Self Cycling Fermentation (SCF) in a stirred tank

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July, 1992.

A thesis submitted to the
Faculty of Graduate Studies and Research
in partial fulfilment of the requirements for the
degree of Master of Engineering

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ABSTRACT

Self cycling fermentations (SCF's) were conducted in an apparatus based on a commercial model two litre stirred tank fermenter. An automated control system and peripheral vessels were used to perform cycling control, medium addition, broth removal and sampling.

The apparatus was used to grow Bacillus subtilis and Acinetobacter calcoaceticus on different media. Both organisms supported a steady state SCF. Measurements of biomass, cycle time and minimum dissolved oxygen all gave repeatable results. Results were comparable to literature values for a cyclone column SCF apparatus.

A gas analyzer was installed to measure oxygen and carbon dioxide concentrations in the effluent gas. The response of the gas analyzer paralleled that of the dissolved oxygen measurements.

RESUME

La fermentation auto-cyclique (FAC) a été appliquée dans un appareil basé sur un fermenteur commercial à deux litres. Un système de contrôle automatisé et des vesseaux additionels ont été utilisés pour faire fonctionner le contrôle des cycles, l'addition des substrats et la prise des échantillons.

L'appareil était utilisé pour cultiver Bacillus subtilis et Acinetobacter calcoaceticus sur des media différents. Les deux organismes ont réussit à obtenir un état stable en FAC. Les mesures de biomasse, la durée de cycle, et le niveau minimum d'oxygène dissout ont tous donne des resultats reproduisables. Les resultats étaient comparables a ceux donnés dans la litérature pour les apareils de FAC de type cyclone.

Un analyseur de gas a été installé pour mesurer les concentrations d'oxygène et de bioxide de carbone dans les effluents gaseux. Les mesures de l'analyseur était paralleles à ceux de l'analyseur d'oxygène dissout.

ACKNOWLEDGMENTS

This work was made possible through grants from the Natural Sciences and Engineering Research Council of Canada (NSERC) and Martinex.

I give my sincere thanks to Dr. D.G. Cooper. I benefitted greatly from his wise and knowledgable direction, and I appreciated his personal commitment to myself and the rest of the Falcon Research Group.

Falcon research receives my thanks for their helpful input and diplomatic camaraderie. A special thanks to my kindred SCF researchers Bill and Bassam for all their help.

Finally, I give my thanks to Kim for her patience and encouragement.

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INTRODUCTION:

1.1 Self cycling fermentation:

A self cycling fermentation (SCF) is a continuously cycling semi-batch fermentation. The cycling of the system is achieved through feedback control determined by the culture's metabolic rate. With this control, the SCF will cycle at a frequency such that the duration of a cycle is equal to the doubling time of the organism (Sheppard, Cooper,1990a; Brown, Cooper, 1991). The cycling of an SCF results in an appreciable degree of synchronous growth of the cells (Brown, Cooper, 1991). Because the cycle timing is determined by the metabolism of the growing culture, the fermentation is said to be self cycling.

The rate of metabolism of the culture is monitored continuously during the experiment. To date, the method used to determine metabolic rate in SCF's has been to measure the dissolved oxygen (DO) (Sheppard, Cooper, 1990a,b,c; Brown, Cooper, 1991). For an aerobic organism, a high DO level in the fermenter indicates that little oxygen in solution is being used for respiration by the organism. Thus the metabolic rate is low. Conversely, low DO indicates vigorous respiration and a high metabolic rate.

When the metabolic rate is observed to slow down--usually as a response to nutrient limitation--half the operating volume in the fermenter is removed. This is referred to as harvesting the fermenter. Half the biomass is removed with the harvest. The

remaining half of the biomass, held in the remaining volume, is replenished with fresh growth medium. The fresh medium brings the volume in the fermenter back up to the original operating volume. The cells in the fermenter then grow and multiply until the nutrient limitation is reached again and the cycle repeats. The cycle patterns for the DO, nutrients and biomass concentrations are shown in figure 1.1.

After a few cycles the biomass concentration in the SCF will reach a cyclic steady state (Sheppard, Cooper, 1990a; Brown, Cooper, 1991). This is to say that sequential harvests will have the same amount of biomass. The actual biomass in the fermenter is oscillating, since with every harvest it is cut in half. Between harvests the organism grows on the freshly supplied medium back to its pre-harvest level. The biomass concentration achieved in an SCF depends on the nutrient concentrations in the medium. The steady state biomass concentration is such that after each harvest, the incoming fresh medium will have sufficient nutrients to allow each cell to reproduce itself once before the nutrients again become exhausted (Sheppard, Cooper, 1990a). Thus at steady state, the duration of the cycle is roughly equivalent to the doubling time of the organism. Also, an organism growing in an SCF at steady state will tend to synchronize growth. This is to say that the doubling of all the cells occurs at approximately the same moment in every cycle.

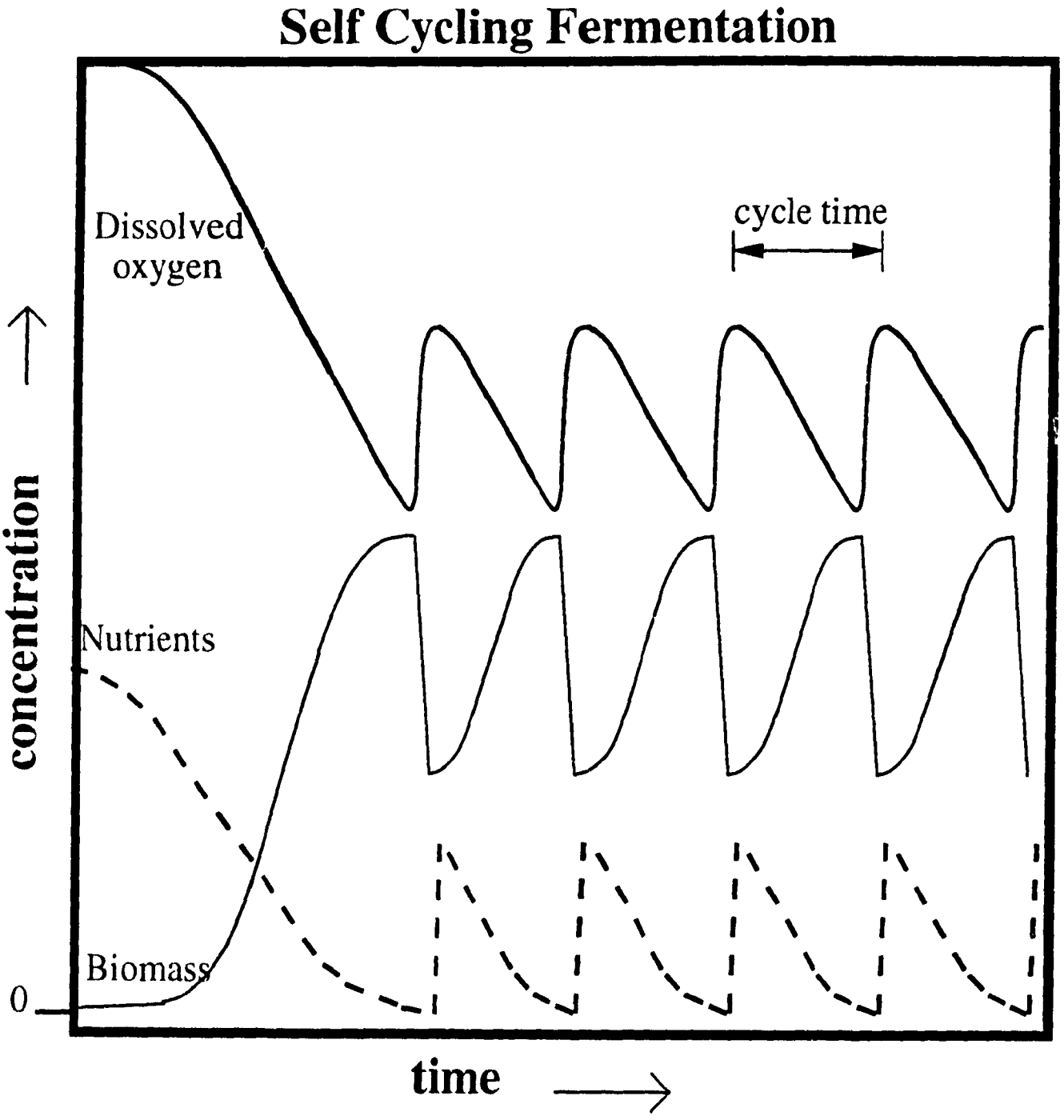

FIGURE 1.1: Fermentation parameters during an SCF

1.2 Characteristics of SCF's:

SCF's offer several unique qualities in comparison to batch or chemostat fermentations. SCF's have high rates of biomass production. The cycling control keeps the culture in its exponential growth phase throughout the fermentation (Sheppard, Cooper, 1990a). Depending on the purpose of the fermentation, this may either be an advantage or a disadvantage.

Productivity rate is high in SCF's. Both primary and secondary metabolites have been produced at accelerated rates using SCF's, in some cases as high as an order of magnitude greater than a comparable batch process (Sheppard, Cooper 1990b; Brown, Cooper 1991). The accelerated production of primary metabolites is related directly to the high biomass production. High rates of secondary metabolite production are attained because each cycle of the fermentation reaches a nutrient limitation. Since nutrient limitation is a stress that often triggers production of a secondary metabolite (Zahner, 1987), SCF's tend to produce secondary metabolites at the end of every cycle. This results in much faster production rates than a typical batch operation.

SCF's give complete utilization of the limiting nutrient. This characteristic may prove useful for the biodegradation of waste materials (Brown, 1991). Brown found that Acinetobacter calcoaceticus reduced residual hydrocarbon concentrations to lower levels in an SCF than in a chemostat system.

The synchrony of the cell growth makes it possible to do

studies on the life cycle of the organism. In an asynchronous culture, it is not possible to distinguish different cell cycle features on the macroscopic level.

There is a direct correlation between the duration of the cycles and the doubling time of the organism. This makes it simple to study the kinetic response of a system to different operating conditions. The relative rates of growth between different operating conditions can be seen immediately by the effect on the cycle time of the organism. The metabolic pathway for a given substrate can also be deduced by studying the changes in growth rate during a cycle (Brown, Cooper, 1991).

SCF's offer very stable operation. Because the medium addition rate is determined by the culture's metabolic rate, there is no danger of washing out the culture with too high a dilution rate or starving it with too low a dilution rate (Sheppard, Cooper, 1990a). The system will adapt to changes in operating conditions by automatically shifting to change the biomass or the cycle time. Most perturbations to the system will only affect one or two cycles before the system resumes its steady state cycling. This stability means that the fermentation can be safely automated and left to run for many cycles with minimal supervision.

The stability of SCF's also improves the reproducibility of results. Once the steady state biomass and cycle times have been attained, the fermentation will continue to give consistent results with every cycle. This is particularly advantageous when

compared to batch experiments, where sequential batch experiments can give widely varying results. Because the cycles follow immediately one after the other, the results can be repeated quickly.

1.3 SCF apparatus:

To date the SCF technique has been applied only in an apparatus based on a recirculating cyclone column fermenter. This fermenter is unique in how it aerates and agitates the culture broth. The fermenter makes use of a centrifugal pump to continuously pump the broth out from the bottom of a tall cylindrical column. The broth is passed through a concentric tube heat exchanger and is recycled back to the top of the column. The broth entering at the top comes in tangentially and swirls around on the inside walls of the column, creating a thin film down the sides of the vessel. The broth collects at the bottom of the column and is then re-pumped around (figure 1.2).

The cyclone column has some advantages over the conventional stirred tank type of fermenter. The recirculation of the broth at relatively high velocity through the smooth inside surfaces of the apparatus allows little opportunity for any build up of adhesive biomass or products on the walls of the apparatus. The culture is completely mixed and the fermenter has no dead spots or short circuit convection channels (Dawson, 1963). The smooth surfaced, highly mixed environment was the original "raison d'etre" for the design, providing good

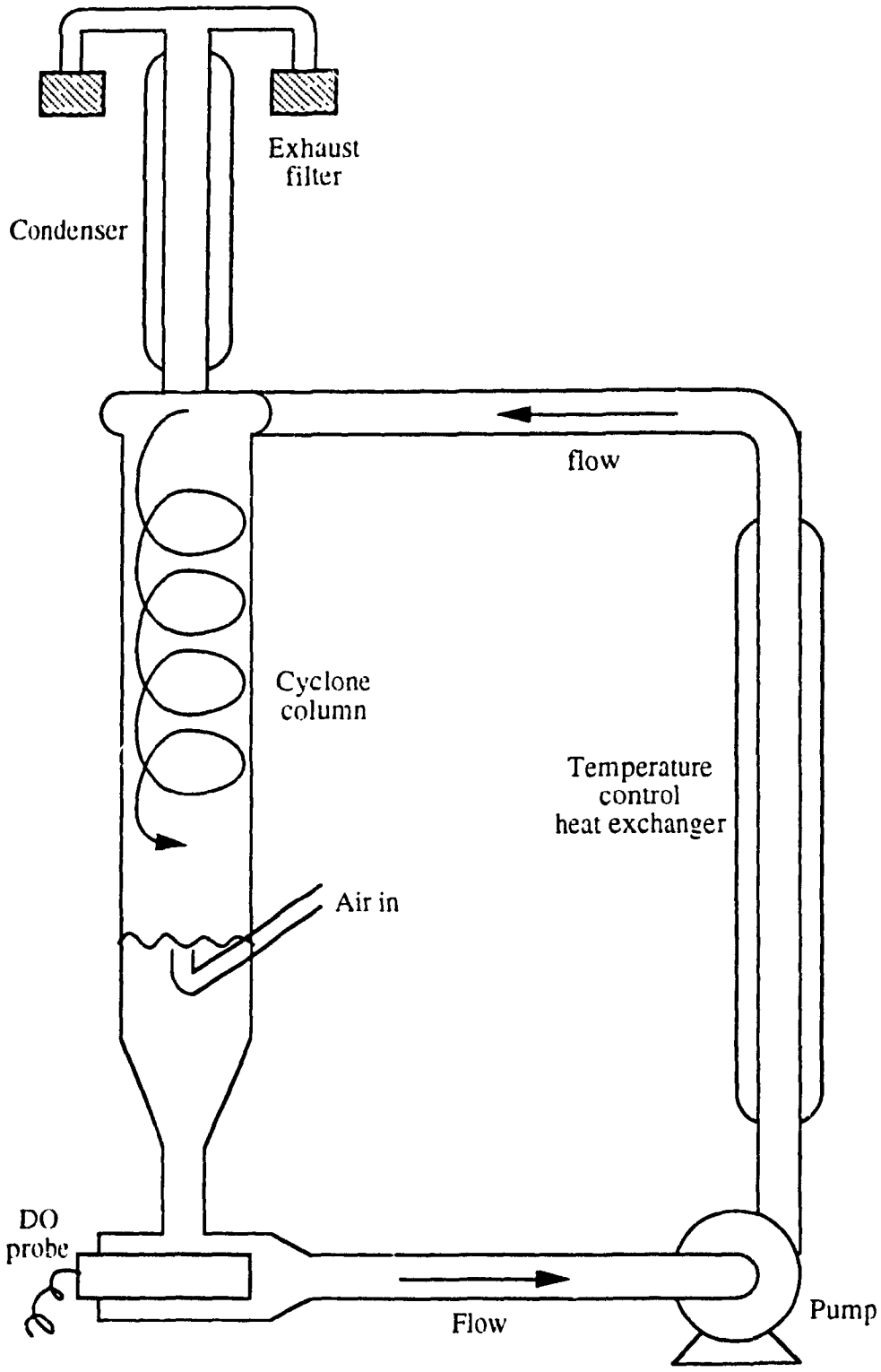

FIGURE 1.2: The recirculating cyclone column fermenter.

conditions for the growth of filamentous organisms (Dawson, 1963). The unique thin film of the column provides excellent oxygen transfer (Sheppard, Cooper, 1990c) to the broth and less air volume is needed to supply the requisite amount of oxygen (Dawson,1971b). Dawson also reports (1971a) that the continuous dropping film collapses foam in the column, although this effect does not guarantee elimination of all foaming problems.

From an experimental point of view, the cyclone reactor has few disadvantages. However, one major drawback is its scarcity in fermentation laboratories. The author knows of only two laboratories using the cyclone column. Because these reactors are rare, the association of SCF's with the reactor may be hindering the attractiveness of SCF's to other researchers.

A second disadvantage of the cyclone reactor as a research tool is that a scale-up of the reactor to industrial size may not prove practical. The energy demands and other operating costs of an industrial scale cyclone fermenter are unknown. It may also be that it is simply too unorthodox to be readily adopted by industry. Although Dawson developed a pilot plant apparatus (Dawson, 1971b), little work has been done in this regard.

The association between SCF's and the cyclone reactor has an arbitrary origin. The cyclone reactor was originally designed for chemostat fermentations of filamentous organisms (Dawson, 1963). SCF is not a chemostat method, nor has it been used with filamentous organisms. There is no "a priori" reason why the

method could not work in a stirred tank reactor.

An anticipated difficulty of transferring the technology to a stirred tank was oxygen transfer, since the control of SCF's have depended on dissolved oxygen (DO) measurements. Another consideration was foaming, since this can have effects on the working volume of the culture broth--also crucial to SCF's.

A second aspect of the SCF apparatus used to date is the DO measurement used to monitor metabolic rate. DO measurement has worked adequately but it is not without its problems. Because the DO probe is inserted directly in the culture broth, it is subject to fouling by adhesive matter. The DO measurement can often be noisy and this creates problems for the control computer (Brown, 1991). DO is sensitive to overhead pressure, which is not always constant during a fermentation. Finally, the DO probe gives readings which reflect the DO concentration at a specific location within the reactor. The reading may not be representative of the average DO concentration of the whole reactor volume.

The measurement of the composition of exhaust gasses from the reactor is an alternative method of monitoring the metabolic rate within the reactor. The carbon dioxide and the oxygen concentrations in the exhaust are directly related to the respiration rate of the organism. By nature of the gas sampling system, exhaust gas analysis would give an average, filtered value for the gas concentrations. Because the sensor would not be immersed in the broth, the fouling problems would be

eliminated.

An anticipated difficulty with gas analysis monitoring was the resolution of the measurement. It was not known whether the variations in the concentration of carbon dioxide and oxygen would have sufficient amplitude to be usable for SCF control. Also, because the sensor is mounted outside the actual fermentation, there was concern over the response time of the control signal.

The utility of the SCF as a research technique will be greatly enhanced if it can be shown to work in the common, conventional type of stirred tank fermenter. The transfer of research findings to applications in industry will be facilitated if the research is known to be valid for a conventional fermenter design. The use of exhaust gas analysis could make SCF's possible in systems that would otherwise be uncontrolable due to fouling of the DO probe.

1.4 Objectives:

The objectives of this study were:

1. To develop an apparatus, based on a conventional stirred tank fermenter, that is capable of performing SCF's.
2. To compare the performance of the new apparatus with that of the cyclone column SCF.
3. To determine the utility of exhaust gas analysis as a control measurement.

APPARATUS:

2.1 Design considerations:

A basic design consideration for the SCF apparatus was that it be sterilizable. SCF's to date have been done only with one or at most two pure cultures. The need for sterility made it desirable to have the apparatus divided into as few sections as possible during sterilization.

Another basic consideration was that the apparatus be assembled from fermentation equipment likely to be found in a typical laboratory. This was to ensure that other researchers who may wish to use SCF's would not be required to acquire specialized equipment. The design of the apparatus was based on a commercial model of stirred tank fermenter.

Design flexibility was important to allow for quick modifications. This led to the choice of using plastic vessels and flexible latex tubing.

2.2 Culture vessel:

The culture vessel used was a New Brunswick two litre Multigen bench top fermenter. This is a standard small volume fermenter that has been available on the market for many years.

The following modifications were made to the fermenter:

Originally, the fermenter consisted of an open top two litre glass vessel mounted on top of a base platform. For this study, the glass vessel was replaced with one made of plexiglass. A drain was mounted at the bottom of the side wall

of the vessel (refer to figure 2.1). A custom built stainless steel top was made for this vessel. It was fitted with: two baffles, two thermowells, a sterile sample port, an air inlet, a concentric tube heat exchanger, an inoculation port, a dissolved oxygen probe connectionand seven other connection ports, three of which were used for the medium addition, the exhaust outlet and the syringe pump injection.

The Multigen fermenter provides agitation by means of magnetically coupled rotating impellers and fixed baffles. The agitator can have up to three six bladed impellers mounted at any desired height on a central shaft. Agitation can be varied between 0 and 500 to 800 rpm, depending on the volume and viscosity of the agitated broth. For this study, two baffles were used and the impellers were mounted at unequally spaced heights as shown in figure 2.1. This spacing of the impellers was done to give less surface turbulence when the reactor vessel was at half its working volume.

The Multigen fermenter is aerated with air sparged into the reactor at the bottom, beneath the impellers. The utility air in the laboratory was used as the air supply.

To minimize changes in liquid volume due to aeration, the inlet air and the exhaust gases were both brought to saturation at the same temperature. The inlet air was humidified by being bubbled through distilled water. At the exhaust of the fermenter a Friedrichs condenser trapped escaping moisture. Both the humidifier and the condenser were cooled to the same temperature

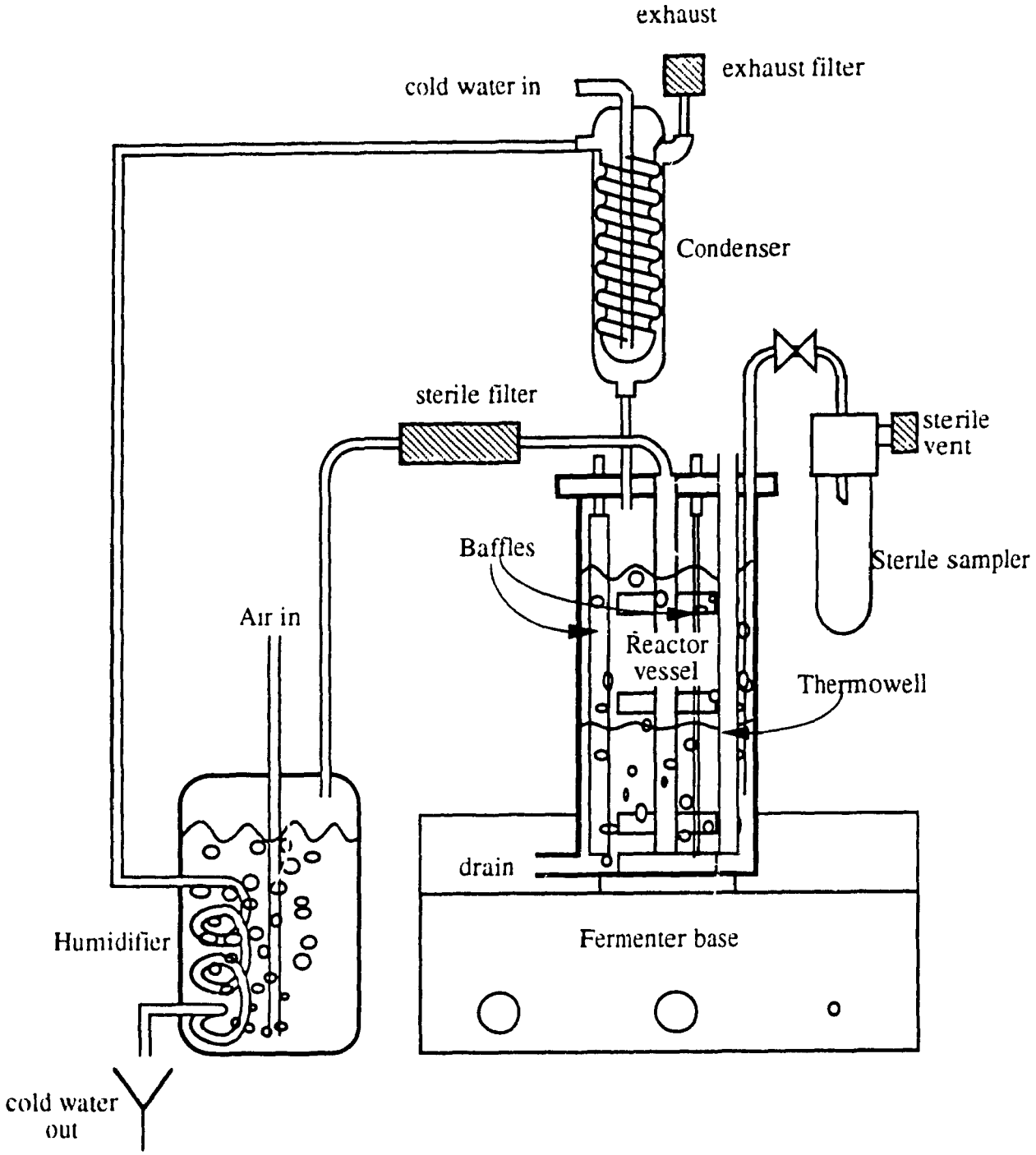

FIGURE 2.1: The stirred tank culture vessel. Operating volume was 1500 ml.The lid was fitted with: two baffles, two thermowells, a heat exchanger, a sterile sample port, a dissolved oxygen probe and connections for medium additions, ventilation, sampling and inoculation. The agitator's middle impeller was installed above the level of 750 ml so that it would not agitate the surface while equilibrating the harvest and reactor volumes.

using the cold tap water in the laboratory.

Sterile samples could be drawn from the culture vessel into a test tube through a sampling tube.

2.3 Medium supply:

The medium supply for the apparatus was gravity fed. It consisted of a magnetically stirred ten litre carboy feeding clear medium to the reactor via an intermediate dosing vessel. (figure 2.2) The carboy was made of polypropylene. It was fitted with a stoppered drain hole at the bottom. A glass rod through the stopper allowed flow through to the dosing vessel. The dosing vessel was an upside down polycarbonate one litre erlenmeyer flask with a screw cap. Connections to the dosing vessel were made with polypropylene tubing connectors. The amount held up in the dosing vessel was adjustable between 0 and 850 grams. This was measured out automatically using an electronic balance (Pennsylvania 5600). The connections between the carboy, dosing vessel and reactor were all made with flexible latex tubing and solenoid valves. Both the carboy and the dosing vessel were vented with glass wool filters.

The opening and closing of the medium supply valves could be controlled automatically by the cycling control computer or by manual override.

