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FERMENTATION METHODS FOR THE PRODUCTION OF POLY(3-HYDROXYBUTYRATE) BY *ALCALIGENES EUTROPHUS* DSM 545

by

© Philippe Marchessault

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the Faculty of Graduate Studies and Research

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ABSTRACT

Production of poly(3-hydroxybutyrate) (PHB) was done in a cyclone bioreactor using various culture methods; including batch (lab and pilot scale) fed-batch and self-cycling fermentation with and without starvation periods. *Alcaligenes eutrophus* DSM 545 was used to accumulate about 87% (wt/wt) PHB to a total of 6.2 g L^{-1} PHB at the end of a 48 hour batch. Similar pilot scale experiments contained a maximum of 96% (wt/wt) PHB with 4.9 g L^{-1} accumulated. Fed-batch culture of *A. eutrophus* produced 96% (wt/wt) PHB with a final PHB concentration of 22.2 g L^{-1} after 54 h. Self-cycling fermentation (SCF) production of PHB resulted in an average of 35% (wt/wt) PHB without starvation periods with production rates reaching 0.24 g L^{-1} hr^{-1}. With starvation periods of 4, 6 and 8 h extended on the cycle times, production of PHB decreased except in the 8 hour starvation period which was 59% (wt/wt). However, the rates of production all decreased to below 0.13 g L^{-1} hr^{-1} as the lengths of the starvation periods were increased.

RÉSUMÉ

La production de 'poly(3-hydroxybutyrate)' (PHB) a été accomplie dans un bioréacteur cyclonal avec des méthodes de culture variées, soit la fermentation fractionné (à l'échelle du laboratoire et de l'usine pilote), la fermentation fractionné alimentée, et la fermentation auto-cyclique avec et sans périodes de jeûne. L' organisme *Alcaligenes eutrophus* DSM 545 a été utilisé pour accumuler environ 87% (m/m) de PHB avec un total de 6.2 g L^{-1} de PHB à la fin de 48 heures. L'expérimentation à l'échelle de l'usine pilote a permis l'accumulation un maximum de 96% (m/m) avec 4.9 g L^{1} de PHB accumulée. La culture de PHB en fermentation fractionnée-alimentée, permis l'accumulation 96% (m/m) de PHB avec une concentration finale de 22.2 g L^{1} après 54 heures. La fermentation auto-cyclique du PHB sans période de jeûne a permis l'accumulation en moyenne 35% (m/m) avec un taux de production de 0.24 g L^{-1} hr^{-1}. Avec des périodes de jeûne de 4, 6 et 8 heures ajoutées à la fin de chaque cycle, la production de PHB a diminué sauf pour la période de 8 heures qui a subit une accumulation de 59%. Cependant, les taux de production ont tous diminués en bas de 0.25 g L^{-1} hr^{-1} en même temps que les périodes de jeûne ont été augmentées.

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CHAPTER I INTRODUCTION

1.1 Poly(3-hydroxybutyrate) and other poly hydroxyalkanoates

Poly(3-hydroxybutyrate) (PHB) is a microbial polyester which is accumulated in the form of amorphous inclusions in certain bacteria (Lausier, *et al*, 1992). It is a biodegradable substitute for thermoplastics. In tests carried out by Krupp and Jewel (1992) PHB was the only so-called biodegradable plastic to show substantial degradation in a bioreactor. When PHB is extracted from the bacteria it crystallizes to form a polymer with similar properties to polypropylene as shown in Table 1.1. PHB is accumulated as a carbon reserve under nutrient limitation. This microbial polyester, which was first discovered by Lemoigne in 1927, was not produced commercially since PHB is more brittle than polypropylene and cannot replace it completely. It was not until 1974 (Whallen and Rohwedder) that PHB was found to be part of a larger family of poly-(hydroxyalkanoates) or PHAs. The general structure of PHA is represented in Figure 1.1. The first industrial production of PHB and PHA did not occur until 1982 when ICI marketed a copolymer under the trade name Biopol™.

The hydroxy acid monomer units depend on the carbon source utilized. Bacteria such as *Alcaligenes eutrophus* have utilized various C_4 and C_5 sources to produce polymers with monomer compositions of 3HB, 4HB, 3-hydroxyvalerate (HV) and 5HV (Anderson and Dawes 1990). *Pseudomonas oleovorans* have produced polymers with 3-hydroxy acids with side chains ranging in length from C_6 to C_{12} which had alkanes such as hexane through dodecane as carbon sources (Anderson and Dawes 1990). Co-polymerization may also occur if more than one carbon source is utilized. Poly(3-hydroxybutyrate-co-valerate) (PHB-co-HV) is an example of co-polymerization which is currently produced by Zeneca, formerly ICI (P.A. Holmes, *et al,* European Patent 69,497, April 1987). It is produced by adding proprionic acid to the glucose rich media as polymer accumulation occurs. This co-polymer is not a blend as are some of the so-called photo-degradable polymers made from starch and polypropylene. The HB and HV monomer units are randomly linked along the polymer chain .

Table 1.1 PHB Compared to Polypropylene.

Property	PHB	PP
Crystaline Melting Point (Deg C)	175	176
Crystalinity (%)	80	70
Molecular Weight (Daltons)	500000	200000
Density (g/cc)	1.25	0.905
Extension to Break %	6	400
Tensile Strength (MPa)	40	38
Cost $/kg	20	0.5
Ultraviolet Resistance	Good	Poor
Biodegradibility	Good	None
Deplete Natural Resources	No	Yes

Comparison of physical properties between Poly-(3-hydroxybutyrate) and Polypropylene. PHB and PP possess similar melting points, crystallinity, and tensile strength. PHB is more brittle than PP but is biodegradable.

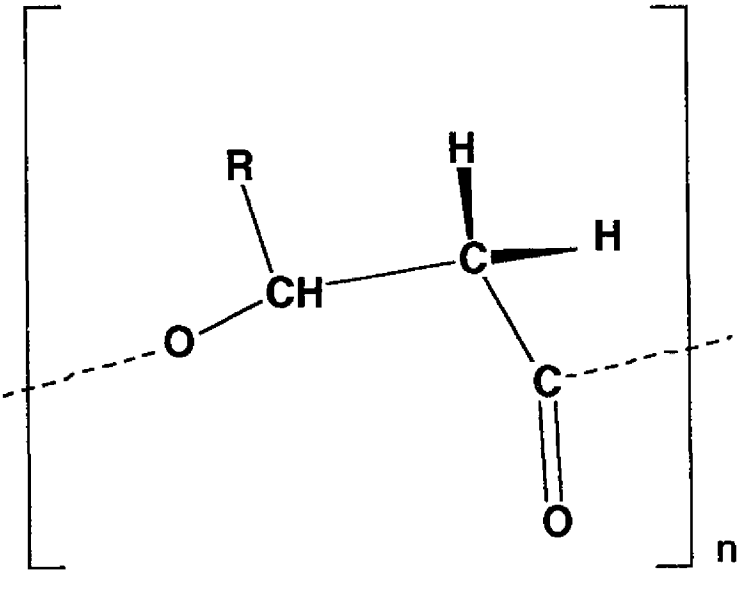

Figure 1.1 The PHB Monomer Structure.

Shown is the molecular structure of monomeric units of poly(3-hydroxybutyrate. The R group is a methyl group for PHB and varies for all other hydroxyalkanoates.

Biopol™ has had limited use because of the high price it demands (20$/kg compared to 0.50$/kg for polypropylene) but has been used for specialty products (biodegradable shampoo bottle) and in the medical field (degradable sutures). Until either the demand becomes so high for biodegradable products that consumers will be willing to pay such a premium or the production costs drop considerably, these plastics will remain too expensive for the average consumer. So, it is of utmost importance that the synthesis of PHAs and PHB in particular be examined carefully and production alternatives be investigated.

1.2 *Alcaligenes eutrophus* DSM 545

Because of its high yield and rapid production rate *A. eutrophus* has been the species of choice for PHB production. It is limited to sugars such as glucose and fructose as its carbon source but does not have the invertase enzyme to degrade sucrose. These sugars are a renewable resource unlike the petroleum needed for the production of synthetic polymers. The optimal growth temperature for *A. eutrophus* is 30°C. It is therefore important to have an efficient cooling system to keep the high-density, rapidly growing culture at the optimal temperature during industrial production (Ramsay, et al, 1990). *A. eutrophus* typically accumulates up to 80% (wt/wt) PHB with glucose as substrate (Holmes 1985). Typical batch fermentation techniques can produce PHB in quantities of only about 10 g L^{-1}. This is due to the inhibitory effect Glucose has on *A. eutrophus* at concentrations above 15 g L^{-1}. Fed-batch cultures of *A. eutrophus* DSM 545 produced 24 g of PHB L^{-1} under ammonium limited conditions (Ramsay *et al*, 1990). A chemostat culture of the same strain resulted in a maximum production of 1.5 g L^{-1} of PHB at a dilution rate of 0.15 h^{-1} (Ramsay *et al*, 1990).

1.3 PHB synthesis

During normal metabolism and cell growth, *Alcaligenes eutrophus* converts glucose into two pyruvate molecules via the glycolytic pathway. Pyruvate is then converted to acetyl coenzyme A which then enters the citric acid cycle, releasing energy in the form of

ATP, and GTP as well as NADH which then enters the electron transport chain and donates its electrons to O_2 where the energy released is trapped in the form of ATP (Prescott *et al*, 1990). However, as the growth becomes limited by an essential nutrient such as nitrogen, *Alcaligenes eutrophus* begins to convert the acetyl-CoA to PHB as a mechanism for storing carbon. PHB is synthesized from acetyl-CoA by a sequence of three reactions catalyzed by 3-ketothiolase, acetoacetyl-CoA reductase, and poly(3-hydroxybutyrate) synthase as shown in Figure 1.3. The two pyruvate molecules which exit the glycolytic pathway are converted to 2 acetyl CoA molecules. These are combined to form acetoacetyl-CoA catalyzed by 3-ketothiolase (acetyl-CoA acetyl transferase) acetoacetyl-CoA is then converted to 3-hydroxybutyryl-CoA with the aid of acetoacetyl-CoA reductase (hydroxybutyryl-CoA dehydrogenase). The final polymerization is catalyzed by the poly(3-hydroxybutyrate) synthase.

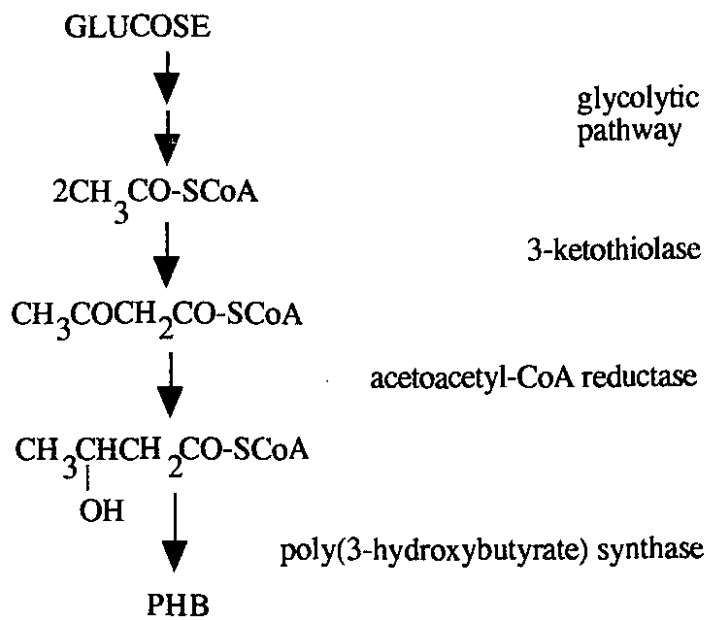

Figure 1.2 Synthesis of Poly(3-Hydroxybutyrate) from Glucose. Shown are the enzymes which catalyze the reactions from glucose to Acetyl-CoA to acetoacetyl-CoA to 3-hydroxybutyryl-CoA to PHB.

1.4 Batch Growth

Growth may be defined as an increase in cellular constituents. It leads to an increase in cell number when microorganisms reproduce by budding or binary fission. In the latter, individual cells enlarge and divide to yield two progeny of approximate equal size. It has not usually been convenient for scientists and engineers to investigate the growth and reproduction of individual microorganisms because of their small size. Therefore, they normally follow changes in the total population numbers when studying growth.

Population growth is studied by analyzing the growth curve of a microbial culture. When microorganisms are cultivated in liquid medium they are usually grown in a batch culture or closed system; that is, they are incubated in a closed culture vessel with a single batch of medium. Because no fresh medium is provided during incubation, nutrient levels decline and concentrations of metabolic byproducts increase. If the growth of microorganisms reproducing by binary fission is plotted as the logarithm of cell number versus the incubation time, the resulting curve is composed of four distinct phases (Figure 1.3).

1.4.1 Lag Phase

When microorganisms are introduced into fresh culture medium, no immediate increase in cell numbers or mass is usually seen, and therefore this period is referred to as the lag phase. Although cell division does not occur right away and there is minimal increase in mass, because new cell components are being synthesized. A lag phase prior to the start of cell division may be necessary for a variety of reasons. The cells may be old and depleted of energy and cell components must be synthesized before growth can begin. The medium may be different from the one the microorganism was growing in previously. In this case, new enzymes would be needed to utilize different nutrients. Possibly, the organisms have been injured and require time to recover. Whatever the cause, eventually the cells replicate DNA, begin to increase in mass, and finally divide.

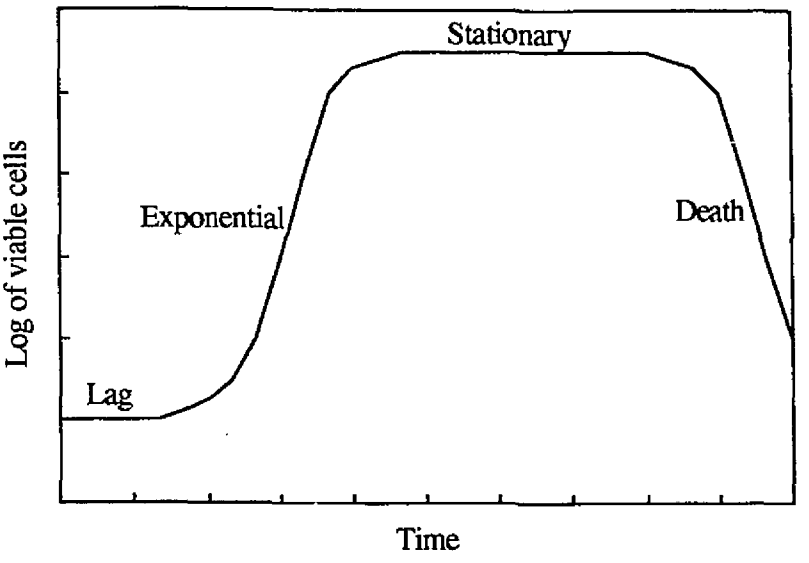

Figure 1.3 Microbial Growth Curve in a Closed System.
Shown, the four phases identified on the curve are each discussed in the text.

The lag phase varies considerably in length with the condition of the microorganisms and the nature of the medium. This phase may be quite long if the inoculum is from an old culture or one that has been refrigerated. Inoculation of a culture into a chemically different medium also results in a lag phase. On the other hand, when a young, vigorously growing exponential phase culture is transferred to fresh medium of the same composition the lag phase will be short or absent.

1.4.2 Exponential Phase

During the exponential or log phase, microorganisms are growing and dividing at the maximum rate possible given their genetic potential, the nature of the medium, and the conditions under which they are grown. Their rate of growth is constant during the exponential phase, the organisms dividing and doubling at regular intervals. Because each individual organism divides at a slightly different moment, the curve rises smoothly rather than in discrete jumps (Figure 1.3). The population is most nearly uniform in terms of chemical and physiological properties during this phase; therefore exponential phase cultures are usually used in biochemical studies.

1.4.3 Stationary phase

Eventually population growth ceases and the growth curve becomes horizontal (Figure 1.3). This stationary phase is usually attained by bacteria at a population level around 10^9 cells mL^{-1} (Prescott *et al*, 1990). Of course, the final population size depends on the nutrient availability and other factors as well as the type of microorganism being cultured. In stationary phase the total number of viable microorganisms remains constant. This may result from a balance between cell division and cell death, or the population may simply cease to divide even though remaining metabolically active.

Microbial populations enter stationary phase for a number of reasons. One obvious factor is nutrient limitation; if an essential nutrient is severely depleted, population growth will slow. It is during this phase where *Alcaligenes eutrophus* cells cease to multiply and begin to accumulate PHB in the form of inclusions. Aerobic organisms are also often limited by O_2 availability because it is not very soluble. As cultures of *A. eutrophus* become more concentrated, O_2 transfer in conventional stirred bioreactors is often not adequate for cell growth. Population growth may also cease due to the accumulation of waste products. For example, *A. eutrophus* can produce so much butyric and other organic acids during its growth phase that its medium becomes acid and growth is inhibited unless pH control is used.

1.4.4 Death Phase

Detrimental environmental changes such as nutrient deprivation and the buildup of toxic wastes lead to the decline in the number of viable cells characteristic of the death phase. The death of a microbial population, like its growth during the exponential phase, is usually logarithmic. Although most of a microbial population dies in a logarithmic fashion, the death rate may decrease after the population has been drastically reduced. This is due to the extended survival of particularly resistant cells. This resistance may come from

sporulation or the storage of energy in the form of PHB in the case of *Alcaligenes eutrophus.*

Alcaligenes eutrophus grown in batch cultures is limited to a maximum yield of 10 g L^{-1} of PHB due to the problem of substrate inhibition, since glucose tends to inhibit cell growth in concentrations above 15 g L^{-1}. The maximum theoretical yield calculated for PHB from glucose is 0.48 (Yamane, 1993). Batch growth is normally achieved in aerated stirred tank fermentors which have limited oxygen transfer at higher concentrations of microorganisms. *A. eutrophus* has been grown in a cyclone column fermentor under nitrogen limitation to produce 6.2 g L^{-1} after 48 h (Sheppard *et al*, 1994a), as these results were obtained for this thesis and were subsequently published they will be discussed further in the experimentation section of this paper. The fed-batch technique is a method similar to batch growth which is used to obtain greater PHB concentrations without inhibition from the substrate.

