,[14,15,16,17] a selected period of nutrient dosing was imposed on the cell cycle. It is obvious that a period of nutrient dosing too short to permit cell doubling will result in the eventual washout of the cells. On the other hand, if the imposed cycle

time is too great the cells will experience a period of nutrient starvation at the end of each cycle. Depending on the duration of this period of starvation, it could have a serious metabolic effect on the cells.[31]

In the technique developed by Sheppard and Cooper,[31] the period of nutrient addition (cycle time) was eliminated as an operator-selected variable. The system was based on the relationship between the concentration of dissolved oxygen and that of the limiting nutrient.[31] Following the depletion of the limiting nutrient, a rapid rise in the dissolved oxygen concentration is observed as a result of the declining respiration rate. Once this minimum in dissolved oxygen is detected, the control sequence is initiated which consists of the harvesting of one half of the fermenter contents and subsequent replacement with an equal volume of fresh medium. An idealized representation of process parameters for this technique is shown in Figure 1.1.[30] After several cycles, a steady state was achieved and the cell population became synchronized. Thus, the cycle time was equal to the doubling time of the microorganism. To date, the self-cycling fermentation technique has been successfully applied to various microorganisms including *Acinetobacter calcoaceticus*, *Bacillus subtilis*, *Candida bombicola* and *Pseudomonas putida*.[10,11,25,31]

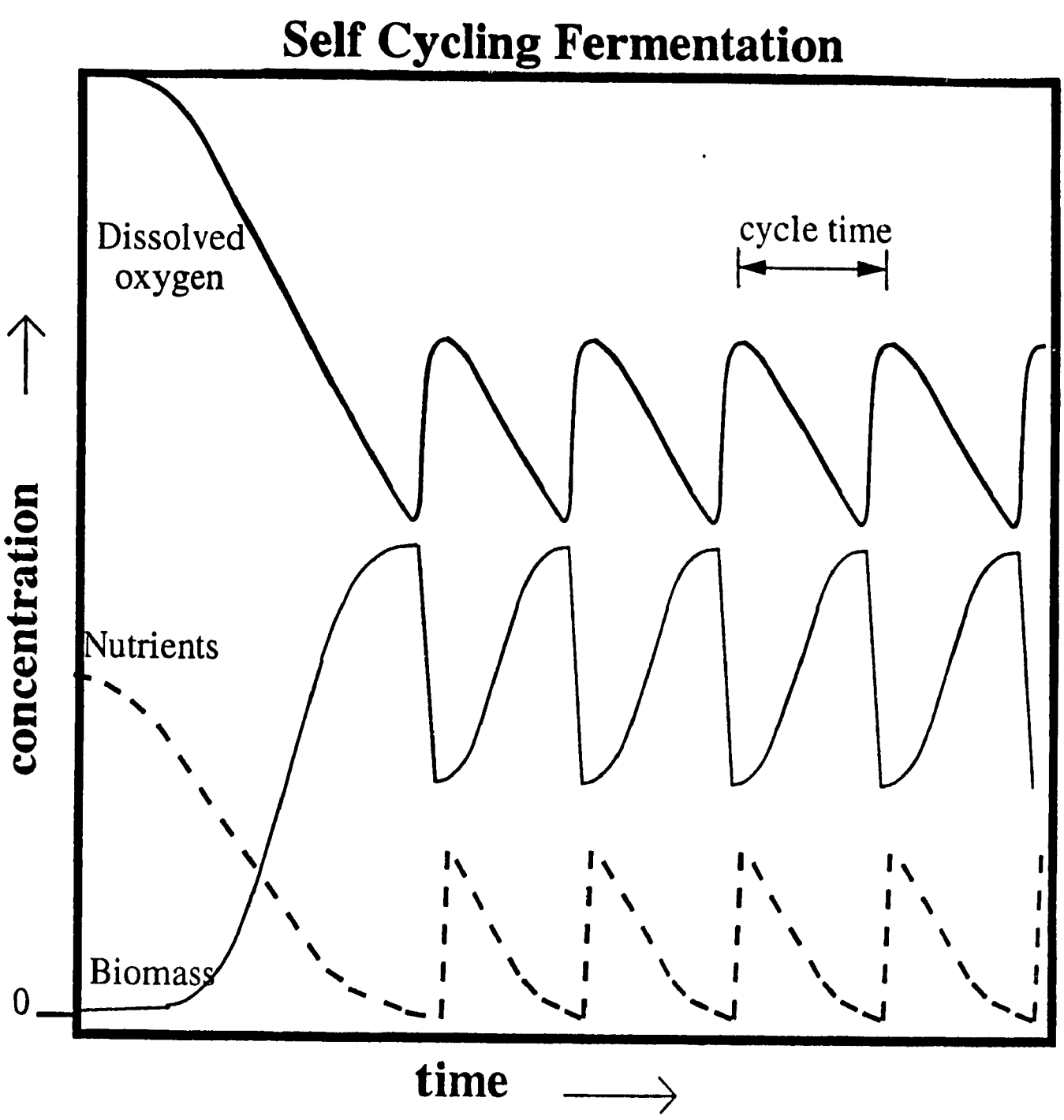

Figure 1.1 Idealized process parameters from a self-cycling fermentation

The major advantages of the self-cycling fermentation system are that no prior knowledge of the cell's growth rate was required and that any change in this growth rate resulted in the immediate compensation in the period of nutrient cycling.[25,31] Also, the portion of the broth which is harvested from the fermenter is available for second stage studies[31] which are important when conducting investigations into post-exponential growth characteristics such as product generation.

1.5 Production of Citric Acid

Citric acid, a natural constituent and common metabolite of plants and animals, is the most versatile and widely used organic acid in the food, beverage and pharmaceutical fields[21]. In the food and beverage industry, citric acid is used extensively in carbonated beverages to provide tartness and to complement fruit and berry flavours. Citric acid is also used in many industrial applications to neutralize bases and act as a buffer. In cosmetics, it is used primarily as a buffer to control the pH in shampoos, hair rinses and setting lotions.

The traditional method of preparing citric acid consisted of extracting the acid itself from the juice of certain citrus fruits, such as lemons, limes or pineapples.[9,21] With the development of fermentation technology such processes quickly became obsolete. The microbial production of citric acid on a commercial scale began in 1923 using a strain of *Aspergillus niger*.[9,21] The fermentation vessel usually consists of a

sterilizable tank of several hundred cubic meters equipped with a mechanical agitator and a means of introducing sterile air. In this case, a complex molasses medium was used as the carbon source.

Until about 1970, *Aspergillus niger* was considered to be the only organism capable of producing citric acid in commercial quantities. At this time it was found that species of yeast, *Candida guilliermondii and Candida lipolytica*, also demonstrated the ability to produce significant quantities of citric acid.[18,21] These cultures were grown in mediums containing either glucose or molasses as the carbon source. The fermentation time with these organisms was found to be shorter than with *Aspergillus niger*.

Candida strains are also used in a novel process which involves the production of citric acid from hydrocarbons. In 1974, Pfizer Inc. patented a continuous process for the production of citric acid by *Candida lipolytica* using a single vessel to which paraffin is continuously added and fermented broth is continuously withdrawn. Citric acid yields (by weight) in excess of 100 % have been observed for this process.[21] For obvious reasons, the uses of such citric acid produced from a hydrocarbon are much more restricted than for citric acid which has been produced from sugars.

The production of citric acid by *Candida lipolytica* from both hexadecane and glucose substrates has been studied extensively in the past.[1,2,3,4,5,7,8,19,20,23,24,28,29,32,33,34] The high yields of

citric acid produced by certain strains of *Candida lipolytica* suggest that these overproducing strains may possess an obstruction in the tricarboxylic acid cycle (Figure 1.2). This barrier was clarified by one study which showed that the aconitate hydratase activity of the cells of the producing strains were about 1% to 10% of those observed in the parent strains.[3,4,5] No prominent differences in any other enzyme activity was observed between those of the parent and the mutant strains.

Candida lipolytica produces citric acid at different times during the fermentation depending on the carbon source used. Citric acid is excreted early in the stationary phase when hexadecane is used.[32] However, when glucose is used, citric acid is excreted for approximately 150 hours after the stationary phase begins.[32] Batch fermentations are currently used with glucose due to the rather long period of acid excretion. The disadvantage which accompanies such a system is the down time which is necessary to cleanup and resterilize the equipment for subsequent batches. The self-cycling fermentation technique would eliminate such inconvenient down times and provide a continuous prcduction of not only cells but citric acid as well.

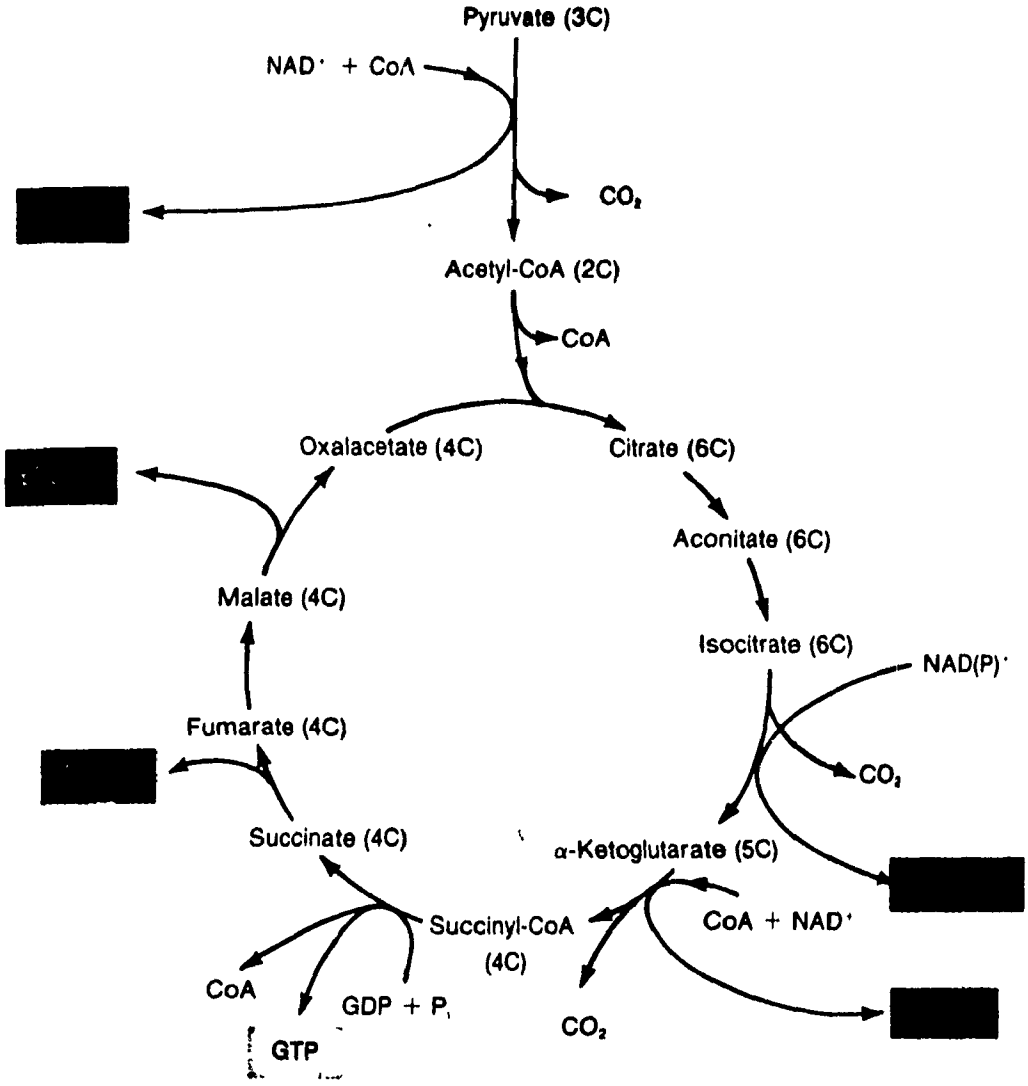

Figure 1.2 The tricarboxylic acid cycle

2. Objectives

This work is part of an ongoing research effort using the self-cycling fermentation technique. The ultimate goals of this effort are to develop the technique and to better define its capabilities as well as its limitations. In order to help accomplish this ultimate goal, the particular objectives of this project were:

1. To apply the self-cycling fermentation technique to *Candida lipolytica*.

2. To compare the specific biomass production rate obtained to those values currently found in the literature.

3. To produce citric acid using the self-cycling fermentation technique and to compare values of specific citric acid production to values in the literature.

3. Materials and Methods

3.1 Microorganism

Candida lipolytica (ATCC 20390), was obtained from the American Type Culture Collection. The culture was maintained on Difco Yeast Malt agar at 4°C. Each month, a single colony was transferred from stored plates to fresh agar plates in order to preserve cell viability.

During the inoculum preparation for the self-cycling fermentation experiments, the culture was first transferred from the agar plates to an aqueous solution of Difco Yeast Malt broth and incubated for two days at 25 °C on a gyratory shaker at 300 rpm (New Brunswick Scientific Company, Incubator Shaker Model G25). The culture was then transferred to the medium used in the experiment and incubated on the shaker at 25 °C for another 3 days. Usually a 1% inoculum was used to inoculate the reactor during start-up.

3.2 Medium and Growth Conditions

The medium used for all experiments was a defined mineral salt medium previously used for the production of citric acid by *Candida lipolytica*.[2] The major carbon and nitrogen sources in the medium were glucose and ammonium sulphate, respectively. For self-cycling fermentation experiments the medium was prepared in 10 L Nalgene reservoirs and had the composition shown in Table 3.1.