2.4 Temperature control:

Temperature control was provided with a recirculating water

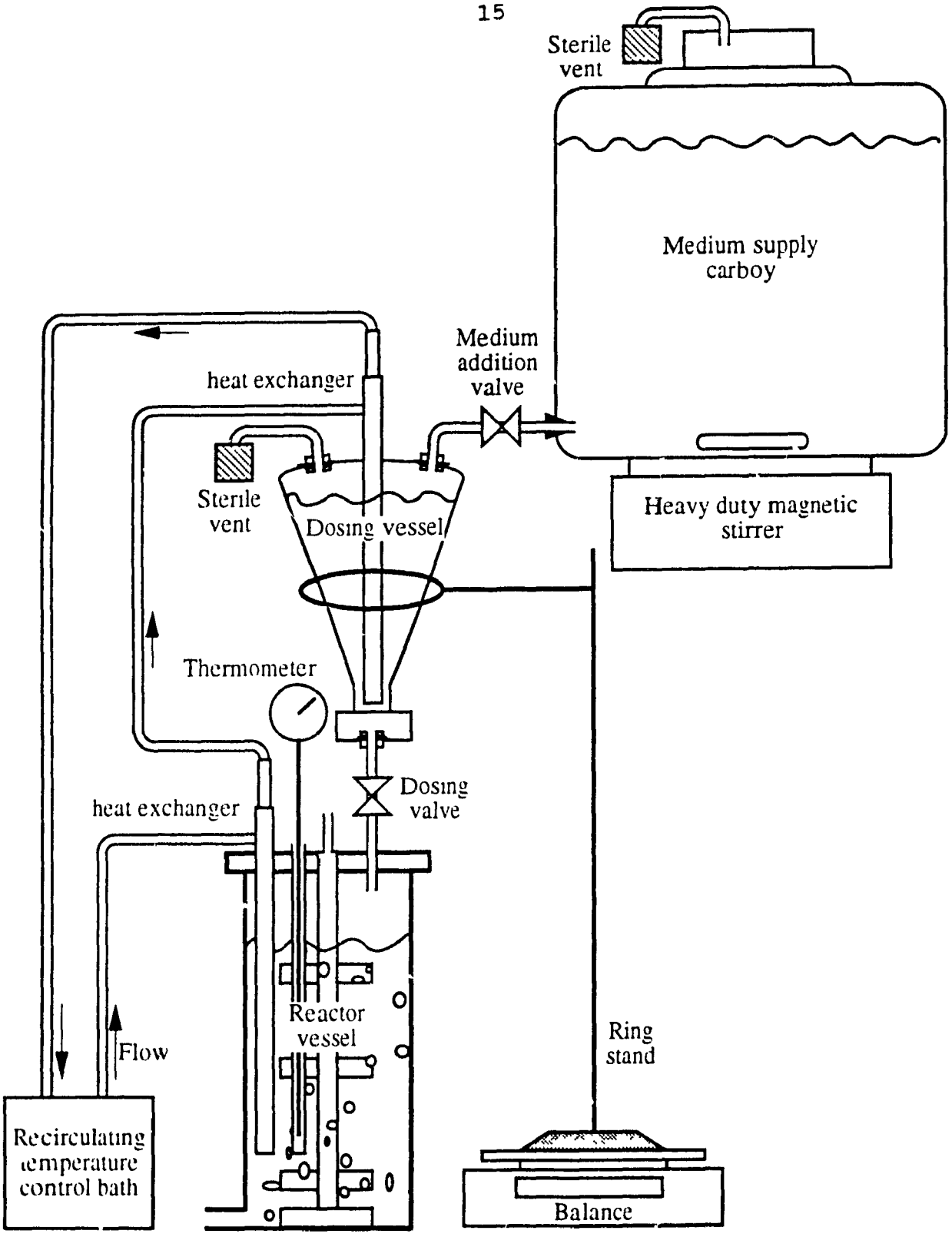

FIGURE 2.2: Medium supply system and temperature control for the reactor and dosing vessels.

bath (Haake model FE 2) and a concentric tube heat exchanger in the fermenter. An identical heat exchanger was mounted in the dosing vessel, in series with the one in the fermenter. This allowed the dosing medium to be brought up to operation temperature before being added to the cycling culture. (figure 2.2)

Temperature of the culture vessel was measured with a bimetallic dial thermometer mounted in a thermowell.

2.5 Harvest system:

The purpose of the harvesting system was to periodically remove half of the growing culture in the culture vessel. This was achieved using hydraulic equilibration between the culture vessel and the harvest vessel (figure 2.3).

A harvest vessel made of plexiglass was mounted next to the reactor tank. This vessel had been constructed with an identical diameter to that of the reactor tank. A displacer was put in the harvest vessel to make up for the volume displaced in the reactor by the various insertions. This made its cross sectional area equal to that of the operating reactor tank. The drain on the reactor tank was connected to a drain on the harvest vessel using latex tubing and a solenoid valve. The solenoid valve had a relatively large orifice (3/8") to promote quick flow between vessels. The harvest vessel was mounted by clamp to a vertical rod. This mounting allowed the height of the harvest vessel to be adjusted so that the volume in the reactor could be

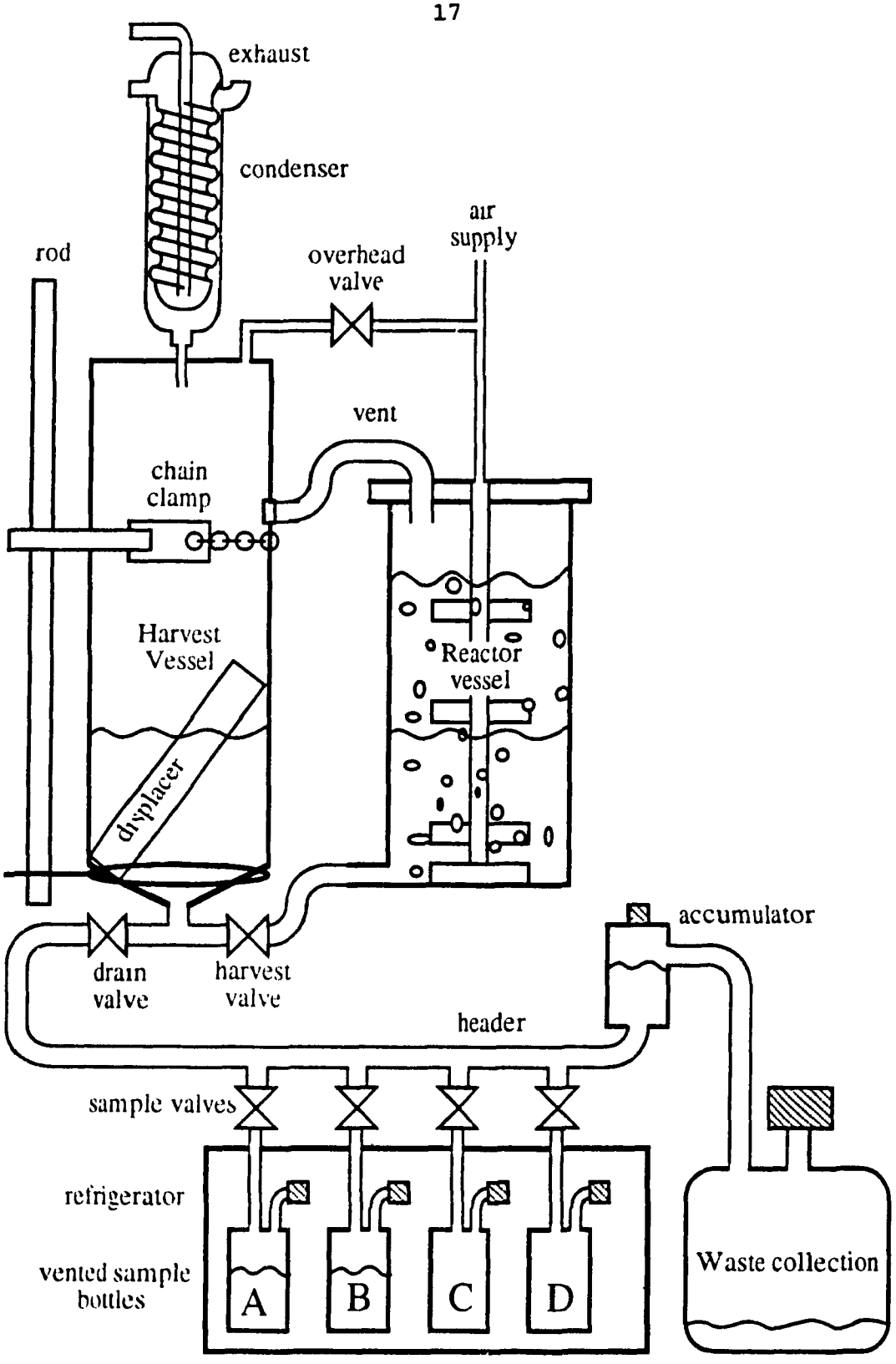

FIGURE 2.3: Harvest and sampling systems of the SCF apparatus.

accurately split into two equal volumes. A second solenoid valve was installed to open a connection between the air supply to the fermenter and the harvest vessel. Opening this valve would divert air away from the sparger. This eliminated air hold up in the reactor volume and assured equal overhead pressure between the vessels.

2.6 Sampling system:

The purpose of the sampling system was to store representative samples for later analysis. This was achieved by diverting a portion of each harvest to a sample bottle kept cool in a refrigerator. (figure 2.3)

The sampling system consisted of a valved drain from the harvest vessel into one end of a header. The header was attached to four different 250 ml glass sample bottles (commercial juice bottles). Each sample bottle had its own solenoid valve attached directly to the header. The sample bottles were kept in a refrigerator (Tatung super deluxe) and vented through glass wool filters. At the other end of the header was fitted a 250 ml polypropylene accumulator bottle. The volume in the accumulator was determined by an overflow drain mounted at the 150 ml level. This drain led to a waste collection carboy. The tops of the accumulator and the waste carboy were vented through foam plugs. 0.3 grams of cupric sulphate was added to the waste carboy. This gave a concentration of 1.2×10^{-4} moles/litre and was done to prevent contaminants from growing in the waste.

The opening and closing of the sample system valves could be controlled automatically by the cycling control computer or by manual override.

2.7 Cycling control hardware:

Refer to figure 2.4 for an illustration of the cycling control hardware. The cycling control of the fermenter was achieved with an IBM PC computer equipped with a data acquisition board (Data Translation model DT-2801). A flat wire connected the DT-2801 to a screw terminal board (Data Translation DT-707) mounted in a separate junction box. The screw terminal board was wired to receive analog signals from the dosing balance and the dissolved oxygen analyzer. Digital signals from the computer passed through the screw terminal board to 16 relays (Data Translation OAC5) mounted on a single circuit board (Data Translation PB-16A). The relay board was also mounted in the junction box. The relays were wired to nine solenoid valves (Skinner model V52LB2052) and a syringe pump (Sage Instruments model 341B). A 5 volt power source was required for the operation of the relays (Archer model 273-1650). Six relays were kept as spares.

Dissolved oxygen measurements are made with a polarographic dissolved oxygen probe (Cole Parmer model L-29020-04) and a dissolved oxygen analyzer (Cole Parmer model L-01971-00).

The digital output signal of the dosing balance (Pennsylvania 5600) was modified to give an analog signal. This

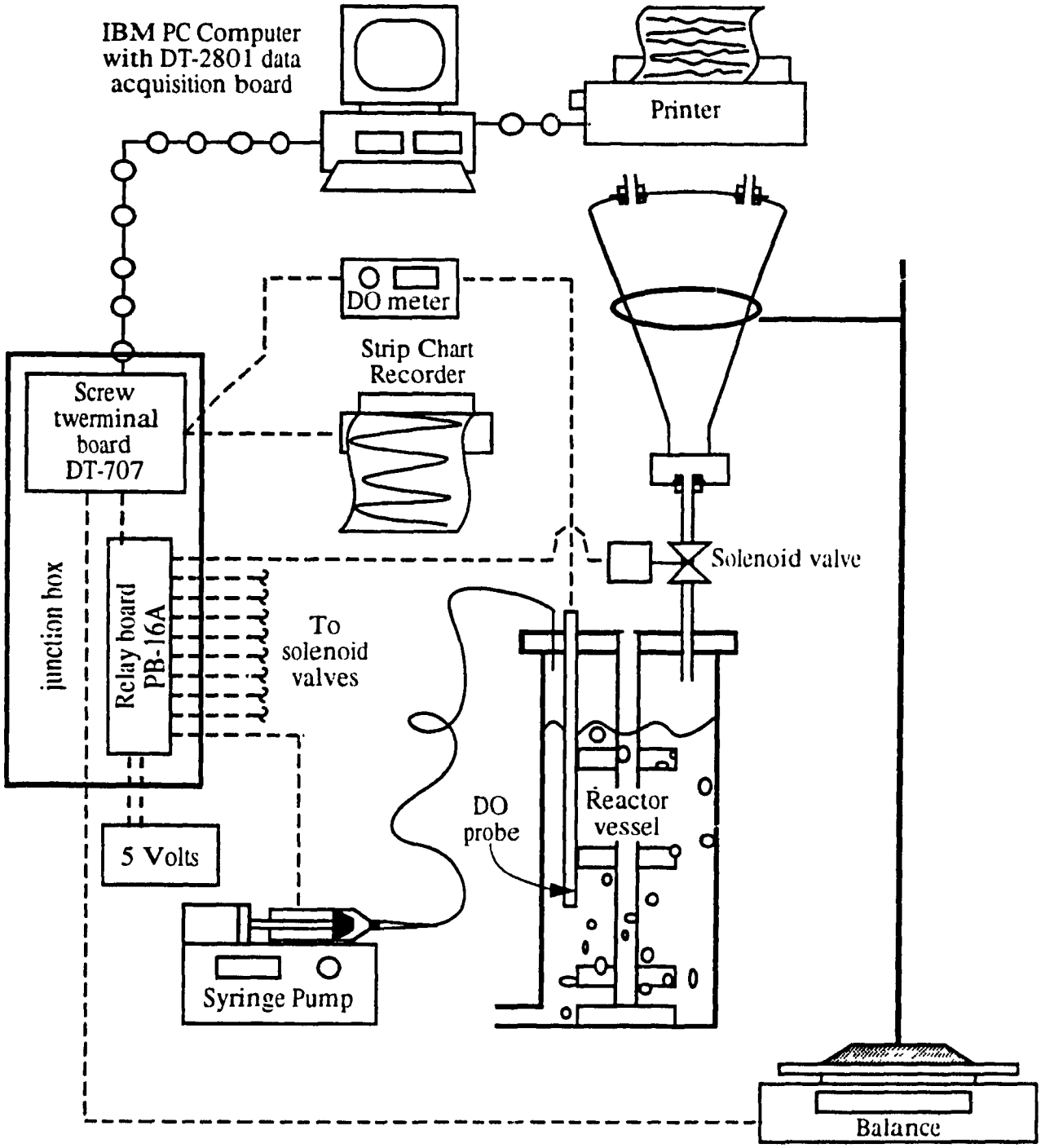

FIGURE 2.4: Cycling control hardware.

saved on the number of digital terminals used on the screw terminal board.

2.8 Cycling control algorithm:

The control program used was specifically written for the application using Turbo Pascal. This program grew out of an earlier version in BASIC, used by Brown (Brown, 1991). Refer to the appendix for a complete printout of the program. A description of the algorithm is found below under "operating techniques."

2.9 Monitoring:

Several process variables were constantly monitored during operation. The dissolved oxygen was recorded on a strip chart recorder (Fisher record all series 5000). The minimum dissolved oxygen level attained for each cycle was logged by the cycling control computer. The control computer also logged the duration of each cycle and the number of samples taken.

Oxygen and carbon dioxide concentrations in the inlet and exhaust gases were logged with a gas analyzer (Columbus Instruments Oxymax system). The analyzer consisted of several modules linked and controlled by an IBM AT compatible computer. Refer to figure 2.5. With its supplied software the computer controlled the operation of the various components and performed all the data processing. The other modules of the analyzer consisted of: a communication junction box, the oxygen analyzer,

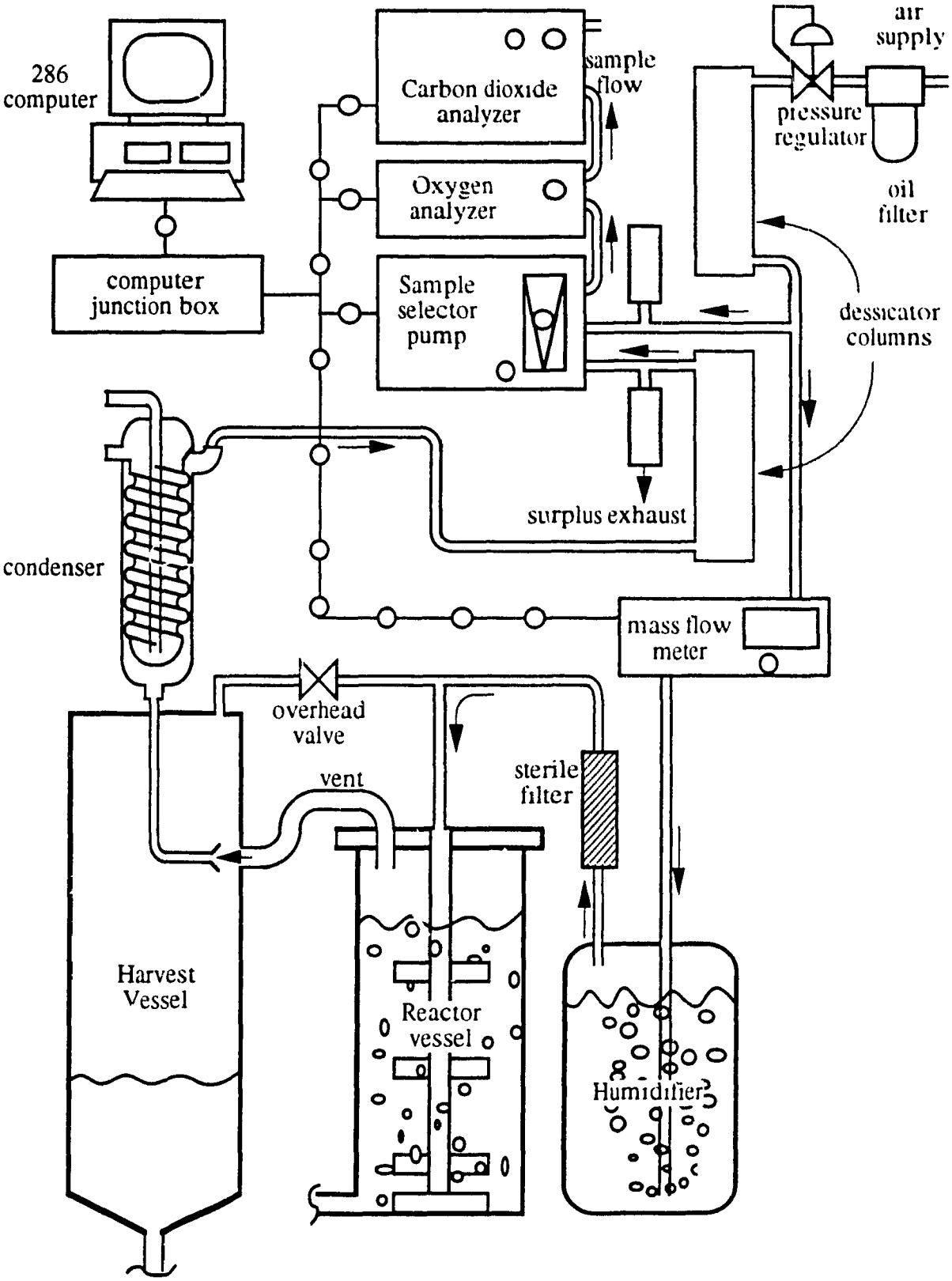

FIGURE 2.5: Gas analysis and monitoring system.

the carbon dioxide analyzer, a sample pump and a mass flow meter. The oxygen analyzer used a chemical cell measuring element and had a range of 0 to 20.5 percent. The carbon dioxide analyzer worked by infra red absorption and had a range of 0 to 0.5 percent. The sample pump drew samples from both the inlet and exhaust gas flows. A mass flow meter accurately controlled the amount of air sent to the fermenter. Air entering the mass flow meter came from the laboratory utility air. It was conditioned by being passed through an oil filter (Falflo), a pressure regulator (Watson) set to 5 psig., and a desiccator column (Drierite). The exhaust gases were also dried through a desiccator column just upstream of the sample pump.

2.10 Assembly:

Figure 2.6 shows the basic assembly of the equipment. The entire system was gravity operated and the five different levels at which liquid were held required about four meters of vertical space.

The fermenter was mounted on a laboratory bench. To one side a ring stand holding the dosing vessel was mounted on the dosing balance. On the other side was the vertical rod on which was mounted the harvest vessel. The temperature control water bath, air humidifier and syringe pump were also put on the bench.

The medium supply carboy was put atop a large magnetic stir plate (Bell Stir heavy duty) mounted on a wooden superstructure

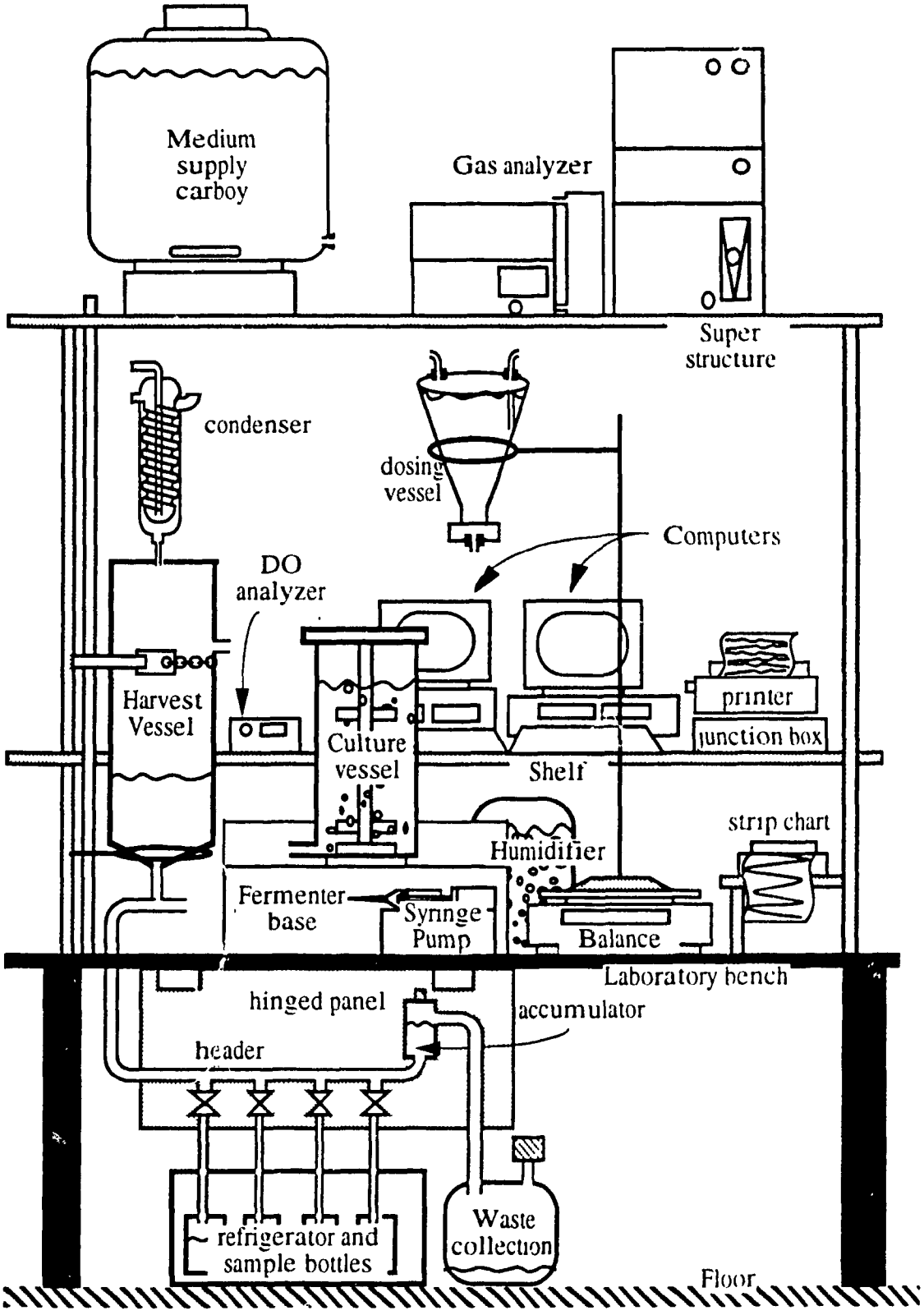

FIGURE 2.6: Laboratory assembly of the SCF apparatus.

rising 1.2 meters above bench level. The latex tubing connecting the medium supply carboy to the dosing vessel passed through a hole in the superstructure platform. The superstructure also supported the gas analyzer, the top end of the harvest vessel's mounting rod, some solenoid valves, wiring and much of the tubing running between vessels.

The header, sample valves and accumulator for the sample system were mounted on a panel attached underneath the bench. This board hung vertically during operation but could be tucked up horizontally when not in use. The waste carboy and the refrigerator holding the sample bottles were put on the floor.

The cycling control computer, the dissolved oxygen instrumentation and the computer running the gas analyzer were mounted on shelves above the bench.

MICROORGANISMS:

3.1 Selection Criteria:

It was decided to limit the choice of organisms to those already grown successfully with SCF in a cyclone fermenter. Using these organisms would make a comparison possible between the new apparatus and the cyclone fermenter. The organisms that had been used previously in SCF's also provided several advantages. They were non pathogenic. They had relatively fast doubling times which would lessen the possibility of contamination. One of the organisms produced a secondary metabolite product, another a primary metabolite product.

3.2 Chosen microorganisms:

Based on the above criteria, two organisms, Bacillus subtilis ATCC 21332 and Acinetobacter calcoaceticus RAG-1 were used.

B. Subtilis had been previously used in SCF's by Sheppard (Sheppard and Cooper, 1990a,b). B. Subtilis produces a powerful bio-surfactant known as surfactin. This product is considered to be a secondary metabolite (Cooper et al., 1981)) and is relatively easy to measure. B. subtilis was cycled successfully by Sheppard using a glucose medium limited in nitrogen.

A. calcoaceticus had been previously used in SCF's by Brown (Brown and Cooper, 1991). A. calcoaceticus produces an emulsifier known as emulsan. This product is contained mostly in the cell wall and is considered a primary metabolite. Brown

succeeded in growing this organism on a variety of carbon substrates under carbon limited conditions. The substrates used included ethanol, acetaldehyde, sodium acetate and hexadecane, among others.

OPERATING TECHNIQUES:

4.1 Sterilization and assembly:

Sterilization of the reactor and all peripheral equipment such as sample bottles, supernatant bottles and sample tubes was done by autoclaving at 121 C for 30 minutes. The plexiglass parts of the apparatus (the culture and harvest vessels) were sterilized separately by soaking in 2000 ppm active chlorine solution overnight (household liquid bleach diluted 4.5 to 1).