1.5 Fed-Batch Growth

Batch culture has some disadvantages that reduce its utility for commercial production for commercial production. It is a non-continuous cell growth method which in some cases, such as with *Alcaligenes eutrophus*, suffers from substrate inhibition. These difficulties are less problematic with the semi-continuous fed-batch technique. Fed-batch extends the normal batch fermentation by supplementing the broth with one or more essential nutrients as they become exhausted. However, products of metabolism are not removed and the working volume in the reactor increases as a result of each nutrient addition (Sheppard 1993). At the end of the batch and fed-batch growth of *Alcaligenes eutrophus* the cells are recovered and the PHB is extracted. Fed-batch culture has been reported to produce over 25 g L^{-1} from glucose although the feeding regimen was not given (Ballard and Senior, 1987). Fed-batch culture of *Protomonas extorquens* was reported to produce up to 149 g of PHB L^{-1} (Suzuki *et al*, 1986). *A. eutrophus* was found to produce up to 24 g of PHB L^{-1} in fed-batch culture. During the accumulation phase, the glucose was kept between 5 and 16 g L^{-1} by the constant addition of a 50 % (wt/vol) glucose solution. The maximum production rate of PHB was 2 g L^{-1} hr^{-1} and the maximum

specific rate of PHB production was 0.14 g L^{-1} h^{-1} g of cellular $protein^{-1}$ (Ramsay *et al*, 1990).

This method is still the most widely used means for production but has certain inherent disadvantages. The major concern is that this is not a continuous process. Once the batch is completed, the process starts over from the initial startup, wasting time during cleanup and sterilization, preparation of new inocula and the lag phase before exponential growth. The reasons for the problematic interpretation of data from batch culture was best described by Sheppard (1989):

1) Each generation of cells is subjected to a different environment as the medium constantly changes.
2) The physiological state or condition of the cells used as the inoculum is not well defined as a result of the source also being a batch culture.
3) A batch culture is a transient phenomenon in which a steady state or equilibrium is never obtained.
4) Control of a batch culture is limited to defining the initial composition of the medium and the end point of the fermentation.

These limitations of the batch culture technique result in generally poor reproducibility and limited understanding of the mechanisms underlying the growth and reproductive process. The need for a more quantitative technique led to the development of an open, continuous method for continuous culture first proposed by Monod (1950).

1.6 Continuous Culture

In closed systems, such as batch and fed-batch (fed-batch is not strictly a closed system since nutrients are added) nutrient supplies are not renewed nor wastes and PHB removed. Exponential growth lasts for only a few generations and soon the accumulation phase has passed and the bacteria are recovered. It is also possible to grow microorganisms in an open system, a system with constant environmental conditions maintained through

continual addition of nutrients and removal of wastes. According to Monod (1950) and later Herbert, *et al,* (1956) these conditions are met in the laboratory by a continuous culture system, and a microbial population can be maintained in the exponential growth phase and at a constant biomass concentration for extended periods of time. The basis for this technique is that a constant nutrient environment is provided to actively growing cells by supplying a continuous feed of nutrient solution, with removal of microbial broth at an equivalent volumetric flow rate. The cells achieve a constant growth rate that is a function of a limiting nutrient concentration, represented by the following empirical equation:

$$\mu = \mu_{max} (S / K_S + S)$$

where : μ is the specific growth rate ($1/x \cdot dx/dt$) (hr^{-1});

: μ_{max} is the maximum specific growth rate at $2 \cdot K_S$ (hr^{-1});

: S is the limiting substrate concentration ($g\ L^{-1}$);

: K_S is the saturation constant ($g\ L^{-1}$).

This system for continuous culture is known as the chemostat.

1.6.1 The Chemostat

A chemostat is constructed in such a way that sterile medium is fed into the culture vessel at the same rate as the broth containing the microorganisms is removed. The culture medium for a chemostat possesses an essential nutrient (e.g., ammonia nitrogen) in rate-limiting quantities. Because of the limiting concentration of this nutrient, the growth rate is determined by the rate at which new media is fed into the growth chamber, and the final cell density depends on the initial concentration of limiting nutrient. The culture is considered to be at a steady state if there is no change in biomass concentration. Under this condition it has been shown that the specific growth rate μ is equal to the rate of nutrient addition expressed as the dilution rate (D). The dilution rate is the rate at which medium flows through the culture vessel relative to the vessel volume, where F is the flow rate ($mL\ hr^{-1}$) and V is the volume (mL).

$$D = F / V$$

Both the microbial population level and the generation time are related to the dilution rate (Figure 1.4). With nutrient concentration constant, an increase in the dilution rate shortens the generation time while the microbial population density remains unchanged. The limiting nutrient will be almost completely used up under these balanced conditions. If the dilution rate rises too high, the microorganisms can actually be washed out of the culture vessel before reproducing because the dilution rate is greater than the maximum growth rate. The limiting nutrient concentration rises at a higher rate because there are fewer microorganisms to use the nutrients.

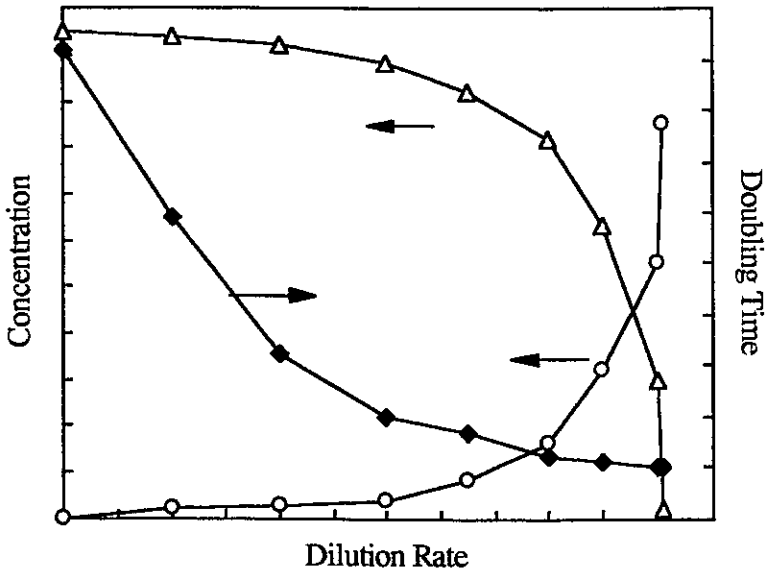

Figure 1.4 The Effects of Dilution Rate on Continuous Cultivation.

Shown is the theoretical relationship at steady state between the bacterial concentration (Δ), the cell doubling time(◆), and the substrate concentration in the broth (o) as a function of the dilution rate; from Herbert, *et al.* (1956).

A. eutrophus has been grown in a chemostat at a dilution rate of 0.15 h^{-1} in a 3.5 L reactor, well below the critical dilution rate D of 0.35 h^{-1} (Ramsay *et al*, 1990). The

glucose concentration was varied from 0 to 20 g L^{-1}. Below 8 g L^{-1} no PHB accumulation occurred, and above this PHB accumulation increased until the glucose concentration exceeded 18 g L^{-1} (Ramsay, *et al*, 1990). At the point of maximum accumulation, 0.6 g of PHB per g of cellular protein was produced. This is an example of the disadvantages of chemostat growth related to PHB accumulation. A final PHB accumulation of 1.5 g L^{-1} was obtained. Most bacteria, such as *A. eutrophus*, do not accumulate high concentrations of PHA in a single stage chemostat because high growth rate and maximum PHA accumulation cannot occur simultaneously (Ramsay, *et al*, 1990). Chemostat growth rates of *A. eutrophus* cannot attain μ_{max} since the cells would spend more of their energy on cell division and less on accumulation. Culturing cells near μ_{max} is also an unstable situation where the growth rate fluctuates. If the rate increases beyond μ_{max} then the cells would be washed out. However, there is a method for continuous cell culturing known as continuous phasing which allows growth near μ_{max} while also producing high concentrations of secondary metabolites.

1.6.2 Continuous Phasing

In response to the limitations of both the batch and chemostat methods, a new technique was developed to further advance the science of cell culturing. This technique is referred to as continuous phasing (Dawson, 1972). It consists of growing the microorganisms for a period equal to their doubling time and then feeding the microorganisms with a fresh supply of nutrients. In order to maintain a constant volume, half of the culture broth is harvested before dosing an equal volume of fresh nutrients to refill the reactor. This ensures that each successive generation is grown under an identical environment. The concept is illustrated in Figure 1.5 by showing the changes in limiting nutrient and biomass concentrations. This technique has the advantages of being a continuous growth method as well as only growing in the exponential phase, removing the lag phase and growing near μ_{max}. It also eliminates certain problems inherent with batch growth by producing two equal, identical samples of harvested broth. One remains in the

reactor, the other is kept for analysis. Every sample is also nearly identical to the next since there is only one doubling time of the bacteria to reach the next cycle. Which leads to the theory of cell synchrony, where the temporal correspondence between the period of nutrient feeding and the biomass doubling time can result in synchronized growth and division of the cells. A synchronized cell culture is able to reveal metabolic phenomenon related directly to the cell cycle, something that is not possible with a random population.

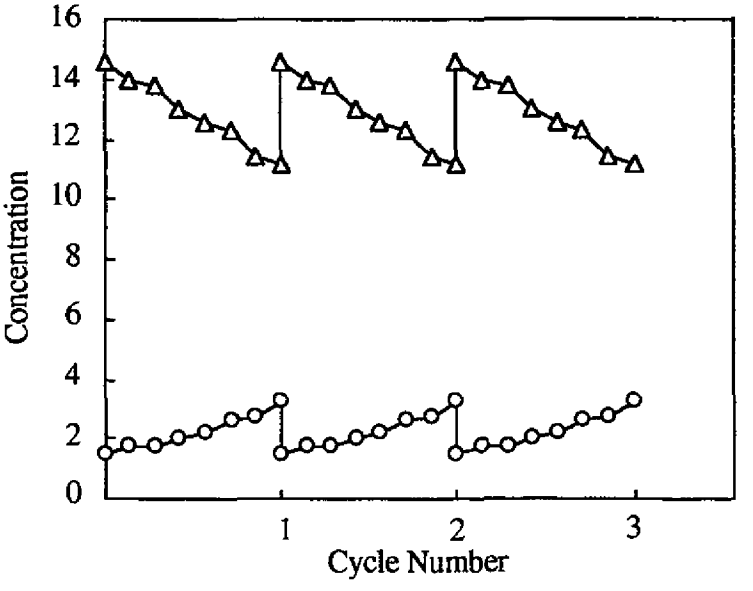

Figure 1.5 The Principles of Continuous Phasing.
Shown are the periodic variations in both the nutrient (Δ) and the biomass (o) concentrations (g L^{-1}). Each nutrient cycle corresponds to one generation of cells.

Continuous phasing has not been widely used compared to batch and chemostat for microbial culture. Edwards (1981) criticized that the method of periodically adding nutrients results in environmental changes that throw doubt on how normal this synchronized cell division and cell cycle events are. This criticism is not valid because the concept of the normal cell cycle is impossible to define (Sheppard, 1989). Metabolic phenomena can only be defined in relation to the environment in which these phenomena

are observed, and almost all we know about the microbial world has been observed under laboratory conditions that are certainly abnormal compared to nature. The principle problem with the phasing technique is not that the technique is abnormal, but rather in the interpretation of the experimental observations. Interpretation is problematic because of the rather arbitrary selection of the period between feeding times. Testing may determine the shortest period over which the microorganisms can double, however small errors in harvesting and dosing quickly result in instability and inconsistent performance as a result of changes in biomass concentration (Sheppard, 1993). Since the cycle time (estimated doubling time) is chosen from batch cycle experiments the exact doubling time may differ as the cycles progress. It is possible to know if too short of a cycle time is chosen since the cells are not allowed enough time to divide and they are washed-out. But with longer cycle times it is not possible to know how close μ_{max} has been approached. An adaptation to this technique was conceived by Sheppard (1989) by adding a feedback control to improve the stability of the culture and eliminate the need for an arbitrary pre-determined cycle time.

1.6.3 Self Cycling fermentation

Self-cycling fermentation (SCF), a term first coined by Sheppard, *et al* (1990) is similar to continuous phasing, except the growth rate of the microorganisms determine the cycle time. The time at which fresh nutrients are added is determined by the time of exhaustion of a limiting nutrient. Since there is a direct correlation between nutrient limitation and oxygen utilization (Figure 1.6), the dissolved oxygen (DO) is monitored until the exact point of nutrient limitation. It is used to trigger the cycle during which half the nutrients are harvested and replenished with an equal volume of fresh nutrients. The limiting nutrient used to trigger the cycle is nitrogen since PHB accumulation occurs after nitrogen limitation. When the nitrogen becomes limiting the cells stop growing and thus their need for oxygen decreases, showing an increase in dissolved oxygen in the media as shown in Figure 1.6. This change in slope of the dissolved oxygen is monitored by the data acquisition system and the computer then initiates a sequence of operations that result

in the removal of half of the broth and the addition of fresh nutrients, thus beginning a new cycle. It has been noted that some PHB accumulation occurs before complete nitrogen depletion is reached (Ramsay, 1990). This procedure has been used to grow *Bacillus subtilis* ATCC 21332 for surfactin production (Sheppard, 1989, Sheppard and Cooper 1990), and *Acinetobacter calcoaceticus* RAG-1 (Brown and Cooper, 1991) for the production of bioemulsifiers (emulsan).

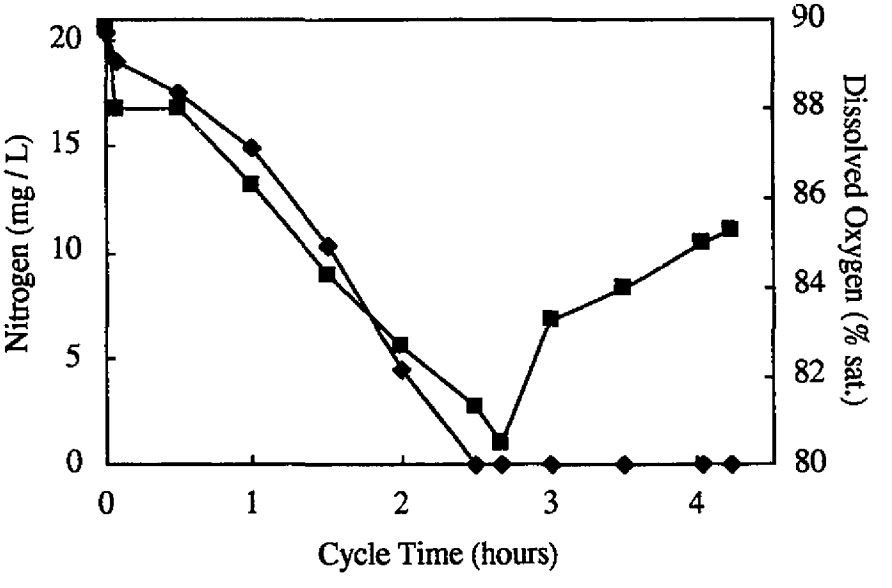

Figure 1.6 The Effect of Nitrogen Concentration on Dissolved Oxygen

Shown is the relation between dissolved oxygen (■) and nitrogen (◆) concentration during an individual SCF cycle. The respiring cells utilize nitrogen until it becomes depleted. As this point is reached the cells no longer grow and the need for oxygen decreases. The computer feedback loop is used to detect the change in slope and thus trigger the next cycle. Each consecutive cycle will undergo the same periodic fluctuation in oxygen.

In conventional SCF systems harvesting will occur directly after nitrogen depletion and a new cycle will start again. At this point, an extended starvation period could be added to the end of each cycle to accumulate greater amounts of PHB. Since the majority of PHB accumulation occurs after nitrogen limitation, an extended period would, in theory produce a higher PHB concentration. There may be a point at which the high concentration of PHB inclusions inhibits the doubling time of the next cycle. This research will attempt to determine the optimum control strategy.

1.7 Objectives

The objectives of this work were to determine the rate of PHB production and yield of PHB from glucose using a culture of *A. eutrophus* grown under the following conditions:

1. In a 1-liter cyclone bioreactor using a batch culture method.
2. In a 75-liter cyclone bioreactor using a batch culture method.
3. In a 1-liter cyclone bioreactor using a fed-batch culture method.
4. In a 1-liter cyclone bioreactor using a self-cycling culture method with various imposed starvation periods.

By achieving these objectives, the utility of the cyclone bioreactor for PHB production at both the lab and pilot scales can be assessed and the potential advantages of the self-cycling technique for PHB production determined.

CHAPTER II MATERIALS AND METHODS

2.1 Biomass concentration

The concentration of biomass in the fermentation broth was determined by dry weight analysis. Between 25 and 30 mL of broth was pipetted and centrifuged at 4000 x g for 10 min (International Equipment Company model #we4q8934). The supernatant was removed and the cells were washed with about 25 mL of distilled water and then recentrifuged. After removing the supernatant the cells were transferred with a small volume of distilled water to pre-weighed aluminum dishes. The dishes were placed in a convection drying oven (Fisher Isotemp) at 105°C and dried until constant weight. Weights were determined using an analytical balance (Saratorius Basic Model BA1108) with an accuracy of 0.1 mg.

There were two potential sources of error from using this method: the inefficient washing of the cells due to poor cell compaction while centrifuging, and measurement errors (volume and weight) due to small sample sizes.

2.2 Glucose Concentration

In order to ascertain the efficiency of glucose utilization (PHB yield), glucose concentration was determined by the DNS method for reducing sugars. The samples were taken from the supernatant (which was frozen after the initial centrifugation) for biomass determination and were diluted to achieve a glucose concentration between 0.5 and 2 g L^{-1}. One mL of the appropriately diluted samples was placed in test tubes while 1 mL of distilled water was used for a blank. A standard curve between 0 and 2.5 g L^{-1} was also prepared. One mL of 0.1 M citrate buffer was added and mixed using a vortex mixer. Three mL of DNS reagent (1.6% (wt/vol) NaOH, 1% (wt/vol) 3,5 dinitrosalicylic acid and 30 % (wt/vol) NaK tartrate) were added and mixed well. The tubes were placed in boiling water for 10 min and allowed to cool. The absorbance was read at 600 nm using a Pharmacia Novaspec II spectrophotometer with the blank as zero. The standard curve was used to calculate the glucose concentration based on the appropriate dilution factors.

In the case of cloudy samples they should be centrifuged to remove the cloudiness. The number of samples to be tested should be limited to approximately 30 at a time (including blank and standards) since the colour degrades within 30 min of removal from the boiling water bath.