Table 3.1: Composition of Medium Used in Self-Cycling Fermentation Experiments

	Concentration (g/L)
Glucose	6.0 - 12.0
$(NH_4)_2SO_4$	1.2
KH_2PO_4	0.5
$MgSO_4 \cdot 7H_2O$	0.5
NaCl	0.1
Na_2HPO_4	6.5
$FeSO_4 \cdot 7H_2O$	0.0010
$ZnSO_4 \cdot 7H_2O$	0.0020
$MnSO_4 \cdot H_2O$	0.0015
EDTA	0.0700

	Concentration (μg/L)
Thiamine·HCl	50
$CuSO_4 \cdot 7H_2O$	50

In order to prevent caramelization of the medium, it was necessary to sterilize the glucose separately. After the glucose solution was cooled, it was then added aseptically to the 10 L medium reservoir.

3.3 The Self-Cycling Fermenter Setup

A schematic of the self-cycling fermenter apparatus, similar to that used by Brown and Cooper,[10,11,14,25,31] is shown in Figure 3.1.[30] The heart of the system consisted of a cyclone column fermenter in which the broth is continuously recirculated by a 0.2 hp pump (March Model MDX). The temperature was maintained at 26 °C by the use of a water

jacket and a recirculating water bath (Haake Model FE 2). Antifoam was added to the system using a syringe pump (Sage Instruments Model 355). Air flow was controlled by a rotameter at 0.55 L/min and sterilized by inline filters. At the end of a cycle, flow of cell laden broth to the harvesting vessel and fresh medium to the dosing vessel was controlled by solenoid valves (Skinner Model V52LB2052). The exact dosing and harvesting volumes were measured using an electronic balance (Precisa 6000D).

Following the harvesting of the broth, the main solenoid valve leading to the sampling system was opened and the broth was passed to an overflow vessel. 250 mL of the broth was retained in this vessel and the remaining volume was discarded into the waste collection jar. Then, the main solenoid was closed and one of the solenoid valves leading to one of the sample bottles was opened. The sampling system was rotated between collection bottles following each completed cycle. The sample bottles were located in a small refrigerator and maintained at a constant temperature of 4 °C.

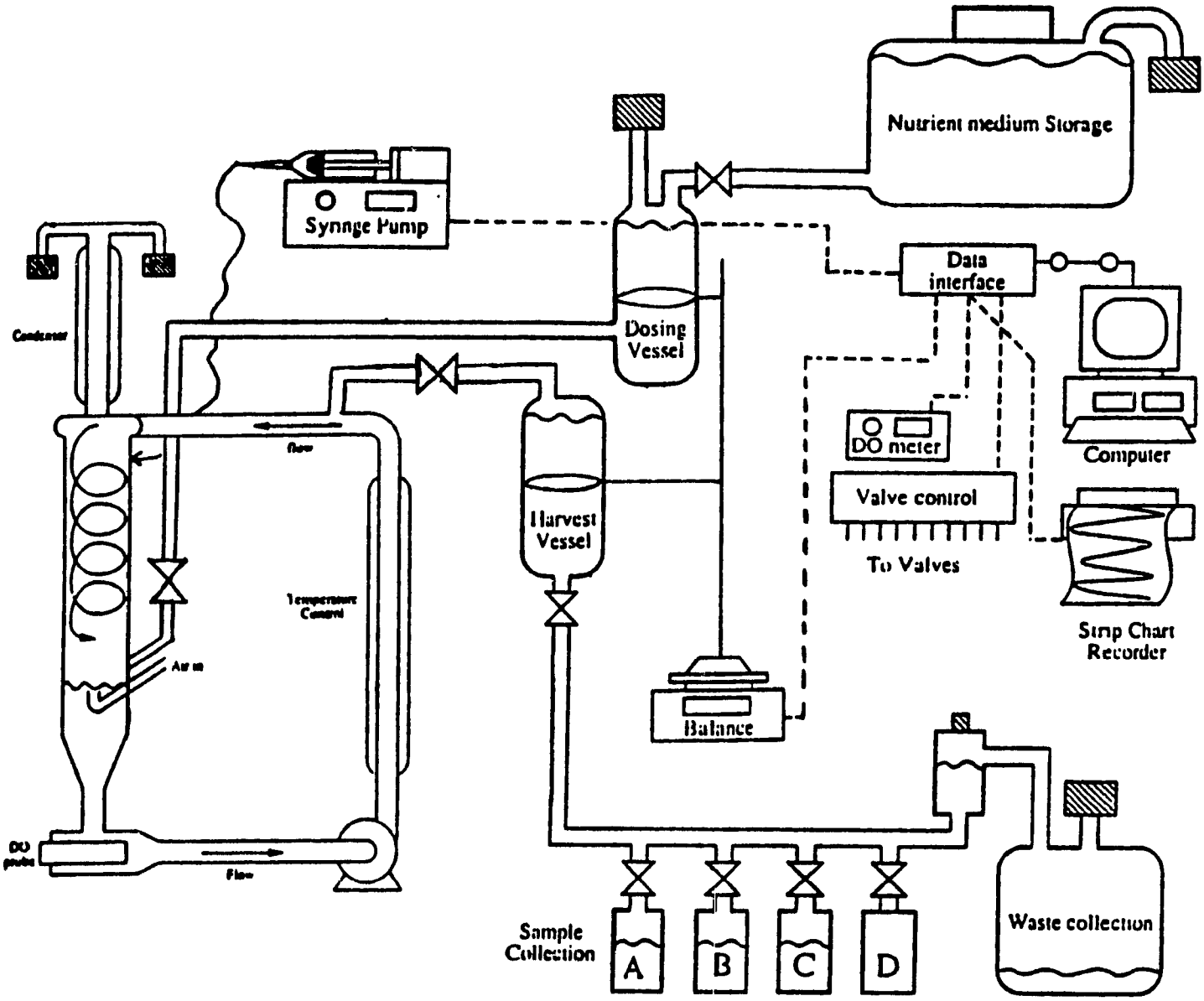

Figure 3.1 The self-cycling fermentation apparatus

3.4 Analysis of Media Components

3.4.1 Ammonia Nitrogen Concentration

Nitrogen analysis was performed by Conway Microdiffusion Analysis.[13] This technique requires special dishes with two concentric chambers. In the inner chamber of the dish, one mL of a boric acid solution containing 1% mixed indicator (0.067% methyl red in alcohol and 0.033% bromocresol green) is added. After the addition of 0.5 mL of saturated Na_2CO_3 and 0.05 mL of the cell free sample to the outer chamber, the dish was covered and sealed with silicone vacuum grease. The dish was then slowly rotated to mix the saturated Na_2CO_3 solution with th sample. The mildly basic conditions result in a release of ammonia gas from the residual ammonium in the sample. The gas released is absorbed by the pink boric acid indicator solution and the colour changes to green. For the purpose of this study, a lack of colour change was sufficient to confirm that the nitrogen had in fact been completely metabolized at the end of the cycle.

3.4.2 Glucose Concentration

The glucose concentration in the fermentation broth was measured using a quantitative glucose enzymatic determination kit by Sigma Diagnostics. This method is based on the following two enzymatic reactions:

$$Glucose + 2H_2O + O_2 \xrightarrow{Glucose\ Oxidase} Gluconic\ Acid + 2H_2O_2$$

$$H_2O_2 + o\text{-}Dianisidine \xrightarrow{Peroxidase} Oxidized\ o\text{-}Dianisidine$$

(colourless) (brown)

The oxidized o-dianisidine has a brown colour. The optical density measured at a wavelength of 450 nm is proportional to the amount of glucose present in the sample. When performing the analysis it was first necessary to prepare the combined enzyme-colour reagent solution. This was accomplished by combining 100 mL of distilled water with 5 units/mL of glucose oxidase, 1 purpurogalin units of peroxidase per mL and 1.6 mL of a 2.5 g/L o-dianisidine solution. In the actual procedure, 0.5 mL of a 20-fold dilution was pipetted into a test tube and mixed thoroughly with 5 mL of the combined enzyme-colour reagent solution. The reaction was allowed to proceed to completion which took approximately 30 minutes at 37 °C or 45 minutes at room temperature. The final colour intensity was measured within 30 minutes of reaction completion, with a dual beam UV-visible spectrophotometer (Varian Model DMS 200) and was proportional to the glucose concentration.

3.4.3 Citric Acid Concentration

Citric acid concentration was measured using a kit distributed by Boehringer Mannheim. Citric acid is converted to oxaloacetate and acetate in the reaction catalyzed by the

enzyme citrate lyase:[26,27]

$$Citric\ Acid \xrightarrow{Citrate\ lyase} Oxaloacetate + Acetate$$

In the presence of the enzymes malate dehydrogenase (MDH) and L-lactate dehydrogenase (L-LDH), oxaloacetate and its decarboxylation product pyruvate are reduced to L-malate and L-lactate, respectively, by reduced nicotinamide-adenine dinucleotide (NADH).

$$Oxaloacetate + NADH + H^+ \xrightarrow{MDH} L\text{-}malate + NAD^+$$

$$Pyruvate + NADH + H^+ \xrightarrow{L\text{-}LDH} L\text{-}lactate + NAD^+$$

The amount of NADH oxidized is proportional to the amount of citric acid present in the sample and was measured by a UV-visible spectrophotometer (Varian Model DMS 200).

The first step in the procedure is the preparation of the reagent solutions. Solution one consists of glycylglycine buffer (pH 7.8), 11.3 units/mL of MDH, 23.3 units/mL of L-LDH and 0.5 mg/mL of NADH, while solution two contains 40 units/mL of citrate lyase. To perform the test, 1.0 mL of Solution 1, 1.8 mL of distilled water and 0.2 mL of the sample solution were pipetted into a cuvette and mixed by inverting the cuvette several times. After approximately five minutes, the absorbance at 340 nm (A_1) was read and the reaction was started by the addition of 0.02 mL of Solution 2. On

completion of the reaction, after approximately five minutes, the absorbance was read again (A_2). The equation for calculating the concentration is given by:[27]

$$c = \frac{V * MW}{\epsilon * d * v * 1000} * \Delta A$$

where c=concentration (g/L)
V=final volume (mL)
v=sample volume (mL)
MW=molecular weight of substance to be measured (g/mol)
d=light path (cm)
ϵ=absorption coefficient of NADH (6.3 @ 340nm (L/mmol/cm))
$\Delta A = A_1 - A_2$

For the concentration of citric acid the above formula reduces to:

$$c = 0.4603 * \Delta A \ (g/L \ citric \ acid)$$

3.4.4 Dissolved Oxygen Concentration

The dissolved oxygen concentration in the fermenter broth was measured using an Ingold Model IL 531 polarographic oxygen sensor distributed by Cole-Parmer. The oxygen probe contains two electrodes immersed in an electrolyte gel which is isolated from the broth by a silicone membrane. Oxygen diffuses through the membrane where it is reduced at the platinum cathode to hydroxyl ions. A current flows between the cathode and the silver/silver chloride anode which is proportional to the concentration of oxygen in the fermenter broth.

3.5 Dry Weight Determination of Biomass

Biomass measurements were made on a dry weight basis. Twenty-five mL samples were used for biomass determination with the deviation between duplicate samples being on average less than ±7%. The samples were centrifuged (Dupont Instrument RC-5 Superspeed Refrigerated centrifuge) for 10 minutes at 9000 RPM and 0°C. The supernatant was decanted and the cells were washed with distilled water and centrifuged again. The supernatant was again decanted, the cell pellet was resuspended in distilled water and transferred into pre-weighed aluminum dishes. These dishes were dried in a convection oven (Fisher Isotemp Oven 100 Series Model 126G) at 105 °C and allowed to dry to constant weight. The dishes were reweighed using an analytical balance (Mettler Model AE 160) and biomass values are reported as dry cell weight in grams per liter of broth.

3.6 Cell Count

In order to perform many cell counts within the short time of a single cycle, it was necessary to develop a quick method of determination. Direct microscope count by hemocytometer (Figure 3.2) was found to be a rapid method for enumerating microorganisms in suspension. This method determined the total number of individual cells (both living and dead) in a suspension by counting the number of organisms in a known volume of fluid contained inside a counting

chamber.[12]

The hemocytometer is a slide with a counting chamber 0.1 mm deep. On the bottom of the chamber there is an etched square divided into nine squares each having an area of 1 mm^2. The central square is divided into 25 smaller squares and each of these in turn are divided into 16 squares (Figure 3.3). When using this method it was important that the suspension be well mixed so that the individual cells were evenly distributed throughout the fluid. A 1/100 dilution of the suspension was made and using a pasteur pipette, one drop was placed in each of the two counting chambers. The hemocytometer was then placed on the microscope stage and a polaroid picture was taken with a camera attachment. The camera provided the opportunity to take more samples since it was not necessary to spend time counting throughout the sample collection period. Cell number was recorded in number of total cells per mL of fermenter broth and deviations between duplicate samples were less than ±5%.