The two plexiglass vessels were rinsed with sterile water to get rid of remaining bleach solution. Their lids were installed under the sterile atmosphere of a laminar flow hood (SteriGARD Hood, the baker company, inc.). The tops and insertions into the vessels were autoclaved in autoclavable plastic bags to keep them sterile in between the autoclave and the laminar flow hood. The rest of the apparatus was assembled at the laboratory bench location. The rest of the apparatus was sterilized in four parts: the air inlet connections, the sample system, the harvest tubing which connects the drains of the two vessels and the dosing vessel with its attached tubing. The exhaust connections were sterilized while attached to the lid of the harvest vessel.

Once the sterile connections were made, peripheral connections such as the water baths and instrumentation were made. Before filling the reactor and inoculating, the whole apparatus was tested for leaks with sterile water. The control system, instrumentation and valve sequencing were also tested

with the sterile water in the apparatus.

4.2 Medium preparation:

Table I lists the ingredients of the various media used for this study. For growing B. subtilis, media previously defined by Sheppard (1989) and Cooper (1981) were used. Sheppard's medium was nitrogen limited and had been previously used to grow B.subtilis in an SCF. Cooper's medium was thought to be iron limited and had been previously used to give high yields of surfactin in batch culture. A third medium (van Walsum) was tried which was based on to Sheppard's formulation but with the same low iron content as Cooper's medium.

For growing A. calcoaceticus some of the media defined by Brown (1991) were used. These media were carbon limited and had been used by Brown to grow A.calcoaceticus in an SCF. The carbon sources used for this study were ethanol and hexadecane.

All media were sterilized in an autoclave at 121 C for periods of time ranging from half an hour to three hours, depending on the volume of the medium. Glucose media had the glucose autoclaved separately to avoid caramelization. Sterile medium ingredients were blended together using sterile technique under a laminar flow hood. When the insoluble hexadecane was used as a substrate, this was added directly to the culture vessel with a syringe pump. This was because the agitation in the medium storage carboy was not sufficient to keep the oil suspended in the aqueous phase.

TABLE I: Different media formulations used.

Ingredient	Coopers moles/L	Sheppard moles/L	vanWalsum moles/L	Brown moles/L
$Na_2EDTA*2H_2O$	4 X 10-6	1 X 10-3	4 X 10-6	7.4 X 10-6
$MnSO_4*H_2O$	0	3 X 10-4	0	9.1 X 10-6
$FeSO_4*7H_2O$	4 X 10-6	1 X 10-4	4 X 10-6	2.5 X 10-5
$MgSO_4*7H_2O$	8 x 10-4	8 x 10-4	8 X 10-4	1.2 X 10-3
$CaCl_2*2H_2O$	7 x 10-6	7 x 10-6	7 X 10-6	4.5 X 10-4
Na_2HPO_4	4 x 10-2	4 x 10-2	4 X 10-2	1.9 X 10-2
KH_2PO_4	3 x 10-2	3 x 10-2	3 X 10-2	2.0 X 10-2
NH_4NO_3	5 x 10-2	0	4 X 10-3	0
$(NH_4)_2HPO_3$	0	2 X 10-3	0	0
NH_4Cl	2.2 x 10-1	0	0	1.9 X 10-2
Nitroacetic acid	0	0	0	1.0 X 10-3
$(NH_4)_6Mo_7O_{24}*4H_2O$	0	0	0	1.5 X 10-4
$Co(NO_3)_2*6H_2O$	0	0	0	8.2 X 10-7
$CuSO_4$	0	0	0	1.6 X 10-6
$Na_2B_4O_7*10H_2O$	0	0	0	4.8 X 10-7
Glucose	2.2 X 10-1	2.8 X 10-2	2.8 X 10-2	0
H_2SO_4	as needed	as needed	as needed	as needed
KOH	as needed	as needed	as needed	as needed
pH	6.7	6.7	6.7	7.0

4.3 Inoculation:

In most cases the inoculum for the fermentations was grown on nutrient broth (Difco), prepared as per the manufacturer's instructions. For some experiments the inoculum was grown on the same defined medium as used in the fermenter. The inocula were grown to maturity in 500 ml shake flasks. The shakers (New Brunswick model # G25) were set at 30 C and 60 rpm. In most cases the inoculum would grow to maturity within 24 hours.

A volume of 25 to 30 ml was used to inoculate the fermentation. In a 1500 ml volume this represented an inoculum of 1.6 to 2 percent. Inoculation was done with a sterile syringe or transfer pipette using sterile technique. The inoculation broth and the reactor medium were tested before and after inoculation for contamination using streak plates. Samples were taken and streaked throughout the experiments to check for contamination of the fermentation.

4.4 Cycling sequence and control:

Figure 4.1 shows a simplified flow sheet of the control algorithm. The program passed through seven sequential steps for each cycle of the fermentation.

Before starting the repeating sequence, the program was primed with basic information pertaining to the particular run. Input variables included: the name of the experiment, the operating volume of the reactor, the desired number of cycles, the time delay needed as a settling time at the start of each

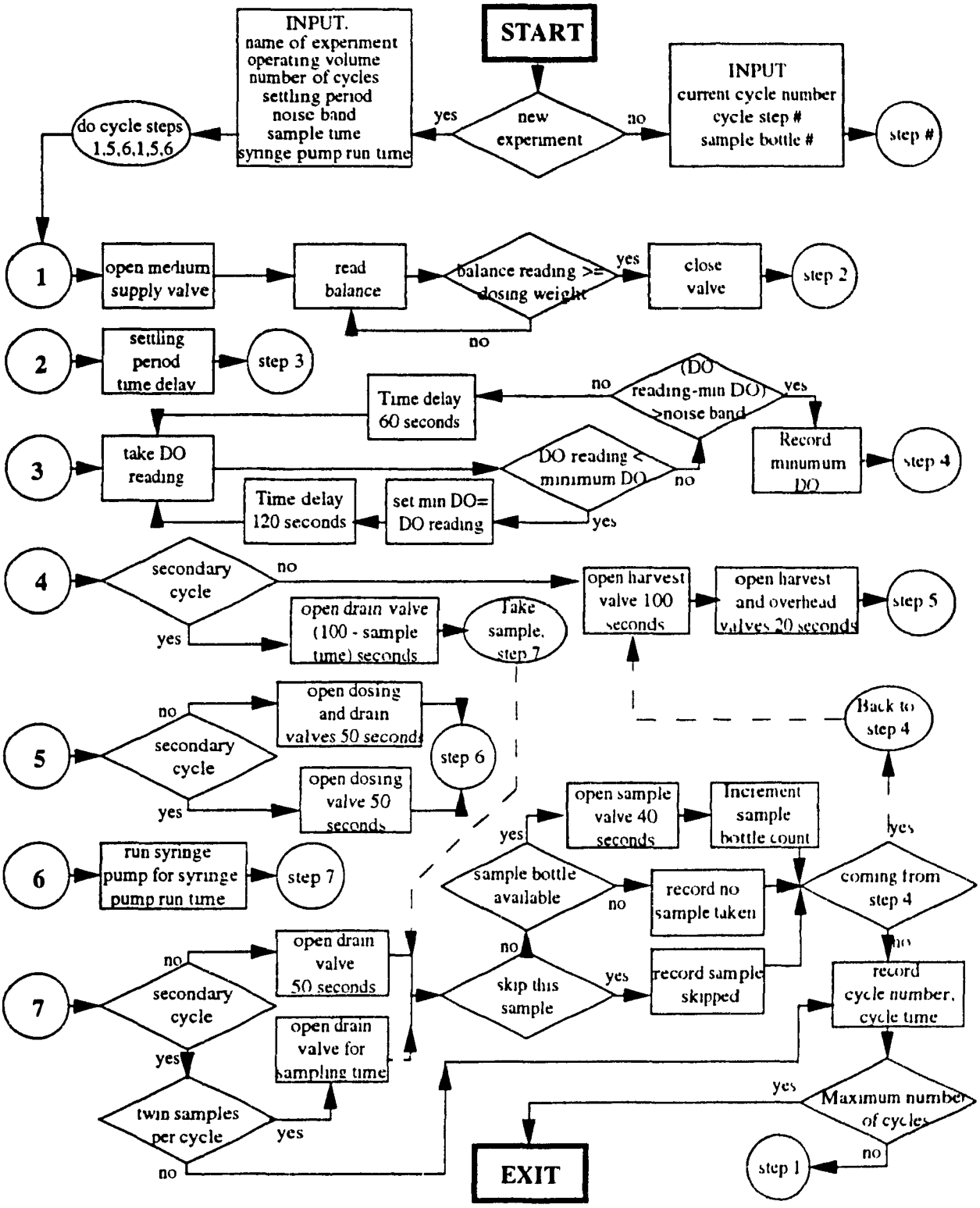

FIGURE 4.1: Cycling control algorithm.

cycle, the width of the noise band applied to the DO readings, the run time for the syringe pump and variables controlling the sampling frequency.

If problems arose during an experiment, ie. a power failure, the program could be restarted at any one of the seven sequential steps. The seven steps were as follows:

step one:

The first step opened the medium addition valve to fill the dosing vessel from the medium storage. The valve was closed when the digital balance measured the desired mass of medium. The mass used for this study was 750 g. Assuming a density of 1 g/ml, this gave half of the fermenter operating volume of 1500 ml.

step two:

The second step was a time delay to allow the dissolved oxygen reading to settle. The length of this time delay depended on how quickly the organism was cycling. The time delay was usually set to between 25 and 30 percent of the full cycle time.

step three:

After the time delay the program started searching for a minimum in the dissolved oxygen level. The computer was programmed to read the DO level once every two minutes. The reading itself was the average of a hundred readings measured by the computer over a period of about two seconds. When the DO was seen to be rising, the computer increased its sampling frequency to once a minute. At this point the computer would compare the

rise in DO to the noise band setting. If the rise in the DO reading exceeded the range of the noise band it was interpreted to indicate a minimum in the DO. The width of the noise band was adjustable. Depending on the experimental conditions, the band was usually set to between 10 and 20 percent of the cycle's DO amplitude. When the minimum in dissolved oxygen was reached it was time to remove half the broth from the stirred tank.

step four:

The removal of half the culture broth was done by opening the harvest valve. This allowed the broth in the reactor to flow into the harvest vessel. The harvest valve was kept open for 120 seconds. For the last 20 of these 120 seconds, the overhead valve was also opened. This diverted air away from the air sparger to eliminate any differences in the hydraulic behaviour of the vessels due to air sparging. It also allowed for the equalization of overhead pressure should the regular vent between the vessels have become plugged with foam.

step four with secondary cycling:

There was additional sequencing work to be done during step four when the SCF was operating in secondary cycle mode. Secondary cycle mode was when the harvest from a cycle was not sampled immediately but was held in the harvest vessel for the duration of the next cycle. The harvest thus underwent a "secondary" cycle while the SCF was continuing onto the next regular cycle in the culture vessel.

If the system was operating in secondary cycle mode then

the removal of broth from the fermenter could not be initiated until the harvest vessel was emptied of the previous harvest, held in its secondary cycle. Under these conditions the secondary cycle would be drained and a sample taken prior to opening the harvest valve. The draining and sampling of the secondary cycle was done as described below under step seven.

step five:

After the equalization of the harvest and fermenter volumes, the harvest valve was closed and the dosing valve was opened to refill the fermenter from the dosing vessel. This valve was programmed to stay open for 50 seconds.

step six:

If an insoluble substrate was being used, the next step was to activate the syringe pump. The activation time of the syringe pump was determined by the injection speed of the pump, the volume of the syringe and the desired volume of substrate. The syringe pump was also used in some experiments to inject antifoam in each cycle. If there was no addition to be made the run time of the pump was set to zero.

step seven:

The final step was to sample the harvest. The drain valve was opened to drain the harvest into the sample header. The sample header let most of the harvest pass through to the waste container, but the last 150 ml were held up in the accumulator. This hold up was then drained to a sample bottle by way of one of the four sample valves.

If the experiment was proceeding very rapidly with a fast cycle time, it was sometimes desirable to sample only every second or third cycle. This was done by skipping the sampling during intermittent cycles, leaving the sampling valves closed. The uncollected samples would sit in the accumulator for the duration of the next cycle. At the end of the next cycle they were flushed to the waste container by the next incoming sample.

step seven with secondary cycling:

If the system was working in the secondary cycle mode then the harvest was not drained during step seven. The harvest was left in the harvest vessel for almost the entire duration of the next cycle period. The harvest vessel would be drained and a sample taken during the next cycle, just prior to the next harvesting (step four). The valve timing sequence used was the same as described above.

step seven with secondary cycling and twin samples:

Sometimes it was desirable to take samples both directly after harvest (step seven), and again after the secondary cycle during the next cycle sequence (step four). This was done by opening the drain valve for a short period of time after the harvest (step seven) and then a longer period of time after the secondary cycle (step four). For our apparatus these times were set to 19 and 81 seconds respectively. This allowed some of the harvest to enter and flush out the sample header, but most of the harvest was kept in the harvest vessel for the secondary cycle.

Before restarting the sequence with the next cycle, the computer logged the cycle number, cycle duration, minimum dissolved oxygen level and sample information. The computer could continue to repeat the cycle sequence as often as required.

At each step of the cycle sequence (except step one) it was possible to divert the program into a manual mode. Manual mode allowed for changing the operating parameters set at the beginning of the experiment or for manually opening and closing valves.

4.5 Analyses:

4.5.1 Biomass

The biomass was determined using the method described in Gerhardt et al., p. 505. The oven used was a Fisher Isotemp 100 series model 126G kept at 105 C. Samples were centrifuged using a Dupont Instruments RC-5 superspeed refrigerated centrifuge. It was operated at 5 C and 10 000 rpm. Sample sizes used were either 25 or 40 ml. Tests were done with duplicate centrifuge samples. Samples of B.subtilis were rinsed with distilled water and centrifuged a second time. This was done because the pellet from the expended broth was not firm enough to collect. Biomass was reported in units of grams per litre.

4.5.2 Carbohydrates:

Total carbohydrate concentrations in broth supernatant were determined using the phenol-sulphuric acid photometric method.

This method is described in Gerhardt et al., p.333. Glucose was used to create the necessary calibration curve (figure 4.2). Carbohydrate concentrations were reported in grams per litre equivalent of glucose.

4.5.3 Emulsan:

For the A. calcoaceticus cultures, emulsan concentrations were determined using the method outlined in Ng et al., 1989. The method was modified to use the phenol test for total carbohydrates, rather than the toluene test as described. Emulsan was reported in grams per litre.

4.5.4 Surfactin:

When growing the organism B. subtilis, surfactin concentration in the broth was measured by determining the critical micelle concentration (CMC), as outlined in Sheppard and Cooper, 1990b. This was done using a surface tensiometer (Fisher Autotensiomat). Sequential dilutions of the broth were tested for surface tension. The measurements were done in duplicate. The values of surface tension were plotted versus the log of the dilution. The resulting graph, see figure 4.3, showed constant, low surface tension at low dilutions and increasing surface tensions at higher dilutions. Tangents were drawn to the constant and the steeply sloping sections of the curve. The ordinate of the intersection of these tangents is the CMC. Surfactin concentrations were reported in units of inverse CMC, CMC^{-1}.

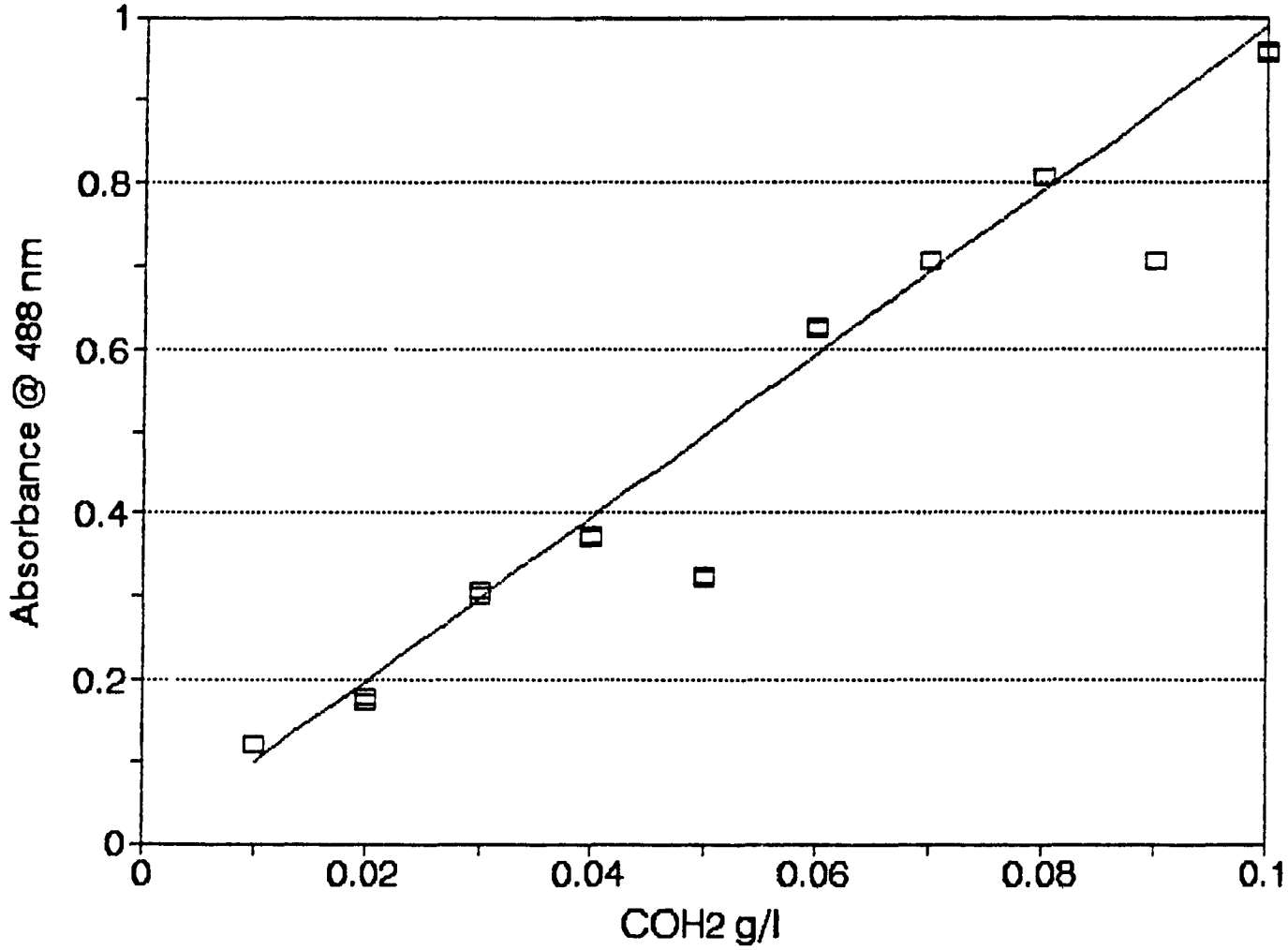

FIGURE 4.2: Calibration curve for the determination of carbohydrate concentration. The two outlying points were ignored for the regression.

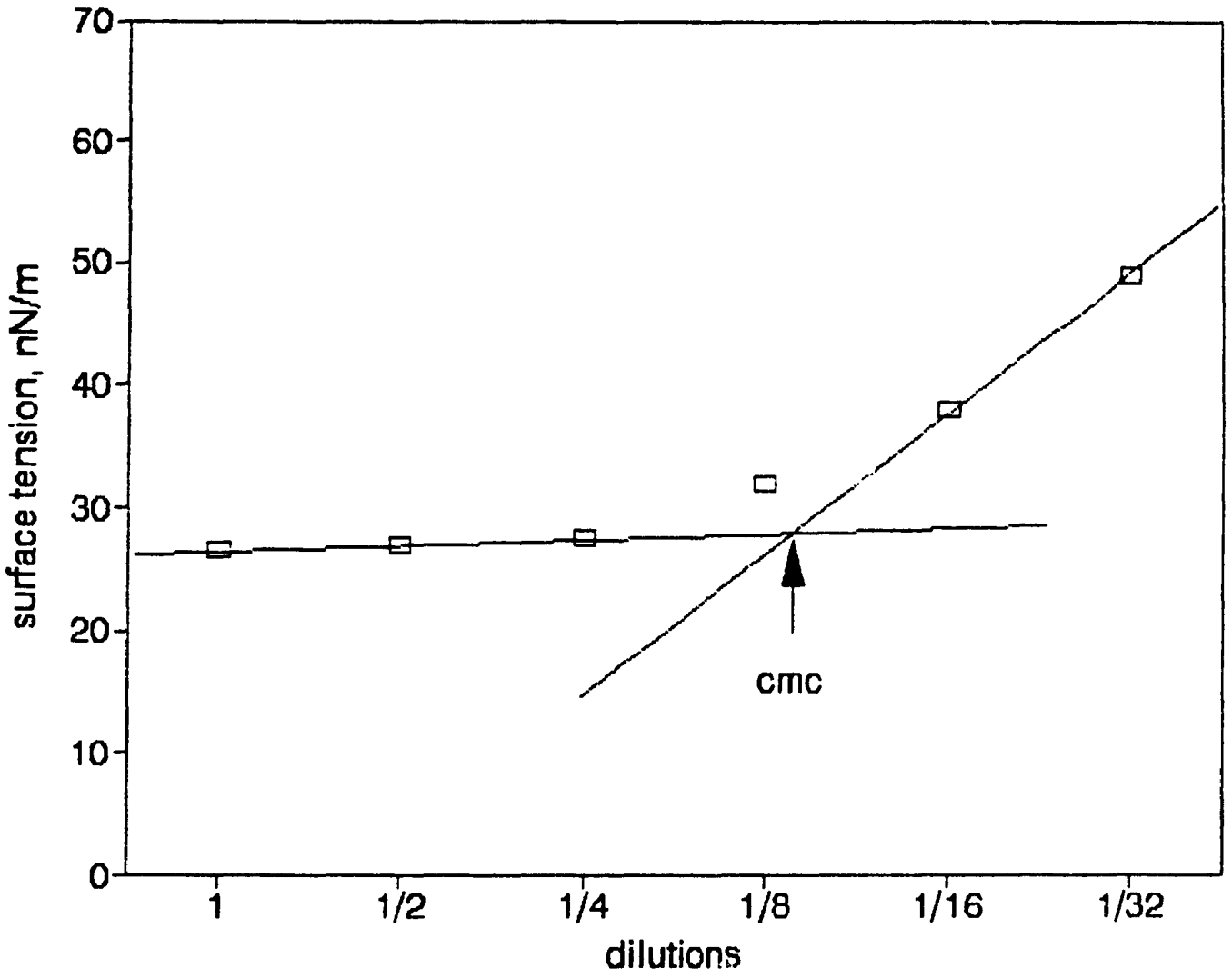

FIGURE 4.3: Determination of critical micelle concentration using sequential dilutions.

4.5.5 Glucose:

Glucose concentrations in broth supernatant were measured with an enzymatic photometric method. The test made use of glucose oxidase and peroxidase to achieve a final concentration of oxidized o-dianisidine, which was quantified using a Varian model DMS200 UV-Visible scanning spectrophotometer. The test used was the Sigma Chemicals enzymatic test 510-A. The method was as described in the test manual. Glucose was reported in grams per litre.

4.5.6 Ethanol:

Ethanol concentrations in broth supernatant were measured with an enzymatic photometric method. The method made use of nicotinamide adenine dinucleotide and alcohol dehydrogenase (NAD-ADH) to produce $NADH_2$, which was quantified using the spectrophotometer. The test used was the Sigma Chemicals enzymatic test 332. The method was as described in the test manual. See figure 4.4 for the calibration curve used. Ethanol was reported in percent weight per volume.

4.5.7 Hydrocarbon:

Residual hexadecane concentration was determined using gas chromatography (Hewlett Packard 5890-A with a Swelco Petrocol B column), as described by Brown (Brown, 1991). The initial temperature for the column was set to 100 C. The final temperature was 170 C. Pentadecane was used as an internal standard. The sample was prepared by vortexing 2 ml of broth with 2.5 µl of pentadecane. Next 5 ml of iso-octane was added

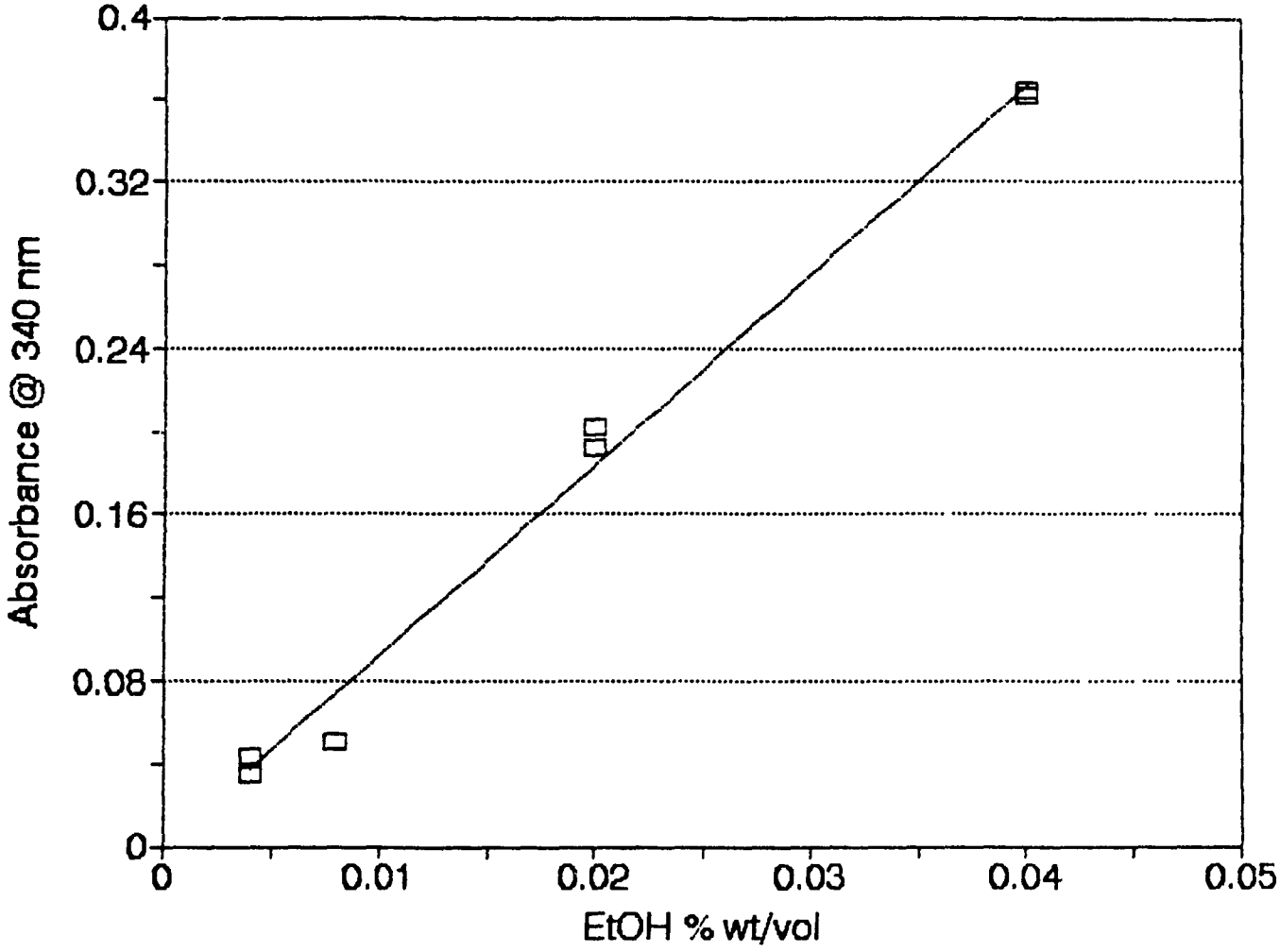

FIGURE 4.4: Calibration curve for the determination of ethanol concentration.

and it was vortexed again. After letting the sample sit for 20 minutes, 0.1 µl of the organic phase was injected into the GC column. The final value was derived from the equation:

$$TSH=(AR/HCF)*TIS$$

where TSH is the hexadecane concentration, AR the area ratio of hexadecane to pentadecane, HCF is a correction factor of 1.0025 and TIS is the concentration of pentadecane in the sample. The correction factor was calculated by Brown (1991) in his earlier work with A.calcoaceticus. Residual hexadecane was reported in units of percent of hexadecane originally added.