2.3 Ammonia Nitrogen Concentration

The nitrogen content of the broth was determined in order to ascertain if nitrogen limitation had occurred and whether the cells had entered the PHB accumulation phase. Five mL samples of supernatant were diluted to 50 mL in order to be within the range of 1.4 to 140 ppm NH_3. Five M NaOH was used to adjust the solution to a measured pH of 13 which shifted the balance of the equilibrium from NH_4^+ to NH_3 for direct quantitation. The nitrogen content was quantified with an Orion specific ion electrode (Model 95-10). Standard solutions of ammonium sulfate were used to construct a calibration curve to correlate mV to NH_3 concentration. The possible sources of error for this technique arise from the dissappearance of the NH_3 from solution since in its gaseous form it tends not to remain in solution over time. Samples must be read immediately after NaOH adjustment.

2.4 PHB Concentration Using Gas Chromatography

The samples for PHB determination were prepared by the method of Riis and Mai, (1988), where 5 mL of broth was centrifuged at 3500 x g for 20 min in screw cap test tubes. The supernatant was removed (centrifuged biomass may be frozen for analysis later) and 2 mL of 1,2 dichloroethane or trichloroethane was added to the tubes. To this 2 mL of a solution containing 4 volumes of propanol to 1 volume of concentrated HCL were added with 0.2 mL of an internal standard solution of 2.0 g of benzoic acid in 50 mL propanol. An external standard solution was prepared in the same way with 0.02 g of PHA containing 78% (wt/wt) PHB. The tubes were vortexed and placed in an oven (Fisher Isotemp) at 105°C for 3 h. They were removed every half hour, vortexed and replaced. After the tubes had cooled, 4 mL of water was added and the tubes were vortexed again for 30 seconds. The organic (dichloroethane-propanol; bottom phase) was removed with Pasteur pipettes for analysis.

The PHB content of the samples was then quantified using an HP 5890 Series II gas chromatograph. The gas chromatograph was fitted with an automatic injector and a flame ionization detector which was supplied with 500 mL of air, 30 mL of hydrogen and 30 mL of helium $min.^{-1}$. The injection split ratio was 100:1 with a helium flow of 0.9 mL $min.^{-1}$ through the 25 m long HP5 capillary column (Hewlett Packard, Palo Alto, Calif.). The injector port temperature was 180°C, and the detector temperature was 200°C. The initial oven temperature was 120°C and increased by 8°C per min. to a final temperature of 210°C. Injections of 5 µl were made and the retention times for the methyl ester of 3-(hydroxybutyrate) and benzoic acid were 4.1 and 7.0 min respectively.

The automatic injection system improves the reproducibility of this method by reducing the error which arises from manual injections. These errors occur as users tend to inject at different rates which may alter retention times or widen peaks. Other errors arise from contaminants which accumulate in the column, however, proper conditioning of the column overnight before a run of samples will eliminate this effect. The conditioning program is similar to the temperature profile for a sample except the final temperature of 210°C is maintained for several hours. The threshold value of PHB was determined to be 0.05 g L^{-1}. Therefore, it was decided that *A. eutrophus* should be grown to produce PHB above 0.5 g L^{-1} to increase the reproducibility of the data.

2.5 Dissolved Oxygen Concentration

In order to determine the on-line time of nitrogen exhaustion, the dissolved oxygen must be monitored *in situ*. This was accomplished using an Ingold model (IL532) polarographic oxygen sensor connected to an O_2 meter (Instrumentation Laboratories Inc. Model 531). The multimeter provided the amplifier and a 4 - 20 mA output was measured by connecting a chart recorder (BBC Goerz Metawatt model SE 420) across a 47 ohm resistor. The data acquisition unit (Chapter 3.2) could also be used to convert the analog output to a digital signal for monitoring by the computer. The IL 532 contains two electrodes suspended in an electrolyte and isolated from the sample by a stainless steel reinforced 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 anode that is proportional to the concentration of oxygen in the sample. The probe can be

sterilized by heating at 120°C for 20 min and has a built in thermistor for temperature compensation between 15°C and 45°C.

The probe was mounted in a stainless steel fitting placed directly below the outlet from the cyclone column for the laboratory scale fermentor (Figure 3.1). The recirculating pump sucked the broth past the probe at high velocity (0.46 m s^{-1}), ensuring no stagnant layer or dead spaces around the membrane. The sensor was installed in the recirculation loop of the pilot scale fermentor and placed perpendicular to the flow (Figure 3.2).

2.6 Viable Cell Count

Viable cell count was measured to determine if there was any degree of cell synchrony, ie. to show if there was a specific point during the cycle when most cell division occurred. The method was a simple enumeration by Plate Count Method (Department of Natural Resource Science, McGill University, 1994). This method involves serial dilutions of the sample followed by dispensing aliquots of the dilutions onto the surface of agar plates. This technique is called the spread plate technique because the aliquot is spread over the entire surface of the agar with a glass rod spreader. A 10^{-2} dilution of the sample was prepared by aseptically transferring 1.0 mL of culture into a 99 mL sterile dilution bottle filled with H_2O. Three subsequent dilutions were made of 10^{-4}, 10^{-6}, and 10^{-7}. Sterile plate count agar plates were inoculated from the 10^{-6} and 10^{-7} dilution bottles with 0.1 mL of aliquot. The aliquot was spread over the plates with a glass rod until the 9 mm diameter surface was completely covered. The plates were inoculated at 30°C for 24 h and those containing between 30 and 300 colonies were individually counted. Viable cell count in the original sample was calculated using the formula:

$$\frac{\text{CFU mL}^{-1}}{} = \frac{\sum \#\text{ of colonies / plate}}{\#\text{ of plates counted}} \text{X} \frac{1}{\text{aliquot (mL)}} \text{X} \frac{1}{\text{decimal dilution}}$$

example:

$$\frac{150+175+164}{3} \text{X} \frac{1}{0.1} \text{X} \frac{1}{10^{-5}} = 163X10^{6} = 1.63X10^{8}\ \text{CFU mL}^{-1}$$

2.7 Medium Preparation

Alcaligenes eutrophus DSM 545 was grown in a medium described by Ramsay *et al* (1990) which was subsequently modified for the scale-up and for use in fed-batch and self-cycling experiments according to Table 2.1a and 2.1b.

Table 2.1a Medium Composition

Ingredient	Concentration (g L^{-1})			
	Ramsay's*	Batch**	Fed-batch	SCF**
Phosphate buffer				
KH_2PO_4	2.5	2.5 or 0.83	2.5	0.83
$Na_2PO_4 \cdot 7\ H_2O$	9.9	9.9 or 3.3	9.9	3.3
Salts solution				
$CaCl_2$	0.01	0.01	0.01	0.01
NH_4 • Fe (III) Citrate	0.06	0.06	0.06	0.06
$MgSO_4$	0.2	0.2	0.2	0.2
$(NH_4)_2SO_4$	0.84	0.28 or 0.84	3.36	0.84 or 1.68
Trace element solution***	1 mL L^{-1}	1 mL L^{-1}	1 mL L^{-1}	1 mL L^{-1}
Glucose • 1 H_2O	16.5	16.5	16.5	5.5 or 16.5

*From Ramsay *et al* (1990) ** Batch growth: phosphate concentration decreased when scaled-up, batch and SCF growth: glucose and nitrogen concentration varied depending on experiment.

Table 2.1b Trace Element Solution

Ingredient	Concentration (mg L^{-1})
H_3BO_3	300
$CoCl_2 \cdot 6H_2O$	200
$NaMoO_4 \cdot 2H_2O$	30
$NiCl_2 \cdot 6H_2O$	20
$CuSO_4 \cdot 5H_2O$	10

***Concentration of trace elements.

During **batch growth**, the excess phosphate buffer was used to maintain the pH between 7 and 7.5. The potassium/sodium phosphate buffer solution, the mineral salts solution and the glucose solution were each autoclaved separately in quantities of 700, 100 and 100 mL respectively, and then allowed to cool to avoid precipitation. The contents of the three flasks were aseptically transferred to the 1 L cyclone bioreactor. The 100 mL inoculum was prepared in the same manner as the medium, with the same concentration of nutrients and also transferred aseptically. The inoculum took between 15 and 24 h to grow in a controlled environment incubator shaker (New Brunswick Scientific Co.) set at 30°C and 300 rpm. The limiting nutrient for the fermentation was the nitrogen source.

The **pilot scale** experiment used the same nutrients in the same proportions to a total of 75 L. Two different ammonium sulfate concentrations were used for the different experiments. A lower phosphate buffer concentration was used since the pH was maintained using an Ingold Model 2301 pH transmitter, Ingold pH electrode Model 405-DPAS-K8S/200 and a Masterflex peristaltic pump adding 2 mol L^{-1} NaOH. The sterilization of the media was more complex, it was performed *in situ* and is explained further in Section 3.2.

The **fed-batch** experiment was done in a laboratory scale cyclone bioreactor. The identical medium was used for the batch growth except the initial $(NH_4)_2SO_4$ concentration was 4 times higher and a 50% (wt/vol) glucose solution was added with a maximum addition of 25 mL (12.5 g glucose anhydrous) when the measured glucose in the reactor fell to 2.5 g L^{-1}.

The **self cycling experiments** also had the same medium except for the glucose, $(NH_4)_2SO_4$ and phosphate buffer concentrations. The glucose concentration for the initial experiment was decreased to 5.5 g L^{-1} since there was no accumulation phase added to the cycle time. The phosphate buffer concentration was kept low (Table 2.1a) for the same reason while the initial $(NH_4)_2SO_4$ concentration remained at 0.84 g L^{-1} for preliminary results and testing of equipment. The medium from the second experiment contained an $(NH_4)_2SO_4$ concentration raised to 1.68 g L^{-1} to increase the number of bacteria, or

biomass concentration. All of the experiments with imposed starvation periods used the initial measured glucose concentration of 16.5 g L^{-1}, while the phosphate buffer remained low since short starvation periods (less than 10 h) were planned. The potassium/sodium phosphate buffer solution (8 L) was autoclaved in a 10 L Nalgene bottle connected to the control solenoid valve on the inlet to the reactor. The mineral salts solution and the glucose solution were each autoclaved separately in quantities of 1 L respectively, allowed to cool and the contents of the two smaller flasks were aseptically transferred to the 10 L Nalgene bottle. The medium was replenished periodically by autoclaving a second Nalgene bottle (same as above) which could be connected with a Swagelock™ stainless steel quick-release tube fitting. The 100 mL inoculum was prepared in the same manner as for batch growth.

CHAPTER III APPARATUS

3.1 The Cyclone Column Reactor

All the experiments were performed in a reactor referred to as a cyclone column. It has been used extensively by Dawson (1963, 1971 and 1974) for the growth of *Candida utilis* at both bench and pilot plant scales and is the subject of a patent licensed to W.H.E. Process Systems Limited. Bench scale units have been used for the study of *Bacillus subtilis* by Sheppard (1989) one of which is illustrated in Figure 3.1. These studies of *Alcaligenes eutrophus* were also done with a bench scale reactor and a modified version of the pilot scale reactor the details of which have been published by Sheppard *et al* (1994a).

3.1.1 Lab Scale Reactor

The laboratory scale bioreactor consisted of a custom made 7.6 cm internal diameter Pyrex cyclone with a total height of 59 cm and a working volume of 0.5 to 2.0 L. The cyclone was connected at the bottom with 1/2" flexible latex tubing to a 14 Watt magnetically driven centrifugal pump (March Model MDX). The pump recirculated the broth through a side-arm and back into a tangential entry point at the top of the vessel at a velocity of 1.0 m s^{-1}. This caused swirling down the inside surface of the column where the mixing occurred.

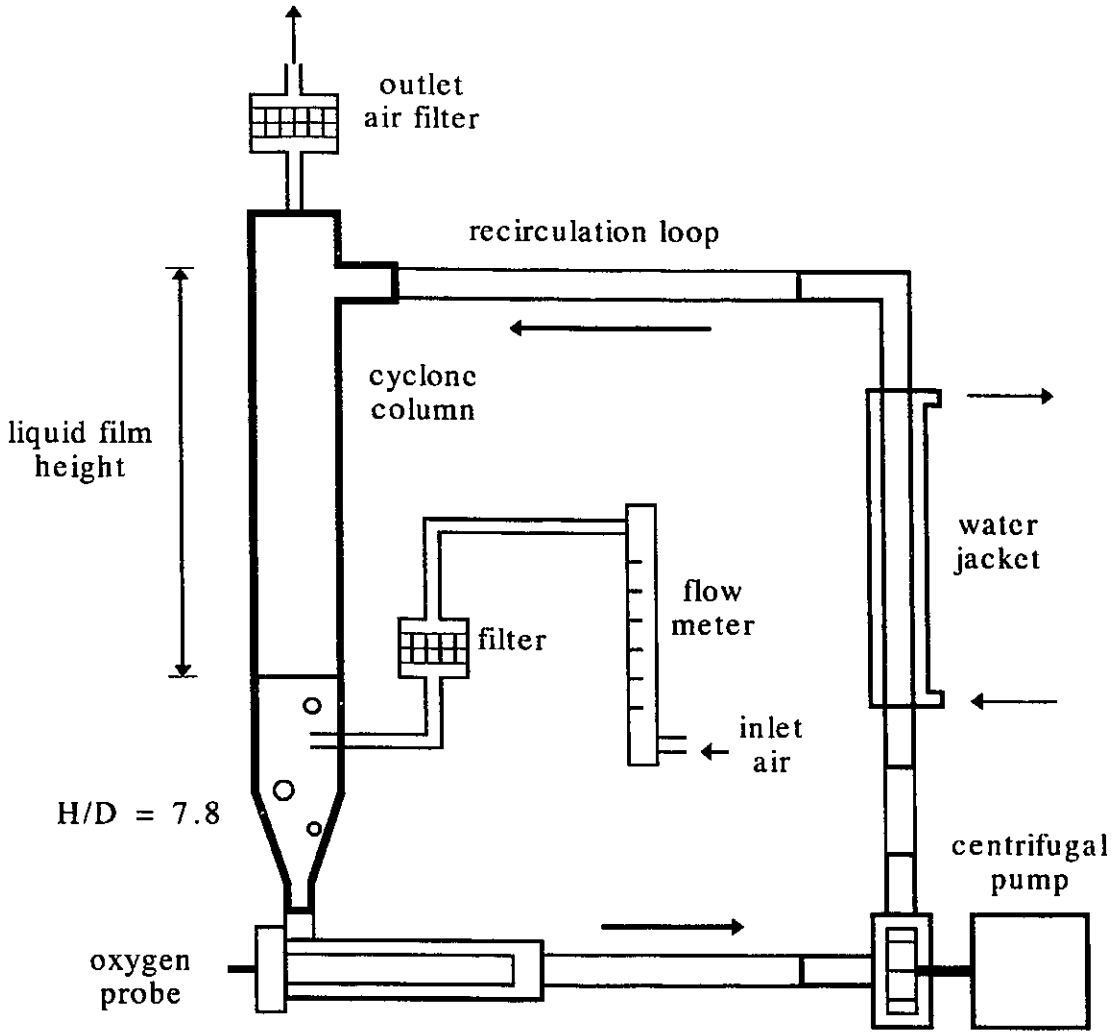

Figure 3.1 The Cyclone Column Bioreactor

Shown is the 1 L bioreactor used for batch and fed-batch growth. (Sheppard *et al*, 1994b)

Filtered air was introduced into the column about 15 cm from the bottom at a rate between 0.5 and 1.0 L min^{-1}. The air rose counter-current to the liquid flow, escaping out the top after passing through a condensor (not shown) and polypropylene filters. Oxygen transfer from the gas into the liquid phase occurred across the falling film and from the bubbles entrained into the recirculation loop. The recirculation loop had a length of 0.6 m, a volume of 0.1 L and a measured liquid residence time of 0.8 s.

The cyclone column was selected for these studies because the units have several advantages over conventional stirred tanks. The advantages include:

1) Uniform mixing without the need for baffles is accomplished with a high measured recirculation rate (7.9 L per minute or a 7.6 second turnover rate for 1.0 L). This ensures that uniform measurements can be obtained for *in situ* analysis with a dissolved oxygen probe and for external laboratory purposes (Sheppard, 1989).

2) The action of pumping around a recirculation requires a power to volume ratio which is considerably higher than that required in a stirred tank, thus ensuring superior mixing that is applied uniformly to the entire broth. Unlike the stirred tank there are no need for coils for temperature control or for an air sparger ring to regulate the environment , (Sheppard *et al*, 1994b).

3) There are no aseptic seals. Sterilization of the complete unit is accomplished by simply unclipping from the mounting board and placing it (including the side-arm, pump-head and dissolved oxygen sensor) in the autoclave for 30 min at 121°C. The magnetically-driven recirculation pumps are rated for continuous duty, the heads being sterilizable after addition of aluminum clamping plates. The absence of seals and gaskets in addition to the light weight, all glass construction results in a simple but reliable reactor (Sheppard, 1989).

3.1.2 Pilot Scale Reactor

A scaled-up version of the cyclone bioreactor is shown in Figure 3.2. The pilot scale reactor consisted of a 316 stainless steel cyclone reactor, a progressing-cavity type recirculating pump (Seepex Model, 17-12NS) with a variable capacity of 50 to 125 L min^{-1} which required a power of 400 to 1700 W and a tube-in-tube heat exchanger. The cyclone and heat exchanger were custom made by Triton Engineering Ltd. (Halifax, N.S.) and Doral Ltd. (Montréal, Québec) respectively. The various instruments for process monitoring and control shown in Figure 3.2 are listed and described in Table 3.1. The working volume of the entire reactor (including recirculation loop) can vary between 40 and 140 L but is usually filled with 75 L, resulting in a scale-up factor of about 100.