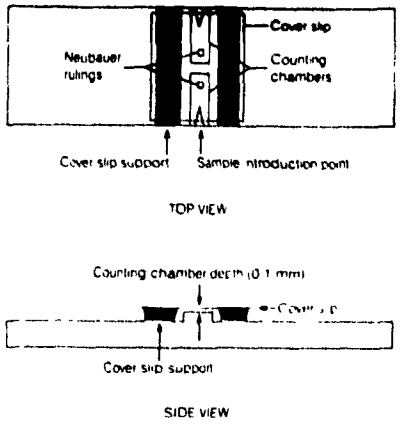

Figure 3.2 The hemocytometer

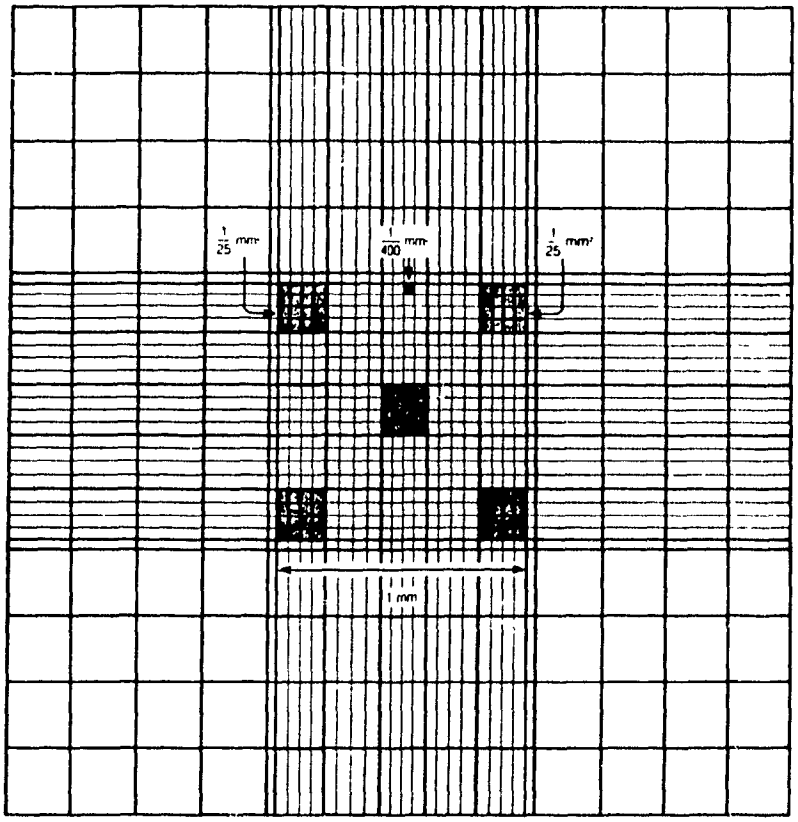

Figure 3.3 Enlarged view of the Neubauer ruling of the hemocytometer

4. Results

4.1 Self-Cycling Fermentations

Two operating advantages of self-cycling fermentations (SCFs) are: the length of time for which they can be run and the variety of different conditions which can be tested during each experiment. Table 4.1 and Table A1 in the Appendix summarize these characteristics for each of the eight self-cycling fermentation experiments performed throughout this study. The longest fermentation was Run #8, which lasted 26 days and 137 cycles. Run #1 ended due to fouling of the dissolved oxygen probe. Contamination resulted in the termination of only one experiment, Run #5.

Table 4.1: Duration of Experiments

Run Number	Duration (days)	Total Number of Cycles
1	9	15
2	13	75
3	9	69
4	17	74
5	7	36
6	16	73
7	20	67
8	26	137

4.1.1 Minimum Nutrient Starvation

Ammonium sulphate was the limiting substrate in all of the self-cycling fermentations. The test organism used was *Candida lipolytica* ATCC 20390. The temperature was kept constant for all experiments at 26 °C while the air flow was maintained at 0.55 L/min.

The dissolved oxygen (DO) profile versus time for *Candida lipolytica* grown on 1.0 g/L $(NH_4)_2SO_4$ is shown in Figure 4.1. The start of each cycle was indicated by a sharp rise in the dissolved oxygen profile. As a cycle progressed the dissolved oxygen concentration gradually decreased for approximately 3 hours. When the nitrogen source became completely exhausted, the organism's respiration rate decreased, resulting in an observed increase in the concentration of dissolved oxygen.

The stability of this fermentation system is demonstrated by Figures 4.2 and 4.3. For these particular experiments, Tables 4.2 and 4.3 show the variation of the medium composition, the corresponding cycle time and end of cycle biomass or minimum dissolved oxygen concentrations. Within two cycles of each change to the medium, the dissolved oxygen concentration, biomass and cycle time had adjusted to new steady state values. The low deviations observed in cycle times for each of the eight runs (see Table A1 in Appendix) illustrate the high degree of stability which is achieved with this system.

The system also exhibited excellent control. Following

process upsets, such as the power shutdown encountered during cycle #13 in Figure 4.2, the system quickly reestablished its former steady state value of 256 minutes (σ=11 minutes).

Table 4.2: Conditions for Run #3

Cycle Number	$(NH_4)_2SO_4$ (g/L)	Glucose (g/L)	Average Cycle Time (min)	Average Min DO Conc (% sat)
1-14	4.0	8.0	256 (σ=11.0)	0.436 (σ=0.018)
15-25	1.0	7.5	187 (σ=6.81)	0.512 (σ=0.018)
26-36	1.0	9.0	226 (σ=6.91)	0.513 (σ=0.015)
37-52	1.0	6.5	197 (σ=7.35)	0.478 (σ=0.012)
53-65	2.0	6.5	219 (σ=3.97)	0.442 (σ=0.023)

Table 4.3: Conditions for Run #7

Cycle Number	$(NH_4)_2SO_4$ (g/L)	Glucose (g/L)	Average Cycle Time (min)	Average Biomass (g/L)
1-16	1.2	12.0	441 (σ=39.0)	4.97 (σ=0.15)
17-30	1.2	10.0	451 (σ=11.6)	4.05 (σ=0.13)
31-42	1.2	7.0	334 (σ=32.1)	2.49 (σ=0.16)
43-59	1.2	6.5	327 (σ=19.6)	---
60-67	1.2	9.0	368 (σ=11.4)	3.84 (σ=0.06)

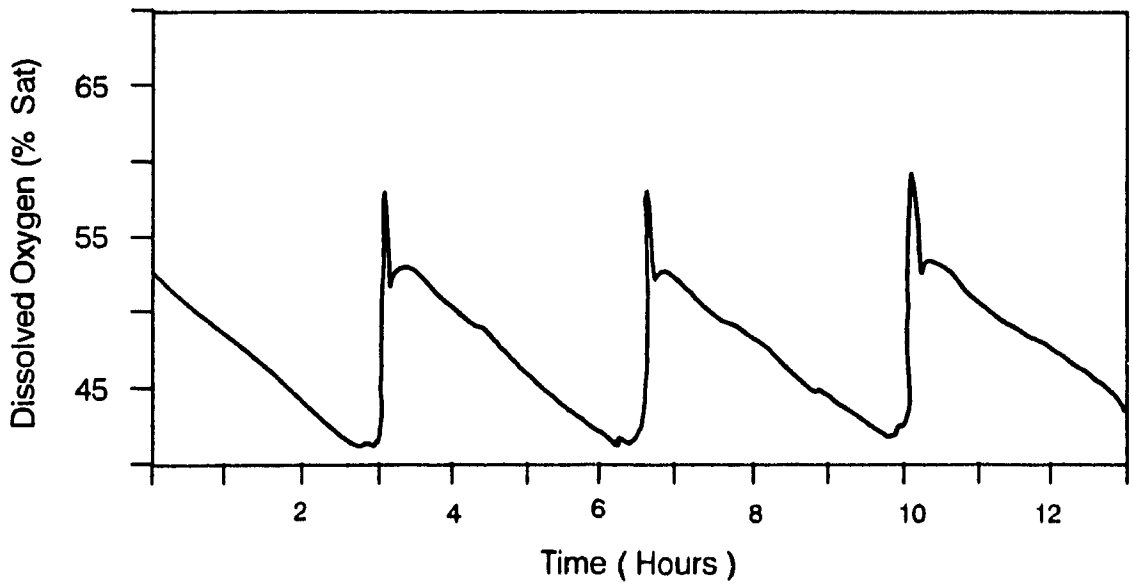

Figure 4.1 Dissolved oxygen utilization with minimum nutrient starvation for cycles 54,55,56 and 57 for Run #3

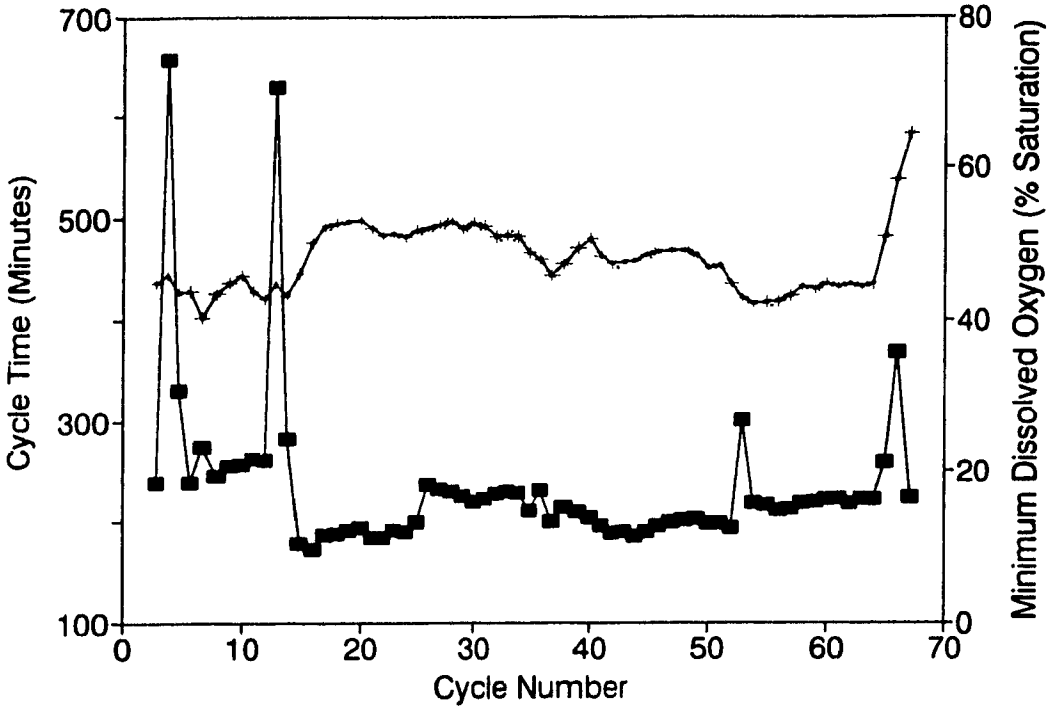

Figure 4.2 Cycle time (■) and minimum dissolved oxygen concentration (*) for Run #3

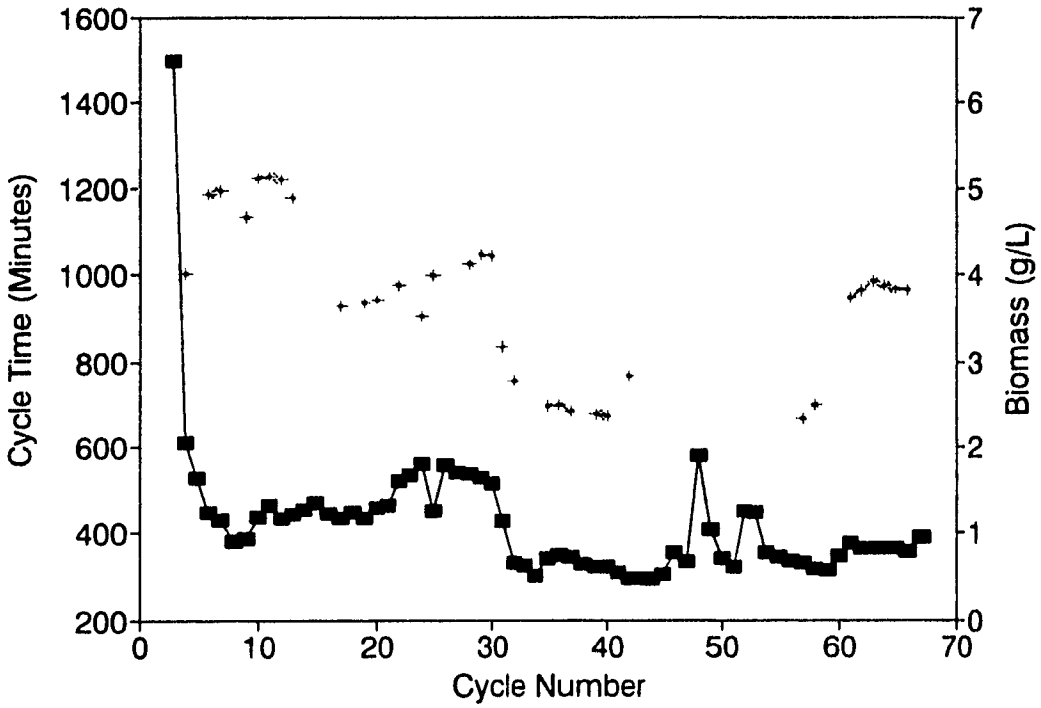

Figure 4.3 Cycle time (■) and biomass concentration (*) for extended cycles of Run #7

4.1.2 Extended Nutrient Starvation

Allowing the cycle to continue beyond the exhaustion of the nitrogen source until a second nutrient is completely consumed is termed extended nutrient starvation. The dissolved oxygen profiles for cycles of extended nutrient starvation using 1.2 g/L $(NH_4)_2SO_4$ with 7.0 g/L glucose and 1.2 g/L $(NH_4)_2SO_4$ with 10.0 g/L glucose are illustrated in Figures 4.4 and 4.5 respectively. In both cases the dissolved oxygen profile is similar to that of Figure 4.1. A manually set time delay, during which the computer does not record any values of the dissolved oxygen concentration, was incorporated into the control program. As a result, the minimum dissolved oxygen concentration, obtained upon depletion of $(NH_4)_2SO_4$ approximately 3 hours into the cycle, was ignored. After the minimum value had been obtained, the computer was put back on line to once again record values of the DO concentration. This allowed the fermentation to continue until the carbon source (glucose) was also completely utilized. At this point, a second sharp rise in the DO concentration was measured and the computer actuated the control sequence allowing the system to cycle.