4.5.8 Carbon Balance:

The closing of a carbon balance was done using the following equations:

$$\% \text{ closure} = \frac{(\text{Carbon in}) - (\text{Carbon out})}{(\text{Carbon in})} \times 100$$

$$\text{Carbon in} = S * f_s$$

$$\text{Carbon out} = (B * f_b) + (CO_2 * f_{co2}) + (Sr * f_s) + (P * f_p)$$

where S is the substrate concentration in g/L

Sr is the residual substrate concentration in g/L

B is the average dry biomass in g/L

CO_2 is the average mass of CO_2 produced per cycle, calculated assuming 22.4 L per mole of CO_2

P is the average product concentration in g/L

f_s, f_b, f_{co2}, f_p are the carbon mass fractions of the subscripted variable. f_s and f_{co2} were derived from the chemical composition of the substrates and carbon dioxide. f_b was assumed

to be equal to that of E. Coli at 0.5. For emulsan, f_p was assumed to be equal to that of a regular carbohydrate, ie. 0.4. To close the carbon balance, average values were drawn from consecutive cycles showing repeatable results.

4.5.9 Nitrogen:

Residual ammonium concentrations in broth supernatant were determined using the micro diffusion and titration method described by Conway (1947). Residual nitrogen was reported in units of percent of nitrogen originally added.

4.5.10 Contamination:

Samples were taken before and during the experiment to verify that the system was free of contamination. A volume of approximately 2 ml was removed from the reactor using the sterile sample port. A loop of this sample was streaked out on nutrient agar (Difco) and incubated for 16 to 30 hours at 30 C (Fisher Scientific low temperature incubator model 146A).

4.5.11 Viable cell count:

Viable cell counts were determined by plating out diluted samples as outlined in Gerhardt. A sample of approximately 2 ml was drawn from the reactor through the sterile sample port. Under the laminar flow hood, 100 µl was extracted by micro pipette and diluted into 100 ml of sterile distilled water. This was shaken and then diluted again, 100 µl into 100 ml, to give a final dilution factor of 10^{-6}. 100 µl of this final dilution was spread on a nutrient agar plate with a glass wand. This

dilution rate gave cell counts in the range of 30 to 150 cells per plate. Each plating was done in triplicate. The used wand was occasionally applied to a second fresh agar plate to count the number of cells adhering to the wand.

The plates were incubated at 30 C. The colonies were counted after about 24 hours in the incubator. The viable cell concentration was reported in units of viable cell count per ml.

4.5.12 Oxygen transfer coefficient:

The oxygen transfer coefficient, (K_1a) was determined using the gassing in/gassing out method, as described in Gerhardt. K_1a is defined by the equation:

$$QO_2 = K_1a(C^*-C)$$

where QO_2 is the transfer rate of oxygen in moles/litre-hour, C^* the oxygen concentration at the gas/liquid interface and C the concentration of oxygen in the liquid.

The reactor was filled to operating volume with water. Sodium sulphite at .002 moles /litre was used as an oxygen scavenger to bring the dissolved oxygen to zero. Air was bubbled in at different flow rates (0.67, 1.0, 1.33 and 1.67 lpm) with three different agitation rates (250, 550 and 800 rpm). The response of the dissolved oxygen probe was recorded on a strip chart. Values of $\ln(C^*-C)/C^*$ were plotted versus time. The slope of the graph is the negative of the K_1a. See figure 4.5 for a sample K_1a determination.

The response time of the dissolved oxygen probe was also determined. The probe was switched between two water filled

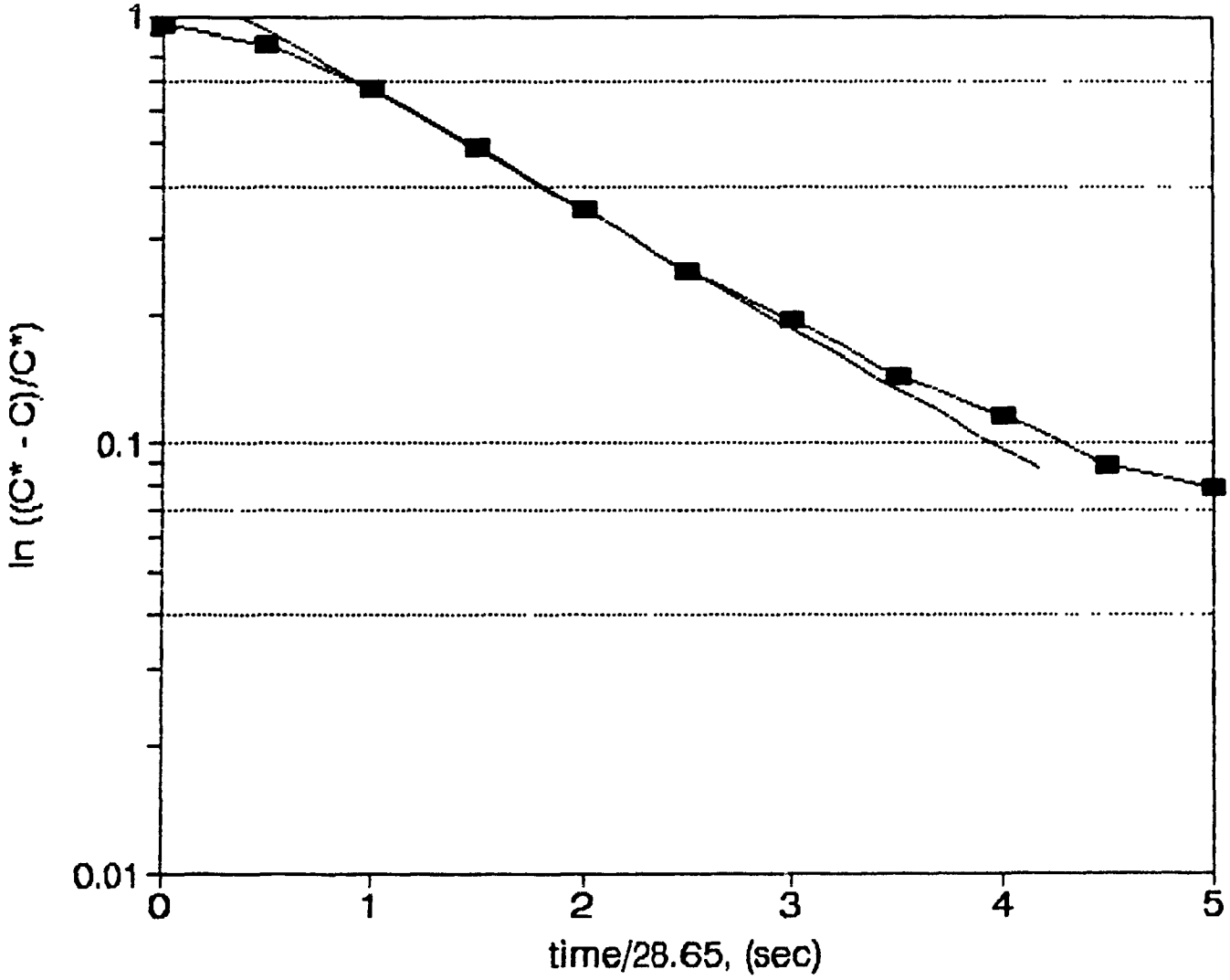

FIGURE 4.5: Determination of K_1a
c^* = saturated DO concentration, C= DO concentration, samples were taken every 28.65 seconds.

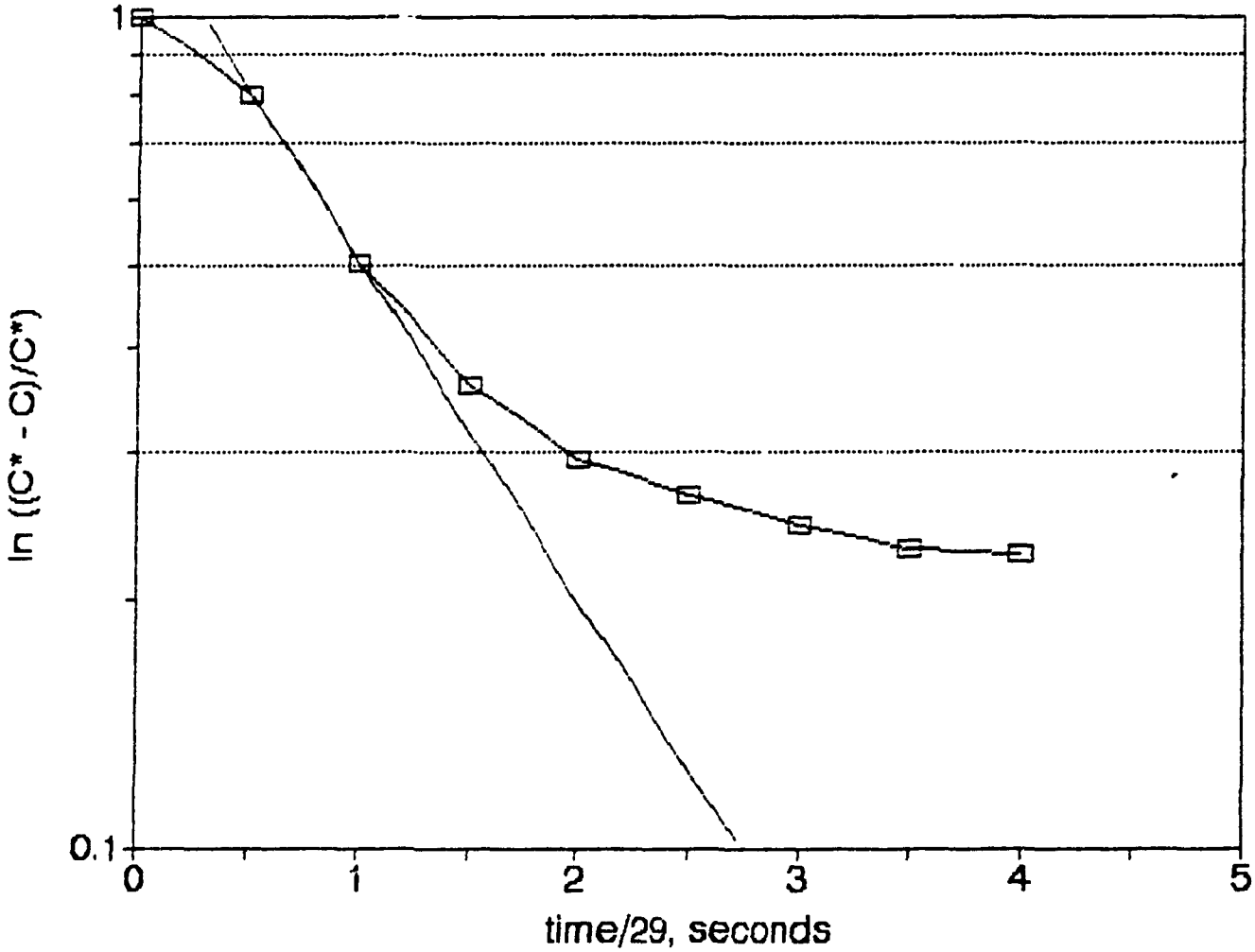

FIGURE 4.6: Determination of DO probe response time.
C^* = saturated DO concentration, C= DO concentration, samples were taken every 29 seconds.

beakers. One beaker was sparged with air, the other with nitrogen. Both were agitated with magnetic stirring bars. The response of the probe between the two beakers was recorded on a strip chart. Values of $\ln(C^*-C)/C^*$ were plotted versus time. The response time was measured off the graph by drawing a tangent to the curve at the inflection point and determining the time for 90 percent response. See figure 4.6 for an illustration of the technique.

4.5.13 Power requirements:

The power requirements of the fermenter were measured using a digital ampere meter connected in series with the power supply to the base of the fermenter. The compressor and temperature control in the fermenter base were switched off. The amperage was measured for different agitation and aeration rates and at different liquid levels. The measured amperage multiplied by the voltage (assumed to be 115 volts) gave the power required at the particular settings. The net power applied to agitating the liquid in the reactor, excluding that lost to internal friction within the equipment, was estimated by subtracting the power requirements for an empty reactor from that of a full reactor at the same agitation setting.

Power used for aeration was estimated from the rate of physical work done by the air forcing its way though the sparger at the bottom of the reactor. The equation used was:

$$p = P * dV/dt$$

where p is power in watts, P is the head of the reactor in

Pascals and dV/dt the volume flow rate of the air in m^3/s. Calculations were done using a head of 20cm and an air flow rate of 1.5 L/min.

RPM values were assigned to the different agitation settings using a hand held digital tachometer.

4.5.14 Harvest Volume:

The volumes of harvest removals were verified by draining the harvested broth into a large graduated cylinder. This was done by disconnecting the sample header from the drain valve and draining from the harvest vessel directly into the graduated cylinder. The measured broth was then poured manually into sample bottles for later analysis. Volumes were also measured on a simulated SCF using water instead of a growing culture.

The percent of volume removed was tested using dilutions. An SCF was simulated with water. Blue dye was introduced to the reactor on one cycle. The dye intensity was measured for each subsequent cycle. The dye concentrations were quantified with a spectrophotometer using a 1 cm path length. The measurements were taken at 629 nm. See figure 4.7 for the dilution calibration curve of the blue dye. Plotting the dye intensity versus cycle number gave a dilution curve.

4.5.15 Determination of inter-sample mixing:

The degree of mixing between samples in the sample system was determined using a dye tracer. The reactor was filled with water and run through simulated phasing cycles. Blue dye was added to the harvest on one cycle. The dye intensity of this

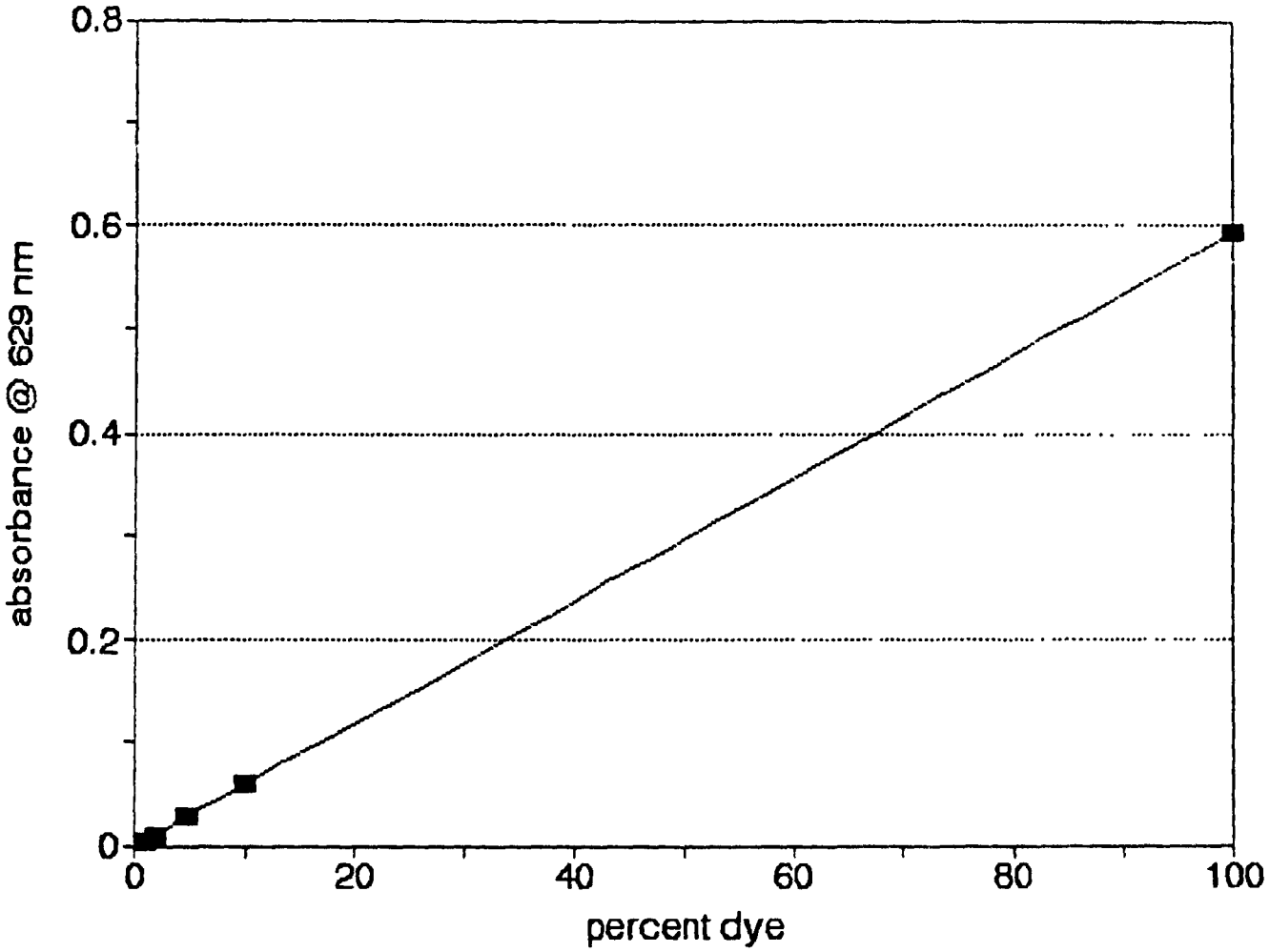

FIGURE 4.7: Calibration curve for the dilution of blue dye.

harvest was measured in the spectrophotometer. Samples from subsequent rinsing harvests were then analyzed for residual blue dye. Measurements were taken up to four cycles after the initial dye cycle. Regular and alternating cycle sampling patterns were tested. Each test was done in triplicate. Results were reported as percent of original dye concentration remaining.

RESULTS:

5.1 Fermenter development:

5.1.1 Volume division:

The reactor design was tested for its accuracy and consistency in removing half of the fermentation broth. Figure 5.1 shows the results of tests done during two different experiments. One set of points are the harvest volumes of an SCF growing B. Subtilis on Sheppard's medium. The other two sets of points show the dosing mass and the harvest volume of an SCF simulated with water.

The three different volume levels produced during the experiment with microbes present were produced at three different aeration rates. This particular experiment was conducted without the inlet air humidifier. The data from the SCF simulation were generated without air flow through the reactor.

A dilution test was done to verify that the volumes removed were actually half the operating volume. Figure 5.2 shows the absorbances of blue dye for successively diluted harvests. It can be seen that the dilutions fall along a straight line--corresponding to accurate 50 % dilutions on each cycle.

5.1.2 Sample differentiation:

The sampling system developed for the fermenter was tested to find out how much mixing was taking place between sequential samples. Figure 5.3 shows the results of experiments done to

simulate the mixing of liquid samples from one cycle to the next. Two modes of sampling operation were compared: sampling every cycle and sampling every second sample. The figure shows the percent carryover from one sample into the next two or four samples. The points shown in the figure represent average values from several tests. Error bars represent three standard deviations. For both regular sampling and intermittent sampling the mixing was on the order of two percent carryover from one cycle to the next sample. The next two samples had very little carry over--less than one percent. It was found that the fourth sample after starting the test had a carry over on the order of two percent. These results are as would have been expected from the older system which did not use an accumulator bottle or waste overflow.

5.1.3 Dissolved oxygen measurements:

Figure 5.4 shows a representative strip chart trace of a DO measurement over several cycles of an SCF. This trace was from an experiment growing B. subtilis on Sheppard's medium. It can be seen by its amplitude that the DO measurement was sufficiently responsive to enable feedback control.

5.1.4 Oxygen transfer coefficient:

$K_l a$, the effective oxygen transfer coefficient, was measured in the stirred tank fermenter at different agitation and air flow rate conditions. The results of the tests are shown in figure 5.5. The vertical axis of the graph shows the $K_l a$ value in $hours^{-1}$. The

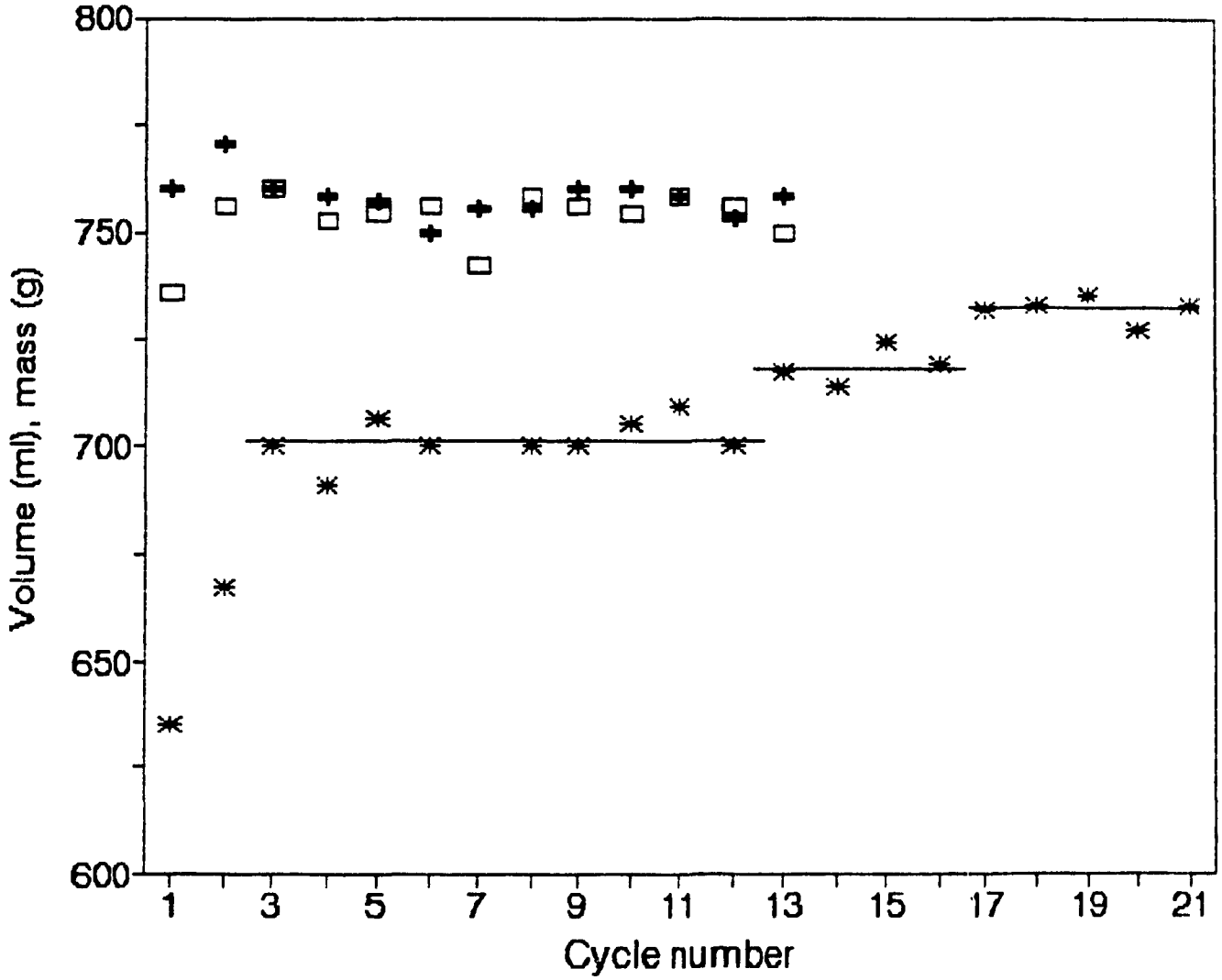

FIGURE 5.1: Successive harvest and dosing volumes for actual and simulated SCF experiments. (✳) growing SCF at three different aeration rates, (✚) simulation harvest volumes, (□) simulated dosing masses.