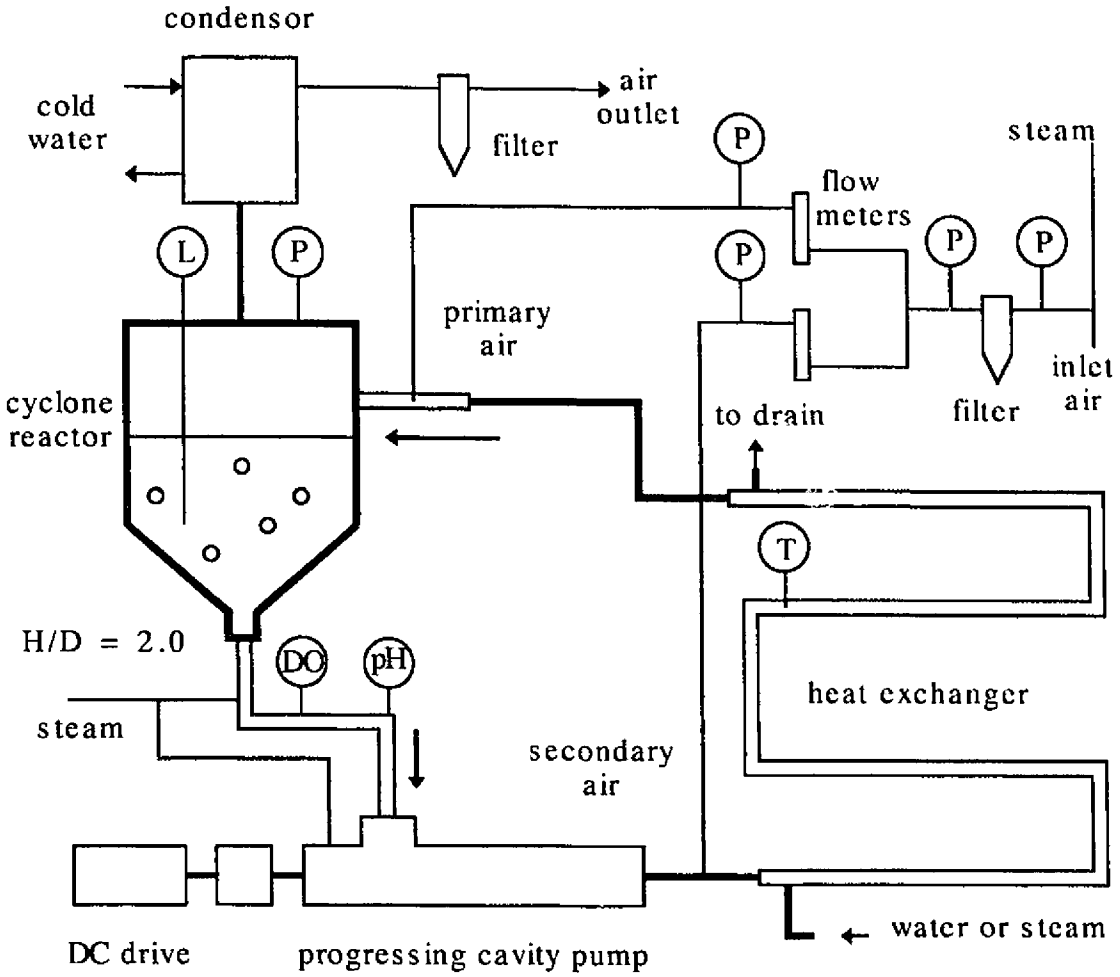

Figure 3.2 The Pilot Scale Bioreactor

The 75 L pilot scale reactor showing the locations of pressure gauges (P), pH probe (pH), level probe (L), thermistor (T) and dissolved oxygen sensor (DO).

In order to obtain the same rate of oxygen in the pilot scale reactor as in the laboratory scale cyclone, the same ratio of interfacial film area to total volume would be required. However as the size of the reactor increases, the working liquid volume increases faster than the area of the liquid film. Since the swirling film area in the 1.0 L laboratory scale bioreactor is about 960 cm^2 (Sheppard and Cooper, 1990), the pilot scale reactor would require 9.6 m^2 with a 100 L volume, resulting in a height to diameter ratio greater

than 15. This was not practical, especially if even larger reactors would be built. Instead, the rate of oxygen transfer relied more on bubbles entrained in an extended recirculation loop, while the reactor itself had a height to diameter ratio of only 2. The recirculation loop was constructed of 5 cm diameter schedule 40 pipe on the suction side of the pump and 2.5 cm diameter schedule 40 pipe on the discharge side, which included the heat exchanger. The total volume of the recirculation loop was 26 L, or about 30 % of the normal working volume, with an overall length of 22.9 m and a residence time ranging from 12 to 31 seconds depending on the pump speed.

Function	*Instrument*	*Specifications*
Temperature control	YSI Model 2158	Thermistor output ± 0.1 °C; control to ±0.5 °C; two relays for hot and cold water solenoids
pH control	Ingold Model 2301 pH transmitter	Dual relays for acid and base pumps; control to ±0.05 pH units
Liquid flow	Controltron System 180	Dual ultra-sonic high temperature heads; 0-1585 L / min ±1% FS
Liquid volume	Drexel Brook Eng. Co. Series 700	Input from capacitance level probe Model 700-2-24; 38-130 L ± 1% FS
Air flow	Cole Parmer Model 32603-00 meter	0.5-4.0 scfm (14-113 L /min) ±10% FS; steam sterilizable
Air pressure	Weksler Model AA44-2 Guage	0-60 psig (0-141 kPa) ±0.5% FS
Dissolved oxygen	Ingold Model 170 % air amplifier	Input from Ingold 120 mm polarographic sensor; 98% response in 20 s at 37°C; 0.3% linearity; temperature compensated

Table 3.1 Instrumentation for the Pilot Scale Bioreactor

In order to attain a power input per unit volume equivalent to that of the laboratory scale reactor (17 W L^{-1}) a pump speed of 350 rpm was required. However, the time in the recirculation loop was still 13 times longer than that of the lab scale reactor, thus the extended recirculation loop required more than one air injection point. The points of air injection are shown in Figure 3.2: just before the broth enters the cyclone (primary aeration), and at the pump discharge (secondary aeration).

3.2 Control Equipment

The 1 L laboratory scale cyclone column reactor was operated in batch and fed-batch mode with minimal control equipment other than the recirculating water bath (Haake Model FE2) for temperature control and a rotameter (Brooks Model 1355BB1B1AAA) for control of the air supply. Samples were obtained manually from a sterile sample tube at the top of the recirculation loop. In the case of fed-batch, a Masterflex peristaltic pump was used to add the 50 % (wt/vol) glucose (anhydrous) feed after the level reached 2.5 g L^{-1}. The periodic addition of the nutrient solution and removal of the cell broth, as occurs during SCF, required considerably more hardware.

For SCF operation sterilizable solenoid valves (Skinner Model V52LB2052) were used to control the flow of fresh medium from the medium storage through 1/4" latex tubing into the reactor (Figure 3.3) and out of the reactor to the sample containers. The media was gravity fed from sterilized media bottles above the reactor. Harvesting was also done by gravity and controlled by a sequence of three solenoid valves which can harvest up to three samples in a row without requiring their immediate removal. There is a fourth valve upstream from the other three to ensure sterility. In order to measure the liquid volume entering and leaving the reactor several methods (Dawson 1972, Sheppard, 1989, Sheppard, *et al* 1990) have been used for volume control as well as to control the cycle itself. Self-cycling fermentation with improved volume control (Sheppard, 1993) requires that the entire cyclone be mounted on a stand placed on an electronic balance (Precisa Model 8000D-24000G).

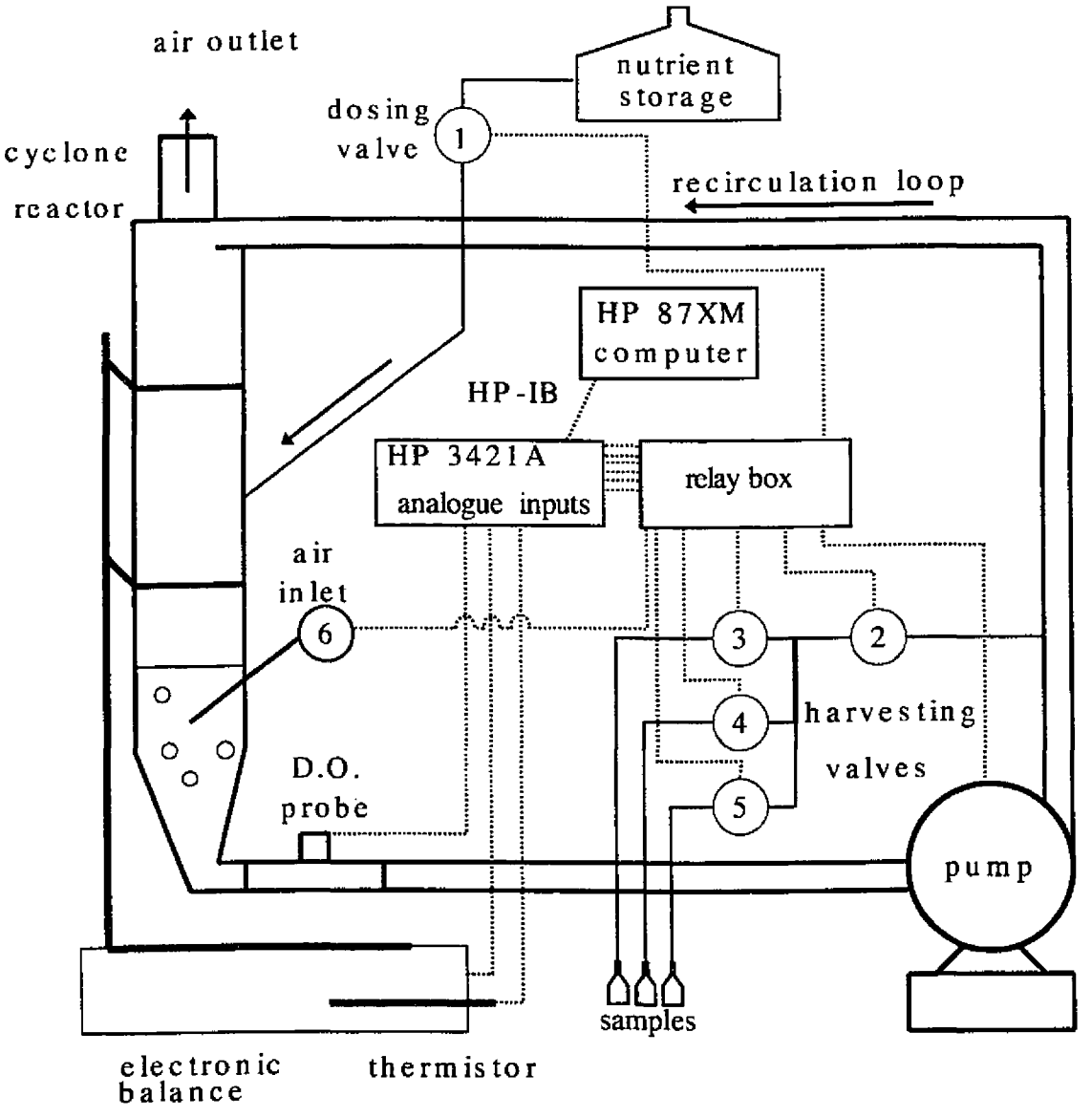

Figure 3.3 Lab Scale Self-Cycling Apparatus

The fermentation apparatus showing the cyclone column reactor mounted on an electronic balance, the dissolved oxygen probe and the six solenoid valves for accomplishing the harvesting, dosing and aeration during the SCF.

Both the control flexibility and accuracy were improved with the use of a computer in conjunction with a data acquisition and control interface connected to the electronic balance in lieu of the relays and timers which had been previously used to control continuous phasing (Sheppard, 1989). The computer was a Hewlett Packard Model 87

XM, connected via an HP-IB parallel interface bus to a Hewlett Packard 3421 data acquisition and control unit which performed the analog to digital conversion of the 0-0.8 V signal from the balance and a 0-0.8 V input from the dissolved oxygen meter. A thermistor was required to compensate for the effect of temperature on the electronic balance. A linear correlation was obtained between the balance temperature and the balance output in volts with a slope of 0.39 % / °C. Even though this is apparently a small effect, the entire reactor plus fermentation broth weighs about 4.7 kg. Thus a three degree change in temperature can result in a change in weight of about 67 grams or 13% of a 500 mL harvest volume. The software was designed to measure the thermistor temperature before every cycle and calibrate the balance input signal to the data acquisition and control unit as shown in the simplified flow chart of the control logic for the program in Figure 3.4.

Peripheral computer equipment consisted of a dual disk drive (HP Model 9121) and a printer (HP Model 82906A) all connected via HP-IB parallel interface. The setup of the system is illustrated in Figure 3.3, showing the HP 3421A acting as a bi-directional interface. The HP 3421A is capable of being connected to up to 30 devices through three I/O cards. Each card can be configured to receive 8, 9, or 10 analog inputs with up to two channels functioning as control relays. One of the cards was connected to a relay box which increased the number of control relays to 10 per card instead of 2 per card. This was done in order to not limit the control capacity in the case of future scale-up of the self-cycling bioreactor since there are many more components to control in the pilot scale bioreactor (Table 3.1). In this system the control relays were attached to six solenoid valves, five of which controlled dosing and harvesting and one which turned the air inlet valve and circulating pump on and off.

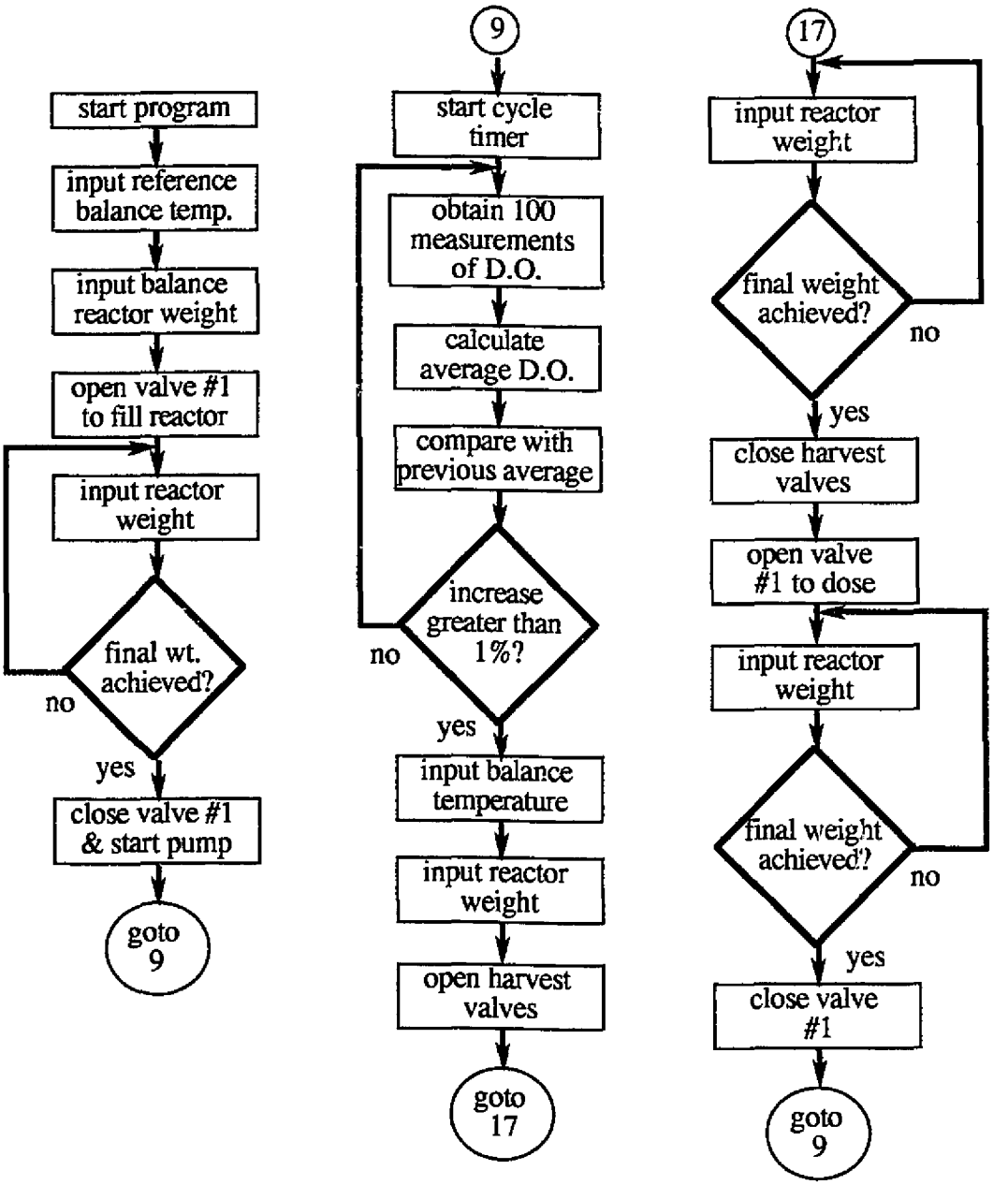

Figure 3.4 The Flowchart of the Computer Program

Simplified flowchart illustrating the logic of the computer program used for control of the broth harvesting and nutrient dosing taken from Sheppard (1993). This flowchart does not demonstrate the possibility of adding a starvation period at the end of each cycle.

The apparatus was controlled with an HP BASIC custom program written for the personal computer. A copy of the program is included as Appendix A. The program used simple instructions to the data acquisition and control unit. To read the balance voltage for example, the command is to output to the data acquisition and control unit device number asking for the DC voltage reading of a specific channel on one of the boards; OUTPUT 709; "DCV02". This stores the value in the internal memory of the data acquisition and control unit which can be read with ENTER 709; BALWT to enter the value as the variable describing the balance weight in volts. The two-wire resistance of the thermistor is read with "TWO04", and the DO probe is read with "DCV03". The solenoid valves and pump are controlled by closing the circuit with relays with the command "CLS12" for example to close the second relay on board number 1. The program is structured with control loops to fill and empty the reactor and also to calculate average values for the dissolved oxygen, thus providing a means for determining the precise time of nutrient exhaustion.

CHAPTER IV EXPERIMENTATION

4.1 Lab Scale Batch Culturing

4.1.1 Procedure

The main purpose of producing PHB in batch cultures using *A. eutrophus* was to compare the results from the lab scale cyclone with the pilot scale reactor. Also, the results could be used to estimate the potential PHB accumulation during the starvation period (after nitrogen limitation) in SCF experiments. The entire bioreactor, including recirculation arm and pump head, was autoclaved at 121°C for 30 min. The same was done for the 900 mL of medium which was prepared in separate flasks and allowed to cool to avoid precipitation of some salts. The 10% inoculum was prepared as stated in Section 2.7 and was aseptically transferred to the reactor along with the medium. Samples were taken every 3 h except for near the point of nitrogen limitation (generally between 10 to 15 h of growth) where samples were collected every 1 hour.

4.1.2 Results

The data from this experiment have been published, (Sheppard *et al,* 1994a). Figure 4.1 illustrates the accumulation of PHB and biomass while showing the point of nitrogen limitation which coincided with the dip in dissolved oxygen concentration. The concentration of PHB was 0.58 g L^{-1} in 1.89 g L^{-1} of biomass when the initial nitrogen supply of 180 mg was exhausted after about 14 h. At this point, 3.1 g L^{-1} glucose was consumed resulting in a biomass yield on glucose of 0.61 g g^{-1}. After this point the cells are respiring less and are accumulating PHB. The glucose was exhausted after 48 h, resulting in a final production of 6.2 g L^{-1} PHB in 7.1 g L^{-1} at the end of the batch corresponding to a PHB content of 87% (wt/wt).