The duration of these extended cycles depended on the amount of glucose that was present. The amount of time from the point of nitrogen exhaustion to that of complete carbon utilization versus initial glucose concentration in the medium is illustrated in Figure 4.6A. In this particular experiment,

the concentration of $(NH_4)_2SO_4$ was kept constant at 1.2 g/L while the glucose concentration was varied between 6.5 g/L and 12.0 g/L (see Table 4.3). Depending on the amount of glucose present, the extended periods lasted between 35 minutes for 6.5 g/L of glucose and 300 minutes for 12.0 g/L of glucose.

The amount of biomass at the end of a cycle was also affected by the glucose concentration in the medium. When the glucose concentration in the fresh medium was increased, the end of cycle biomass increased in a linear fashion as shown in Figure 4.6a. For a glucose concentration of 7.0 g/L, the end of cycle biomass was found to be 2.5 g/L (dry weight). The end of cycle biomass doubled to 5.0 g/L when the glucose concentration was increased to 12.0 g/L. Figure 4.6b shows the specific biomass production rate, in units of grams of biomass produced per gram of glucose consumed per hour, versus the extended cycle time. The greatest production rate of 0.33 g/g/hr was measured for an extended cycle period of one hour.

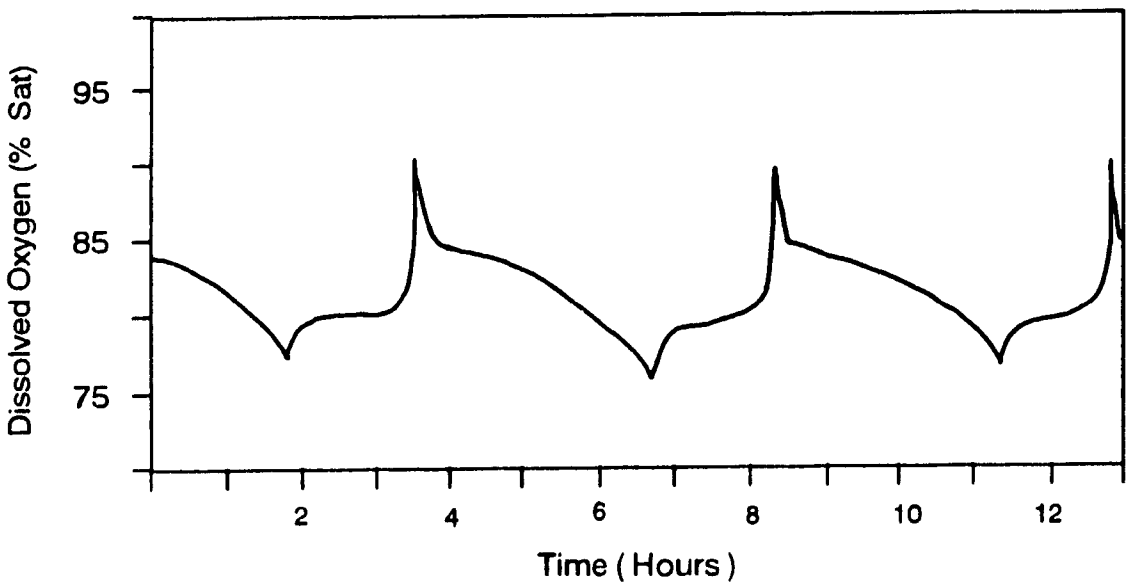

Figure 4.4 Dissolved oxygen utilization for cycles 19,20 and 21 for Run #7 using 1.2 g/L $(NH_4)_2SO_4$ and 7.0 g/L glucose

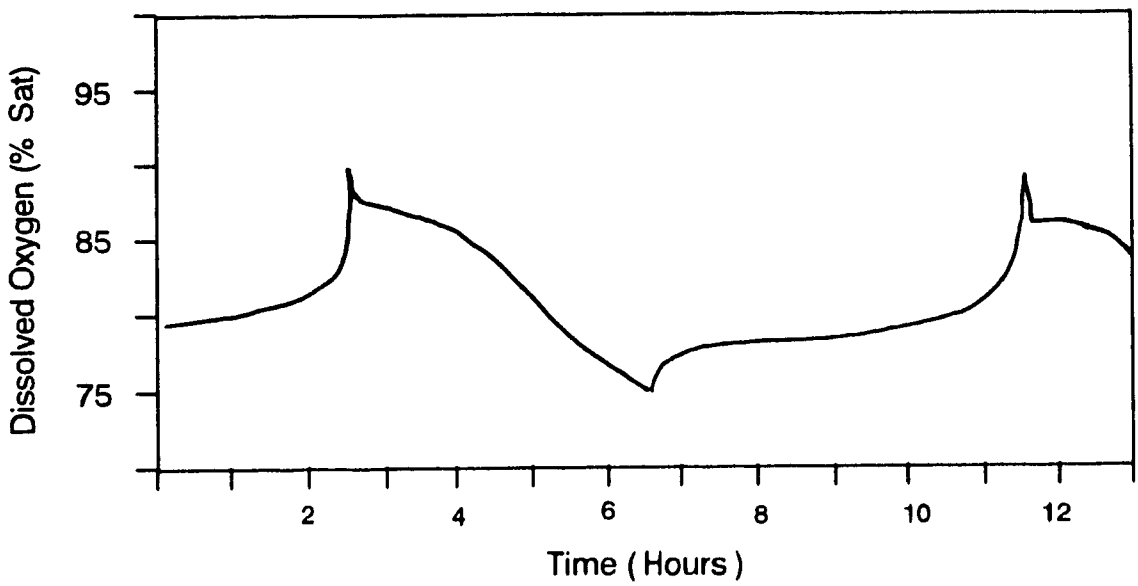

Figure 4.5 Dissolved oxygen utilization for cycles 26,27 and 28 for Run #7 using 1.2 g/L $(NH_4)_2SO_4$ and 10.0 g/L glucose

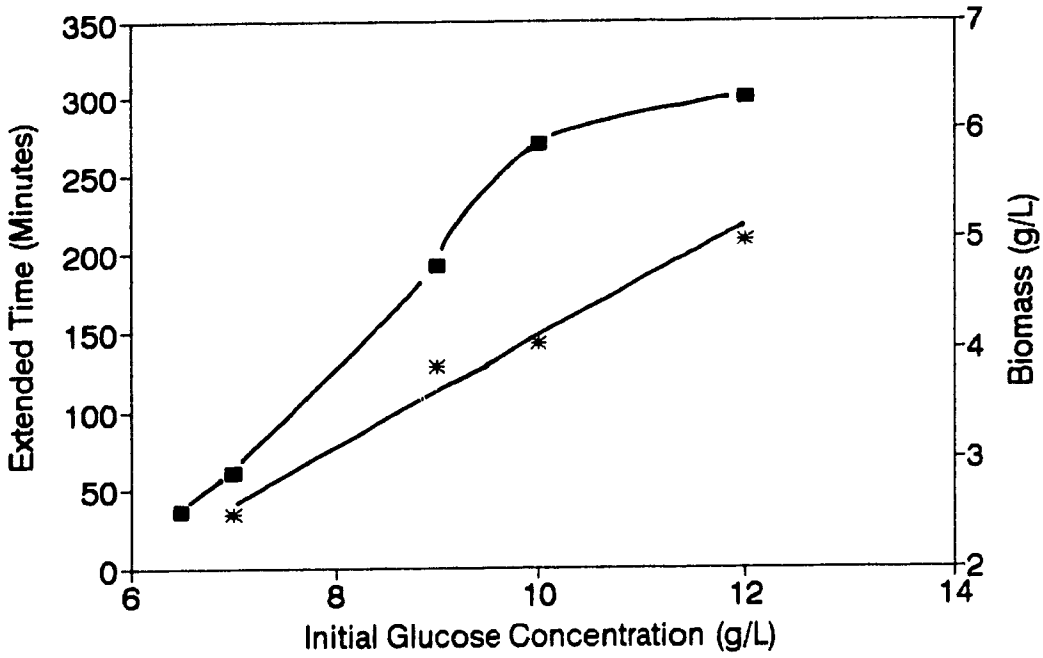

Figure 4.6a Extended time (■) and biomass concentration (*) versus initial glucose concentration for Run #7

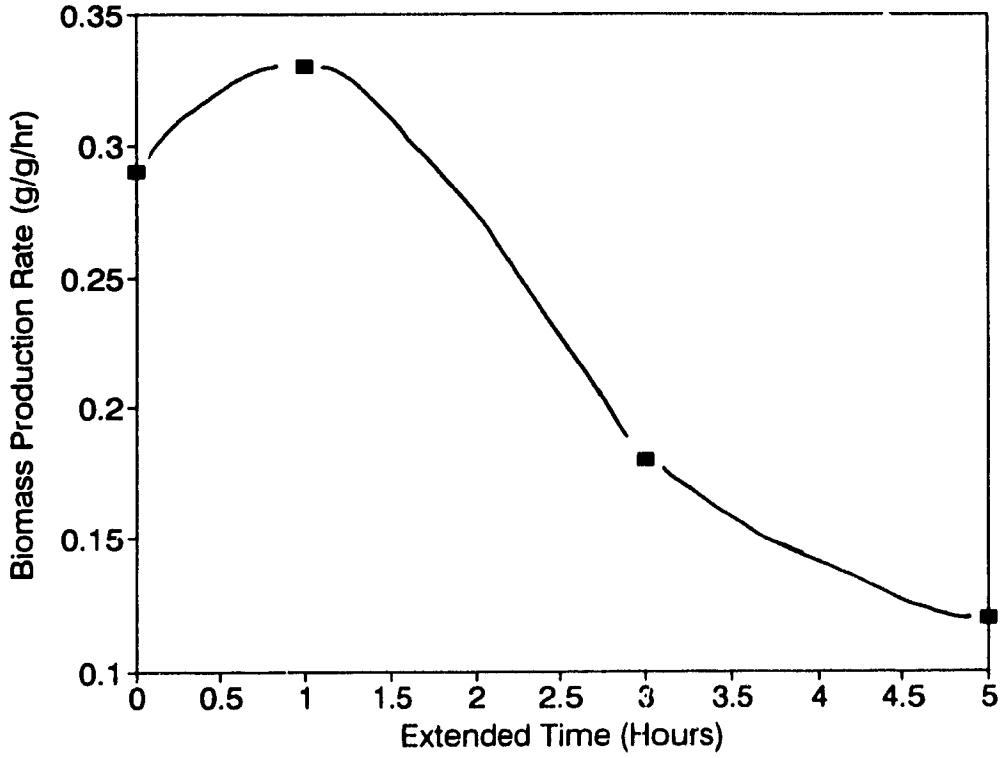

Figure 4.6b Biomass production rate versus period of extended nutrient starvation for Run #7

4.1.3 PH Control

It was necessary to have either a heavily buffered system or periodically add base to the system to maintain a constant pH. The system was extremely stable when 6.5 g/L of Na_2HPO_4 buffer was present in the medium. The DO profile for the last three cycles of Run #3, which were run without the presence of a buffer, is shown in Figure 4.7. In Figure 4.2, the minimum dissolved oxygen concentration increased from 45 % saturation to 65 % saturation throughout these last three cycles of Run #3. This absence of buffer in the medium resulted in the death of the organism. Run #7 and Run #8 (see Tables A1g and A1h) show different cycle times for the same medium compositions. The sole difference in operating conditions was the initial pH of the medium, 5.2 for Run #7 and 5.8 for Run #8. This effect of initial medium pH on cycle time was not studied in further detail.

Another method used to control the pH was the periodic addition of 2.0 N NaOH. Figure 4.8 shows the pH profile obtained for a pH controller set point of 5.2. However, the ceramic diaphragm of the pH probe quickly became fouled and resulted in a profile typical of that shown in Figure 4.9. When fouling was observed, a significant amount of drift would occur. The pH measured by the probe and the true pH of the culture broth would commonly differ by as much as 3. This made pH control of the system by periodic addition of a basic solution virtually impossible.