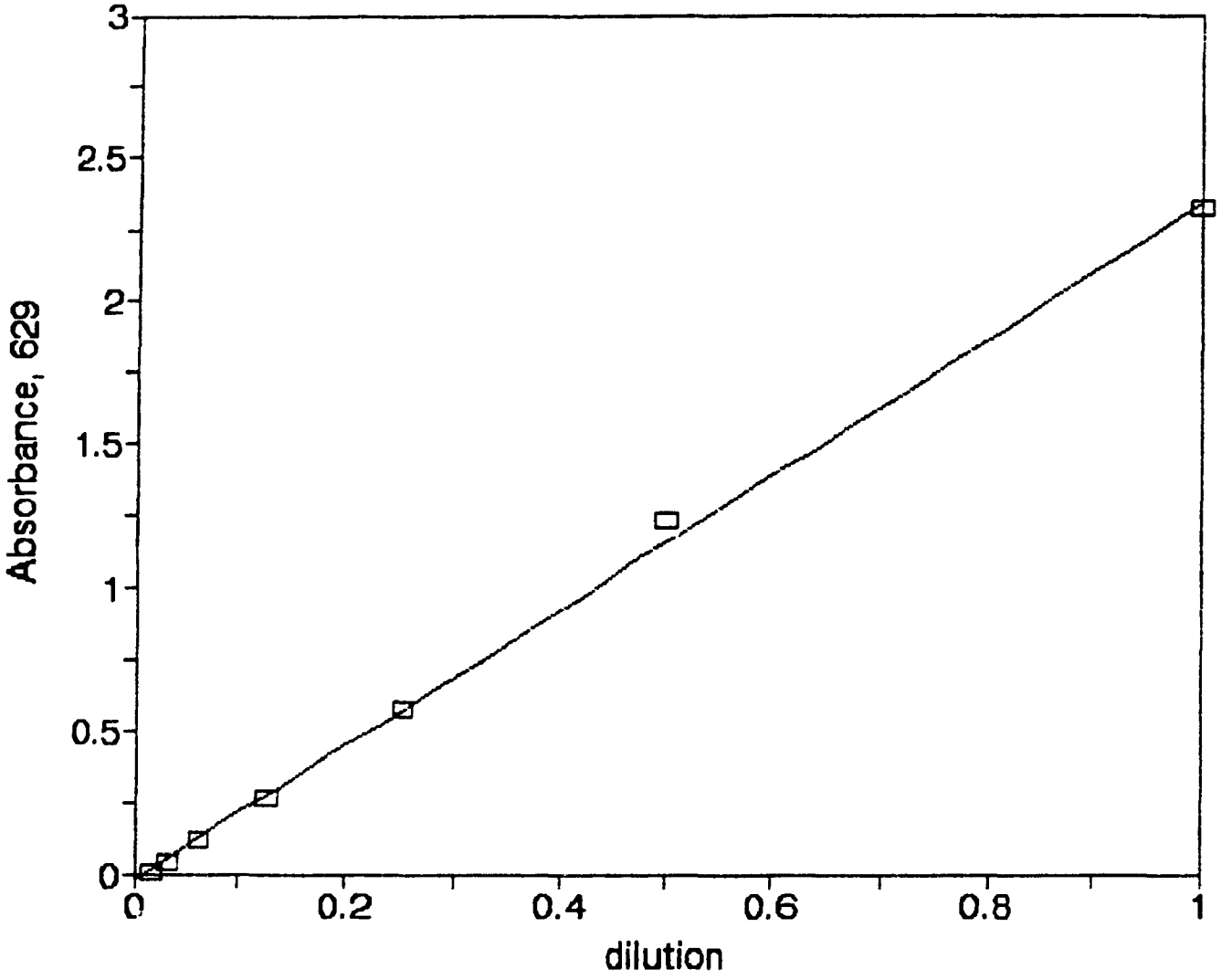

FIGURE 5.2: Absorbance of blue dye in successive harvests taken from a simulated cycling experiment.

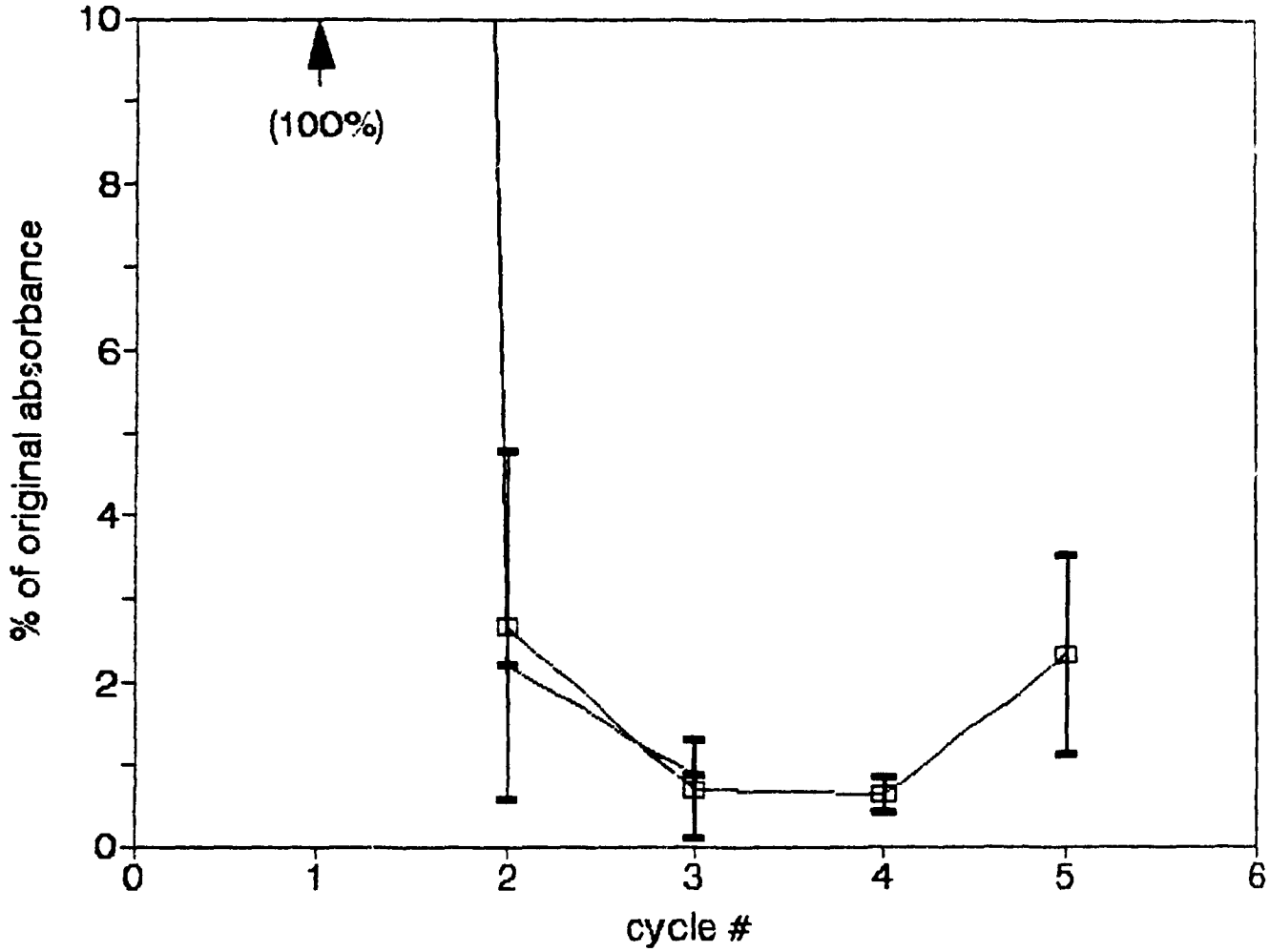

FIGURE 5.3: Carry over of liquid from one sample to subsequent samples. (□) regular sample sequence (✚) skipped sample sequence. Error bars represent three standard deviations.

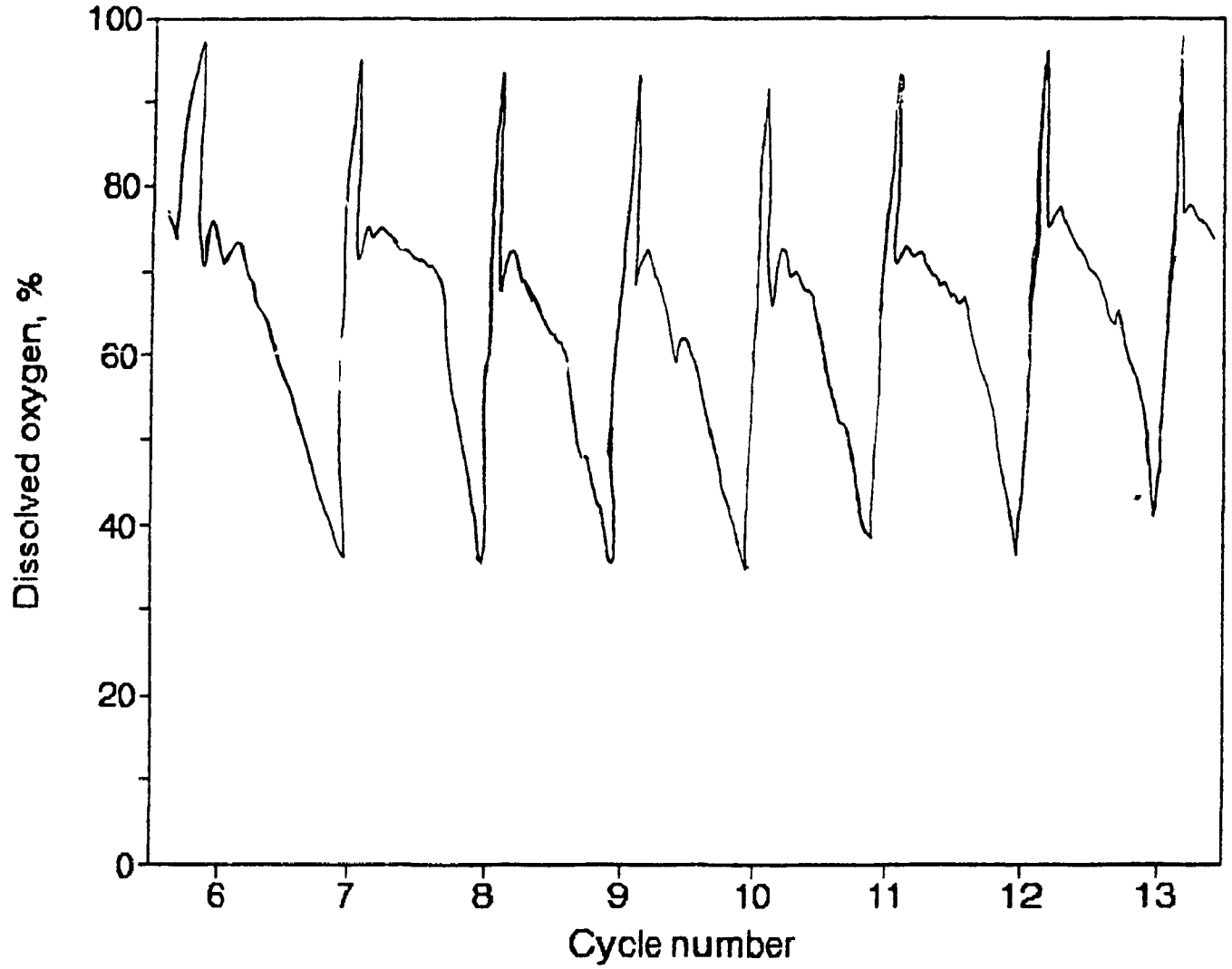

FIGURE 5.4: Dissolved oxygen readings during an SCF in a stirred tank fermenter. B.Subtilis growing on Sheppard's medium.

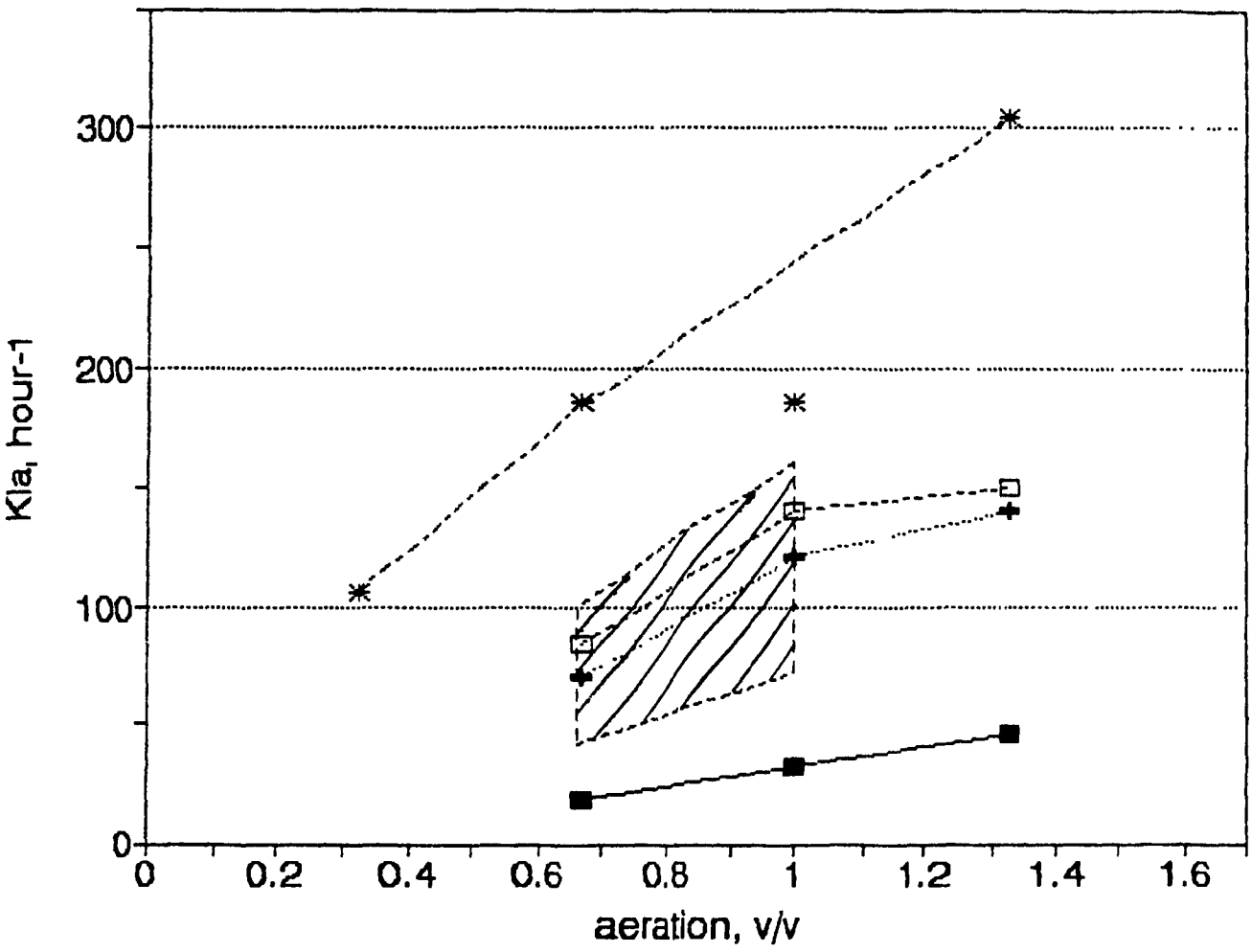

FIGURE 5.5: Values of Kla at different agitation and aeration rates. (✳) agitation = 800 rpm, (✚) agitation = 550 rpm, (■) agitation = 250 rpm, (□) cyclone fermenter with 1000 ml working volume, (▨) usual operating range for stirred tank.

horizontal axis shows the different aeration rates in volume of air per volume of water per minute. This ratio corresponded to different air flow rates with the volume of water in the reactor kept constant at 1.5 litres. The different plots represent different agitation rates of 250, 550 and 800 rpm. K_la measurements were done at each agitation rate with three different air flow rates: 0.67 v/vm, 1.0 v/vm and 1.33 v/vm. An additional measurement was taken at a lower aeration rate of 0.33 v/vm. It was done only with the highest agitation level because the response of the system was too slow at the lower agitation levels.

Figure 5.5 shows the usual range of operating conditions for the SCF experiments. The agitation rates used for the SCF varied between 310 and 600 rpm. The air flow rates varied between 0.67 and 1.0 v/vm. Therefore the K_la during experiments can be estimated to have ranged between 40 and 160 hours^{-1}. The experimental K_la results are for a pure water system. The actual K_la values during a fermentation would differ because of different surface tension, viscosity, temperature, pressure or other factors.

5.1.5 Power and air requirements:

The power used by the reactor for agitation is shown in figure 5.6. The horizontal axis shows power in watts delivered into the liquid as agitation. The power values were normalized to units of watts per litre to allow for comparison to the cyclone reactor. The operating volume for the tests was 1.5

litres. The vertical axis shows the observed oxygen transfer. The tests were done at four different air flow rates. The power added by air compression was calculated to be approximately 0.33 watts and was omitted from the information contained in figure 5.6. The figure shows that the K_1a is directly affected by power input.

The relationship between power input and agitation rate is shown in figure 5.7. As expected, there is a close correlation of power input to RPM. The points on this graph represent power required at an aeration rate of 1 v/vm. Changing the air flow had only a minor effect on the relationship of agitation rate to power input.

The power required for the pump of the cyclone reactor was also measured. The results of these tests are shown in figure 5.6. The different K_1a points represent different operating volumes and correspondinglydifferent liquid to air volume ratios. The amount of air used was 1 litre per minute for each operating volume. It can be seen that there is a nearly direct correlation between cyclone power consumption per litre and K_1a.

5.2 Biological measurements:

5.2.1 Repeatability of cycles:

The stability of an SCF can be determined by observing the changes in cycle time and biomass from cycle to cycle. Figure 5.8 shows the biomass and cycle time values for an experiment using A. calcoaceticus growing on hexadecane. It can be seen

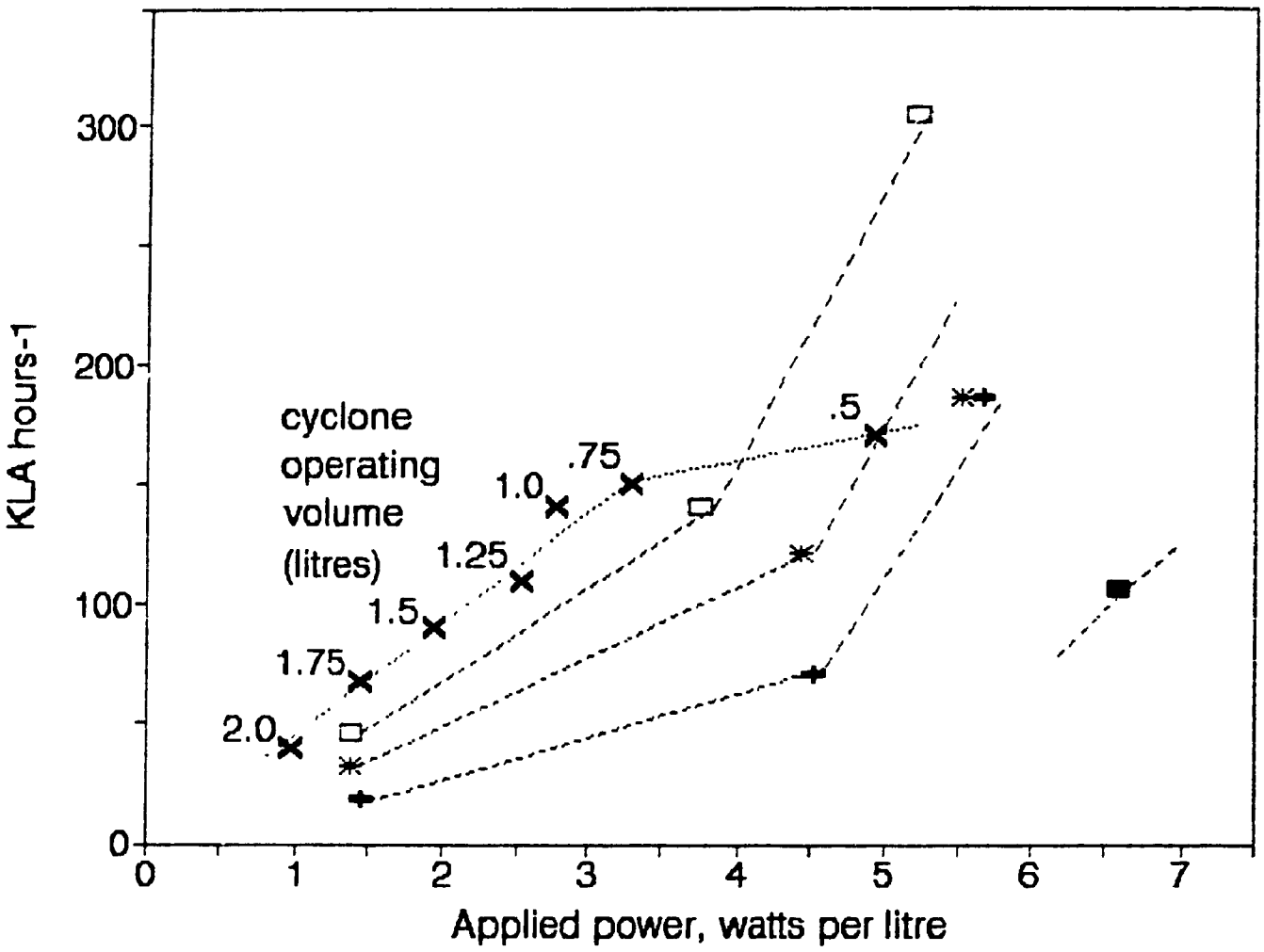

FIGURE 5.6: K_1a values delivered at different agitation power inputs. (□) aeration = 1.33 vol/vol, (✳) aeration = 1.0 vol/vol, (✚) aeration = 0.67 vol/vol, (■) aeration = 0.33 vol/vol, (✕) cyclone fermenter at indicated operating volumes. Cyclone K_1a values from Sheppard and Cooper, 1990c.

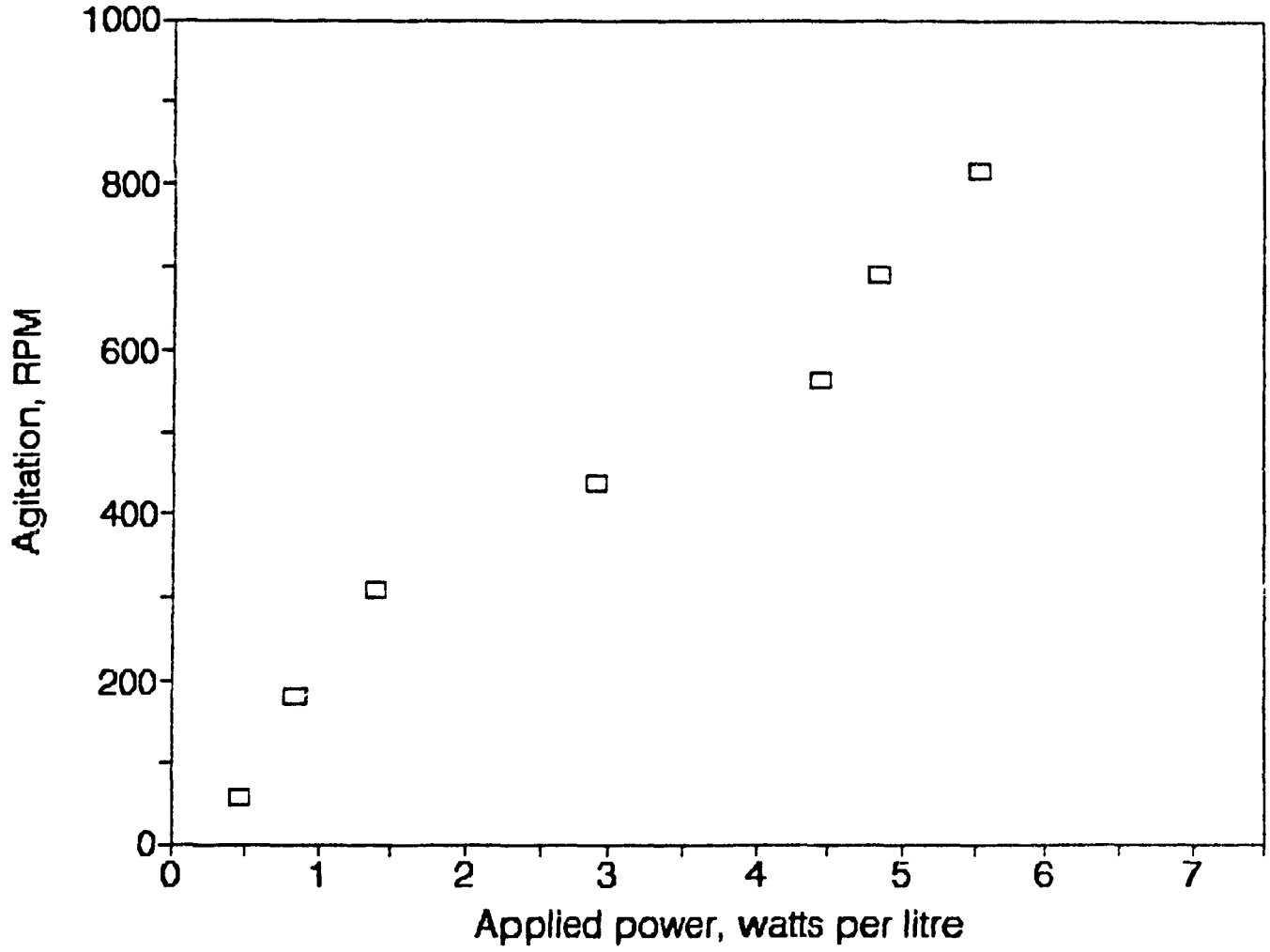

FIGURE 5.7: Agitation RPM versus applied power per litre in a Multigen stirred tank fermenter. Operating volume 1.5 L of water.

that once steady state was achieved, the biomass and cycle times were quite constant. Replicates were taken of each biomass reading. Error bars on the biomass values represent three standard deviations. Biomass measurements were not taken for the later cycles in this experiment.

5.2.2 Cycle time:

Table II shows average cycle time values for repeated cycles of B. subtilis and A. calcoaceticus growing on various media in different experiments. Not surprisingly, the cycle times were found to be different for different organisms and different substrates. Cycle times usually showed very good reproducibility. Once steady state cycling was achieved, standard deviation in cycle time from cycle to cycle was on the order of 5 to 10 percent.

5.2.3 Biomass:

Table II shows average biomass values for repeated steady state cycles of B. subtilis and A. calcoaceticus growing on various media in different experiments. A. calcoaceticus gave higher biomass growth on its given medium.The standard deviation of the biomass values were on the order of 10 to 25 percent. The larger biomass measurements of A.calcoaceticus had better repeatability than the measurements of B.subtilis.