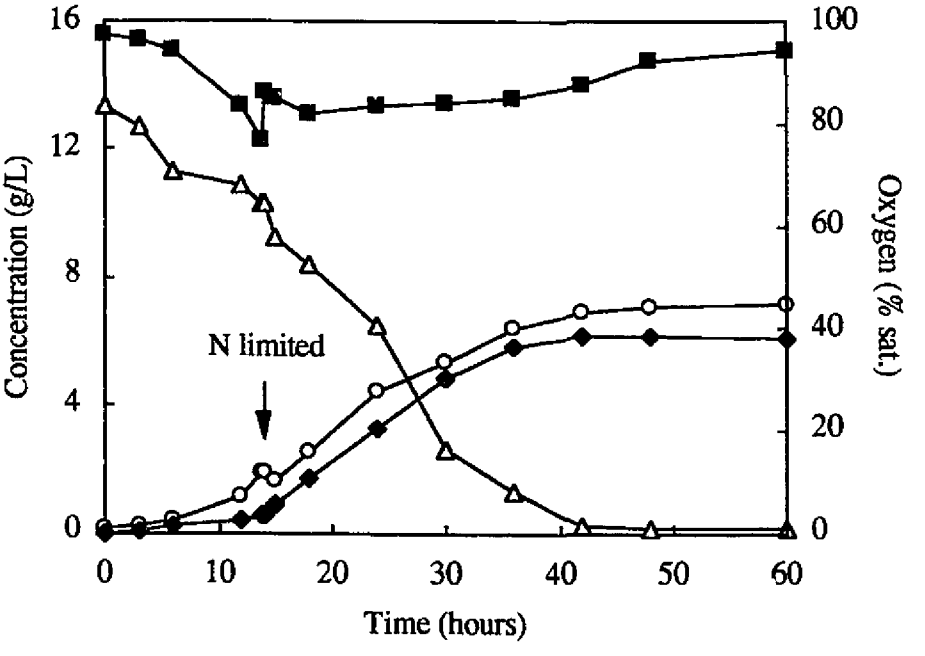

Figure 4.1 Batch (B1) Growth of *A. eutrophus*
Accumulation of PHB. while growing *A. eutrophus* DSM 545 in the laboratory scale bioreactor showing the changes in dissolved oxygen (■), glucose (Δ), biomass dry weight (o), and concentration of PHB (◆). Growth of the culture was limited by 0.84 g L^{-1} ammonium sulfate.

The overall yields from glucose were 0.53 and 0.46 for total biomass and PHB respectively. This is very close to the maximum theoretical yield calculated for PHB from glucose of 0.48 (Yamane, 1993). The overall PHB accumulation rate was determined to be 0.13 g L^{-1} hr^{-1} over the 48 h, corresponding to an accumulation rate of 0.018 g PHB g $biomass^{-1}$ hr^{-1}. The minimum concentration of dissolved oxygen, 78%, occurred just prior to nitrogen limitation.

A duplicate of this batch was done, producing similar results as shown in Figure 4.2. The 180 mg of nitrogen was exhausted after 15 h and the glucose was exhausted after 48 h. The final PHB content was found to be 6.1 g L^{-1} with a biomass concentration of 6.8 g L^{-1} after 48 h.

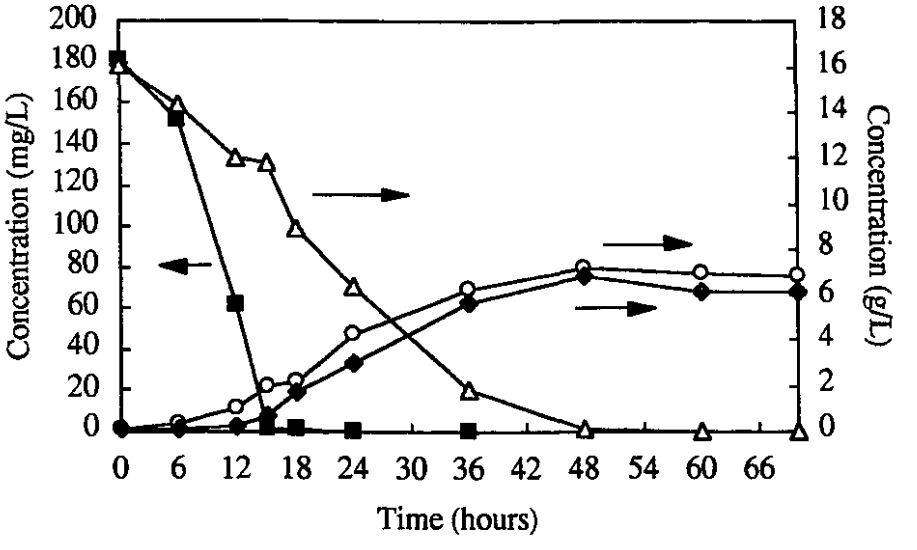

Figure 4.2 Batch (B2) Growth of *A. eutrophus*

Accumulation of PHB while growing *A. eutrophus* DSM 545 in the laboratory scale bioreactor showing the changes in nitrogen (■), glucose (Δ), biomass dry weight (o), and concentration of PHB (◆). Growth of the culture was limited by 0.84 g L^{-1} ammonium sulfate.

The overall PHB accumulation rate was 0.128 g L^{-1} hr over 48 h, corresponding to an accumulation rate of 0.018 g PHB g biomass^{-1} hr^{-1} because little accumulation took place after 48 h. The minimum concentration of dissolved oxygen, 86.5%, occurred just prior to nitrogen limitation. The kinetics for both growth and PHB production for experiments B1 and B2 are very similar to those reported by Sonnleitner *et al* (1979) for *A. eutrophus* H 16 growing on lactate as a carbon source in a conventional stirred reactor, although only 15% (wt/wt) PHB based on dry weight was achieved during the growth phase, with a maximum accumulation of 78% (wt/wt) PHB.

4.1.3 Discussion

As expected, most of the production of PHB in a conventional batch culture using *A. eutrophus* DSM 545 occurs after the point of nitrogen limitation. There was an accumulation of 30% (wt/wt) PHB in biomass dry weight before the point of nitrogen limitation was reached which agrees with Ramsay *et al,* (1990). The maximum specific growth rate, μ, during the exponential growth phase prior to nutrient limitation was determined to be 0.21 hr^{-1} which would be equivalent to a 3.3 hour doubling time for the bacteria. This was below the critical μ_{max} which had previously been determined to be 0.35 hr^{-1} (Ramsay *et al,* 1990).

The final PHB level of 87% (wt/wt) of the biomass dry weight were substantially higher than those reported by P.A. Holmes (1985) and Sonnleitner *et al* (1979) of 80% (wt/wt) and 78% (wt/wt) respectively. It has been previously demonstrated that *A. eutrophus* is influenced by the concentration of dissolved oxygen for both controlling respiration rate during growth (Sonnleitner *et al,* 1979) and affecting the synthesis of PHB (Steinbüchel and Schleigle, 1989; Steinbuchel and Pieper, 1992). The uniform mixing and high levels of dissolved oxygen may account for the higher PHB content with respect to biomass dry weight. Another possible factor may be a result of using the pumped recirculation loop for achieving agitation which results in plug flow hydrodynamics, unlike the uniform mixing of a stirred tank (Sheppard *et al,* 1994b). The culture is continuously

cycled through variations in both the pressure and concentration of dissolved oxygen, with a frequency determined by the pumping rate and the total working volume. In the laboratory scale cyclone, the cycling frequency was about 7.5 min^{-1}. The cycling frequency, as well as the aeration, would have an effect on the on the magnitude of changes in the dissolved oxygen concentration. The higher the frequency, the closer the agitation will approach perfect mixing with correspondingly less change in the level of dissolved oxygen. Therefore, a possible explanation for the high PHB percentages is the extra stress imposed on the cells as a result of fluctuations in the oxygen supply. However, this hypothesis requires further investigation.

4.2 Pilot Scale Batch Culturing

4.2.1 Procedure

Three experiments were done using the 75 L reactor at different pump speeds and aeration in order to compare the results with the laboratory scale bioreactor. The sterilization procedure was more complex than with the pilot scale reactor since it was performed *in situ*. The sterilization consisted of 2 stages. The first stage was performed when the reactor was empty. Steam was sparged into the system at two points: in through the inlet filter and flow meters, and in the bottom of the cyclone through the air exhaust filter. After sparging for 20 mins, the steam supply was closed and the filters were cooled to ambient temperature with about 30 L min^{-1} of air. After completion of this stage, the air lines were isolated from the reactor by closing ball valves. The reactor was then filled with 70 L of water, the recirculating pump was started, the glucose powder was added and steam was supplied to the heat exchanger. The composition of the pump stator, Buna-N, limited the sterilization temperature to a maximum of 95°C. This temperature was maintained by regulating the rate of steam supplied to the heat exchanger. After 1 h the steam was replaced by cooling water, regulated by a YSI Model 2158 controller to maintain the fermentation temperature at 30±0.5°C. Before inoculation of the pilot scale reactor, the mineral salt solutions were sterilized as two separate 2 L fractions in an autoclave, cooled and then aseptically pumped into the reactor to obtain a working volume of 74 L. The inoculum was grown in the laboratory scale cyclone for 15 h and then aseptically pumped

into the large reactor to give a final volume of 75 L. Samples were taken at similar time intervals to the laboratory scale reactor, however the method for removing samples differed. The sampling system on the pilot scale reactor consisted of a sample pipe, perpendicular to the flow of the recirculation loop (Figure 4.3). The sampling took place by first opening the steam valve and the outer sample valve (2) to sterilize the outlet. The steam valve was then shut and the sample pipe was allowed to cool. The upstream sample valve was then opened to remove a sample and closed again. The steam was opened again and valve 2 and the steam valve were then closed.

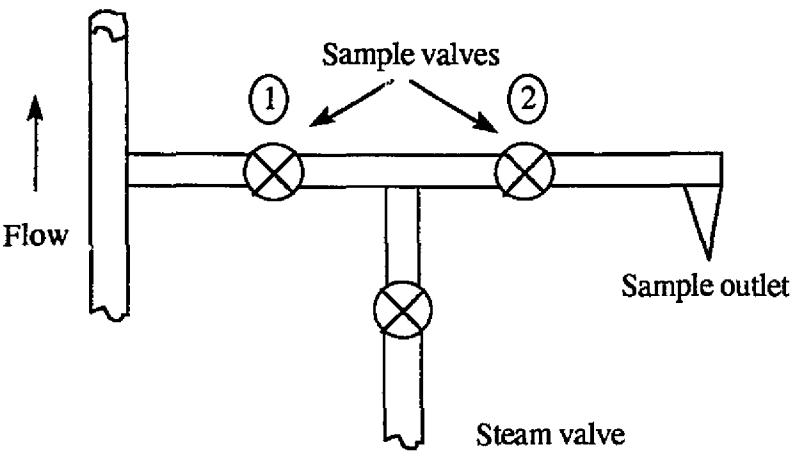

Figure 4.3 Sampling Valve Diagram

The sample pipe consisted of two ball valves with steam injection in between.

The three pilot scale experiments differed by the amount and place of aeration, pump speed and initial concentration of nitrogen in the growth medium. In the first and second experiments, the 0.28 g L^{-1} was used with a pump speed of 200 rpm and either 57 L min^{-1} of primary aeration or 107 L min^{-1} of secondary aeration. For the third experiment, approximately 0.84 g L^{-1} of ammonium sulfate was used. Since the increased nitrogen content increases the amount of growth, it increased the need for oxygen and hence a higher pump speed was used, but aeration was reduced to a total of 45 L min^{-1}, split between the primary and secondary injection points to ensure more uniform aeration.

4.2.2 Results

The results for the pilot scale experiments have also previously been published (Sheppard *et al*, 1994a) and are summarized and compared in Table 4.1. In the pilot scale experiments the growth kinetics closely resembled those obtained in the laboratory scale cyclone, with the nitrogen being exhausted between 15 and 20 h. Accumulation of PHB continued for an additional 40 to 45 h or until the time of glucose exhaustion. In the third experiment with the higher initial nitrogen concentration (Figure 4.4), the final biomass and PHB concentrations were 5.1 and 4.9 g L^{-1}. Although in the larger reactor the accumulation of PHB as a percentage of biomass remained very high, the yield of PHB based on total glucose consumed decreased to between 0.28 and 0.38 g PHB g $glucose^{-1}$, less than what was obtained in the laboratory scale cyclone.

Experiment	PHB Conc. (g / L)	PHB Yield (g / g glucose)	PHB Yield (% of Biomass)	Aeration* (vvm)	Power** (W / L)	Min. D.O. (% sat.)
P1	2.0	0.28	80	0.76/0.0	8	0
P2	2.0	-	89	0.0/1.4	8	74
P3	4.9	0.38	96	0.2/0.4	17	84

Table 4.1 Summary of PHB production in pilot scale reactor.

* Refers to both primary/secondary aeration and vvm is volume of air per volume of liquid per minute.

** Data refers to electrical power consumed for pumping.

The PHB accumulation rate was determined to be 0.10 g L^{-1} hr^{-1} at the end of the experiment which was similar to that of the batch growth in the lab scale cyclone. There was a PHB accumulation rate of 0.020 g PHB g $biomass^{-1}$ hr^{-1}, which was an improvement over batch growth in the lab-scale cyclone due to the higher concentration of PHB as a % of biomass dry weight.

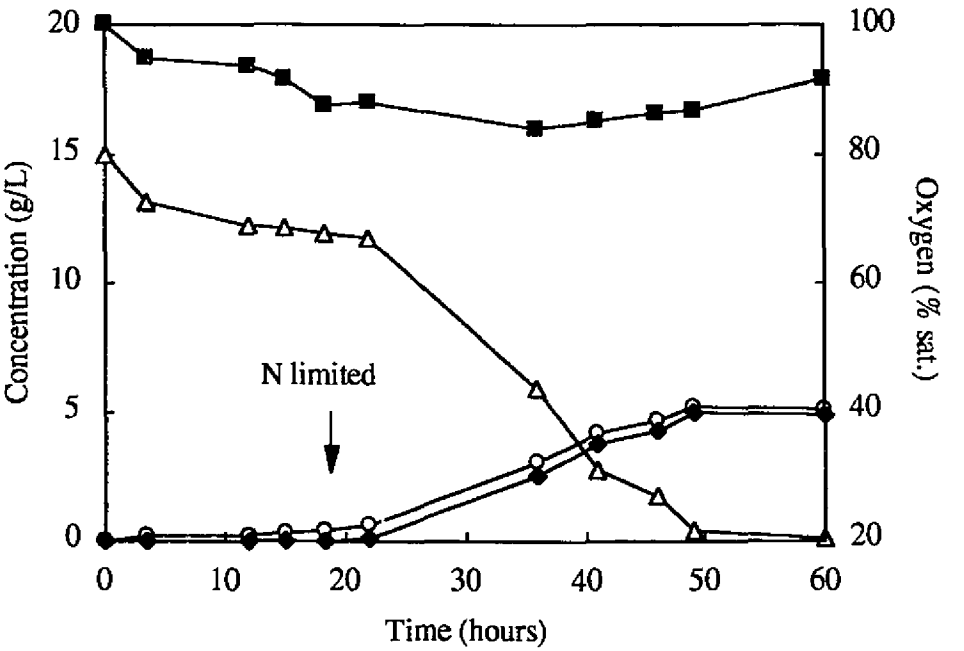

Figure 4.4 Batch growth (P3) in Pilot Scale Reactor

Accumulation of PHB while growing *A. eutrophus* DSM 545 in the pilot scale bioreactor with 15 L min^{-1} of primary aeration plus 30 L min^{-1} of secondary aeration and a pump speed of 350 rpm, showing the changes in the dissolved oxygen (■), glucose(Δ), biomass dry weight (o), and the concentration of PHB (◆). Growth of the culture was limited by 0.84 g L^{-1} of ammonium sulfate.

4.2.3 Discussion

The results have provided some preliminary information with respect to the scale-up criteria of the cyclone bioreactor. The lower height to diameter ratio of the 75 L bioreactor and the long recirculation loop had a significant effect on the concentration of dissolved oxygen that could be maintained without using secondary air injection. Adequate oxygen supply for high densities of bacteria could not be maintained with a pump speed of 200 rpm and injection of primary air alone. The concentration of oxygen in solution, however, was increased by increasing either the pump speed or secondary aeration at the pump discharge. Increasing the pump speed resulted in a lower retention time in the cyclone reactor and greater liquid velocity and turbulence in the recirculation loop, albeit with increased power consumption.

With a pump speed of 350 rpm and a recirculation rate of 118 L min^{-1}, the broth spends only about 20 s in the loop. This was adequate to maintain in excess of 84% saturation of dissolved oxygen with 5.1 g L^{-1} of biomass. It is apparent that the most important criterion for scale-up of PHB production in a cyclone bioreactor was the power input per unit volume. This power requirement will vary depending on the concentration of biomass and it will require further study to characterize this relationship.

As was the case with the laboratory scale bioreactor, there were high levels of PHB with respect to biomass dry weight ranging from 80 to 95% (wt/wt). The relationship between the cycling of the oxygen in the recirculation loop may be even more pronounced in this case since the cycling frequency of the broth in the pilot scale reactor varied between 1.6 min^{-1} and 1.2 min^{-1}. This would have an even greater effect on the magnitude of changes in the dissolved oxygen. Another possible explanation for the high PHB content comes from the gentle action of the progressive cavity pump which has no sharp edges and rotates much slower than conventional larger scale mixers used in stirred tanks which produce high shear forces which may rupture bacteria.

4.3 Fed-Batch Culturing

4.3.1 Procedure

The procedure for the fed-batch was essentially identical to that of the lab-scale batch experiment except that glucose was added periodically and the initial nitrogen concentration was increased to 3.36 g L^{-1} for increased biomass before the PHB accumulation phase. Glucose was added with a Masterflex peristaltic pump in quantities of 25 mL at a time. The subsequent additions of 50% (wt/vol) glucose were added when the glucose concentration fell below 2.5 g L^{-1}. Sterilization, production duration and sampling were all done the same way as in the lab-scale batch growth experiments.