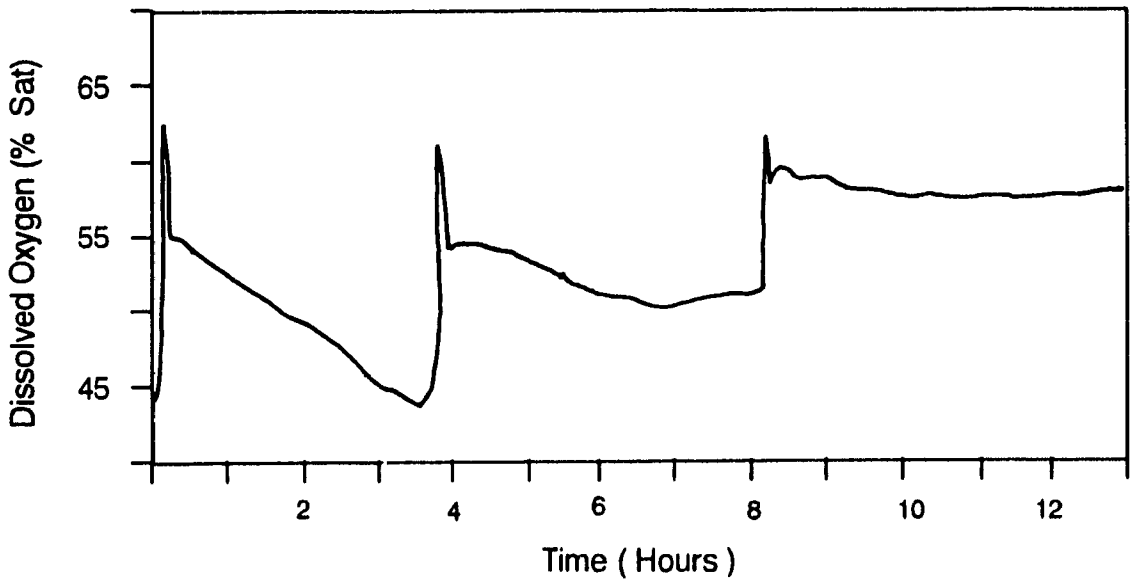

Figure 4.7 Dissolved oxygen utilization for cycles 64,65 and 66 of Run #3 with no buffer in the medium

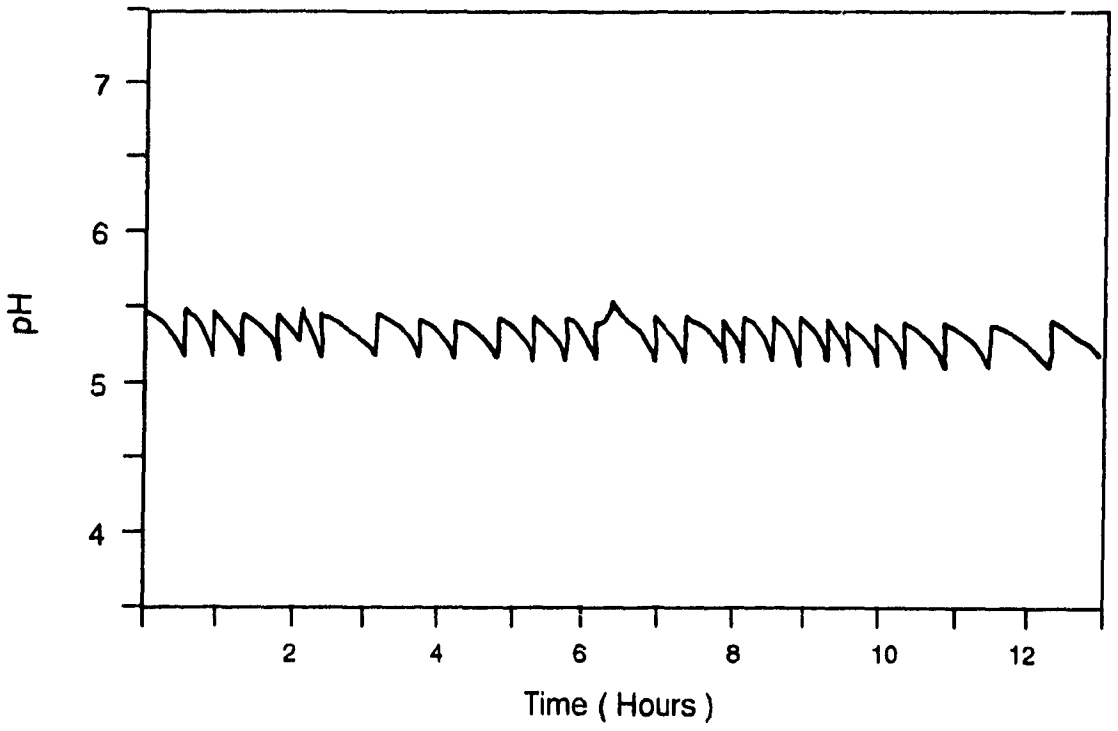

Figure 4.8 PH profile for cycles 32,33,34 and 35 for Run #4 using a controller set point of 5.2

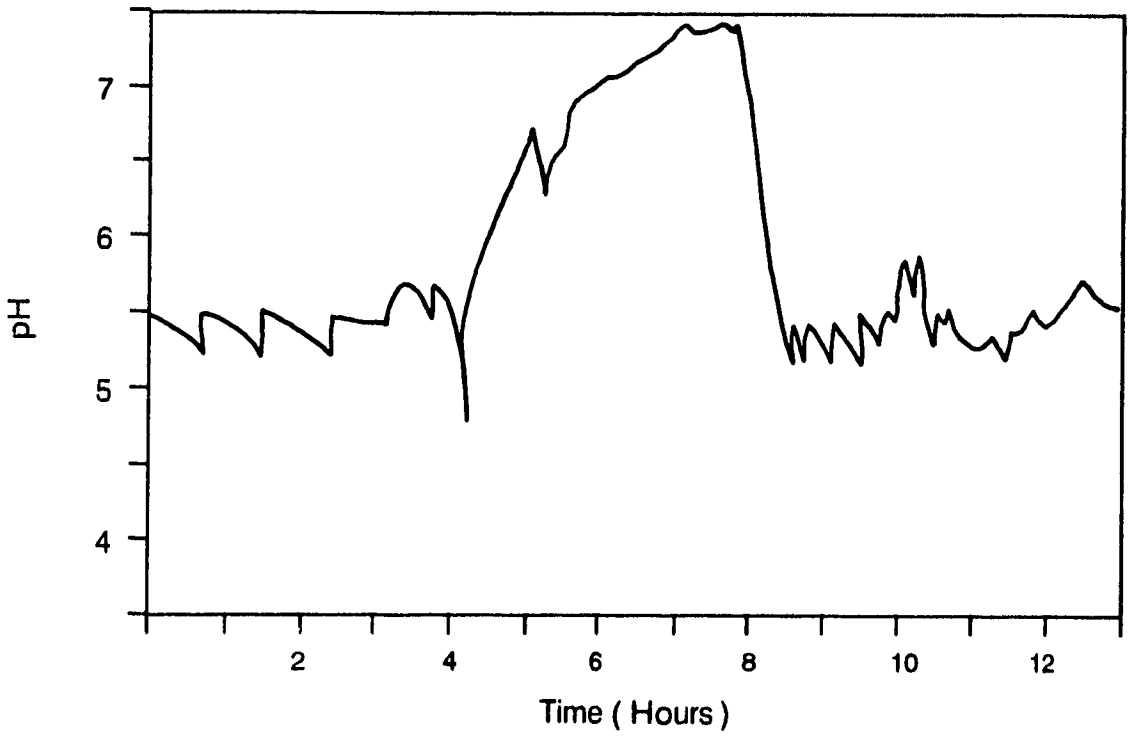

Figure 4.9 PH profile for cycles 35,36 and 37 of Run #4 after probe became fouled

4.1.4 Intracycle Cell Count Study

Intracycle cell counts were obtained for cycles with extended nutrient starvation. The method of counting consisted of direct microscope counts using a hemocytometer. This method determines the total number of cells (both living and dead) in a suspension.

Figure 4.10 shows the cell count and dissolved oxygen trace versus time for cycle 38 of Run #7. The ammonium sulphate concentration was 1.2 g/L and the glucose concentration was 7.0 g/L. Prior to the minimum dissolved oxygen concentration, the cells were increasing in size but not in number, which remained constant at approximately $9.5*10^7$ cells/mL. Once the organism began to run out of nitrogen (ie. the minimum DO was approached), the cells started to divide and within approximately 1 hour had doubled in number. Upon exhaustion of the ammonium sulphate, the cells entered a phase during which they further increased in size but not in number until the end of the cycle.

Figure 4.11 shows the same sort of trend for cycles 24 and 25 of Run #7. The concentrations of ammonium sulphate and glucose were 1.2 g/L and 10.0 g/L respectively. The cells again proceeded through the cycle remaining fairly constant in number at $9.5*10^7$ cells/mL until the minimum DO concentration was approached. At this point, the cells started to divide and had again doubled in number within a period of approximately 1 hour.

Figure 4.12 illustrates the dissolved oxygen trace for an ammonium sulphate concentration of 0.6 g/L and a glucose concentration of 3.5 g/L. The same sort of trends which were observed in the previous two figures also held true for Figure 4.12. It can be seen that after suffering an upset during the previous cycle, the synchrony of the system was disturbed to a certain extent.

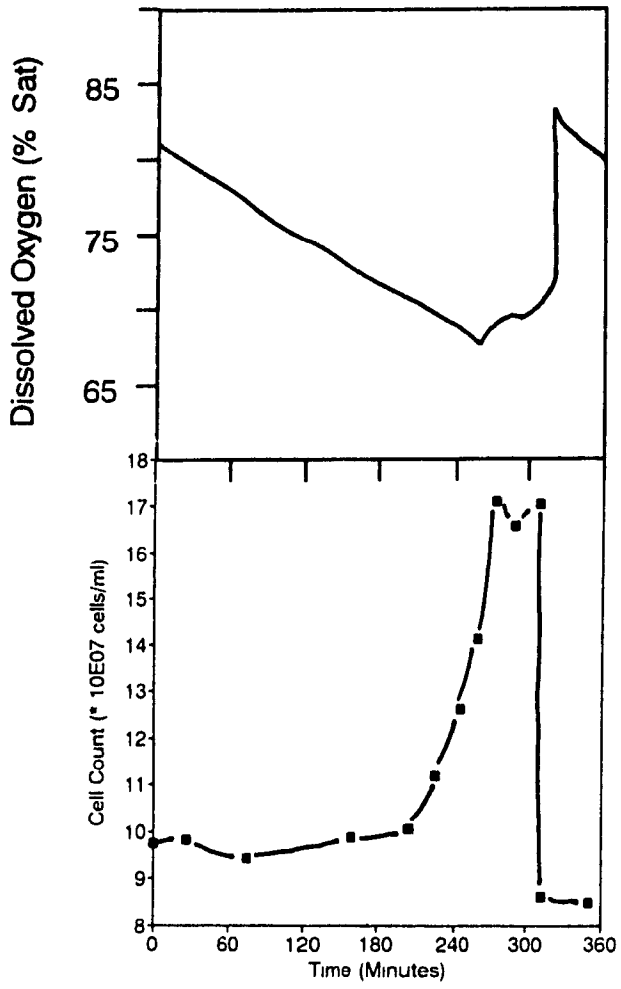

Figure 4.10 Total cell count and dissolved oxygen profile for cycle 38 of Run #7 using 1.2 g/L $(NH_4)_2SO_4$ and 7.0 g/L glucose

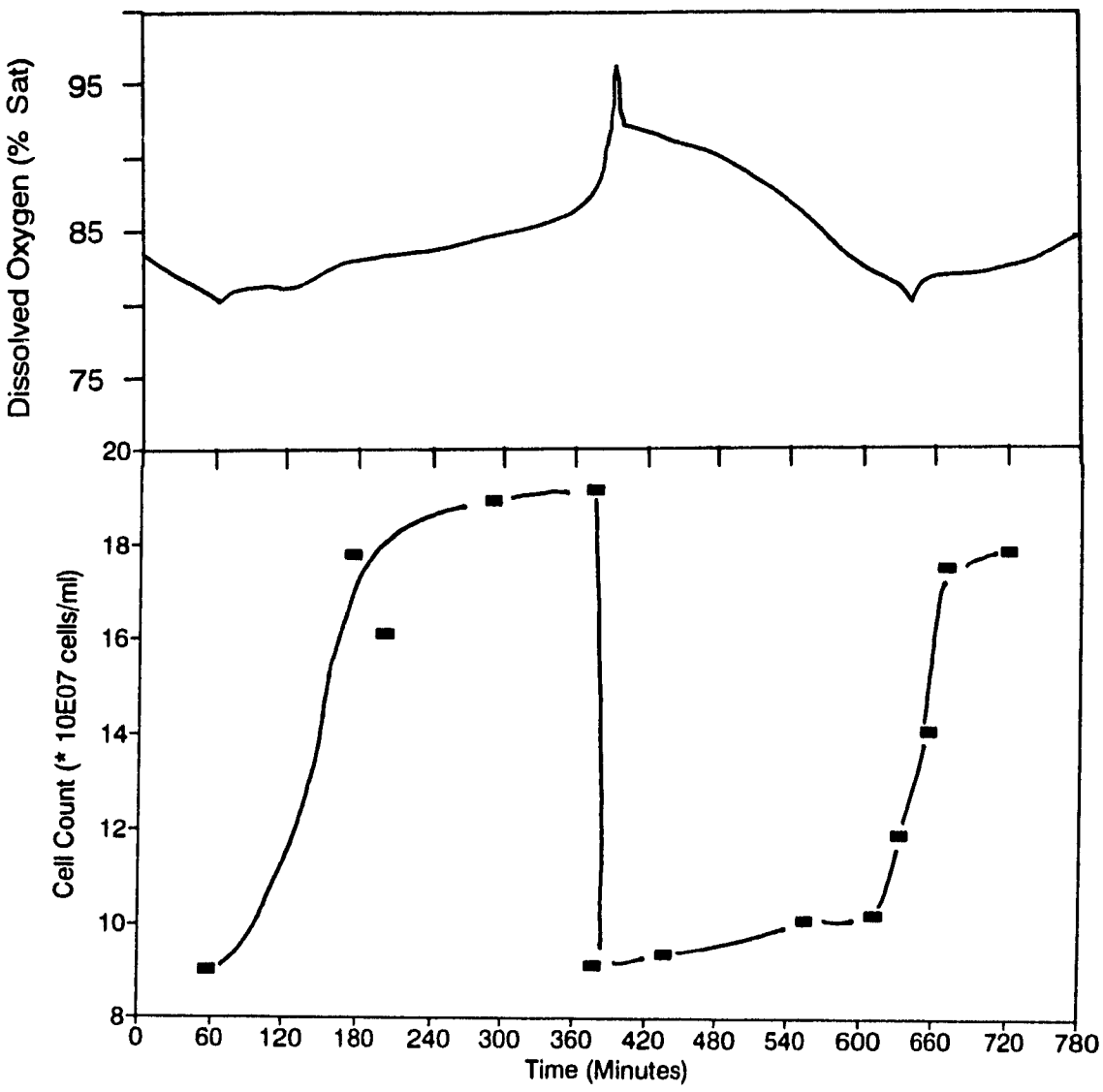

Figure 4.11 Total cell count and dissolved oxygen profile for cycles 24 and 25 of Run #7 using 1.2 g/L $(NH_4)_2SO_4$ and 10.0 g/L glucose