5.2.4 Residual substrate:

The residual substrate measurements verify that the limiting nutrient is exhausted by the end of a growth cycle. Figure 5.9 shows the results of two experiments. One experiment

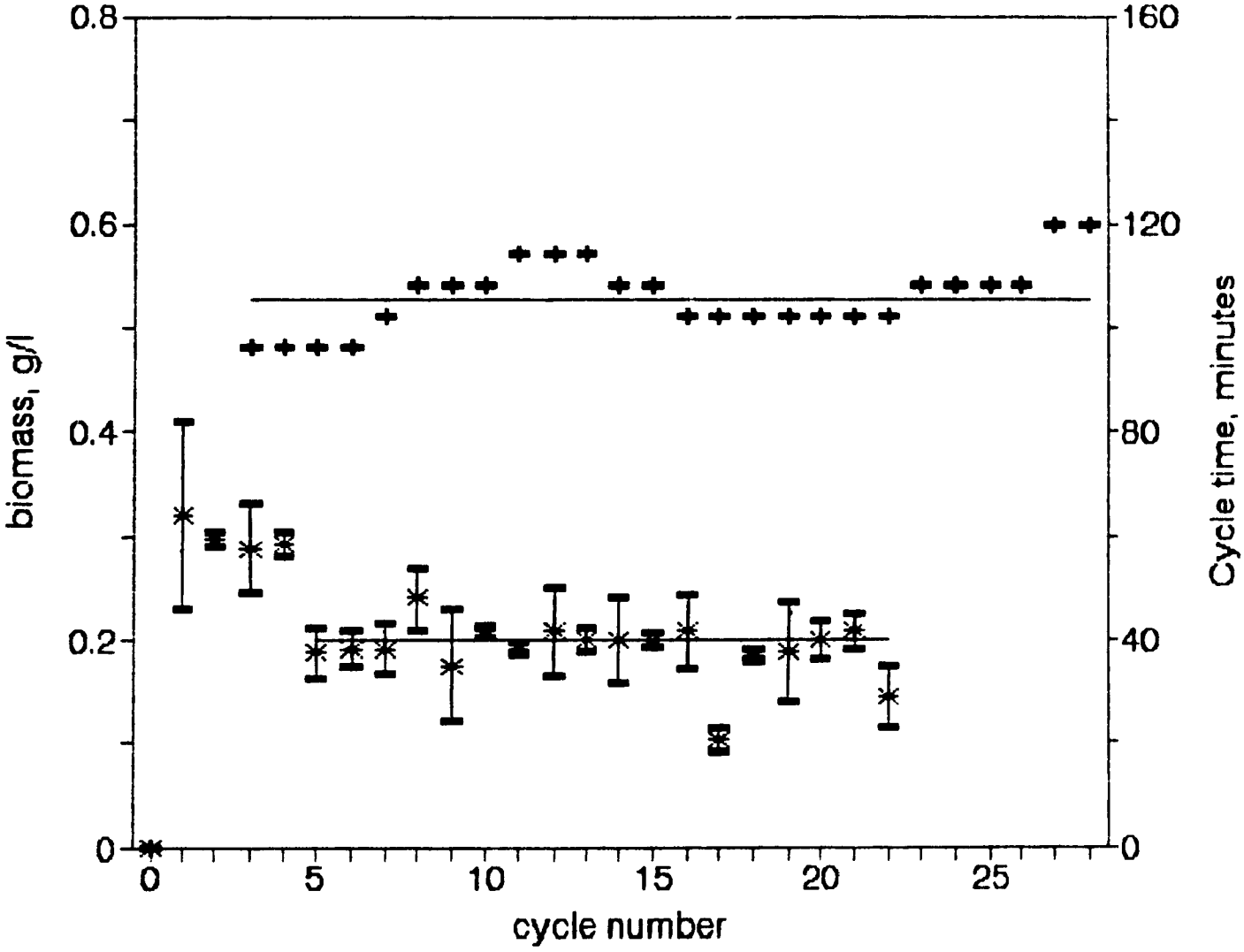

FIGURE 5.8: Biomass and cycle time values during an SCF in a stirred tank. (✳) biomass, g/L, (✚) cycle time, minutes. Error bars represent three standard deviations.

TABLE II: Biomass, cycle time and productivity for different organisms and growing conditions.

Organism Medium	Experiment L=late E= early	Biomass (g/L)	std. dev.	Cycle time (minutes)	std. dev.	Surfactin (CMC^{-1}) or Emulsan (g/L)	std. dev.
<u>B. Subtilis</u> Sheppard medium	Ex 4	0.26	0.04	110	9.7	2.2	0.85
	Ex 7	0.19	0.03	105	5.0	0.5	0.14
	Ex 8	0.15	0.06	8[illegible]	13.1	0.4	0.12
secondary cycle <u>B. Subtilis</u> Sheppard medium	Ex 7			211	10.0	4.5	1.8
	Ex 8			176	23.2	1.0	1.2
<u>A. Calcoaceticus</u> Brown medium hexadecane	Ex 9	0.83	0.05	54	5.3	0.04	0.008
	Ex 10	0.60	0.09	57	4.9		
	Ex 12L	0.70	0.01	69	3.1		
<u>A.Calcoaceticus</u> Brown medium ethanol	Ex 11E	0.30	0.04	157	14.8		
	Ex 11L	0.41	0.03	80	5.0		
	Ex 12E	0.23	0.03	159	22.6	0.01	0.002

was with B. subtilis on a nitrogen limited glucose medium, the other was A. calcoaceticus on a carbon limited hexadecane medium. The two plots are of the residual nitrogen and hydrocarbon concentrations for several cycles of each experiment. As can be seen from the plots, for most cycles the residuals were between zero and six percent of the initial substrate concentration.

Glucose measurements were also taken during the B.subtilis fermentations. The glucose concentration was decreased to approximately 80 percent of its original level.

5.2.5 Change in substrate concentration:

Figure 5.10 shows the cycle time and biomass of sequential cycles in an experiment growing A. calcoaceticus on ethanol. The ethanol concentration was switched in a step change from 0.08 percent by volume to 0.04 percent. After 12 cycles it was brought back up to 0.08 percent. The SCF responded by halving the biomass from 0.4 g/L to 0.2 g/L and then coming up again to its original level. Biomass measurements were done in duplicate. Error bars represent three standard deviations. The cycle time showed no corresponding shift over the same period.

5.2.6 Change in substrate:

Figure 5.11 shows the response of an SCF growing A. Calcoaceticus. In this experiment, the culture was started on ethanol at a concentration of 0.08 % volume per volume. On cycle 12 this was switched to hexadecane at 0.05 % volume per volume. These two carbon sources provided the same amount of carbon on

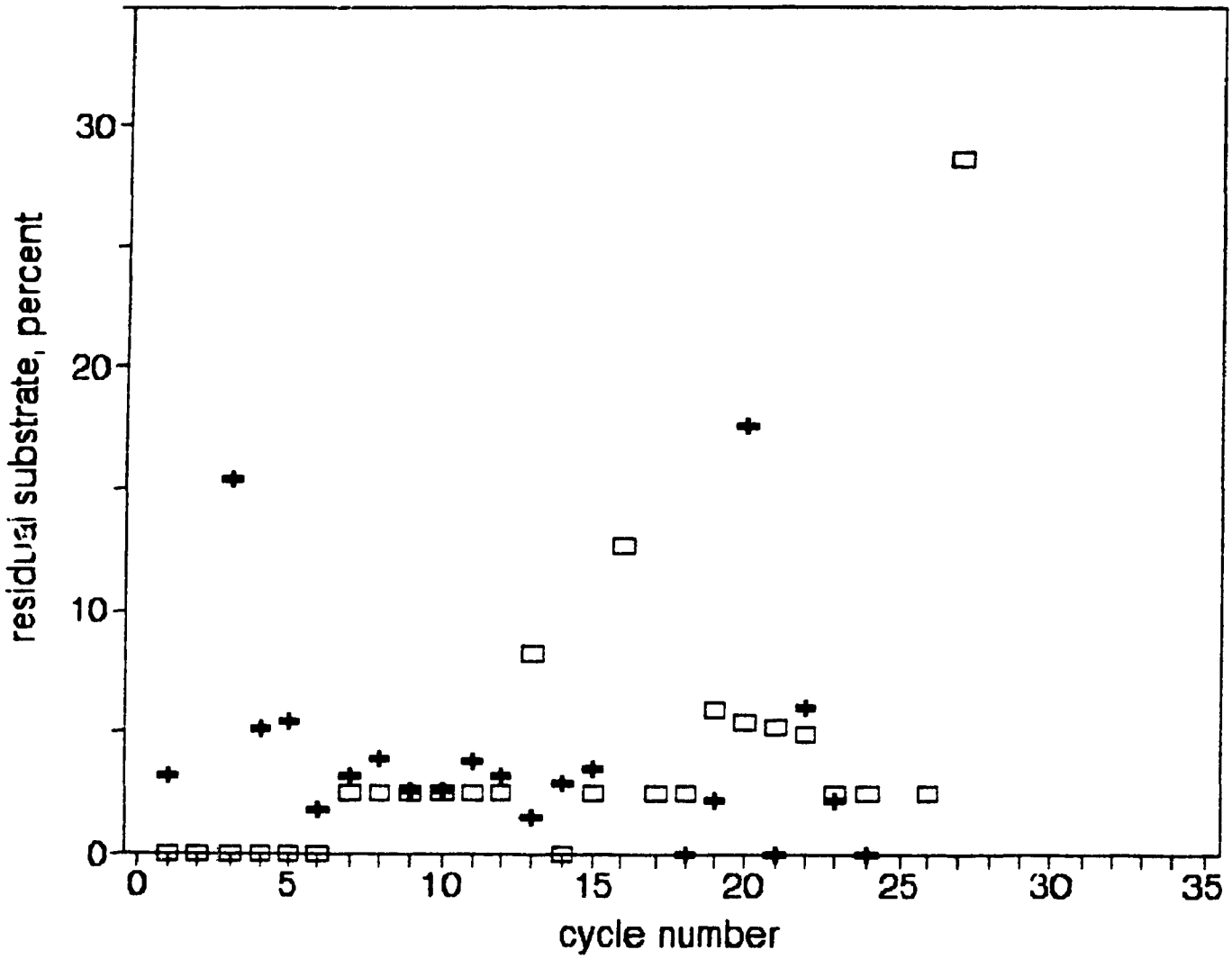

FIGURE 5.9: Residual limiting substrate concentrations at the end of SCF cycles. (□) A.Calcoaceticus on hexadecane (✚) B. Subtilis on nitrogen.

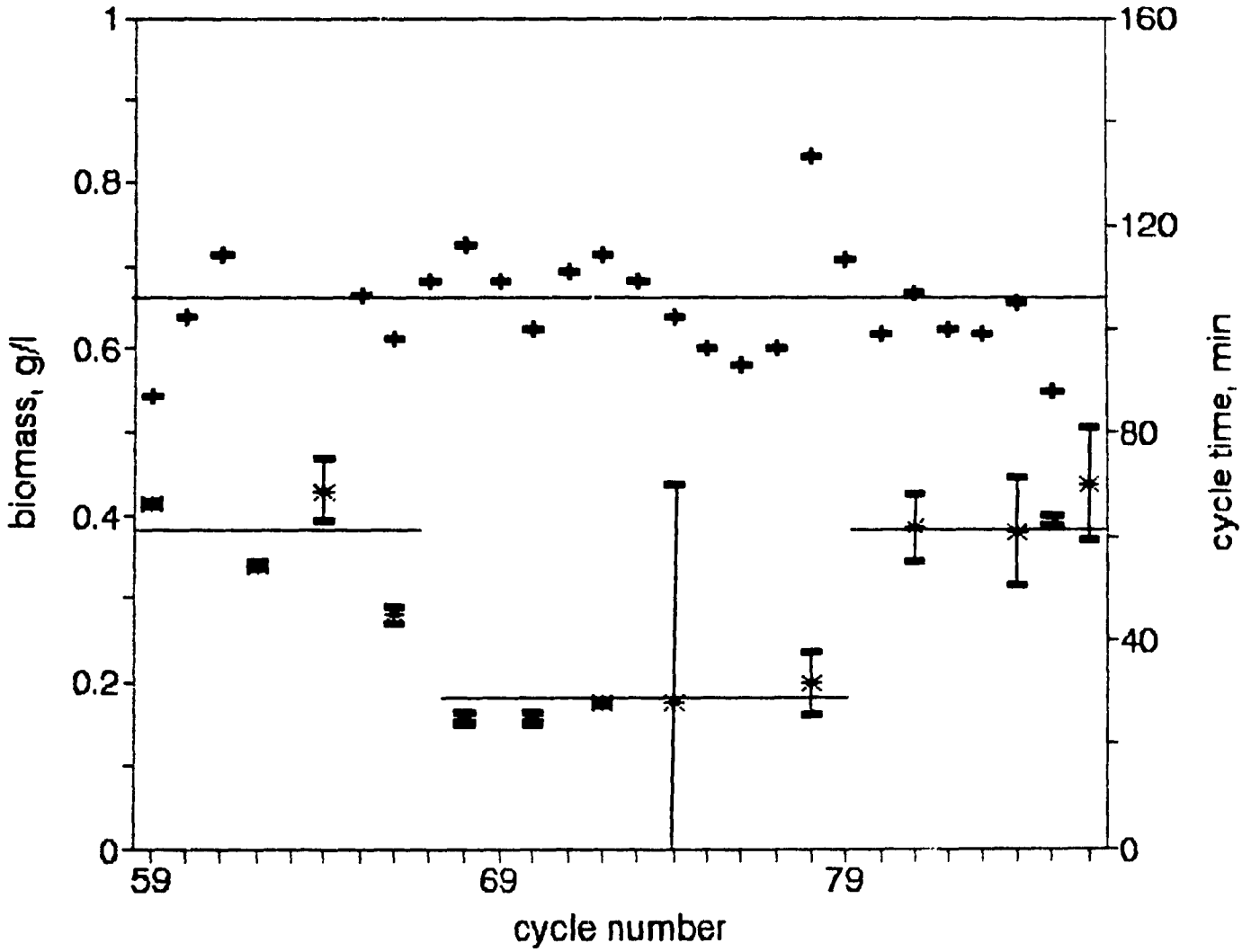

FIGURE 5.10: Variation of biomass and cycle time in response to a step change in limiting nutrient concentration. (✱) biomass, g/L, (✚) cycle time, minutes. Error bars represent three standard deviations.

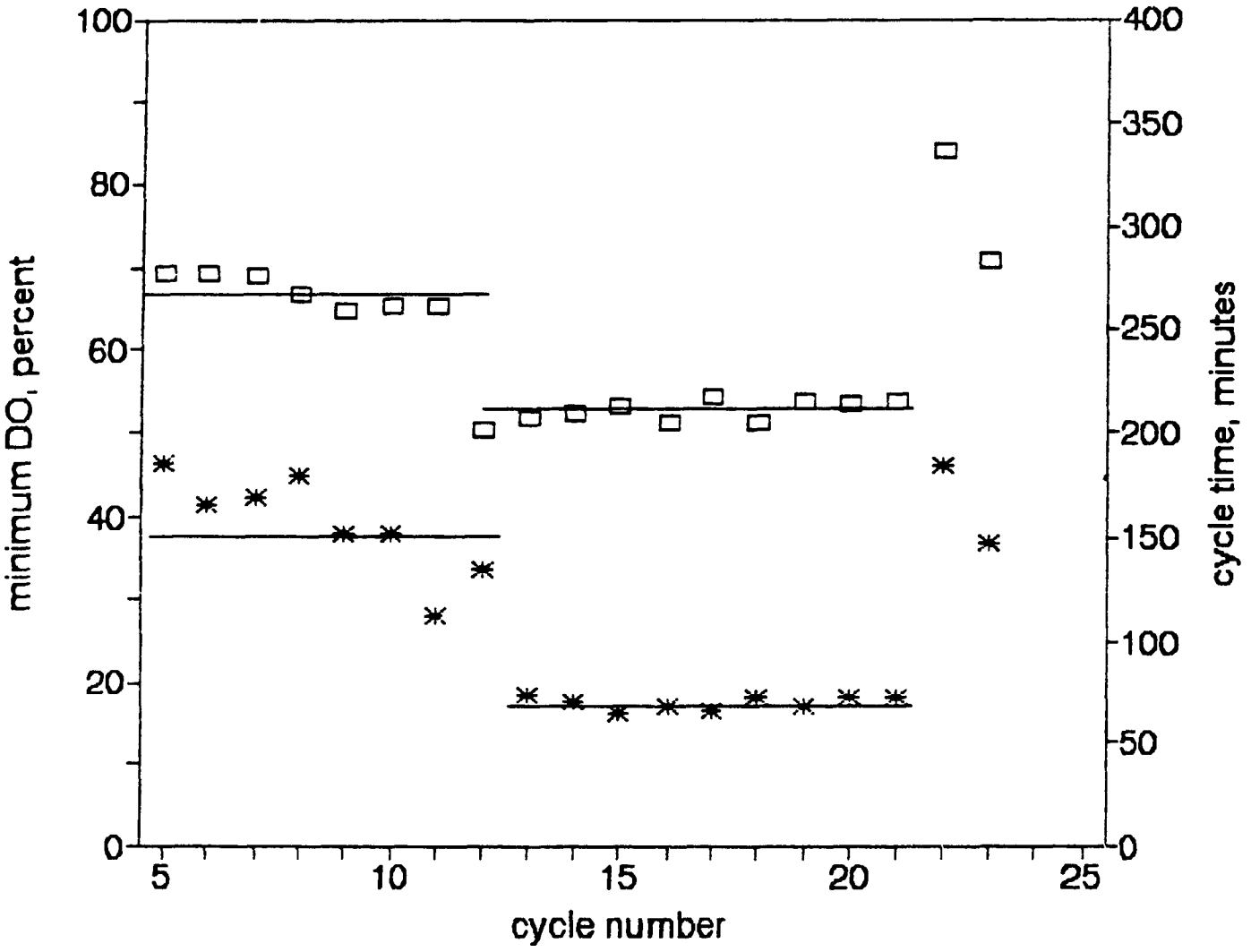

FIGURE 5.11: Response of the cycle time and minimum dissolved oxygen levels in an SCF growing A. Calcoaceticus to a change from ethanol to hexadecane carbon sources. (□) cycle time, minutes, (✳) minimum dissolved oxygen, percent.

a molar basis. It can be seen from the figure that the equilibrium of the system shifted. The culture started growing faster with the switch to hexadecane, reducing the cycle time. It respired more vigorously, lowering the minimum DO level.

5.2.7 Productivity:

Table II shows average product production rates for repeated cycles of B. subtilis and A. calcoaceticus growing on various media in different experiments. Standard deviations for productivity were on the order of 30 percent for B.subtilis and 20 percent for A.calcoaceticus. As shown on the table, growing B subtilis with a secondary cycle tends to increase the surfactin production. Figure 5.12 shows data from an experiment in which samples were taken before and after the secondary cycle. Although there is considerable scatter in the data from the secondary cycles, the increase in productivity is clearly visible as a difference between a regular and a secondary cycle.

5.3 Exhaust gas analysis:

5.3.1 Exhaust gas measurements:

Figure 5.13 shows curves similar to a typical DO trace from an SCF. These curves were generated from the exhaust gas analysis of an SCF growing A.calcoaceticus on hexadecane. The three traces are the oxygen concentration in the exhaust gases, the carbon dioxide concentration in the exhaust gases and the dissolved oxygen concentration in the fermenter. It can be seen

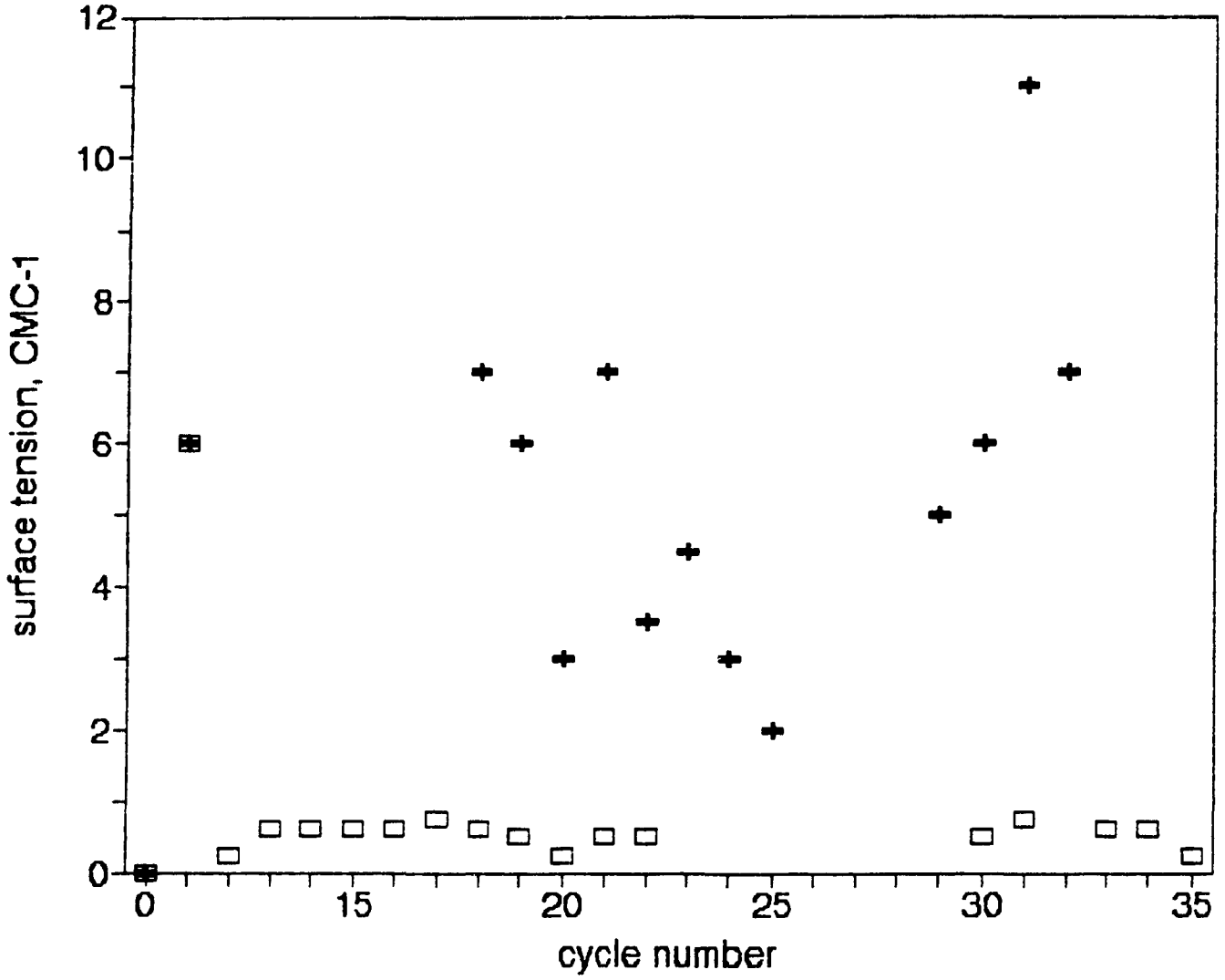

FIGURE 5.12: Results of surfactin production before and after secondary cycles. (□) regular cycle CMC^{-1} (✚) secondary cycle CMC^{-1}.

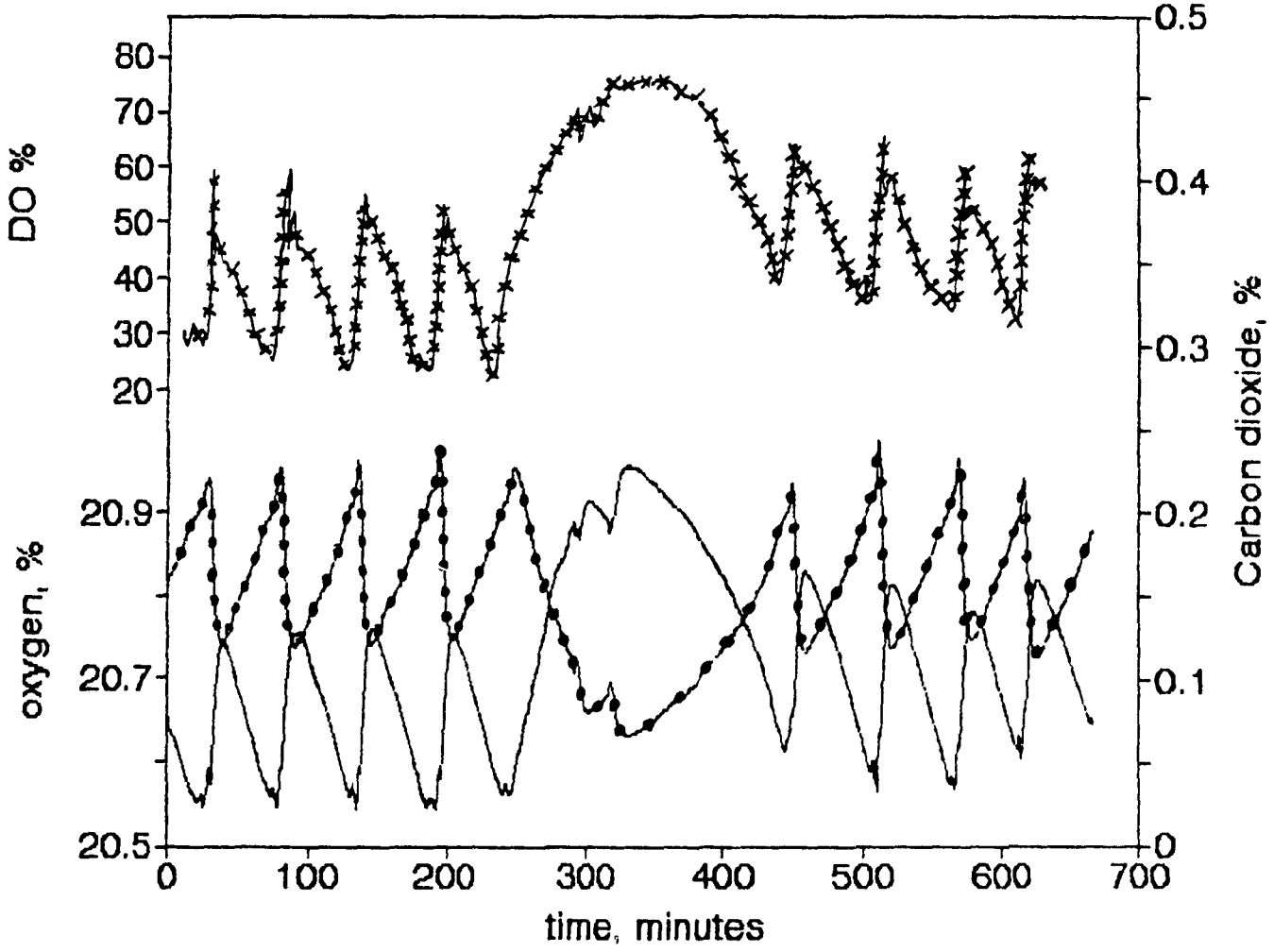

FIGURE 5.13: Oxygen and Carbon dioxide concentrations in fermenter exhaust gasses. (——) oxygen (•—•) carbon dioxide (*—*) DO

that the DO and the oxygen concentrations changed in the same manner. The carbon dioxide concentration inversely mirrored the other two. Both gas measurements showed appreciable variation in magnitude during a cycle. Either one would have had sufficient resolution to function as a feedback control signal. For this experiment, the response time of the analyzer was slower than the DO probe by about four minutes. This is visible as a phase shift between the DO and the gas curves. The analyzer signal did not suffer from noise.