4.3.2 Results

Shown in Figure 4.5 are the results from the fed-batch experiment. This technique differs from a conventional batch technique by the periodic addition of supplemental glucose at the times shown by the arrows in Figure 4.5. The biomass dry weight was increased by the higher initial nitrogen concentration to about 6 g L^{-1} before any significant PHB production began.

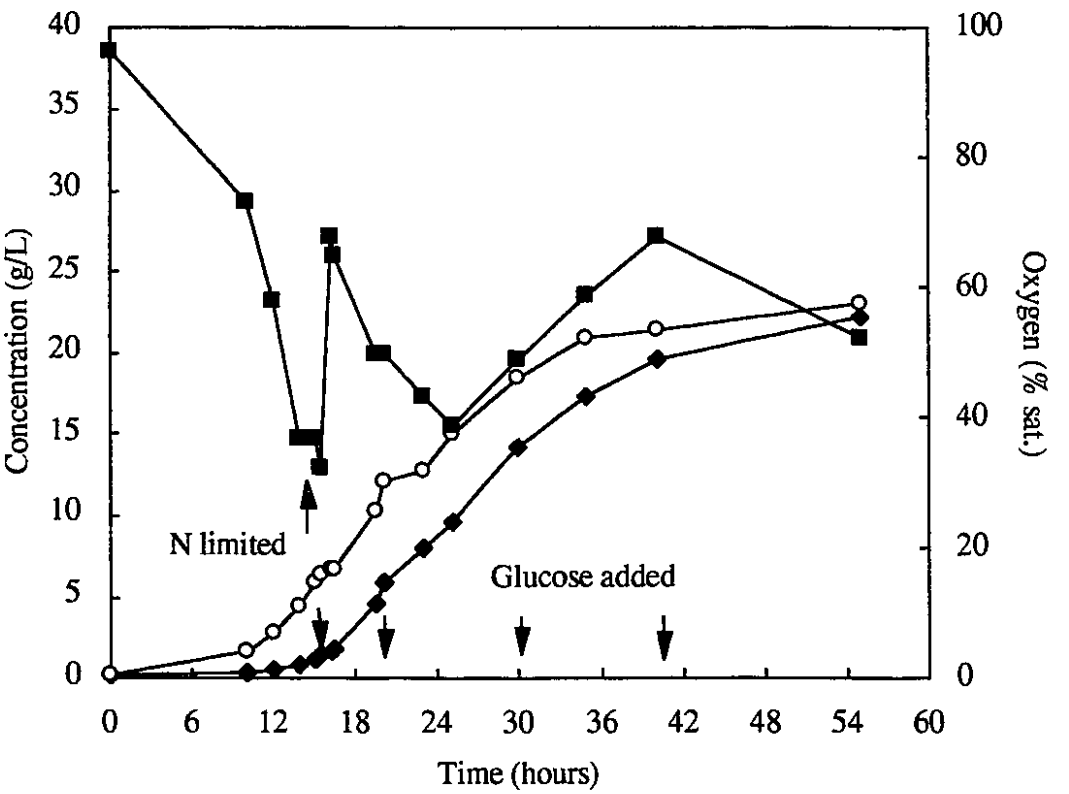

Figure 4.5 Fed-Batch Experiment (F1)

Production of PHB using *A. eutrophus* DSM 545 in the laboratory scale reactor with a fed-batch technique, showing changes in the dissolved oxygen (■), biomass dry weight (o), and the concentration of PHB (◆). Growth of the culture was limited by 3.36 g L^{-1} of ammonium sulfate.

The growth kinetics were similar to the previous laboratory and pilot scale batches with the nitrogen being exhausted after about 15.5 h, coinciding with the minimum concentration of dissolved oxygen, 32 % of saturation. The PHB concentration at the end of 54 h was 22.2 g L^{-1}, which was 96% (wt/wt) of the biomass dry weight. The overall yields of PHB and biomass were also very high, 0.41 and 0.43 g g of glucose^{-1}, respectively.

4.3.3 Discussion

The concentration of 22.2 g L^{-1} PHB attained was similar to fed-batch experiments performed by Ramsay *et al* (1990) except for the high yields of PHB with respect to glucose which were again near the theoretical maximum yield for PHB of 0.48 (Yamane, 1993). The PHB production rate was determined to be 0.41 g L^{-1} hr^{-1} at the end of the experiment (54 hrs) which was 4 times higher than the batch growth. It also had an accumulation rate of 0.018 g PHB g biomass^{-1} hr^{-1}. All the batch experiments had similar growth kinetics. The kinetics are, however, highly dependent on the inoculum since a long lag phase tends to retard cell division. This is why self cycling is such a useful experimental tool. It results in near identical, consecutive cycles which have little or no lag phase. This is due to the cycling of half the reactor volume, which leaves another half of the broth to inoculate fresh medium. Thus, each cycle is inoculated with the same fresh, viable, and highly concentrated inoculum eliminating the conditioning of the medium by the bacteria which causes long lag phases.

4.4 Self Cycling Fermentation

4.4.1 Procedure

Six experiments were carried out (SCF1, 2, 3...), three of which had no starvation period added imposed nitrogen depletion. The other three had starvation periods of 4, 6, and 8 h respectively. The medium chosen for the first two was identical, using the same nutrient concentrations used for batch growth except for a decreased amount of glucose (5 g L^{-1}). This amount was chosen since the cycle time would be short enough that there would be little PHB accumulation and thus no need for high concentrations of glucose.

The experiment SCF3 used an increased concentration of glucose (to 15 g L^{-1}) and nitrogen (to 1.68 g L^{-1}), thereby increasing PHB concentrations and achieving more

reproducible analysis from the gas chromatography. The final three experiments all had the same concentrations of nutrients as experiment SCF3. A decreased phosphate buffer concentration was used for all six experiments since, even with the 8 hour starvation period, there were not enough hydroxy acids to drastically affect the pH. The sterilization procedure was similar to that used for batch experiments; the cyclone reactor, side-arm, pump head, dissolved oxygen probe, dosing and harvesting solenoid valves, and air filters were all assembled and placed in an autoclave at 121°C for 30 min. The medium was autoclaved as described in Section 2.7. After the reactor was cooled, the data and control acquisition unit was attached to the solenoids, pump, D.O. sensor, and the program was started.

The user is prompted by the program with questions concerning the date and time of the experiment, the desired reactor volume when full, the cycle volume and the initial inoculum volume. The choice of imposing a cycle time or using the self cycling feedback loop controlled by the dissolved oxygen is then offered. If the feedback loop is chosen, the user is then prompted to choose a starvation period after nutrient limitation (zero if none is desired). The reactor is automatically filled to 900 mL and the user is prompted to add the 100 mL of inoculum. The pump is automatically started and the dissolved oxygen is monitored by the computer until the point of nitrogen exhaustion. At this point, either a timer is started equal to the starvation period or one half of the broth is harvested if the starvation period is set to zero.

4.4.2 Results

4.4.2.1 Minimal Starvation with Decreased Glucose

The results for all six experiments are summarized in Table 4.2. Figures 4.6 and 4.8 represent the PHB production by self-cycling fermentation using no starvation period after the point of nitrogen limitation. The experiments were concluded after 18 and 36 cycles respectively. The harvested PHB averaged 0.41 and 0.23 g L^{-1} respectively, producing a yield of 37.5 and 27.9 % (wt/wt) PHB with respect to biomass. The average residual glucose levels for each were 2.56 and 1.65 g L^{-1} respectively. Thus having a yield on glucose of 0.17 g PHB g $glucose^{-1}$ and 0.45 g biomass g $glucose^{-1}$ for SCF 1 and 0.07 g PHB and 0.25 g biomass g $glucose^{-1}$ for SCF 2.

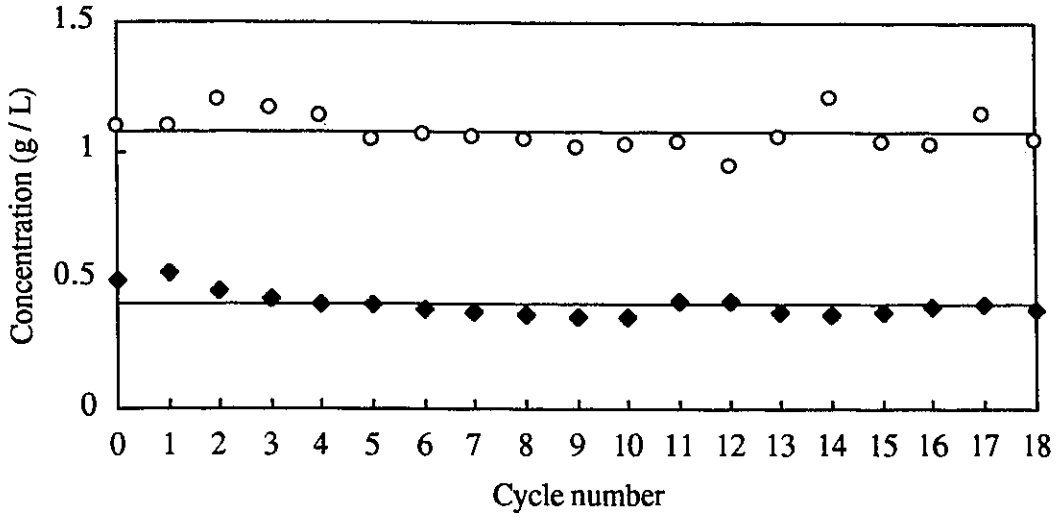

Figure 4.6 PHB and Biomass Concentrations from SCF1

Self cycling fermentation of *A. eutrophus* DSM 545 in the laboratory scale bioreactor showing the PHB (◆) and biomass dry weight (o) accumulation. With a straight line through the average. Growth of the culture was limited by 0.84 g L^{-1} ammonium sulfate. Starvation time was minimal.

Figures 4.6 and 4.8 show the cycle times and volume harvested for every cycle of SCF1 and SCF2. These values were calculated and printed by the program. The average cycle time for each were 157 and 148 min respectively. During SCF2 (Figure 4.8), the initial startup volume was inaccurately dosed due to an error in dead volume calculations and had to be corrected by placing a 50 g weight on the balance until the next cycle where the program corrected itself. Therefore, the average cycle time was calculated based on the stabilized section of cycles. The production rates for PHB in each experiment were 0.08 and 0.5 g L^{-1} hr^{-1} respectively. The production with respect to biomass was 0.072 and 0.053 g PHB g $biomass^{-1}$ hr^{-1} respectively. The specific growth rate, μ for each of the two experiments were 0.26 hr^{-1} and 0.28 hr^{-1} respectively, higher than the 0.15 hr^{-1} used in chemostat growth (Ramsay *et al,* 1990).

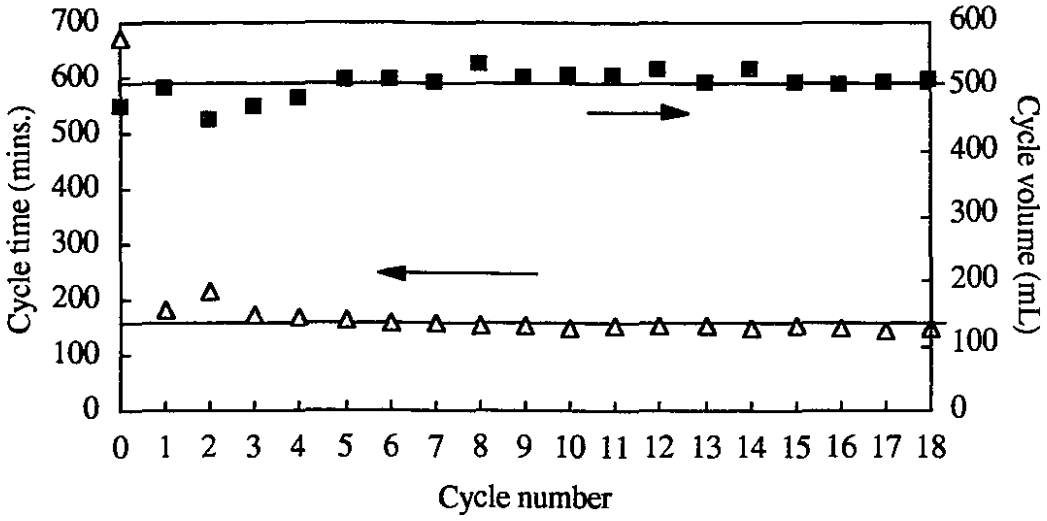

Figure 4.7 Volume and Cycle Time from SCF1

Self cycling fermentation of *A. eutrophus* DSM 545 in the laboratory scale bioreactor showing the cycle volume (■) and cycle time (Δ) with straight line through the average values. Starvation time was minimal.

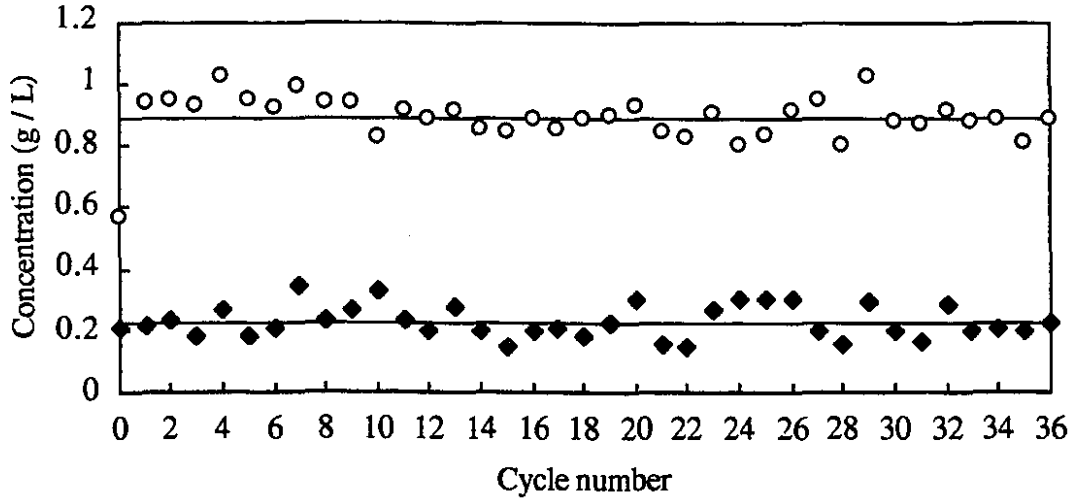

Figure 4.8 PHB and Biomass Concentrations from SCF2

Self cycling fermentation of *A. eutrophus* DSM 545 in the laboratory scale bioreactor showing the PHB (◆) and biomass dry weight (o) accumulation. With a straight line through the average. Growth of the culture was limited by 0.84 g L^{-1} ammonium sulfate. Starvation time was minimal.

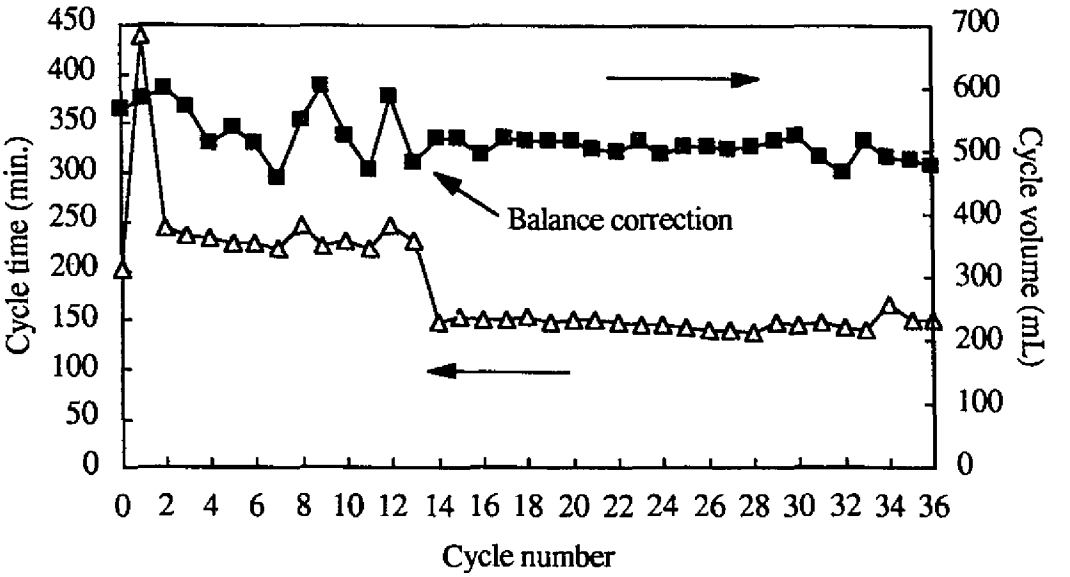

Figure 4.9 Volume and Cycle Time from SCF2

Self cycling fermentation of *A. eutrophus* DSM 545 in the laboratory scale bioreactor showing the cycle volume (■) and cycle time (Δ), with minimal starvation time.

4.4.2.2 Minimal Starvation with Increased Glucose and Nitrogen

Figure 4.10 shows the self-cycling production of PHB with *A. eutrophus* DSM 545 equal to an average concentration of 1.2 g L^{-1} and biomass dry weight equal to 3.0 g L^{-1}. The experiment ran for 26 cycles without contamination. The PHB content with respect to biomass was 40% (wt/wt) at a production rate of 0.47 g L^{-1} hr^{-1} with an accumulation of 0.15 g PHB g $biomass^{-1}$ hr^{-1}. The specific growth rate, μ, for SCF3 was determined to be 0.27 hr^{-1}.

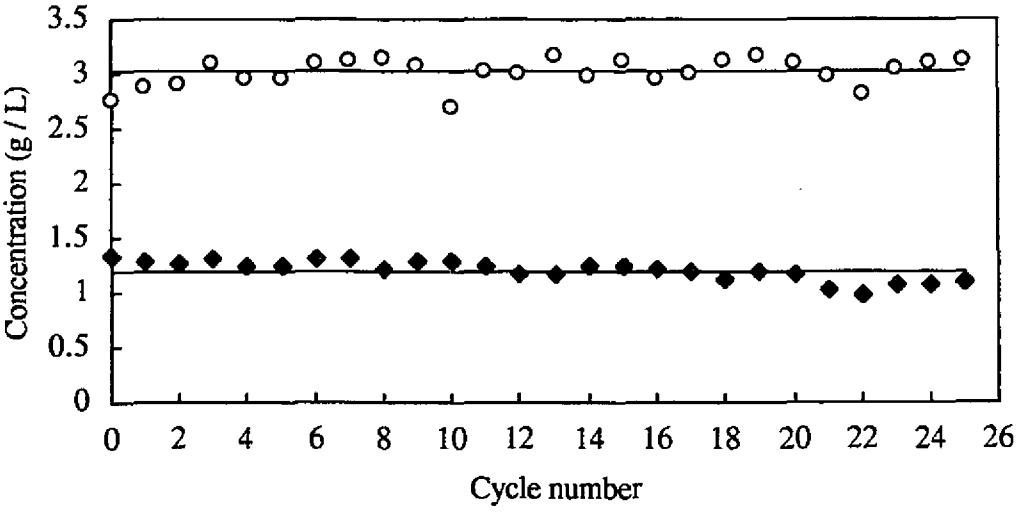

Figure 4.10 PHB and Biomass Concentrations from SCF3

Self cycling fermentation of *A. eutrophus* DSM 545 in the laboratory scale bioreactor showing the PHB (◆) and biomass dry weight (o) accumulation. With a straight line through the average. Growth of the culture was limited by 1.68 g L^{-1} ammonium sulfate. Starvation time was minimal.