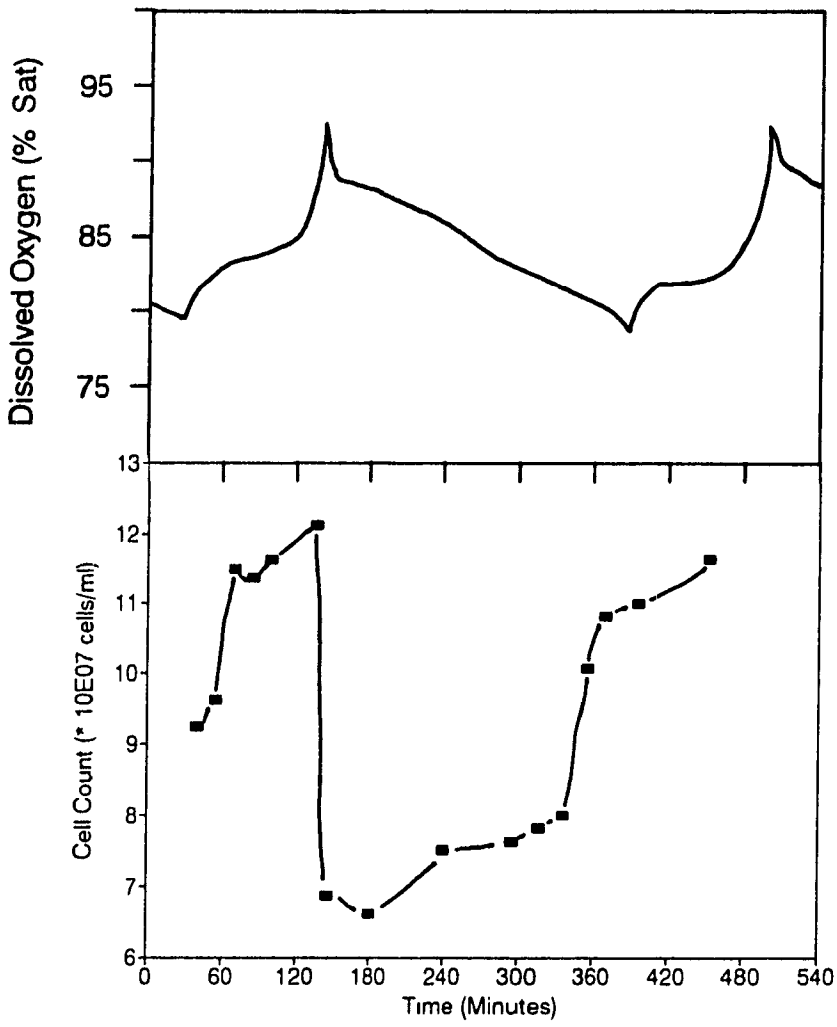

Figure 4.12 Total cell count and dissolved oxygen profile for cycles 14 and 15 of Run #8 using 1.2 g/L $(NH_4)_2SO_4$ and 10.0 g/L glucose

4.2 Production of Citric Acid

Experiments were done with a two stage fermentation. The biomass collected from the reactor at the end of a cycle was put into shakeflasks containing varying amounts of glucose and 10 g of $CaCO_3$. Samples were taken periodically and analyzed for citric acid.

Figure 4.13 shows the result of one of these time studies. The initial concentration of glucose in the shakeflask was 6.67 g/L. The maximum concentration of citric acid measured was 0.09 g/L after approximately 72 hours, while the residual glucose in the shakeflask had decreased to 0.04 g/L at that time.

Figure 4.14 illustrates the results of increasing the initial concentration of glucose to 23.3 g/L. The maximum concentration of citric acid then observed was 3.10 g/L after 130 hours, while the glucose concentration had decreased linearly to 6.90 g/L.

The results for an initial glucose concentration of 33.3 g/L are shown in Figure 4.15. After 97 hours, a maximum concentration of 5.97 g/L of citric acid was measured. The glucose concentration decreased linearly to a level of 6.10 g/L after 125 hours. In all three of these figures, the citric acid concentration decreased significantly after the maximum value was obtained.

Figure 4.16 shows the maximum citric acid produced versus the initial concentration of glucose present in the

shakeflask. The maximum concentration of citric acid obtained was 11.0 g/L for an initial glucose concentration of 50.0 g/L. Figure 4.17 and Table 4.4 show the production rates which were calculated using the data presented in Figure 4.16. The production rate levelled off for the glucose concentration above 33.3 g/L.

Table 4.4: Experimental Results (ATCC 20390)

Initial Concentration of Glucose (g/L)	Specific Production Rate (g citric acid/g glucose hr)
6.67	0.000094
16.67	0.000351
23.33	0.00100
33.33	0.00185
50.00	0.00186

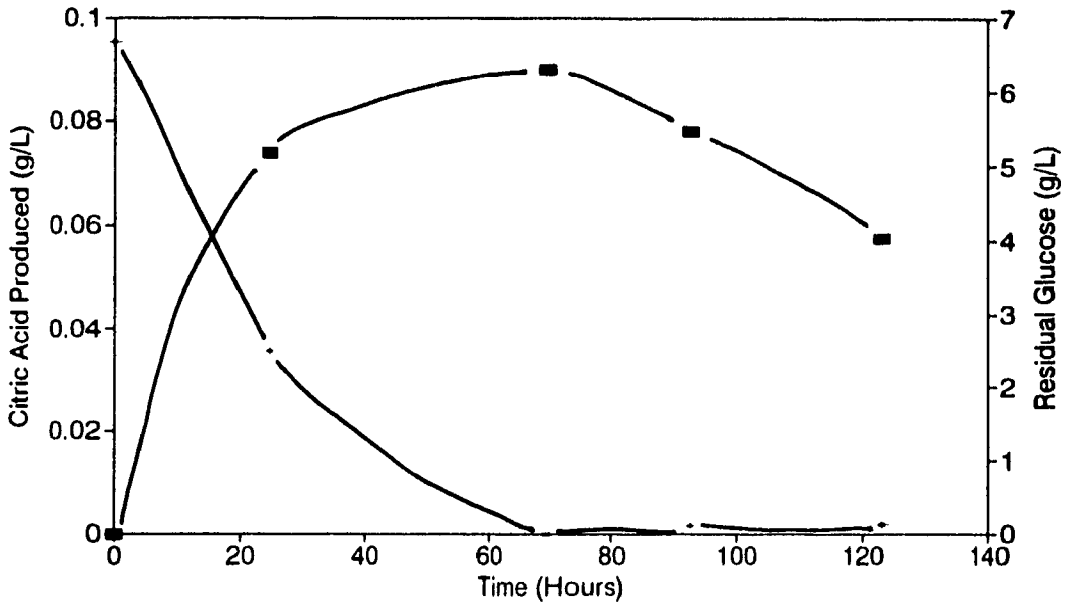

Figure 4.13 Citric acid (■) and residual glucose (*) concentrations for second stage shakeflask experiments using 6.67 g/L glucose

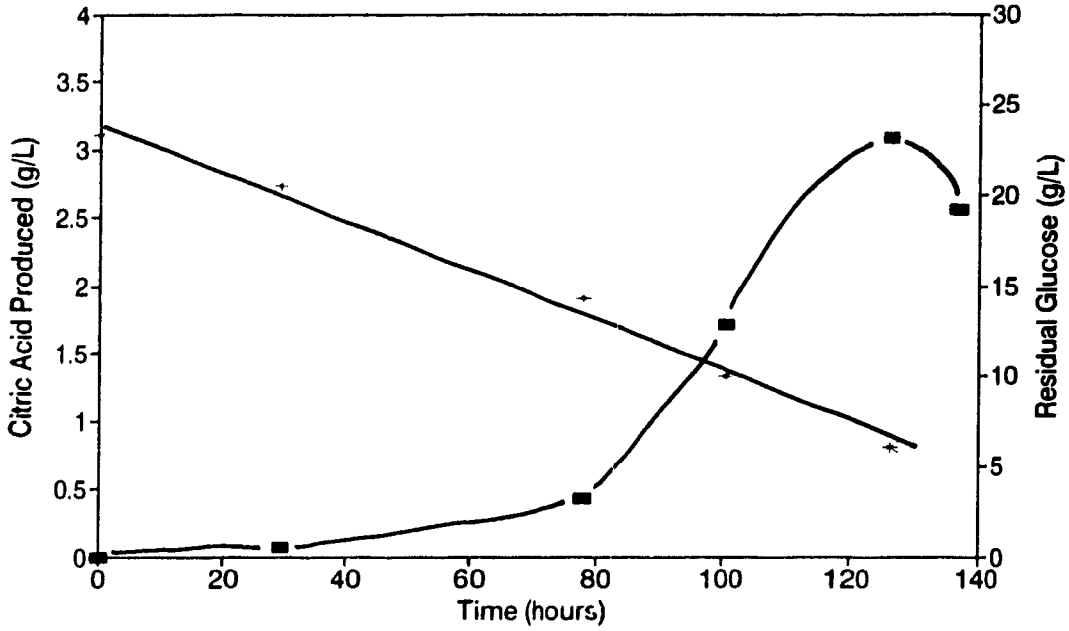

Figure 4.14 Citric acid (■) and residual glucose (*) concentrations for second stage shakeflask experiments using 23.3 g/L glucose

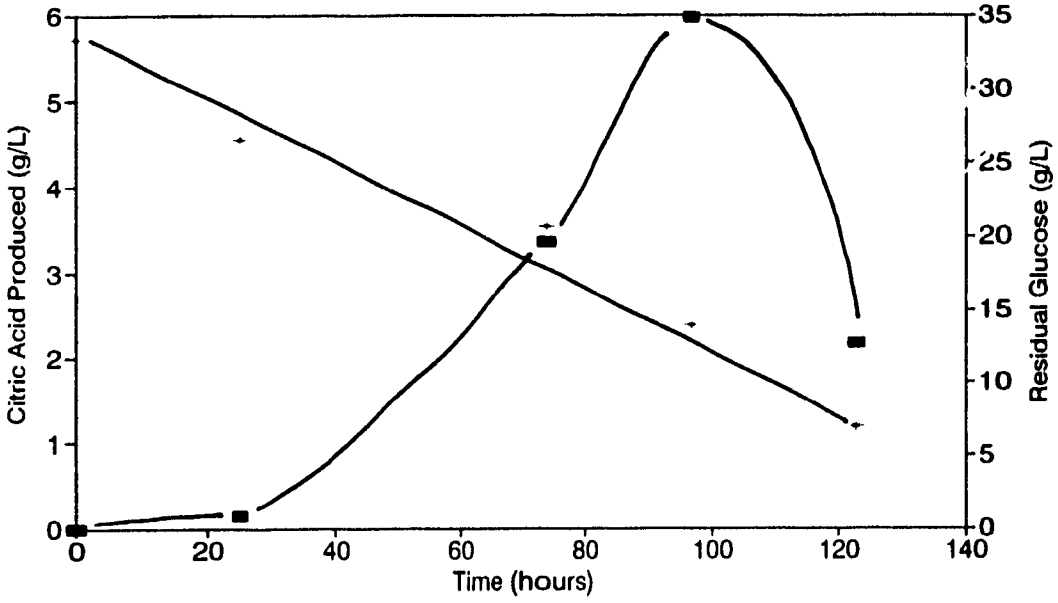

Figure 4.15 Citric acid (■) and residual glucose (*) concentrations for second stage shakeflask experiments using 33.3 g/L glucose

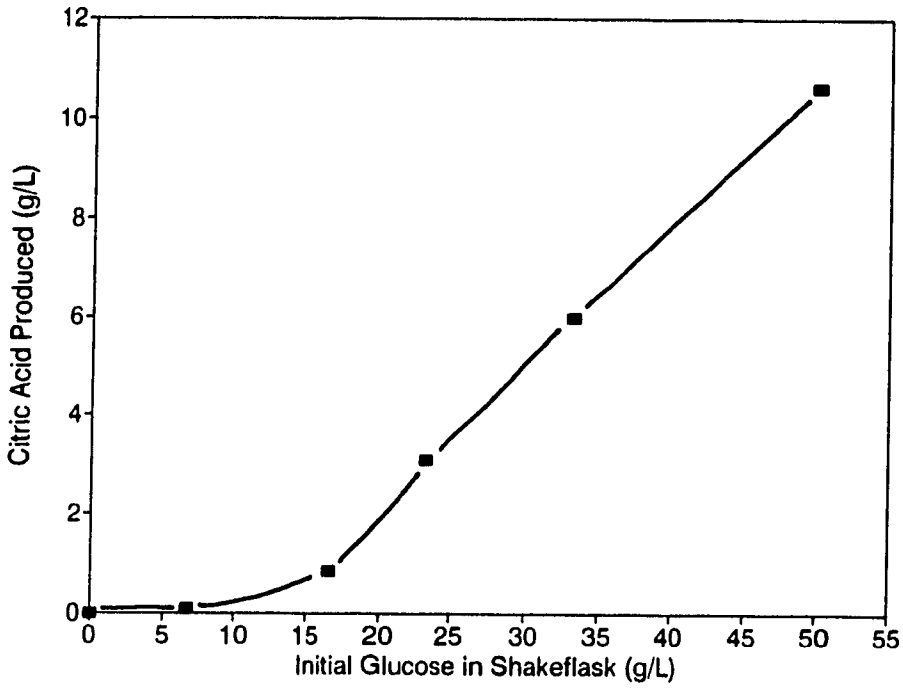

Figure 4.16 Citric acid produced in second stage shakeflask experiments

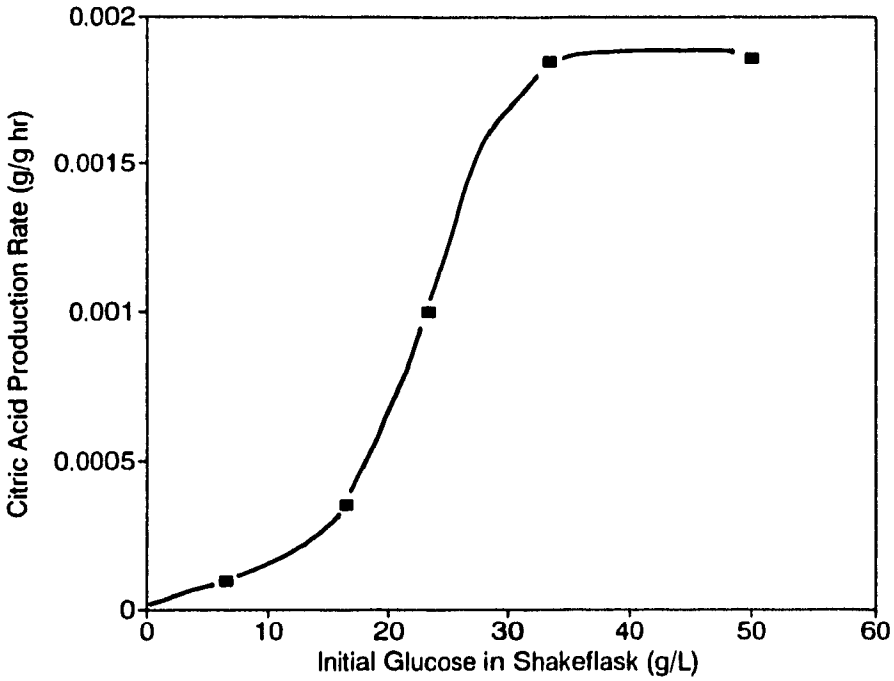

Figure 4.17 Specific production rate of citric acid in second stage shakeflask experiments