5.3.2 Carbon balance:

For experiments where both the gas analyzer and biomass readings were taken over several cycles, carbon balances were calculated. Table III shows the results of one of these experiments. This experiment was done using A. calcoaceticus growing on ethanol. The gas analyzer could log data for periods of time up to 24 hours. Because of this, the carbon balance calculations were done on the groups of cycles corresponding to each analyzer logging cycle. The average closure of the balance was on the order of 12 to 15 percent of the supplied carbon unaccounted for.

TABLE III: Results from carbon balance experiments done on A.calcoaceticus growing on ethanol.

	avg. grams carbon per cycle			combined cycle	total grams carbon		Carbon "lost"
cycles tested	EtOH	Biomass	Carbohydrate	CO2	IN	OUT	percent
4-9	0.25	0.12	0.001	0.32	1.29	0.95	26.5
9-18	0.25	0.12	0.001	0.66	2.38	1.82	23.6
18-29	0.25	0.15	.0008	0.77	2.68	2.37	11.6
29-45	0.25	0.14	.0007	1.16	3.88	3.36	13.2
51-63	0.25	0.16	.0006	1.10	3.12	3.01	3.50
65-76	0.13	0.07	.0004	0.59	1.59	1.48	7.25
79-90	0.25	0.14	.0007	0.98	3.32	2.80	15.6

DISCUSSION:

6.1 Characteristics of the new apparatus:

To date, all work in self-cycling fermentations has been done using a cyclone column fermenter. Although the cyclone column fermenter works very well, it is seldom used in fermentation research. The intention of this study was to determine if SCF's could be successfully implemented in the common stirred tank type of fermenter. This would allow researchers in other laboratories to use the SCF technique with minimal modifications to existing equipment.

The design of the new apparatus was based on a commercial stirred tank fermenter. The design appropriated many features of the existing cyclone SCF apparatus. Aspects of the apparatus which were different from the cyclone design were tested to verify their ability to perform the tasks required of them.

6.1.1 Harvest system:

In switching from the cyclone fermenter to a stirred tank design, the harvesting system was changed. The cyclone fermenter had used a balance to measure the amount of harvest removed from the fermenter. The stirred tank design made use of hydraulic equilibration for harvesting the broth.

In using the balance to measure the harvest, the cyclone SCF had suffered difficulties in maintaining a steady state operating volume. It had been found that the volume in the reactor would steadily change from cycle to cycle if the

fermenter was operated such that the measured input dosing mass was equal to the measured output harvest mass (Brown, 1991). Changes in density between dosing and harvesting and loss of moisture in the reactor meant that the required harvest mass was not exactly equal to the dosing mass. In addition, liquid hold up in various lengths of tubing that were not supported by the scale meant that the measured mass of a dosing or harvest volume was not completely representative.

To compensate for this volume drift, the cyclone control program had correction factors added into the balance readings. These factors were adjusted by trial and error to suit a given experiment. Even with these correction factors, some occasional manual volume adjustments were still necessary (Brown, 1991).

In view of this difficulty with volume control, the harvesting system was changed in the new design. Hydraulic equilibration was an attractive method because of its inherent simplicity. It used no control equipment other than a valve and a time delay. It had the advantage of being an intrinsically stable control method--its volume was self adjusting. Provided that it was set up correctly, it would consistently remove half of the volume of the fermenter. This would be true no matter what the dosing volume or the working volume of the fermenter.

In practice the hydraulic equilibration system lived up to the theoretical predictions. It was simple and reliable. The self adjusting control of the harvest system assured that the steady state harvest volume of the apparatus would be equal to

the dosing volume. Figure 5.1 shows the consistent volumes removed by the hydraulic system. The slight oscillations in harvest volume were no larger than the oscillations in the dosing mass, which was still measured out by means of a balance. To assure in addition that the harvest volume was also half the operating volume, dilution tests were done. The results of a test using blue dye are shown in figure 5.2. The dilution points are plotted at their theoretical value of 50 % dilution. The linear alignment of the dye concentrations with the theoretical dilutions confirmed that the dilution of each cycle was indeed 50 %.

The time required for the hydraulic harvest was slightly greater than that used for the cyclone mass harvest. Harvesting 500 ml from the cyclone required about 80 seconds. Harvesting 750 ml from the stirred tank required 120 seconds. The extra time was needed partly for the extra volume, but mostly because flow from one vessel to the other became slower as the system approached equilibrium. It was found by that 120 seconds were sufficient to ensure that the volume harvested was constant.

Some problems did arise from the hydraulic harvesting. One was that the overhead pressures in each of the two vessels had to be exactly the same. In some experiments, high foam production restricted flow through the vent connecting the two vessels. This created a pressure differential of several inches of water and resulted in extra large harvest volumes. This problem was regulated by installing an oversized vent tube and

connecting a second vent with an overhead valve. This valve would be open only during the harvest.

Another problem was that the harvest vessel had to be installed at precisely the right height to give proper halving of the operating volume. The height was checked by isolating the two vessels and filling each one with identical volumes of water. The liquid level in each vessel was marked. The harvest valve was then opened to let the two levels equilibrate. By adjusting the height of the harvest vessel, the liquid level in the two vessels could be brought back to the original markings. This was difficult to do under sterile conditions; a final check of the harvest calibration was not done after the final sterile assembly.

6.1.2 Sample system:

The sampling system that had been developed for use with the cyclone fermenter was modified to reduce the size of the sample. This was done because the harvest volume of the stirred tank was larger than that of the cyclone. Storing entire harvests as 750 ml samples would quickly use up the available space in the refrigerator. The analyses done on the samples did not require more than 100 ml. The final sampling system sampled a volume of 150 ml out of the harvest.

The modification consisted of an accumulator installed at the end of the sample header. The accumulator would hold up 150 ml of the harvest while allowing the rest of the harvest to overflow to a waste container. The accumulator and overflow were

situated at the exit end of the header so as to allow for a flushing of the sample system by the incoming harvest (see figure 2.3).

Since the total harvest volume was 750 ml and the accumulator held only the last 150 ml, the first 600 ml of the harvest were used as a flush to sweep clean the sample system. The effectiveness of this flushing is shown in figure 5.3. The carry over from one sample to the next was about 2 %. This error did not rise appreciably in cases when the sampling of the harvest was omitted intermittently. In these cases the omitted samples, which remained in the header for the duration of a complete cycle, were flushed out nearly as well as the residue remaining from of a regular cycle.

Figure 5.3 also shows results of carry over into the second, third and fourth following cycles. It can be seen that the second and third samples show little carry over. The fourth cycle shows an increase in sample carry over. This was presumably because the cycle falling fourth after the initial cycle used the same sample valve and sample tubing connection as the initial cycle. By sharing more tubing, the hold up and resulting carry over was greater than that observed in the second and third cycles.

Having smaller samples meant that they were cooled down more quickly in the refrigerator. This was an improvement over the old system since several of the analytical tests done were time sensitive. A rapidly cooled sample was more representative

of the actual reactor conditions.

The overflow system allowed for the voluntary omitting of samples. This was useful for rapidly cycling experiments where it was sufficient to take only every second or third sample. Having a large waste carboy also allowed for the experiment to continue even if all the sample bottles were full. Without the overflow and waste carboy, full sample bottles would have overflowed if the researcher failed to replace them. With the accumulator and overflow system the computer was able to discard samples if there was no sample bottle available. This resulted in the loss of a few samples but allowed for the continuation of the experiment.

6.1.3 Oxygen transfer:

Because an SCF is controlled by the level of dissolved oxygen in the fermenter, it was crucial to verify that the oxygen transfer rate of the apparatus was suitable for SCF's. This was of particular concern since the cyclone fermenter is considered to have especially good oxygen transfer qualities. Figure 5.5 shows the results of K_1a experiments done on the stirred tank filled with water. Also plotted on figure 5.5 are the K_1a results found by Sheppard (Sheppard, Cooper, 1990c) for the cyclone fermenter. The plot of the cyclone K_1a falls close to the plot of the line representing an agitation rate of 550 rpm, which was representative of the typical stirred tank SCF experiment. The stirred tank was found to be capable of operating in the same oxygen transfer regime as the cyclone

fermenter.

The testing methods used to determine the K_1a in the two reactors were basically the same. Both tests were done using water. Both tests were done by lowering the DO level to zero using sulphite and then aerating the reactor. The different aeration rates for Sheppard's experiments were found by varying the operating volume of the cyclone and keeping the air flow rate constant at one litre per minute. This was the inverse of how the tests were done on the stirred tank, where the water volume was kept constant and the aeration rate was changed. The resulting air to water ratios were the same. The agitation in the cyclone, that is to say the pumping rate, was unchanged between volume settings.

The fact that the K_1a values for the two reactors were found to be similar was not accidental. The operating agitation and aeration rates of the stirred tank were determined by conditions that gave a DO signal that was suitable for control. The same was true for the earlier cyclone experiments. From these corroborative results it may be concluded that for a rapidly growing organism such as A. calcoaceticus or B. subtilis, a K_1a value of 100 to 150 hours^{-1} is required for DO control in an SCF. Note that the agitation rate needed to equal the performance of the cyclone was in the upper half of the operating range of the stirred tank fermenter.

6.1.4 Power requirements:

There has been some discussion about the power requirements

of the cyclone fermenter as compared to the stirred tank. It has been generally claimed that the stirred tank requires less energy than the cyclone column, but that the cyclone delivers superior oxygen transfer (Sheppard, 1989, Brown, 1991). Some tests were conducted on both apparatus to determine which reactor gave better oxygen transfer per litre of operating volume per watt of power. The power requirements were calculated as the net power applied to the liquid. The values for net power were derived by subtracting power requirements of the fermenters running empty from the power consumed running at operating volume. It was found that the cyclone was more energy efficient in driving oxygen transfer.

Figure 5.6 shows the power usage per litre of operating volume by the two types of fermenter. It was found that K_1a and power input per litre were strongly related in the stirred tank. This was also true for the cyclone, but for different reasons.

In the stirred tank, the total power requirements are correlated to the agitation. As agitation increases, so do the total power requirements. This is illustrated in figure 5.7. The operating volume in the reactor was held constant for all the tests. Since the operating volume was constant, the ratio of power to operating volume followed the same trend as the total power requirements--it increased with increasing agitation. Therefore, for the stirred tank, the correlation between the K_1a and the ratio of power to operating volume resulted from changing agitation rates.

For the cyclone reactor, the pump speed (the agitation) was not adjustable and was kept constant. Different K_1a values were obtained by using different operating volumes instead of different agitation rates. Because the pump speed was constant, the total power required by the cyclone pump was close to being constant--it varied only slightly with the operating volume of the reactor. Since the total power usage changed very little, the ratio of power to operating volume changed mostly as a function of the changing volume. Therefore, for the cyclone the correlation between the K_1a and the ratio of power to operating volume resulted from changing operating volume.

As can be seen in figure 5.6, the cyclone delivered more K_1a for less power in all but the most extreme operating conditions. Only at high agitation and air to liquid volume ratios did the stirred tank perform more efficiently.

The typical operating volume for the cyclone during an SCF was about one litre with an air to liquid volume ratio of 1.0. According to the observed results, it would normally have consumed about 2.7 watts per litre. The stirred tank required 4.5 watts per litre to deliver the same K_1a at the same air to liquid volume ratio. Thus on the laboratory scale, the stirred tank SCF required more power than the cyclone.

The total aeration power requirements should also include the power used for air compression. This power was not measured but was estimated from simple physical laws. The theoretical amount of power required for air compression was less for the

cyclone. This was because the cyclone air enters the reactor at approximately atmospheric pressure. The air injected in at the bottom of the stirred tank necessarily has a pressure at least equal to the head in the fermentation vessel. Thus, the stirred tank compressor is forced to work harder.

The question of power requirements is not of great concern at the laboratory scale of operation. For scale up of SCF processes, however, it may be necessary to further investigate the trade offs between oxygen transfer and power requirements when selecting the type of culture vessel.

6.2 Self cycling in a stirred tank fermenter:

The new apparatus successfully performed self cycling fermentation. The distinguishing characteristics of SCF's are repeatable fermentation parameters with each cycle, complete utilization of the limiting substrate, and stable, metabolically controlled cycling. These characteristics were all present in the experiments conducted in the new apparatus design.

6.2.1 Repeatability

Repeatability of the SCF cycles was demonstrated in all the fermentation parameters. Figure 5.8 shows an example of end of cycle biomass and cycle time data observed in one experiment. The error bars represent three standard deviations of the biomass measurements. The average steady state biomass values from various experiments are listed in table II. The standard

deviation of biomass values between cycles of an SCF was on the order of 10 percent for A.calcoaceticus and 25 percent for B. subtilis.

The discrepancy between the repeatability of the measurements for the two organisms can be explained. A. calcoaceticus had a higher biomass, especially when growing on hexadecane. This made it easier to measure. Also, the size of the centrifuge samples was larger for A. calcoaceticus--40 ml compared to 25 ml. Finally, the B. subtilis samples were rinsed before drying, the A. calcoaceticus samples were not.

A 50 % change in biomass can be seen between the early and the later stages of experiment 11. Experiment 11 lasted for over one hundred cycles. During the early cycles the organism had difficulty growing on the ethanol substrate. As the experiment progressed, the organism had many generations to adapt to the difficult substrate. It may have thereby been able to increase its biomass.

When an SCF is cycling at steady state, the cycle time is one of the most repeatable variables. The close correspondence of almost all cycle times in table II testifies to its repeatability from one experiment to the next. The standard deviation of cycle times within an experiment was on the order of ten percent. The difference between experiments was also about ten percent.

The only anomaly is the shortening cycle time of A.calcoaceticus during experiment 11. As with the biomass

concentrations, this effect is assumed to be a result of the length of experiment 11. Slowly, as the cycles progressed, the organism seemed to become accustomed to the substrate. Its cycle time shortened and its biomass increased. It can be seen that experiment 12, which grew on ethanol for only 12 cycles, resembled the early stage of experiment 11. Because experiment 12 then switched substrate to hydrocarbon, it never showed the same adjustment to the ethanol substrate.

The shape of the DO curve, which is an indication of the metabolic rate of the organism, was also repeated over the span of an experiment. This is seen in figure 5.4. Between experiments, too, the DO trace would produce a similar pattern for the same growing conditions. This was true even for the very difficult growth of A. calcoaceticus on ethanol. In both experiments 11 and 12 the early growth cycles on ethanol showed a DO pattern of four or five small local minima during each cycle.

Finally, the minimum DO level achieved during each cycle was also a constant value. This can be seen in figure 5.11 both before and after the change in carbon source.

In general, it was found that the whole system functioned as designed. When the SCF was at steady state, all the parameters showed excellent repeatability. Problems that occasionally prevented steady state operation included equipment failures (loss of agitation), contamination and power failures. Power failures usually affected only a limited number of cycles

if the control system was restarted as soon as the power was restored. The power failures suffered during these experiments usually lasted no longer than 20 minutes.

6.2.2 Stability:

The control of the fermenter gave a robust cycle stability. At the beginning of an experiment, the cycle time would reach a steady value within three to five cycles. Later in an experiment, if the timing was thrown off for some reason, the system was sufficiently stable to re-establish itself after the perturbation. An example of this can be seen in figure 5.13 where one cycle was intentionally extended too long. This threw off the cycle timing only for one additional cycle. Recovery after short power failures also demonstrated the ability of the SCF to maintain its steady state through temporary disturbances.

If the SCF was subjected to a change in operating conditions, it responded by shifting some or all of its parameters to a new steady state. This was the result of the feedback control allowing the system to be self cycling. For example, the temporary shift in nutrient concentration shown in figure 5.10 only temporarily altered the steady state. In this example, the biomass changed to a lower value but the cycle time remained the same. When the nutrient concentration was restored to its original level, the biomass was also restored. Another demonstration of shifting equilibrium was when an organism was switched from one substrate to another, as is shown in figure 5.11. Here the cycle time and minimum DO levels changed to new

levels. Again, the new parameters settled easily into a controlled steady state.

5.2.3 Residual substrate:

The SCF left very little residual substrate. Figure 5.9 shows the residual amounts of limiting nutrient remaining in the harvest of two different runs. With the exception of a few outlying points, the range of residual nutrients was from zero to six percent. Both experiments registered some zero residual points, confirming that the measured substrate was indeed limiting. For the non-zero points, the amount of residual nutrient remaining in the harvest was largely controlled by the shape of the DO curve and the width of the programmed noise band. For curves with relatively flat DO minima, the cut off time for the cycle control was somewhat arbitrary. It was the width of the noise band that determined how long the program would wait before ending the cycle.

By comparison, the glucose concentration in B. subtilis SCF's was measured to be approximately 80 percent of its original concentration by the end of a cycle. This confirmed that for these experiments, glucose was not limiting.

6.2.4 Contamination:

Most experiments were unaffected by contamination. In some experiments a contaminant was identified, but it did not become well established in the fermentation. An indication of this phenomenon is shown in figure 6.1. In this experiment with B. subtilis, a contaminant was identified on the second cycle. It

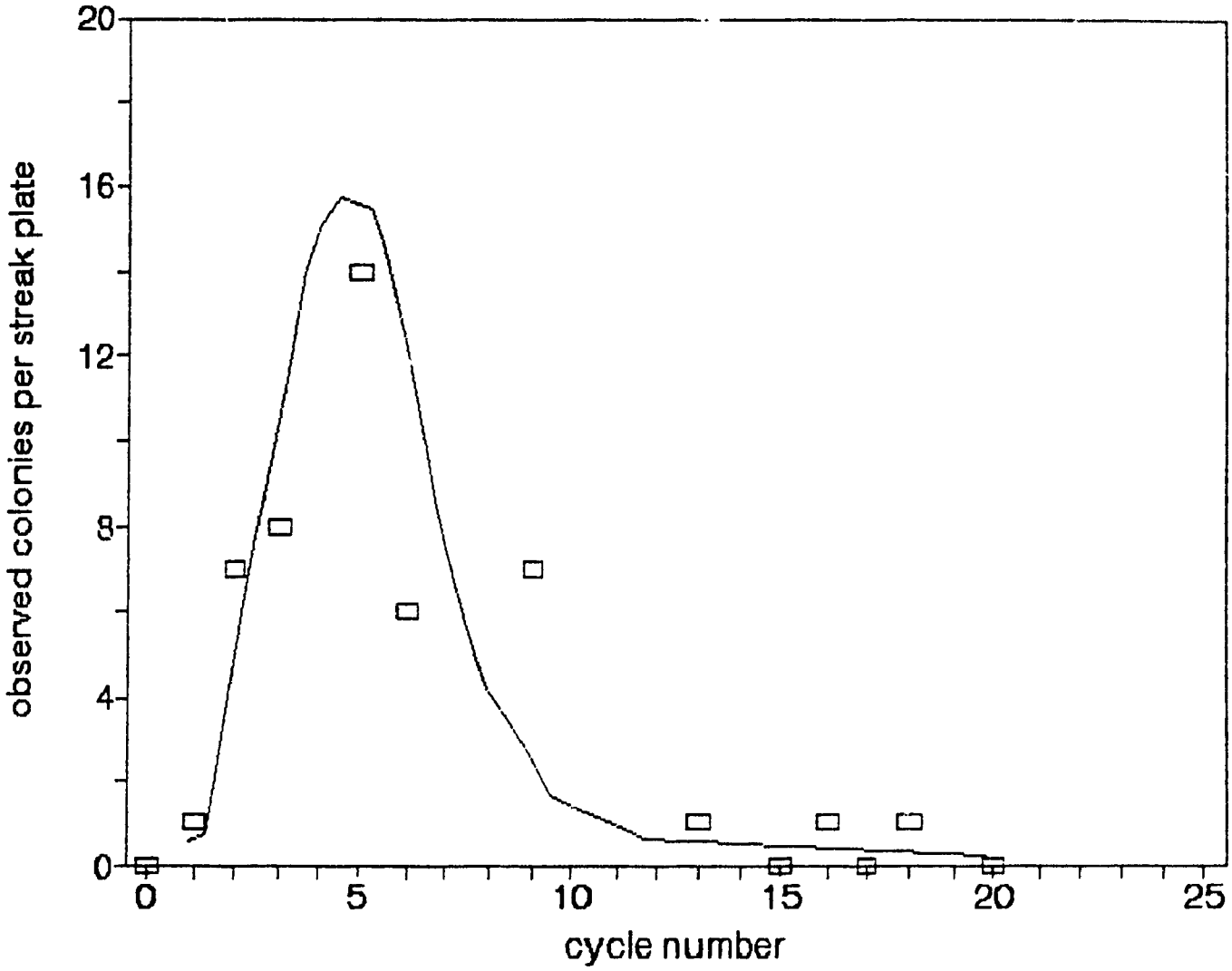

FIGURE 6.1: Number of contaminant colonies observed growing on streak plate samples taken during an experiment with B.subtilis

persisted and increased until about the fifth cycle. After this it started to lose its hold and was finally reduced to a very low level. It is proposed that because the inoculated culture was so well established, and because it dictated the cycle time of the experiment, the contaminant was not able to persist.

There was one run where what looked like a contaminant turned out to be a mutation of the inoculated culture. It may be that some mutations will grow well in SCF's because the rigorous cycling schedule can select for certain abnormal growth characteristics.

6.3 Productivity:

6.3.1 Surfactin production:

The striking result in surfactin production was the dramatic increase during experiment 7 from the regular cycling SCF to the secondary cycles. This result was not nearly so dramatic in experiment 8, although the difference was still there. Figure 5.12 shows the data from experiment 7. The secondary cycle was achieved by allowing the harvested broth to sit in the harvest vessel for the duration of the next complete cycle. Surfactin is considered to be a secondary metabolite. One means of triggering its production is nutrient limitation. Thus the secondary cycle, a period of nutrient limitation, is a period of high product production. This was also confirmed through the observation that foam production rose dramatically just as the minimum in dissolved oxygen was reached.

The application of extended cycles in an SCF is not new. In his experiments with B.Subtilis, Sheppard had experimented with running a time delay after the detection of the minimum in the DO curve and before the harvest step (Sheppard, Cooper, 1990a). This prolonged the period of low oxygen utilisation and nutrient starvation. Sheppard found that delaying the harvest of a regularly cycling SCF did not throw off the steady state cycling of the system. As with our findings, while growing B. subtilis this delay led to increased surfactin production.

The difference between the two methods of extending the cycle lies in the location of the maturing culture. In Sheppard's work the extension was done in the reactor. In this study it was in the harvest vessel. This meant that the cycle time of the reactor was not increased. The same higher product production was achieved without slowing down the reactor throughput. For B. subtilis the time saving was actually not very large, since a long starvation period was not necessary to trigger good production. There may be other products, however, where the extra time needed for product production is much higher, and the secondary cycle growth may well be a means of improving throughput for these processes.

6.3.2 Emulsan production:

Emulsan was barely produced from the experiments growing A. calcoaceticus. The reason for this is unknown. Two different methods of measuring total carbohydrates were used for the emulsan assay but their results were similar. It may simply have

been that our strain of A.calcoaceticus had lost its ability to express emulsan production.

6.4 Exhaust gas analyzer:

6.4.1 Gas analysis:

The gas analyzer was installed on the apparatus for two reasons. First, it was anticipated that analysis of the exhaust gases would provide more information on the metabolism of the organism. Second, the possibility of metabolism control with the gas analyzer was attractive. The gas analysis signal would be less noisy than the DO signal. The analyzer would not be susceptible to fouling. It would not be sensitive to changes in overhead pressure. Also, analysis of carbon dioxide may make it possible to monitor and cycle anaerobic growth, which would not be possible with dissolved oxygen control.

The analyzer did function well to provide extra data. The carbon dioxide values were used to close a carbon balance around the SCF. Both the oxygen and the carbon dioxide readings were useful to check the operation of the DO probe. In one experimen a slowly drifting DO reading was verified to be just that through the readings of the gas analyzer, which registered no such drift.

Figure 5.13 shows some of the results from the gas analyzer. The parallel between the analyzer and the DO readings is obvious. There is a slight phase shift between the DO trace and the analyzer readings. This was due to a delay in the

response of the analyzer, caused mostly by dead time in the sample system. The total volume of the vessels and tubing between the culture vessel and the analyzer was about six litres. The actual path travelled by the moving gases was probably more on the order of three litres. The gas flow was usually between 1.0 and 1.5 litres per minute, thus giving a dead time of at least two to three minutes. The volume of the sample line was reduced as much as possible by keeping the tubing lengths to a minimum and installing a short circuit sample tube inside the harvest vessel. The total observed time delay between the two instruments still amounted to about four minutes.

As can be seen in figure 5.13, the signals from the analyzer were clean and gave good resolution. The anticipated advantages of a low noise signal, pressure independence and a non fouling installation were all realized. Although control by exhaust gas analysis was not used for this study, there seems to be no reason why this could not be done. The problem of slower response should be addressed, although even the current delay of four minutes is not intolerable for an SCF.

6.4.2 Carbon balance:

Table III shows the results of carbon balances around the SCF. As is obvious from the results, the carbon balance does not close very consistently. The best closure was 3.5 percent of the input carbon unaccounted for, the worst was 26.5 percent lost. It may be possible that the first two balances calculated,

giving the two highest deviations, had a particular source of error not present in the other balances.

Possible explanations for the poor closing include inaccurate calibration of the analyzer, undetected aqueous carbon or an incorrect carbon ratio for the biomass carbon contribution. The biomass of A. calcoaceticus was assumed to be equal to that of E. coli. This may not have been an accurate assumption. It is also possible that the measurement of input carbon was not accurate.

6.5 Comparison with literature values:

For the most part, the results from the SCF's conducted in the stirred tank were similar to those found by previous researchers using the cyclone reactor. Table IV lists the results of biomass, cycle time and productivity for stirred tank and cyclone SCF's of B. subtilis and A. calcoaceticus. The cyclone results were drawn from the studies done by Sheppard (Sheppard, Cooper, 1990a) and Brown (Brown, Cooper, 1991). The results for the two reactor configurations were roughly similar except for the emulsan production of A.calcoaceticus.

Under regular cycling control, the cycle times for B. subtilis were faster in the stirred tank than in the cyclone. The value of 135 minutes listed in the table is an average of the cycle times observed by Sheppard. Sheppard had found that the cycle time gradually decreased during his experiments from 150 minutes down to 120 minutes. It may have been that the

TABLE IV: Results from stirred tank and cyclone type of SCF apparatus. B.subtilis cyclone results from Sheppard and Cooper, 1990a. A.calcoaceticus cyclone results from Brown and Cooper, 1991.

organism medium	Reactor Type	Biomass (g/L)	std. dev.	Cycle time (minutes)	std. dev.	Surfactin (CMC^{-1}) or Emulsan (g/L)	std. dev.
B. Subtilis Sheppard medium	STR	0.21	0.04	100	8.0	1.05	
	Cyclone	0.28		135		0.75	
B. Subtilis Prolonged cycle Sheppard medium	STR			200	16.0	4.5	
	Cyclone	0.28		178 + 30		1.0	
A. Calcoaceticus Brown medium hexadecane	STR	0.71	0.09	60	6.6	0.04	
	Cyclone	0.92		55		0.65	
A.Calcoaceticus Brown medium ethanol	STR	0.31	0.07	158 (80)	1.0	0.01	
	Cyclone	0.55		52		0.2	

culture used in the stirred tank experiments was more adapted to the growing conditions and thus produced a faster cycle time.