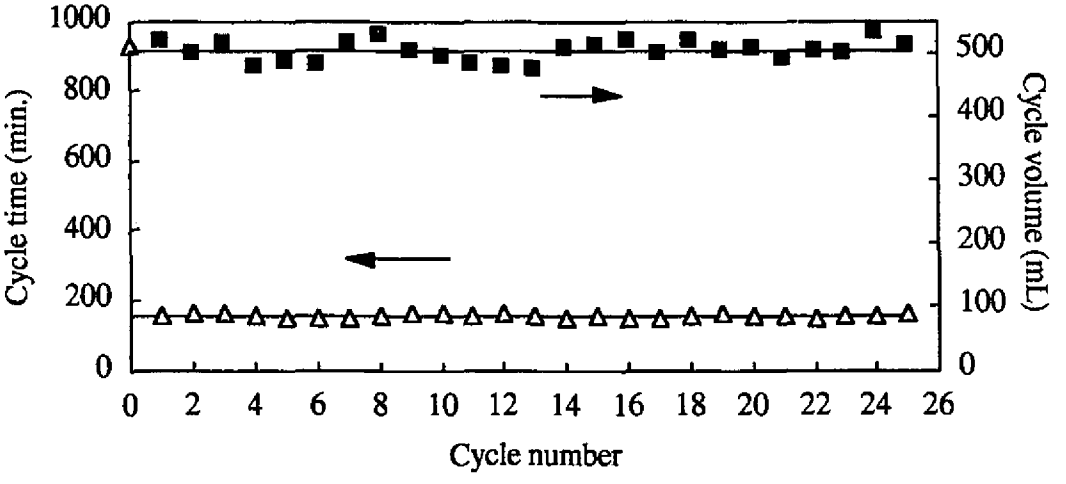

Figure 4.11 Volume and Cycle Time from SCF3

Self cycling fermentation of *A. eutrophus* DSM 545 in the laboratory scale bioreactor showing the cycle volume (■) and cycle time (Δ) with straight line through the average values. Starvation time was minimal.

4.4.2.3 Intercycle cell growth

During cycle 23 of the SCF3, samples were taken directly from the reactor every 20 min for analysis and plate-count in order to verify if any cell synchrony was occurring. The data presented in Figure 4.12 shows the cell numbers double midway through the cycle. As shown in Figure 4.12, the PHB and biomass concentrations double throughout the cycle. Since there was 3.45 g L^{-1} of glucose utilized during the cycle, the yield of PHB on glucose was equal to 0.18 g PHB g $glucose^{-1}$ and the yield of biomass on glucose was 0.44 g biomass g $glucose^{-1}$.

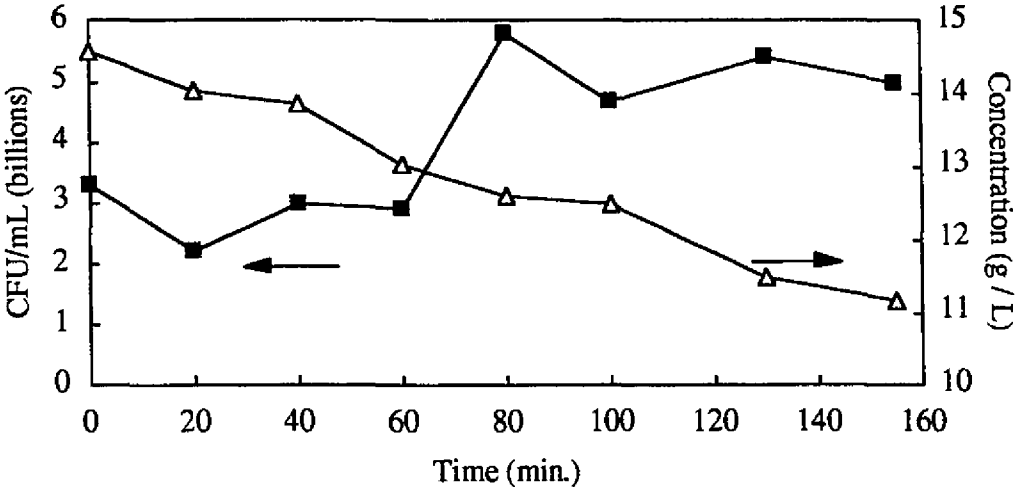

Figure 4.12 Inter Cycle Glucose Concentration and viable cell count from SCF3

Single cycle of self-cycling fermentation. Shown is the glucose (Δ) concentration and number of colony forming units mL^{-1} (x 10^9) (■) of *A. eutrophus* during the 23rd cycle with a limiting nitrogen concentration of 1.36 g L^{-1}.Starvation time was minimal.

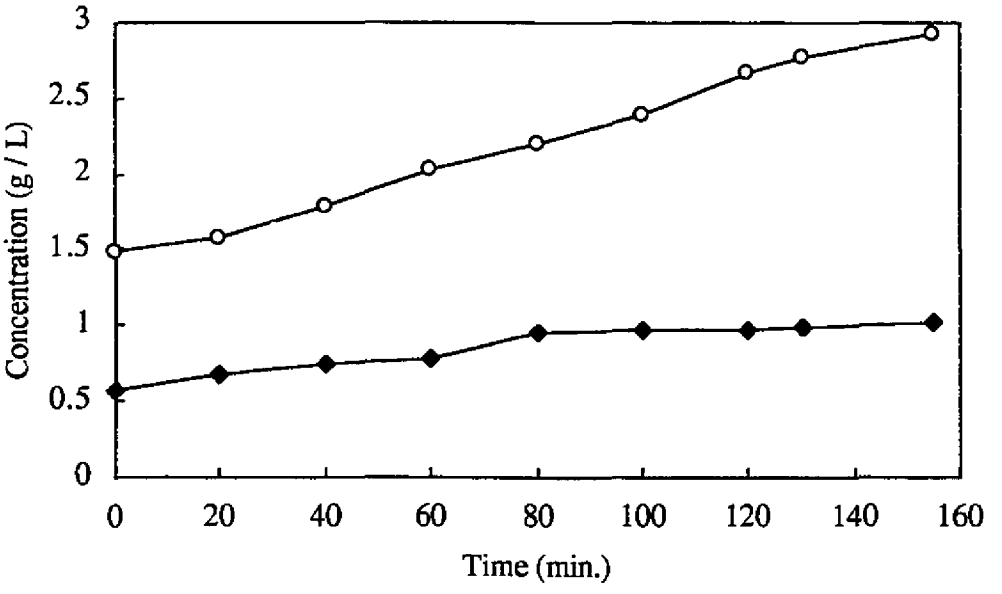

Figure 4.13 Inter Cycle PHB and Biomass Conc. from SCF3

Single cycle of self-cycling fermentation of *A. eutrophus* DSM 545. Shown are the PHB (◆) and biomass dry weight (o) concentration during the 23rd cycle with a limiting nitrogen concentration of 1.36 g L^{-1} with minimal starvation time.

Shown in Figure 4.12, the glucose is not completely utilized, its concentration is increased to 14.6 g/L at the beginning of every cycle. The PHB concentration (Figure 4.13) increases gradually throughout the cycle but not uniformly, after the point where the cells double, the slope of PHB increase decreases slightly, thus accumulating less PHB after the time of cell division.

4.4.2.4 Four Hour Starvation Period

The results for SCF4 are shown in Figure 4.14 where there was a PHB accumulation of 0.95 g L^{-1} and a final biomass concentration of 3.30 g L^{-1} resulting in a PHB content of 29% (wt/wt). SCF4 ran for 11 cycles before it became contaminated. The doubling time increased to an average of 167 min (Figure 4.15) which is equivalent to a

specific growth rate of 0.25 hr^{-1}. The PHB was produced at a rate of 0.14 g L^{-1} hr^{-1} and accumulated at a rate of 0.04 g PHB g $biomass^{-1}$ hr^{-1}.

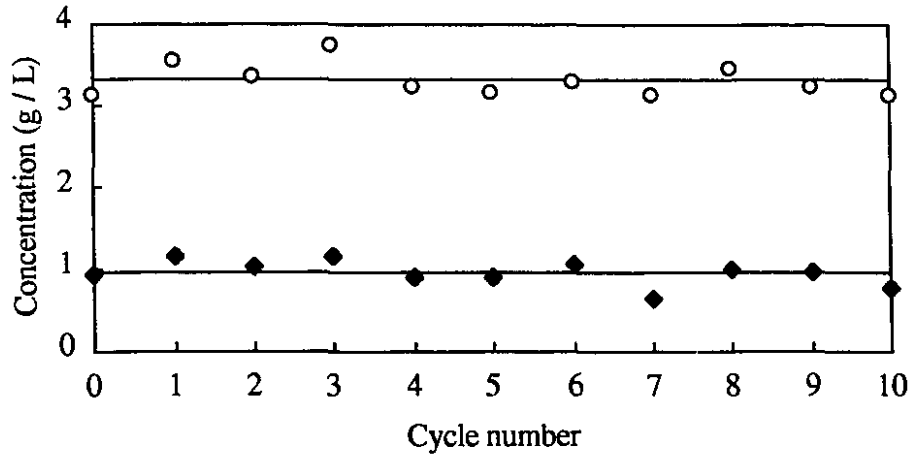

Figure 4.14 PHB and Biomass Concentrations from SCF4

Self cycling fermentation of *A. eutrophus* DSM 545 in the laboratory scale bioreactor showing the PHB (◆) and biomass dry weight (o) accumulation. With a straight line through the average. Growth of the culture was limited by 1.68 g L^{-1} ammonium sulfate. A four hour period of starvation was imposed after each cycle.

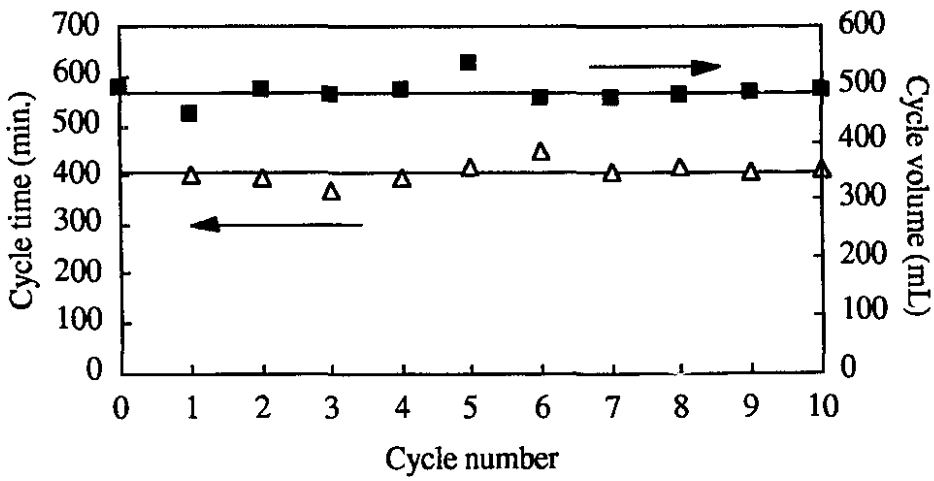

Figure 4.15 Volume and Cycle Time from SCF4

Self cycling fermentation of *A. eutrophus* DSM 545 in the laboratory scale bioreactor showing the cycle volume (■) and cycle time (Δ) with straight line through the average values. Note, the cycle time includes 240 min of imposed starvation, so the average doubling time is 167 min.

4.4.2.5 Six hour starvation period

SCF5, shown in Figure 4.16 accumulated 0.71 g L^{-1} PHB and had a final biomass concentration of 2.91 g L^{-1} resulting in a PHB content of 25% (wt/wt). SCF4 ran for 23 cycles before becoming contaminated. The doubling time was on average 176 min which is equivalent to a growth rate of 0.23 hr^{-1}. The PHB was produced at a rate of 0.08 g L^{-1} hr^{-1} and accumulated at a rate of 0.03 g PHB g $biomass^{-1}$ hr^{-1}.

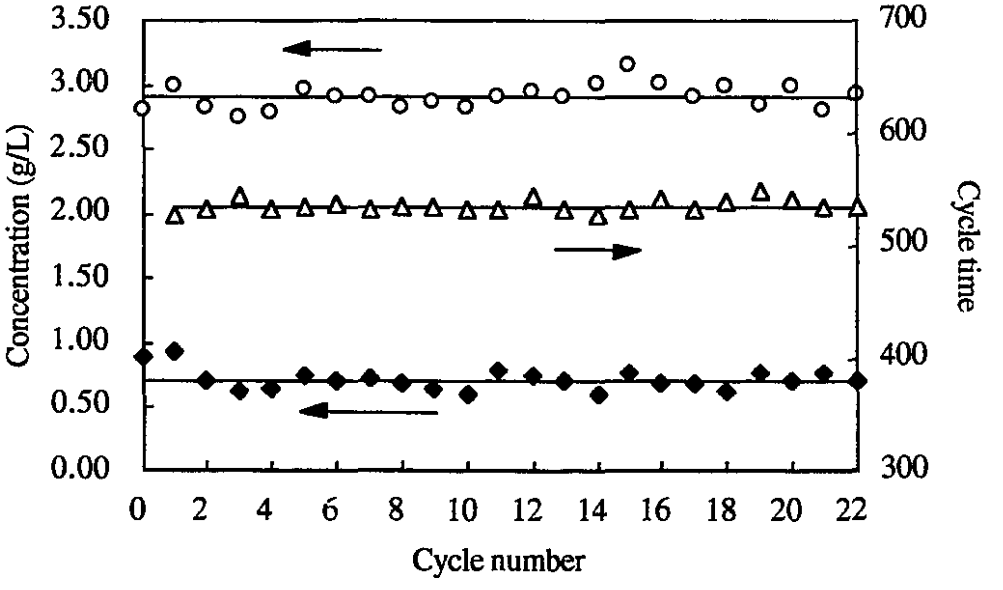

Figure 4.16 PHB and Biomass Concentration with Cycle Time for SCF5

Self cycling fermentation of *A. eutrophus* DSM 545 in the laboratory scale bioreactor showing the PHB (◆), biomass dry weight (o) and cycle time (Δ) with straight line through the average values. Note, the cycle time includes 360 min of imposed starvation, so the average doubling time is 176 min.

4.4.2.6 Eight hour starvation period

The results for SCF6 are shown in Figure 4.17 where there was a PHB accumulation of 2.72 g L^{-1} and a final biomass concentration of 4.60 g L^{-1} resulting in a PHB content of 59% (wt/wt) at the end of each cycle. SCF6 ran for 9 cycles before it

became contaminated. The doubling time of the *A. eutrophus* was 164 min which is equivalent to a growth rate of 0.25 hr^{-1}. The PHB was produced at a rate of 0.31 g L^{-1} hr^{-1} and accumulated at a rate of 0.07 g PHB g $biomass^{-1}$ hr^{-1}.

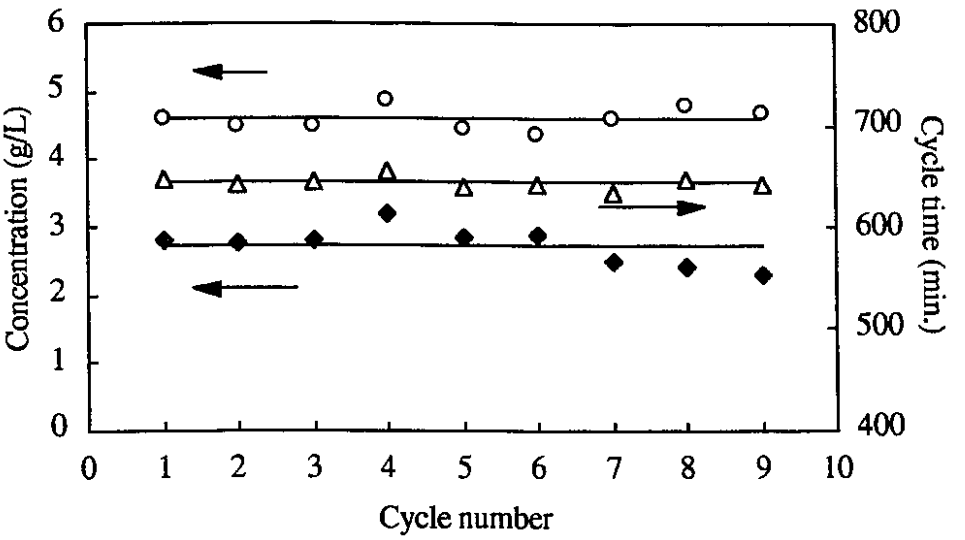

Figure 4.17 PHB and Biomass Concentration with Cycle Time for SCF6

Self cycling fermentation of *A. eutrophus* DSM 545 in the laboratory scale bioreactor showing the PHB (◆), biomass dry weight (o) and cycle time (Δ) with straight line through the average values. Note, the cycle time includes 480 min of imposed starvation, so the average doubling time is 164 min.

Exp. #	Nmbr. of Cycles	PHB conc. g/L	PHB st. dev. g/L	Biomass conc. g/L	Biomass st. dev. g/L	%PHB in Bio.	Prod. rate* g/g·hr	Prod. rate* g/L·hr	Doubling time mins.	Starv. period mins.
SCF1	19	0.41	0.05	1.08	0.07	38	0.072	0.08	157	-
SCF2	37	0.23	0.05	0.89	0.08	26	0.053	0.05	148	-
SCF3	26	1.20	0.09	3.02	0.13	40	0.077	0.23	155	-
SCF4	11	0.95	0.16	3.30	0.20	29	0.021	0.07	167	240
SCF5	23	0.71	0.08	2.91	0.09	25	0.014	0.04	176	360
SCF6	9	2.72	0.28	4.60	0.17	59	0.028	0.13	164	480

Table 4.2 SCF Experiment Results

* The rate of PHB production is in units of g PHB g $biomass^{-1}$ hr^{-1} and of g PHB L^{-1} hr^{-1}.