5. Discussion

5.1 Selection of a Suitable Growth Medium

Earlier self-cycling fermentation work with hexadecane has demonstrated an advantage of using a hydrocarbon - it inhibits foaming.[10,11,25] Thus, the first experiment used this substrate. Hexadecane has been used commercially as a substrate for the production of citric acid by *Candida lipolytica*.[18] However, hexadecane caused a problem with the morphology of the microorganism. *Candida lipolytica* initially grew in small clumps, as opposed to being evenly distributed throughout the broth when glucose was the substrate. This was not a problem in the shakeflasks but, when the cyclone column fermenter was inoculated with *Candida lipolytica*, the apparatus was found to be incompatible with these growth characteristics of the organism. The shear force of the pump was too great to permit the organism to grow in spherical clumps. This appeared to force the organism into a survival mode of growth. It is well known that microorganisms under stress will alter surface properties and become more likely to attach to surfaces.[9] In this case, *Candida lipolytica* stuck to the rubber tubing, the base of the cyclone column fermenter, the pH probe and the dissolved oxygen probe. The dissolved oxygen probe soon became completely fouled with growth and control of the system was rendered impossible (Figure A1).

As a result of the above problems associated with the

hexadecane substrate, all subsequent work was done with glucose as the carbon source. These media resulted in excessive foaming which also caused severe problems. One problem involved the solenoid valve which controlled flow to the harvesting vessel. This valve is located in a line which bleeds off from the side arm after the pump. The foam became tightly packed in the tubing leading to the solenoid valve. When the control sequence was initiated, flow through the valve was impossible. Another effect of the foaming was that it became extremely difficult to determine the actual volume of liquid in the reactor. These problems were easily solved by the addition of antifoam. A 70 % silicone based antifoam (Antifoam B) from Sigma Chemicals was used and tests indicated that there was no inhibitory effect of the antifoam on the growth of the organism.

Another important aspect of this fermentation was pH control. Since the product is a relatively strong acid, without pH control, the pH would typically reach values of 2.2. Such a low pH environment is toxic for the microbe and must be avoided (Figure 4.7). PH control was first attempted by the periodic addition of a 2.0 N NaOH solution, which worked very well for a period of up to three or four days. Following this period of excellent pH control, the ceramic diaphragm of the probe always became fouled causing a significant amount of drift to occur (Figure 4.8 and Figure 4.9). After this fouling, the actual pH of the culture broth

and that measured by the probe itself would commonly differ by as much as 3. This made pH control of the system with periodic addition of a basic solution impossible for an entire run. Therefore, it was deemed necessary to control the pH of the system by adding a sufficient amount of phosphate buffer to the initial medium. This resulted in an end of cycle pH of approximately 3.0.

5.2 Self-Cycling Characteristics of *Candida lipolytica*

After the development of a suitable medium, it was possible to achieve the primary goal of this project which was to apply the self-cycling fermentation technique to *Candida lipolytica*. Use of this technique resulted in a system which was found to exhibit excellent stability, demonstrated by Figures 4.1, 4.2 and 4.3.

Figure 4.2 includes minimum dissolved oxygen and cycle time data for five changes in the composition of the medium (Table 4.2) and in each case the system quickly reached a new steady state after only a couple of cycles.[10,11,25,31] Within each composition the average cycle times (< 10%) and average minimum dissolved oxygen concentrations (< 5%) exhibit very low deviations. However, what was surprising was that the average cycle time was found to depend on the composition of the medium used (Table 4.2). The cycle time is equal to the doubling time of the organism and should be a constant.[10,11,25,31] If there is no inhibitory substrate effect, an increase in

substrate concentration leads to an increase in biomass concentration with no increase in cycle time.[30]

The results obtained and shown in Figure 4.2 and Table 4.2 can be explained by considering the limiting substrate. In this study, it was discovered that the system was under carbon limitation for cycles 15-25 and cycles 37-52 with cycle times of on average 193 minutes (σ=9.01 minutes). However, the system was under nitrogen limitation for all remaining cycles with corresponding cycle times of on average 231 minutes (σ=17.1 minutes). Therefore, when the limiting nutrient was switched from the carbon source to the nitrogen source, a change in cycle time was observed.

It was not expected that the self-cycling fermentation technique would greatly increase the yield of citric acid obtained from a given amount of glucose. The advantage of the technique would be the increase in overall rate of production. This increase is in part due to the ability of the system to continuously generate biomass for extremely long periods of time (Table 4.1). This eliminates down time needed for cleaning and sterilization necessary in the operation of conventional reactors between batches. In addition, no lag phase is observed at the beginning of each cycle because the organism is always in the exponential growth phase. Furthermore, the inocula are very large - 50 % of the biomass.

Thus, if it could be shown that *Candida lipolytica* could grow in the self-cycling fermentation mode, it was only

necessary to establish whether the biomass produced was suitable for citric acid production. The first step was very successful. A comparison with the literature[7] of specific biomass production rate, given in units of grams of biomass produced per gram of glucose consumed per hour, is found in Table 5.1. Almost a full order of magnitude improvement in the specific biomass production rate was observed.

Table 5.1: Comparison of Specific Biomass Production Rate

Organism	Specific Production Rate (g biomass/g glucose hr)
Candida lipolytica (ATCC 20390) Briffaud and Engasseur[7]	0.033
Candida lipolytica (ATCC 20390) Our experimental results	0.29

Citric acid production could be done either in one vessel or by going to a two stage process. One vessel production entails allowing the cycle to continue until a second rise in the dissolved oxygen concentration is detected (Figures 4.4 and 4.5). This rise was found to coincide with the complete consumption of a second nutrient (glucose) and is referred to as extended nutrient starvation.[25] After the nitrogen source has been depleted, *Candida lipolytica* should use most of the remaining glucose to produce citric acid and to produce further biomass.[7] In this study, the biomass was found to increase in the absence of a nitrogen source with the duration

of tne extended cycles but the levels of citric acid measured remained extremely low (< 0.2 g/L) even for the longest extended cycle. It was found that a higher initial concentration of glucose in the medium at the beginning of each cycle corresponded to longer extended cycle times and higher measured end of cycle biomass (Figures 4.4, 4.5 and 4.6A). When all conditions, except glucose concentration, were held constant, the end of cycle biomass doubled from approximately 2.5 g/L for the minimum nutrient starvation cycles to as much as 5.0 g/L for extended cycles of 7 hours. It was interesting that even though the biomass increased, the cell number remained constant during the extended period of the cycle, indicating that the cells were increasing only in size and not in number (Figures 4.10 and 4.11) and that synchrony was maintained throughout these long extended cycles.

The yield of citric acid at the end of these extended cycles was poor. This is not surprising as the production of citric acid by *Candida lipolytica* on glucose continues at a constant rate after the nitrogen source has been depleted and the total time for this step is usually about a week.[2,7,8,42] It was not practical to allow the system to run with cycle times of one week, however, it was important to see if the increase in biomass in the extended period led to an increase in the specific biomass production rate. This would allow the opportunity to choose the optimum time to harvest the cells

which would then be transferred to a second vessel for citric acid production. It was found that an extended period of one hour further increased the specific biomass production rate to 0.33 g/g/hr compared to 0.12 g/g/hr for an extended period of five hours and 0.29 g/g/hr for no extended period (Figure 4.6B). Therefore, all samples for the citric acid production second stage experiments were harvested after an extended period of one hour. The reduced rate in the longer extended cycles stems from the fact that the glucose is no longer being used primarily towards the production of biomass.

Previous studies have shown that one of the properties of the self-cycling fermentation system is the presence of a synchronous cell population.[10,25,31] With the use of such a system, cell populations can be studied that are essentially all at the same stage of development.[17,22,35] It is interesting to investigate how cell synchrony was affected by the extended periods of nutrient starvation. The curves in Figures 4.10 and 4.11 illustrate a situation in which it is clear that the cells are all in the same stage of development. Normally, at the point of minimum nutrient exhaustion, the system would cycle resulting in a halving of the cell number. In this study the cycle was allowed to continue until a second nutrient (glucose) was completely consumed. This also led to an incredibly stable system over many cycles (Figures 4.4 and 4.5). Within each cycle, the cell number remains constant until the first limiting nutrient is depleted - doubling at

this point (Figures 4.10 and 4.11). The cell number then remains constant until the second limiting nutrient is exhausted, at which point the control sequence is initiated and half the cells are removed. The fact that cells of *Candida lipolytica* have a lengthy period of viability, capable of surviving for extended periods of time without a nitrogen source, contributes to the highly stable extended cycles.[2,7,8,20,42] However, if grown anaerobically for this extended period the organism loses its ability to produce citric acid.

There exists an index for the measurement of the synchrony of cell populations.[6] This index is equal to the fraction of cells which divide during a measured interval in excess of the fraction that would divide during random growth in the same interval and is given by the following formula:

$$F = \left(\frac{N}{N_0} - 1\right) - 2^{\frac{t}{g}}$$

where: N_0 - cell number at beginning of the measured interval
N - cell number at the end of the measured interval
t - the measured time interval
g - one generation time

The maximum value of this index is 1 if the entire population divides in an infinitely short period of time. It has positive values of less than 1 if the entire population divides in the measured time interval. Previous studies have shown that for a culture of *Acinetobacter calcoaceticus*, this synchrony index was found to be 0.77.[10] When this formula was applied to the data for *Candida lipolytica* in Figures 4.10 and

4.11, the index was calculated to be approximately 0.82 indicating, according to Blumenthal et al,[6] a highly synchronous population. The highly ordered, synchronous nature of the system must therefore be partially responsible for the high level of stability observed in studies involving the self-cycling fermentation technique.[10,11,15,16,17,25,30]

5.3 Production of Citric Acid

In our experiments, the second stage fermentations for citric acid production were done in shakeflasks. No attempt was made to optimize the production of citric acid. The purpose, at this time, was merely to prove that the biomass produced in the cyclone column fermenter still possessed the ability to generate significant amounts of citric acid. As can be seen in Figure 4.16, citric acid levels as high as 11.0 g/L were measured in this second stage of the fermentation. The highest specific production rate calculated was 0.0019 for glucose concentrations of 33.3 g/L and 50.0 g/L. This specific rate increased until the concentration of glucose reached 33.3 g/L, levelling off at the higher concentration of 50.0 g/L (Figure 4.17 and Table 4.4). Furthermore, it appears that when the concentration of glucose decreased below values of approximately 6.0 g/L, the citric acid concentration began to decrease, indicating consumption of the citric acid (Figures 4.14 and 4.15). Treton et al[32] discovered that in the presence of glucose or glycerol, citric acid is not consumed

as long as the concentration of the major carbon source exceeds a certain threshold. It is not clear what their threshold concentration was, however, from experimental results obtained in this study, it appears that the concentration should be maintained above 6.0 g/L (Figures 4.14 and 4.15). This also fits in quite well with other work,[7,8] which suggests that in a fed batch system, the glucose concentration be kept between 10 g/L and 40 g/L during the citric acid production stage of the fermentation. It must be kept above 10 g/L to prevent consumption of the citric acid by the organism and below 40 g/L as the production rate, above this concentration, appears to level off.

Table 5.2 shows a comparison of the citric acid

Table 5.2: Comparison of Specific Citric Acid Production Rate

Organism	Production Rate (g citric acid/g glucose hr)
Saccharomycopsis lipoytica (IFP 29 Derivative)[32]	0.0028
Candida lipolytica (Thiamine Auxotroph)[2]	0.0038
Candida lipolytica (ATCC 20390)[7]	0.0039
Candida lipolytica (ATCC 20390)[7]	0.0037
* *Candida lipolytica* (ATCC 20324)[23]	0.0015
* *Candida lipolytica* (ATCC 20346)[23]	0.0013
Experimental Results (ATCC 20390)	0.0019

* Total acid: both citric acid and isocitric acid

production rates among the results of this study and the values found in the literature.[2,7,23,32] Our specific citric acid production rate compares very well with those values quoted in the literature. No attempt was made to optimize the second stage of our system. However, this optimization has been dealt with in detail in the literature concerning batch fermentations and these results would be applicable to this process. Fed-batch has been used. This involves periodically adding glucose while keeping the concentration between 10 g/L and 40 g/L for reasons mentioned earlier.[7,32] Another alternative is to use a technique called "Vigorous Stationary Phase Fermentation" which studies conditions allowing the microbe to maintain its maximum fermentation rate while remaining viable without cell growth for long periods of time.[20] For *Candida lipolytica*, this technique involves the minute addition, after nitrogen depletion, of 56 micrograms of NH_4NO_3 per 10^{10} cells per hour to increase the cell viability from approximately 150 hours to 600 hours.

6. Conclusion

This study showed that the application of the self-cycling fermentation technique to a culture of *Candida lipolytica* resulted in an extremely stable system. An investigation into the variance of cell number within a cycle showed that synchronous growth was maintained even throughout long periods of extended nutrient starvation.

A specific rate of production of biomass, exceeding values in the literature by up to a full order of magnitude, was obtained. In a second stage process, this biomass was found to be suitable for the production of citric acid. Keeping in mind that only half of the reactor contents are harvested after each cycle, this would lead to an increase in overall citric acid production of at least five times that observed in conventional batch fermenters of the same size.

7. References

1. Aiba, S. and M. Matsuoka. 1978. **Citrate Production From n-Alkane by *Candida lipolytica* in Reference to Carbon Fluxes in vivo.** Eur. J. Appl. Microbiol. Biotechnol. **5**:247-261.

2. Aiba, S. and M. Matsuoka. 1979. **Identification of Metabolic Model: Citrate Production from Glucose by *Candida lipolytica*.** Biotechnol. Bioeng. **21**:1373-1386.

3. Akiyama, S., T. Suzuki, Y. Sumino, Y. Nakao and H. Fukada. 1972. **Production of Citric Acid from n-Paraffins by Fluoroacetate Sensitive Mutant Strains of *Candida lipolytica*.** Agr. Biol. Chem. **36**:339-341.

4. Akiyama, S., T. Suzuki, Y. Sumino, Y. Nakao and H. Fukada. 1973. **Induction and Citric Acid Productivity of Fluoroacetate-sensitive Mutant Strains of *Candida lipolytica*.** Agr. Biol. Chem. **37**:879-884.

5. Akiyama, S., T. Suzuki, Y. Sumino, Y. Nakao and H. Fukada. 1973. **Relationship Between Aconitate Hydratase Activity and Citric Acid Productivity in Fluoroacetate-sensitive Mutant Strain of *Candida lipolytica*.** Agr. Biol. Chem. **37**:885-888.

6. Blumenthal, K. and S. Zahler. 1962. **Index for Measurement of Synchronization of Cell Populations.** Science **135**:724.

7. Briffaud, J. and M. Engasseur. 1979. **Citric Acid Production from Glucose - Growth and Excretion Kinetics in a Stirred Fermenter.** Biotechnol. Bioeng. **21**:2083-2092.

8. Briffaud, J. and M. Engasseur. 1979. **Citric Acid Production from Glucose - Growth and Excretion Kinetics in a Trickle-Flow Fermenter.** Biotechnol. Bioeng. **21**:2093-2111.

9. Brock, T.D. and M.T. Madigan. 1991. **Biology of Microorganisms - 6th Edition.** Prentice Hall Inc. New Jersey.

10. Brown, W.A. and D.G. Cooper. 1991. **Self-Cycling Fermentation Applied to *Acinetobacter calcoaceticus* RAG-1.** Appl. Environ. Microbiol. **57**:2901-2906.

11. Brown, W.A. and D.G. Cooper. 1992. **Hydrocarbon Degradation by *Acinetobacter calcoaceticus* RAG-1 Using the Self-Cycling Fermentation Technique.** Biotechnol. Bioeng. **40**:797-805.

12. Cano, R.J., J.S. Colomé, D.V. Grady, A.M. Kubinski. 1986. **Laboratory Exercises in Microbiology.** West Publishing Co. New York.

13. Conway, E.J. 1957. **Microdiffusion Analysis and Volumetric Error - 4th Edition.** Crosby Lockwood Co. London.

14. Dawson, P.S.S. 1963. **A Continuous-Flow Culture Apparatus: The Cyclone Column Unit.** Can. J. Microbiol. **9**:671-687.

15. Dawson, P.S.S., M. Anderson and A.E. York. 1971. **The Cyclone Column Culture Vessel for Batch and Continuous, Synchronous or Asynchronous, Culture of Microorganisms.** Biotechnol. Bioeng. **13**:865-876.

16. Dawson, P.S.S. 1971. **A Pilot Plant Apparatus for Continuous Phased Culture - Some Observations on Oxygen Usage by *Candida utilis* During the Cell Cycle.** Biotechnol. Bioeng. **13**:877-892.

17. Dawson, P.S.S. 1972. **Continuously Synchronised Growth.** J. Appl. Chem. Biotechnol. **22**:79-103.

18. Demain, A.L. and N.A. Solomon. 1981. **Industrial Microbiology.** Sci. Amer. **245**:67-73.

19. Enzminger, J.D. and J.A. Asenjo. 1986. **Use of Cell Recycle in the Aerobic Fermentative Production of Citric Acid by Yeast.** Biotech. Lett. **8**:7-12.

20. Kim, E.K., J.R. Ambriano and R.S. Roberts. 1987. **Vigorous Stationary Phase Fermentation.** Biotechnol. Bioeng. **30**:805-808.

21. **Kirk-Othmer Encyclopedia of Chemical Technology - 3rd Edition.** 1979. John Wiley & Sons Inc. **6**:150-167.

22. Lloyd, D., R.K. Poole and S.W. Edwards. 1982. **The Cell Division Cycle: Temporal Organization and Control of Cellular Growth and Reproduction.** Academic Press Publ. New York.

23. MacKenzie, C.R., S.J. Davids, G.R. Lawford and H.G. Lawford. 1981. **Citric and Isocitric Acid Production from Hydrocarbons by Yeast Fermentation.** Adv. Biotechnol. **2**:57-62.

24. Marchal, R., O. Chaudé and M. Metche. 1977. **Production of Citric Acid from n-Paraffins by *Saccharomycopsis lipolytica*: Kinetics and Balance of the Fermentation.** Appl. Microbiol.

25. McCaffrey, W.C. 1992. A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfilment of the requirements of the degree of Master of Engineering. Department of Chemical Engineering - McGill University, Montreal.

26. Moellering, H. and W. Gruber. 1966. **Determination of Citrate with Citrate Lyase.** Anal. Biochem. **17**:369-376.

27. Moellering, H. 1985. **Citrate.** Methods of Enzymatic Analysis, **7**:2-12.

28. Moldonado, P. and M. Charpentier. 1976. **Fermentation Process for the Production of Citric and Isocitric Acids.** U.S. Pat. **3,997,399.**

29. Moo-Young, M., T. Shimizu and D.A. Whitworth. 1971. **Hydrocarbon Fermentations Using *Candida lipolytica*: Basic Growth Parameters for Batch and Continuous Culture Conditions.** Biotechnol. Bioeng. **13**:741-760.

30. Sarkis, B. 1992. A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfilment of the requirements of the degree of Master of Engineering. Department of Chemical Engineering - McGill University, Montreal.

31. Sheppard, J.D. and D.G. Cooper. 1990. **Development of Computerized Feedback Control for the Continuous Phasing of *Bacillus subtilis***. Biotechnol. Bioeng. **36**:539-545.

32. Tréton, B., M. Le Dall, and H. Heslot. 1978. **Excretion of Citric and Isocitric Acids by the Yeast *Saccharomycopsis lipolytica***. European J. Appl. Microbiol. Biotechnol. **6**:67-77.

33. Wayman, M. and E. Wein. 1969. **The Effect of Air Pressure on the Growth of *Candida lipolytica* on n-hexadecane**. Can. J. Microbiol. **16**:1255-1261.

34. Whitworth, D.A. 1974. **Hydrocarbon Fermentation: Protein and Enzyme Solubilization from *Candida lipolytica* Using an Industrial Homogenizer**. Biotechnol. Bioeng. **16**:1399-1406.

35. Zeuthen, E. 1964. **Synchrony in Cell Division and Growth**. John Wiley & Sons Inc. New York.

Appendix

Table A1a: Conditions for Run #1

**** RUN ABORTED ****

Table A1b: Conditions for Run #2

Cycle Number	$(NH_4)_2SO_4$ (g/L)	Glucose (g/L)	Yeast Extract (g/L)	Average Cycle Time (min)
1-14	3.0	10.0	0.1	344 (σ=57.3)
15-29	3.0	10.0	0	228 (σ=7.19)
30-45	3.0	5.0	0	179 (σ=5.18)
46-60	3.0	5.0	0.1	176 (σ=7.03)
61-68	3.0	7.5	0	173 (σ=10.0)
69-75	3.0	9.0	0	194 (σ=9.81)

- system cycled at point of minimum nutrient starvation

Table A1c: Conditions for Run #3

Cycle Number	$(NH_4)_2SO_4$ (g/L)	Glucose (g/L)	Average Cycle Time (min)
1-14	4.0	8.0	256 (σ=11.0)
15-25	1.0	7.5	187 (σ=6.81)
26-36	1.0	9.0	226 (σ=6.91)
37-52	1.0	6.5	197 (σ=7.35)
53-65	2.0	6.5	219 (σ=3.97)

- system cycled at point of minimum nutrient starvation

Table A1d: Conditions for Run #4

Cycle Number	$(NH_4)_2SO_4$ (g/L)	Glucose (g/L)	Average Cycle Time (min)
1-16	0.5	9.0	235 (σ=6.88)
17-32	0.65	9.0	256 (σ=15.0)
33-47	0.65	9.0	246 (σ=11.8)
*48-67	0.5	15.0	688 (σ=7.02)

- system cycled at point of minimum nutrient starvation
* system cycled at point of extended nutrient starvation

Table A1e: Conditions for Run #5

**** RUN CONTAMINATED ****

Table A1f: Conditions for Run #6

Cycle Number	$(NH_4)_2SO_4$ (g/L)	Glucose (g/L)	Average Cycle Time (min)
1-13	1.5	12.0	309 (σ=44.2)
14-30	1.5	12.0	357 (σ=28.5)
31-47	0.3	12.0	217 (σ=5.17)
48-62	0.9	12.0	253 (σ=13.0)
63-65	1.2	12.0	226 (σ=22.4)

- system cycled at point of minimum nutrient starvation

Table A1g: Conditions for Run #7

Cycle Number	$(NH_4)_2SO_4$ (g/L)	Glucose (g/L)	Average Cycle Time (min)
1-16	1.2	12.0	441 (σ=39.0)
17-30	1.2	10.0	451 (σ=11.6)
31-42	1.2	7.0	334 (σ=32.1)
43-59	1.2	6.5	327 (σ=19.6)
60-67	1.2	9.0	368 (σ=11.4)

- system cycled at point of extended nutrient starvation

Table A1h: Conditions for Run #8

Cycle Number	$(NH_4)_2SO_4$ (g/L)	Glucose (g/L)	Average Cycle Time (min)
1-137	1.2	7.0	272 (σ=24.2)

- system cycled at point of extended nutrient starvation

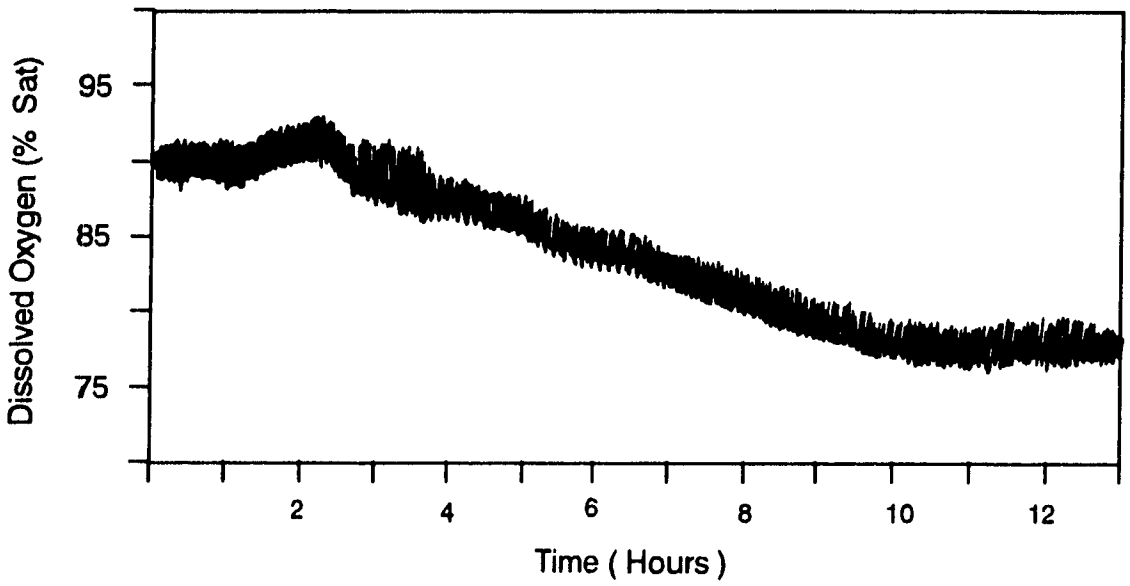

Figure A1 Typical dissolved oxygen profile for hexadecane experiment