4.4.3 Discussion

The SCF results had a high degree of reproducibility with respect to consecutive cycles, the three SCF experiments which had no starvation period had a PHB content of between 26 and 40% (wt/wt) based on biomass (Table 4.2). The difference between SCF2 and SCF1 and SCF3 is probably a result of inacurate control of cycle volume. It seems also that because growth rate was faster the medium was probably somewhat different. Figure 4.7 shows that the first cycle of SCF1 is a batch cycle of 14 h and the subsequent cycles are equal to the doubling time of *A. eutrophus*. The cycle times did stabilize immediately and the averages are based on the cycle times subsequent to the first cycle. In Figure 4.8 however, there was a temperature fluctuation in the initial cycle which affected the dissolved oxygen concentration and caused the computer to cycle the reactor prematurely. The subsequent cycle was extended automatically to fully utilize the excess nitrogen in the reactor left over from the first cycle. It may be that the initial glucose concentration was lower as a result of mixing the medium before it had a chance to cool properly. This could cause the formation of a precipitate and reduced the availability of glucose. This theory cannot be verified since the samples were taken automatically at the end of the cycle and no data on initial concentrations are available.

The effects of starvation on PHB accumulation were noticed only when starvation periods of 8 h were used. The growth rate (doubling time) remained constant throughout the experiments and the overall production rate decreased as cycle times increased. The SCF3 experiment which had double the nitrogen as the medium for batch growth (B1-2) produced double the amount of PHB / g of biomass. The rate of PHB accumulation of 0.23 g L^{-1} hr^{-1} was also much higher than 0.03 g L^{-1} hr^{-1} after nitrogen limitation (14 hrs) or compared to the 0.13 g L^{-1} hr^{-1} at the end of the batch (B1). This rate was similar to that obtained in chemostat growth by Ramsay, *et al* (1990) which had a rate of 0.225 g L^{-1} hr^{-1}. The accumulation rate of PHB of 0.077 g PHB g biomass^{-1} hr^{-1} during SCF3 was also higher than the batch by a factor of six at the point of nitrogen limitation and a factor of 4.3

at the end of the 48 h . This was because the biomass and PHB were harvested every 2.7 h (i.e. each cycle) instead of after 14 h of batch growth or after the 34 h of accumulation at the end of the batch. The accumulation rate of PHB during SCF3 was also 1.5 times greater than that of the chemostat accumulation rate of 0.05 g PHB g biomass^{-1} hr^{-1} (Ramsay *et al*, 1990).

Although experiments SCF4 and SCF5 produced significant amounts of PHB they were not equivalent to the amounts produced after an equivalent amount of starvation period during the batch growth (B1). The batch cycle (B1), after 18 h produced 1.72 g PHB L^{-1} hr^{-1} with a PHB content of 68% (wt/wt) of biomass which was 1.8 times higher than the amount of PHB accumulated after a 4 hour starvation (Table 4.2). The rate of PHB accumulation in SCF4 decreased compared to SCF3 because the PHB content remained the same while the cycle time increased. This also affected the rate during the longer cycle times of SCF5 and SCF6. It appears that forcing the cells to start reproducing again after accumulating PHB may affect the subsequent cycles by slowing the growth and also inhibiting the accumulation of PHB early in the cycle. Experiment SCF6, however, did show an increase in PHB production compared to SCF3, similar to PHB accumulation during batch growth after 22 h, where the rate of PHB accumulated in B1 (obtained through extrapolation) was 2.77 g L^{-1} hr^{-1}. Perhaps with longer imposed periods of starvation PHB accumulation is less inhibited by the effect of cycling. However, as cycle times increase the accumulation rate decreases. In SCF6 where the PHB accumulation was the highest at 59 % (wt/wt) of biomass; the production rate was half of that of the SCF3 experiment with no starvation period.

Although the data presented concludes that shorter starvation periods are more productive, it is important to remember that product recovery costs must be factored into production costs. In order to reduce recovery costs, higher concentrations of PHB must be accumulated, at the expense of greater productivity. Further study could use this method to study the control of PHB molecular weight, the utilization of stored PHB, or cell division in the presence of high concentrations of PHB.

CHAPTER V CONCLUSIONS

Several methods of microbial cell culture were used to produce poly-3-hydroxybutyrate (PHB) in a cyclone bioreactor using *Alcaligenes eutrophus* DSM 545. The batch (lab and pilot scale), fed-batch and self-cycling fermentation (SCF) procedures were used to determine the utility of the cyclone bioreactor and SCF technique for PHB production. The following conclusions have been drawn based upon the objectives set forth in this study:

1) PHB was grown reproducibly in a 1-liter lab-scale cyclone bioreactor using a batch culture of *A. eutrophus* DSM 545. Generally PHB concentration increases significantly during the final generations of cells, achieving an overall production rate of 0.13 g PHB g biomass^{-1} hr^{-1} with 6.2 g L^{-1} of PHB.

2) A scale-up of the bioreactor to 75 L produced results with similar growth kinetics to the batch growth when producing PHB. The PHB concentrations obtained in the pilot and lab-scale batches were also similar with the same medium and power input for recirculation.

3) The fed-batch growth of *A. eutrophus* in a 1 - liter cyclone bioreactor was attained with similar growth kinetics to and higher PHB concentrations than previously published work, achieving an overall production rate of 0.018 g PHB g biomass^{-1} hr^{-1} with 22.2 g L^{-1} of PHB.

4) Self-cycling fermentation of *A. eutrophus* DSM 545 produced PHB while still retaining the ability for cells to divide during subsequent cycles. Inter-cycle data shows how the PHB and biomass are accumulated during cell growth and the point at which the cells divide. The production rate is optimal when the reactor is cycled just after nitrogen limitation and decreases when higher starvation periods are chosen even though higher PHB concentrations are obtained. The maximum overall production rate achieved was 0.077 g PHB g biomass^{-1} hr^{-1} with 1.2 g L^{-1}.

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CHAPTER VII APPENDIX

Computer Program for SCF

```
10 REM ***PHASING CONTROL PROGRAM MAC 17*** MAY 1995
20 REM ***SINGLE NUTRIENT SUPPLY. 3 SAMPLE VALVES***
25 REM ***TEMPERATURE COMPENSATED. SETUP DUMMY PROOF***
27 REM ***SETUP FOR HP 87XM WITH RELAY VALVES***
30 DISP "ENTER FIRST CYCLE NUMBER. 0 FOR BATCH"
40 INPUT CCOUNT
50 SVALVE=0
60 MINOXY=2
70 PRINTER IS 701
80 DISP "ENTER REACTOR VOLUME IN ML"
90 INPUT RVOL
100 DISP "ENTER INOCULUM VOLUME IN ML"
110 INPUT IVOL
120 DISP "ENTER CYCLE VOLUME IN ML"
130 INPUT CVOL
132 DISP "ENTER DENSITY OF MEDIUM. 1.0 FOR WATER"
134 INPUT DENSE
140 DISP "FOR AUTO CYCLING ENTER 1. IMPOSED CYCLING ENTER 0"
150 INPUT ACYCLE
160 IF ACYCLE=1 THEN GOTO 200
170 DISP "ENTER IMPOSED CYCLE PERIOD IN MINUTES"
180 INPUT CPERIOD
190 GOTO 230
200 DISP "ENTER STARVATION PERIOD IN MINUTES"
210 INPUT SPERIOD
220 SSPERIOD=SPERIOD*60000+1
230 DISP "ENTER TIME USING FORMAT: HRS, MINUTES SINCE MIDNIGHT"
240 INPUT HRS,MINS
250 DISP "ENTER DATE USING FORMAT: DAY, MONTH, YEAR"
260 INPUT DAY,MNTH,YR
270 DISP "ENTER EXPERIMENT NUMBER"
280 INPUT ENUM
290 PRINT USING "10A,3D,18A,4D,19A,4D,3A" ; "EXPT. NO. ".ENUM,"; REACTOR VOLUME:
 ".RVOL," ML; CYCLE VOLUME: ",CVOL," ML"
300 PRINT USING "17A,DD,A,DD,4A,DD,A,DD,A,DD" ; "EXPT. STARTED AT ".HRS,":",MINS
," ON ",DAY,"/".MNTH."/",YR
310 ON KEY# 2 GOTO 890
320 DISP "IF STARTUP, ENTER 99"
330 INPUT STARTUP
340 IF STARTUP=99 THEN GOTO 410
350 DISP "INPUT INITIAL WT IN VOLTS"
360 INPUT RWTI
370 DISP "INPUT REFERENCE TEMP IN DEG C"
380 INPUT RTEMP
385 RWT=RWTI*(1-(RTEMP-32.2)*.0039) !       *CORRECT FOR TEMPERATURE
395 BALVDC=.000038865*DENSE
400 GOTO 620
410 OUTPUT 709 :"DCV2" !                    *MEASURE INITIAL WEIGHT OF REACTOR
420 ENTER 709 ; RWTI
430 OUTPUT 709 ;"TWO3" !                    *READ THERMISTOR RESISTANCE
440 ENTER 709 ; THERM
450 TEMP=.003123+.000307*LOG (THERM/1000)-.000024*LOG (THERM/1000)^3
460 RTEMP=1/TEMP-273 !                      *CALCULATE REFERENCE TEMPERATURE
462 RWT=RWTI*(1-(RTEMP-32.2)*.0039) !       *CORRECT FOR TEMPERATURE
470 BALVDC=.000038865*DENSE !                   *CONVERSION FACTOR VOLTS/ML
480 PRINT USING "13A,D.5D,10A,2D.1D,6A" ; "REACTOR WT = ".RWTI," VOLTS AT ",RTEM
P," DEG C"
490 FWT=RWT+(RVOL-IVOL-140)*BALVDC
500 OUTPUT 709 :"CLS11" !                   *OPEN MEDIUM CONTROL VALVE
502 WAIT 30000 !                            *WAIT 30 SECONDS
```

```
504 OUTPUT 709 :"OPN11" !                    *STOP DOSING
506 OUTPUT 709 :"CLS10" !                    *START PUMP
508 WAIT 10000 !                             *WAIT 10 SECONDS
510 OUTPUT 709 :"OPN10" !                    *STOP PUMP
512 OUTPUT 709 :"CLS11" !                    *CONTINUE DOSING
520 OUTPUT 709 :"DCV2" !                     *MONITOR REACTOR WEIGHT GAIN
530 ENTER 709 ; IWTI
535 IWT=IWTI*(1-(RTEMP-32.2)*.0039)
540 EWT=FWT-IWT-9*BALVDC
550 IF EWT<= 0 THEN GOTO 570
560 GOTO 520 !                               *CONTINUE MONITORING LOOP
570 OUTPUT 709 :"OPN11" !                    *CLOSE MEDIUM CONTROL VALVE
580 DISP "REACTOR FILLED, ADD INOCULUM"
590 DISP "PRESS CONTINUE KEY WHEN FINISHED"
600 PAUSE
610 OUTPUT 709 :"CLS10" !                    *TURN ON PUMP AND TEMP CONTROL
620 SETTIME 0.1
630 IF ACYCLE=1 THEN GOTO 670
640 SPERIOD=CPERIOD*60000
650 ON TIMER# 1.SPERIOD GOTO 890 !           *DELAY CYCLING FOR SELECTED PERIOD
660 GOTO 680
670 ON TIMER# 1.1800000 GOTO 690 !           *DELAY O2 SAMPLING FOR 1/2 HOUR
680 GOTO 680
690 OFF TIMER# 1
700 MINOXY=2 !                               *INITIALIZE O2 VALUE
710 SCOUNT=0 !                               *SET COUNTER TO ZERO
720 OXY1A=0 !                                *SET O2 CONC TO ZERO
730 SCOUNT=SCOUNT+1 !                        *ADVANCE COUNTER
740 OUTPUT 709 :"DCV04" !                    *READ O2 CONCENTRATION
750 ENTER 709 ; OXY1
755 OXY1=-OXY1 !                            *DUMMY PROOF DO CONNECTION
760 OXY1A=OXY1A+OXY1 !                       *SUM O2 READINGS
770 IF SCOUNT=100 THEN GOTO 790 !            *CHECK COUNTER
780 GOTO 730 !                               *CONTINUE LOOP IF < 100
790 OXY1=OXY1A/SCOUNT !                      *CALCULATE AVERAGE O2 VALUE
800 IF OXY1<MINOXY THEN GOTO 840 !           *CHECK IF MIN O2 REACHED
810 DELTAOXY=OXY1-MINOXY !                   *CALCULATE CHANGE IN O2
820 IF DELTAOXY>.006 THEN GOTO 860
830 GOTO 710 !                               *CONTINUE O2 LOOP
840 MINOXY=OXY1
850 GOTO 710
860 ON TIMER# 2.SSPERIOD GOTO 880 !          *START STARVATION PERIOD
870 GOTO 870
880 OFF TIMER# 2
890 MTIME=TIME /60 !                         *CALCULATE CYCLE TIME
892 OUTPUT 709 :"DCV2" !                      *MEASURE TOTAL WEIGHT
894 ENTER 709 ; PUMP
900 OUTPUT 709 :"OPN10" !                    *TURN OFF PUMP
910 WAIT 60000 !                             *WAIT 60 SECONDS
920 OUTPUT 709 :"DCV2" !                     *MEASURE TOTAL WEIGHT
930 ENTER 709 ; PHWT
932 OUTPUT 709 :"CLS10" !                    *TURN ON PUMP
940 OUTPUT 709 :"TWO3" !                     *MEASURE THERMISTOR RESISTANCE
950 ENTER 709 ; THERM
960 TEMP=.003123+.000307*LOG (THERM/1000)-.000024*LOG (THERM/1000)^3
970 ITEMP=1/TEMP-273 !                       *CALCULATE INDICATED TEMPERATURE
975 PHWTC=PHWT*(1-(ITEMP-32.2)*.0039) !      *CORRECT FOR TEMPERATURE
976 PUMPC=PUMP*(1-(ITEMP-32.2)*.0039) !      *CORRECT FOR TEMPERATURE
978 CORR=(.0068-PHWTC+PUMPC)/BALVDC !        *CORRECT FOR FOAMING
979 FOAM=CORR*.29 !                          *CALCULATE FOAM PRODUCTION
```

```
980 OUTPUT 709 :"CLS15"
990 SVALVE=SVALVE+1 !                          *INCREMENT SAMPLE VALVE NO.
1000 IF SVALVE<4 THEN GOTO 1020
1010 SVALVE=1 !                                 *RESET SAMPLE VALVE TO NO. 1
1020 IF SVALVE>1 THEN GOTO 1050
1030 OUTPUT 709 :"CLS12" !                      *OPEN SAMPLE VALVE NO. 1
1040 GOTO 1090
1050 IF SVALVE=3 THEN GOTO 1080
1060 OUTPUT 709 :"CLS13" !                      *OPEN SAMPLE VALVE NO. 2
1070 GOTO 1090
1080 OUTPUT 709 :"CLS14" !                      *OPEN SAMPLE VALVE NO. 3
1090 OUTPUT 709 :"DCV2" !                       *MEASURE TOTAL WEIGHT
1100 ENTER 709 ; NWT
1110 NWTC=NWT*(1-(ITEMP-32.2)*.0039)
1135 HWTC=RWT+(RVOL-CVOL-295+CORR)*BALVDC ! *CALCULATE POST-HARVEST WT
1140 EWT=NWTC-HWTC-5*BALVDC
1150 IF EWT<= 0 THEN GOTO 1170
1160 GOTO 1090 !                                *CONTINUE HARVESTING
1170 OUTPUT 709 :"OPN15"
1180 WAIT 15000
1190 OUTPUT 709 :"OPN12" !                      *CLOSE SAMPLE VALVE NO. 1
1200 OUTPUT 709 :"OPN13" !                      *CLOSE SAMPLE VALVE NO. 2
1210 OUTPUT 709 :"OPN14" !                      *CLOSE SAMPLE VALVE NO. 3
1220 WAIT 10000 !                               *WAIT 10 SECONDS
1230 OUTPUT 709 :"DCV2" !                       *MEASURE FINAL POST HARVEST WT.
1240 ENTER 709 ; PWT
1245 PWTC=PWT*(1-(ITEMP-32.2)*.0039)
1250 HVOL=(PUMPC-PWTC)/BALVDC !                 *CALCULATE HARVESTED VOLUME
1252 OUTPUT 709 :"OPN10" !                       *TURN OFF PUMP
1260 OUTPUT 709 :"DCV2" !                       *MEASURE TOTAL WEIGHT
1265 ENTER 709 ; PDWT
1270 OUTPUT 709 :"CLS11" !                      *OPEN MEDIUM DOSING VALVE
1275 OUTPUT 709 :"DCV2"
1280 ENTER 709 ; PDWT
1285 PDWTC=PDWT*(1-(ITEMP-32.2)*.0039)
1290 EWT=RWT-PDWTC+(RVOL-140)*BALVDC
1300 IF EWT<= 0 THEN GOTO 1320
1310 GOTO 1270 !                                *CONTINUE DOSING
1320 OUTPUT 709 :"OPN11" !                      *CLOSE MEDIUM DOSING VALVE
1340 OUTPUT 709 :"DCV2" !                       *MEASURE FINAL WEIGHT
1350 ENTER 709 ; PDWT
1355 PDWTC=PDWT*(1-(ITEMP-32.2)*.0039)
1360 DVOL=(PDWTC-PWTC)/BALVDC !                 *CALCULATE DOSED VOLUME
1370 FINAL=(PHWTC-RWT)/BALVDC+100 !             *CALCULATE FINAL VOL BEFORE HARVEST
1380 OUTPUT 709 :"CLS10" !                      *RESTART PUMP AND TEMP CONTROL
1390 SETTIME 0.1 !                              *RESET CYCLE TIMER
1400 PRINT USING "6A.3D.2A.4D.5A.3D.20A.4D.3D.9A.3D.D.A" ; "CYCLE ".CCOUNT."; ".
MTIME." MIN ".HVOL." HARVEST: FINAL VOL ".FINAL.FOAM." ML FOAM ".ITEMP."C"
1410 CCOUNT=CCOUNT+1 !                          *ADVANCE CYCLE COUNTER
1420 GOTO 630 !                                 *START A NEW CYCLE LOOP
1430 END
```