Sheppard conducted experiments extending the cycle time by fixed intervals of 30, 60, 90 or more minutes. The total duration of the secondary cycles of B. subtilis in the stirred tank were approximately the same as the cyclone cycles extended for 60 minutes.

A calcoaceticus grew rapidly on hexadecane in both the cyclone and the stirred tank. This was not true for its growth on ethanol. Brown found the cycle time on ethanol to be roughly equal to the cycle time on hexadecane. In the stirred tank, the ethanol growth was much slower. The DO trace found by Brown in the cyclone experiments was much simpler than that found in the stirred tank. Brown found each cycle of the SCF gave one small local minimum in the DO trace, appearing as a bump on the descending DO curve. In our experiments, the DO trace had as many as five local minima per cycle. These minima eventually disappeared from the DO trace as the experiment progressed, but the growth never reached the same speed as was found in the cyclone reactor.

B.subtilis gave similar biomass production in the regular cycle stirred tank and in the cyclone. The biomass in the cyclone was higher but the difference was within one standard deviation of the stirred tank experiments. The biomass of A. calcoaceticus was lower in the stirred tank than in the cyclone for both carbon substrates.

The productivity results from the earlier works by Brown and Sheppard are also shown in table IV. Sheppard found surfactin production comparable with the stirred tank results. The extended cycle experiment done by Sheppard showed an increase in surfactin production for an extention time of 60 minutes. This increase was comparable to that found in the stirred tank secondary cycles. One secondary cycle experiment, number seven, exceeded Sheppard's results (see table II). These corroborative results underscore the connection between increased cycle time and productivity for B subtilis. Of the two methods used to increase cycle time, the secondary cycle is superior in so far as it retains maximum throughput of the SCF.

For A.calcoaxceticus, Brown found much higher emulsan production under all conditions.

6.6 Apparatus improvements:

The apparatus worked well and accomplished that which it was designed to do. There are however several improvements that could still be made to the apparatus.

The repeatability of results could be improved with a few changes to improve the consistency of growing and sampling conditions. A sample cooler installed on the sample system would improve sample preservation. As it is the samples cool slowly in a refrigerator. A short concentric tube heat exchanger on the drain line or the header would hasten the cooling time. The header itself, constructed of polypropylene "T" fittings

connected in a line with latex tubing, should be replaced with a rigid piece of glass or stainless steel. This would eliminate some of the intra cycle carry over. The electric temperature control supplied with the fermenter should be used to supplement the temperature control already supplied by the heat exchangers. This will require the installation of a bigger thermowell on the lid of the culture vessel. Finally, the gas analyzer sample circuit needs to be further reduced in volume. Failing this perhaps an inert carrier gas could be used to speed up the sample dead time.

The sterility of the set up could also stand for some improvement. One big sterility hazard is the need to assemble the culture and harvest vessels after sterilization. These vessels should be rebuilt of glass to allow them to be sterilized with the rest of the apparatus in the autoclave. The seals of the polypropylene tubing connectors used on the dosing vessel are not trustworthy. They are difficult to test for leaks. These connectors should be replaced. A sterile drip tube connection should be installed between the dosing vessel and the culture vessel. This would prevent the "pre-inoculation" experienced in one experiment. The risk of contamination in the medium storage carboy and the dosing vessel would be reduced if the limiting nutrient was always supplied to the culture vessel directly with the syringe pump or a peristaltic pump. This worked well for hydrocarbons and would probably work well for other substrates as well.

The control of the cycling would be much cleaner if the gas analyzer could be used instead of the dissolved oxygen. It would also streamline the set up of the equipment if the two computers and their respective functions could be combined into one machine.

CONCLUSION:

Self cycling fermentations (SCF's) were successfully run in an apparatus based on a stirred tank fermenter. The SCF's reached steady state operation and produced reproducible results. It is hoped that the technique of SCF may now be more accessible to researchers desiring to make use of its distinct qualities.

The new apparatus gave results comparable to those found by other researchers using a cyclone column SCF. There is no apparent reason why most or all results found using a cyclone reactor could not be reproduced in a stirred tank. The stirred tank was found to require more power than the cyclone to achieve the oxygen transfer rate necessary for dissolved oxygen control on an SCF.

A gas analyzer measuring oxygen and carbon dioxide concentrations in the effluent gas was found to give equal resolution to that of the dissolved oxygen (DO) probe. It is possible that gas analysis may prove to be a more suitable control variable for SCF's than the DO.

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Appendix I: Cycling control program

```
{$N+}
Program body;
USES Dos,CRT,printer;
VAR Alternate,Altcount,End_cycle,cycle,choice:byte;
    Replenish_time,Sample_volume,Valve_code,Available_jars:integer;
    Reply,Manual_on: char;
    In_weight,Minimum_DO,Sensitivity: real;
    DiskFile,Disk,run_data: Text;
    Title: String;
    New_run,skip: boolean;
    Time_Open,Volume,Start_time, Lag_time,Harvest_time:Real;
    Previous_cycle_time:Real;
    Cycle_time: real;
    Tare:double;

{$I PHASEII.INC}     { Include all of the DT2801 procedures  }


BEGIN
   Initialize_Board;
   Assign(run_data, 'phaserun.dat');
   Append(run_data);

   ClrScr;
   Sensitivity:= 0.03;
   Lag_time:=1200;
   Harvest_Time:=100;
   Sample_volume:=100;
   Available_Jars:=4;
   Replenish_time:=50;
   Skip:= False;
   Alternate:=1;
   Altcount := alternate;

   Write('Do you want manual control? Y or N -->');
   Readln(manual_on);
   If (Manual_on = 'Y') or ( Manual_on = 'y') then Manual_Mode;

   Get_user_data(End_cycle,In_weight,Volume,New_run,Cycle);
   If New_run=False then
      Begin
         ClrScr;
         Writeln('Choose point of entry into program');
         Writeln;
         Writeln('1 - Fill Dosing Vessel');
         Writeln('2 - Early Cycle Time Delay');
         Writeln('3 - Sample Dissolved Oxygen');
         writeln('4 - Pre-Harvest drain');
         Writeln('5 - Fill Harvesting Vessel');
         Writeln('6 - Replenish Reactor');
         Writeln('7 - Syringe addition');
         Writeln('8 - Fill Collection Jar');
```

```
          Writeln;
          Write('Make a selection -->');
          Readln(choice);
          Writeln;
       End
    Else
       Begin
          Start_up_reactor(in_weight,Volume,Start_Time);
          Previous_Cycle_time:=Start_time;
          Fill_dosing_Vessel(In_weight);
          Time_delay(2*Lag_time,true);
          Choice:=3;
       End;
    While Cycle <= End_Cycle do
       Begin
          While Choice <= 8 do
             Begin
                Case Choice of
                   1: Fill_Dosing_Vessel(In_weight);
                   2: Time_Delay(Lag_Time,True);
                   3: Sample_Dissolved_Oxygen(Minimum_DO, Sensitivity);
                   4: Fill_Collection_jar(sample_jar);
                   5: Fill_Harvesting_Vessel(Harvest_Time);
                   6: Add_Fresh_Medium_to_Reactor(start_time);
                   7: Syringe_Addition(Volume);
                   8: Fill_Collection_Jar(Sample_jar);
                end;
                Choice:=Choice+1;
             End;
          Cycle_time:=Elapsed_Time(Start_time, Previous_Cycle_Time)/60;
          Previous_Cycle_Time:=Start_Time;

          Writeln(run_data);
          Writeln(run_data,'                Cycle = ',Cycle);
          Writeln(run_data,' Minimum_DO = ',Minimum_DO:4:4,'    Cycle_Time = ',
                                          Cycle_Time:5:2);
          If available_jars < 0 then Writeln(run_data,'   No sample collected');
          If alternate=2 then Writeln(run_data,'       Alternate sample not
collected');
          Writeln;
          Writeln('                Cycle = ',Cycle);
          Writeln(' Minimum_DO = ',Minimum_DO:4:4,'    Cycle_time = ',
                                          Cycle_time:5:2);
          If Available_jars < 0 then Writeln('     No sample collected');
          If Alternate=2 then Writeln('     Alternate sample not collected');
          Writeln;

          Choice:=1;
          Cycle:=Cycle + 1;
       End;
    Close(run_data);
END.
```

```
CONST
   Base_address = $2EC;
   Command_register = Base_address + 1;
   Status_register  = Base_address + 1;
   Data_register    = Base_address;

   Command_wait = $4;       {  all of these global variables are for  }
   Write_wait   = $2;       {  the data  acquisition board            }
   Read_Wait    = $5;

   CReset       = $0;
   CError       = $2;
   CStop        = $F;
   CClock       = $3;
   DIOPort      = $2;
   CDOut        = $5;
   CSAD         = $D;
   CDIOut       = $7;
   CRAD         = $E;

   Max_Conversions = 100;

TYPE Loop_Range = 1 .. Max_Conversions;

VAR
   number : integer;
   Solenoid_0, Solenoid_1 : Byte;  { variables to actuate valves }
   Sample_jar : Byte;
   Conversions:Byte;

PROCEDURE Wait(Port_number: Word; n, j: Byte);
VAR Data: word;
BEGIN
   Repeat
      Data:=Port[ Port_number ];
   Until ( (Data XOR j) AND n ) <> 0;
END;

PROCEDURE Wait_Status;  { this just saves a lot of repetitive typing }
BEGIN
   Wait( Status_register, Write_wait, Write_wait );
   Wait( Status_register, Command_wait, 0 );
END;

FUNCTION Digit( n: byte ) : char;
{ Used in conjunction with "Hex" to convert binary to hexadecimal }
BEGIN
     if n < 10
        then digit := chr( ord('0') + n )
        else digit := chr( ord('A') + ( n - 10 ))
END;
```

```
FUNCTION Hex( Number : Integer ) : string;
{ Used in conjuntion with "Digit" to convert binary to hexadecimal }
BEGIN
     Hex:= Concat( Digit(Hi(Number) DIV 16 ),
                   Digit(Hi(Number) MOD 16 ),
                   Digit(Lo(Number) DIV 16 ),
                   Digit(Lo(Number) MOD 16 ) );
END;

PROCEDURE Read_Error_Register(VAR Err1, Err2: Integer);
VAR Temp: Integer;
BEGIN
   Port[ Command_register ]:= CStop;   { Stop the DT2801 and empty   }
   Temp:= Port[ Data_register ];       { the data register           }

   Wait_Status;                        { Wait until DATA IN FULL is clear }
   Port[ Command_register ]:= CError;  { and READY flag is set, then      }
                                       { write READ ERROR register to     }
                                       { command register                 }
   Wait( Status_register, Read_wait, 0 );  { Wait for READY flag and get  }
   Err1:=Port[ Data_register ];            { Error register low byte      }

   Wait( Status_register, Read_wait, 0 );  { Wait for READY flag and get  }
   Err2:=Port[ Data_register ];            { Error register high byte     }
END;

PROCEDURE Report_Error(Status:integer);
VAR Error1, Error2 : Integer;
BEGIN
   WriteLn('Fatal Board Error');
   WriteLn('Status Register Value is ', Hex( Status ) );
   Read_Error_Register(Error1, Error2);
   WriteLn;
   WriteLn('Error Register Values Are:');
   WriteLn('BYTE 1 = ', Hex( Error1 ) );
   WriteLn('BYTE 2 = ', Hex( Error2 ) );
   HALT;
END;

PROCEDURE Check_For_Error;
VAR Status: Integer;
BEGIN
   Wait_Status;
   Status:=Port[ Status_register ];
   If ( Status AND $80 ) = $80 then Report_Error( Status );
END;

PROCEDURE Wait_Write;  { another procedure to save typing }
BEGIN
   Wait( Status_register, Write_wait, Write_wait );
END;
```

```
PROCEDURE Illegal_Status_Register(Status: Integer);
{ Report illegal status register value and halt the program }
BEGIN
   WriteLn;
   WriteLn('Fatal Error -- Illegal Status Register');
   WriteLn('Status Register Value is ',Hex( Status ) );
   WriteLn;
   HALT;
END;

PROCEDURE Actuate_Valve( Solenoid_0, Solenoid_1 : Integer );
{ Open or close a solenoid valve }
BEGIN
{ Wait until DATA IN FULL is clear and READY FLAG is set, then       }
{ write the WRITE DIGITAL PORT IMMEDIATE command to Command register }
   Wait_Status;
   Port[ Command_register ]:= CDIOut;

{  Wait until DATA IN FULL flag is clear, write DIGITAL PORT SELECT  }
{  byte to the Data In Register                                      }
   Wait_Write;
   Port[ Data_register ]:= DIOPort;

{  Wait until DATA IN FULL flag is clear, write DIGITAL PORT VALUE   }
{  byte to the Data In Register                                      }
   Wait_Write;
   Port[ Data_register ]:= ( 255 - Solenoid_0 );
   Wait_Write;
   Port[ Data_register ]:= ( 255 - Solenoid_1 );

   Check_For_Error;
END;

PROCEDURE Set_AD_Sampling( Start_Channel, End_Channel, Conversions:Integer);
BEGIN
   Wait_Status;
   Port[ Command_Register ]:= CSAD;

   { Set A/D gain byte to 8 }
   Wait_Write;
   Port[ Data_register ]:= 3;

   { Set A/D start and end channel bytes }
   Wait_Write;
   Port[ Data_register ]:= Start_channel;
   Wait_Write;
   Port[ Data_register ]:= End_channel;

   { Write high and low bytes of the number of
     conversions desired }
   Wait_Write;
   Port[ Data_register ]:= Conversions;
```

```
    Wait_Write;
    Port[ Data_register ]:= 0;

    Check_For_Error;
END;

FUNCTION Read_Data( Conversions: Integer ):Real;
TYPE Loop_Type  = array[Loop_Range] of Double;
VAR  Factor, Total_Value : Double;
     High, Low: Loop_Type;
     Loop : Byte;
BEGIN
   Wait_Status;
   Port[ Command_register ]:= CRAD;
   For Loop:= 1 TO Conversions DO
   Begin
      Wait( Status_register, Read_wait, 0 );
      Low[Loop]:= Port[ Data_register ];
      Wait( Status_register, Read_wait, 0 );
      High[Loop]:= Port[ Data_register ]
   End;
   Check_For_Error;
   Factor:= (10/4096)/8;
   Total_value:= 0;
   For Loop:=1 TO Conversions DO
      Total_value:=Total_value + High[Loop]*256 + Low[Loop];
   Read_Data:= Total_value * Factor / Conversions;
END;

FUNCTION Vessel_Weight(Desired_weight, Tare: Double;
                       Conversions:Integer;Fudge:real): Double;
VAR  Average_value,Average_weight: Double;
BEGIN
   REPEAT
      Average_value:= Read_Data( Conversions );
      Average_weight:= Average_value * 9072 - Tare
   UNTIL (Average_weight >= Desired_weight - Fudge)
         OR (Desired_Weight = 0);
   Vessel_Weight:= Average_weight;
END;

FUNCTION Reset_Tare(Conversions:Byte): Double;
VAR Set_Tare,Desired_weight,Fudge : REAL;
BEGIN
   Set_Tare:= 0;
   Desired_weight:=0;
   Fudge:=0;
   Reset_Tare:= Vessel_Weight( Desired_weight, Set_Tare, Conversions, Fudge );
END;

PROCEDURE Reset_DT2801;
VAR Temp,Status : Integer;
```

```
BEGIN
   Status:=Port[ Status_register ];
   If NOT (( Status AND $70 ) = 0 ) THEN Illegal_Status_Register(Status);
   Port[ Command_register ]:= CStop;
   Temp:= Port[ Data_register ];
   Wait_Status;
   Port[ Command_register ]:= CReset;
   Wait( Status_register, Read_wait, 0 );
   Temp:= Port[ Data_register ];
   Check_For_Error;
END;

PROCEDURE Set_Clock;
BEGIN
   Wait_Status;
   Port[ Command_register ]:= CClock;
   Wait_Write;
   Port[ Data_register ]:= 0;
   Wait_Write;
   Port[ Data_register ]:= 58;
   Check_For_Error;
END;

PROCEDURE Set_Digital_Port;
BEGIN
   Wait_Status;
   Port[ Command_register ]:= CDOut;
   Wait_Write;
   Port[ Data_register ]:= DIOPort;
   Check_For_Error;
END;

PROCEDURE Close_all_valves;
BEGIN
   Solenoid_0:=0;
   Solenoid_1:=0;
   Actuate_Valve(Solenoid_0,Solenoid_1);
END;

PROCEDURE Initialize_Board;
BEGIN
   Reset_DT2801;
   Set_Clock;
   Set_Digital_Port;

   { Close Solenoids and Turn off Pump }
   Close_all_valves;
END;

FUNCTION Timer : Real;
VAR Hour, Minute, Second, Sec100: Word;
    TTime: Real;
```

```
BEGIN
   GetTime(Hour, Minute, Second, Sec100);
   TTime:=Hour*60*60 + Minute*60 + Second +Sec100/100;
   Timer:=TTime;
END;

PROCEDURE Open_selected_valves(valve_code:integer);
BEGIN
   Solenoid_0:=Valve_code DIV 256;
   Solenoid_1:=Valve_code MOD 256;
   Actuate_valve(Solenoid_0,Solenoid_1);
END;

PROCEDURE Time_delay(delay:real;access:Boolean);
FORWARD;

PROCEDURE Actuate_selected_valves(time_open:real; valve_code:integer);
VAR  access:boolean;
BEGIN
   Open_selected_valves(valve_code);
   Access:=true;
   Time_delay(time_open,access);
   Close_all_valves;
END;

PROCEDURE Manual_Mode;    { Procedure to change global  }
VAR Selection: char;      { control values              }

 Procedure Ask_Question(VAR Selection:char);
 BEGIN
   ClrScr;
   Writeln;
   Writeln('What do you want to do?');
   Writeln;
   Writeln('1  - Change Reactor Volume');
   Writeln('2  - Change Time Delay    ');
   Writeln('3  - Change Volume of Syringe Addition');
   Writeln('4  - Change Minimum Dissolved Oxygen Sensitivity');
   Writeln('5  - Change Maximum Number of Cycles');
   Writeln('6  - Change Harvest time');
   Writeln('7  - Change Number of Availble Jars');
   Writeln('8  - Change Sample Volume');
   Writeln('9  - Change Replenish time');
   Writeln('0  - Change Alternating sampling');
   Writeln('V  - Open selected Valves');
   Writeln('T  - Actuate Selected Valves');
   Writeln('C  - Close all valves');
   Writeln('S  - Skip to end of time delay');
   Writeln('R  - Return to Automatic Control');
   Writeln;
   Writeln('Q  - Quit Phasing Program');
   Writeln;
```

```
  Writeln;
  Write('So, what''s your choice?   -> ');
  Readln(Selection);
  Writeln;
END;

Procedure  Change_In_Weight;
BEGIN
  Writeln;
  Writeln('Present value is - ',In_weight:4:1,' grams.');
  Writeln;
  Write('Enter a new value -> ');
  Readln(In_weight);
  Writeln;
END;

Procedure  Change_Alternate;
BEGIN
  Writeln;
  Writeln('Present value is - ',alternate,'.');
  Writeln(' alternate = number of cycles per sample');
  Write('Enter a new value -> ');
  Readln(alternate);
  Altcount := alternate;
  Writeln;
END;

Procedure  Change_Time_Delay;
BEGIN
  Writeln;
  Writeln('Present value is - ',Lag_time:4:1,' seconds.');
  Writeln;
  Write('Enter a new value -> ');
  Readln(Lag_time);
  Writeln;
END;

Procedure  Change_Volume;
BEGIN
  Writeln;
  Writeln('Present value is - ',Volume:3:1,' seconds.');
  Writeln;
  Write('Enter a new value -> ');
  Readln(Volume);
  Writeln;
END;

Procedure  Change_Sensitivity;
BEGIN
  Writeln;
  Writeln('Present value is - ',Sensitivity:1:3);
  Writeln;
```

```
  Write('Enter a new value -> ');
  Readln(Sensitivity);
  Writeln;
END;

Procedure Change_Maximum_Cycles;
BEGIN
   Writeln;
   Writeln('Current cycle number is   - ',Cycle);
   Writeln('Present maximum number is - ',End_cycle,' cycles');
   Writeln;
   Write('Enter a new maximum value -> ');
   Readln(End_cycle);
   Writeln;
END;

Procedure Change_Harvest_time;
BEGIN
   Writeln;
   Writeln('Current Harvest time is  - ',Harvest_Time:3:1, ' seconds');
   Writeln;
   Write('Enter new Harvest time -->');
   Readln(Harvest_Time);
   Writeln;
END;

Procedure Change_Available_Jars;
BEGIN
   Writeln;
   Writeln('Current Number of available jars is  - ',Available_Jars);
   Writeln;
   Write('enter new number of available jars (max 4) -->');
   Readln(Available_jars);
   Writeln;
END;

Procedure Change_Sample_Volume;
BEGIN
   Writeln;
   Writeln('Current Sample Volume is  - ',Sample_volume,' seconds');
   Writeln;
   Write('enter new Sample volume -->');
   readln(Sample_Volume);
   Writeln;
END;

Procedure Change_Replenish_Time;
BEGIN
   Writeln;
   Writeln('Current Replenish Time is  - ',Replenish_time,' seconds');
   Writeln;
   Write('enter new Replenish Time (max 100) -->');
```

```
   Readln(Replenish_time);
   Writeln;
END;

Procedure Select_valves_To_Open;
VAR Valve_code,i,j:integer;
    Valve_ID:array[1..10] of char;
BEGIN
   Writeln;
   Writeln('Choose which valves you want to open:');
   writeln;
   writeln('0 - Harvest Valve');
   Writeln('1 - Drain Valve');
   Writeln('2 - Dousing Valve');
   Writeln('3 - Air By-Pass Valve');
   Writeln('4 - Medium Addition Valve');
   Writeln('5 - Sample Valve A');
   Writeln('6 - Sample Valve B');
   Writeln('7 - Sample Valve C');
   Writeln('8 - Sample Valve D');
   Writeln('S - Syringe pump');
   Writeln;
   Writeln('X - Cancell Selection');
   Writeln;
   Write('Select a valve -->');

   i:=0;
   Valve_code:=0;
   REPEAT
   Begin
      i:=i + 1;
      Readln(Valve_ID[i]);
      if (Valve_ID[i] = 'x') or (Valve_ID[i] = 'X') then
         begin
            Valve_code:=0;
            Valve_ID[i]:='P';
         end
      else
         begin
            Case Valve_ID[i] of
               '0': j:=1;
               '1': J:=2;
               '2': j:=4;
               '3': j:=8;
               '4': j:=16;
               '5': j:=32;
               '6': j:=64;
               '7': j:=128;
               '8': j:=256;
               'S','s': j:=16384;
               'P','p': j:=0;
            end;
```

```
            Valve_code:=valve_code + j;
            write('Select another valve or type P to pop the valve(s) -->');
          end;
    End;
    Until(Valve_ID[i] = 'p') or (Valve_ID[i] ='P');
    if Valve_code > 0 then Open_selected_valves(valve_code);
END;

Procedure  Determine_time_to_open;
VAR  time_open:real;
     access:boolean;
BEGIN
    Writeln;
    Write('enter time for valves to be open (sec) -->');
    Readln(time_open);
    access:=true;
    Select_valves_to_open;
    Time_delay(time_open,access);
    Close_all_valves;
END;

Procedure Skip_to_End_of_Time_Delay;
VAR  skipalong:char;
BEGIN
    Writeln;
    Writeln('Do you really wany to skip to the end of this time delay?');
    Write('           Y or N -->');
    Readln(skipalong);
    If (skipalong = 'Y') or (skipalong = 'y') then
        skip:=true;
    Writeln('program will skip to end of time delay');
    Writeln;
END;

Procedure  Return_to_Program;
BEGIN
    Writeln;
    Writeln('returning to program');
END;

Procedure  Quit_Everything;
VAR i:char;
BEGIN
  ClrScr;
  Writeln('CONFIRM TERMINATION OF PROGRAM');
  Writeln;
  Write('Exit Program    Y or N  ---> ');
  Readln(i);
  Writeln;
  If (i = 'Y') or (i = 'y') then
  Begin
     Close_all_valves;
```

```
      Halt(1);
   End;
END;

Procedure  Try_Again;
BEGIN
END;

BEGIN
   ClrScr;
   Repeat
   Begin
      Ask_Question( Selection );
      CASE Selection OF
         '1':  Change_In_Weight;
         '2':  Change_Time_Delay;
         '3':  Change_Volume;
         '4':  Change_Sensitivity;
         '5':  Change_Maximum_Cycles;
         '6':  Change_Harvest_Time;
         '7':  Change_Available_Jars;
         '8':  Change_Sample_Volume;
         '9':  Change_Replenish_Time;
         '0':  Change_Alternate;
         'V','v':  Select_Valves_To_Open;
         'T','t':  Determine_Time_To_Open;
         'C','c':  Close_All_Valves;
         'R','r':  Return_to_Program;
         'S','s':  Skip_to_End_of_Time_Delay;
         'Q','q':  Quit_Everything;
      Else Try_Again;
      END
   End;
   Until (Selection = 'R') or (Selection = 'r');
END; { Procedure manual mode }

PROCEDURE Time_Delay_Manual( Delay : Real; Access : Boolean;
                             Low_DO: Real;
                             VAR New_DO: Real );
VAR Initial, Current : Real;
    Clear_buffer,key,reply: Char;
BEGIN
   Initial:= Timer;
   Write(' In Time_Delay_Manual.');
   Writeln(' Access=',access,'. Delay=',delay:4:1,'. Alt F1, Alt F10 ');
   skip:=false;
   Repeat
      If Access = True
         THEN If Keypressed
            THEN If ReadKey = Chr(0)
               THEN
               BEGIN
```

```
                    Key:=ReadKey;
                    Case Key of
                    'h':    {  alt F1  }
                        BEGIN
                          WHILE Keypressed DO     { Clears keyboard buffer so
}
                          Clear_buffer:= ReadKey;{ than no accidental characters
}
                          Manual_Mode             { are passed to Manual_Mode
}
                        END;
                    'q':   {   alt F10 }
                        BEGIN
                          WHILE Keypressed DO
                          Clear_buffer:= ReadKey;
                          Writeln;
                          Write('Do you REALLY want to manually phase? ');
                          Readln(Reply);
                          Writeln;
                          If (Reply = 'Y') or (Reply = 'y')
                          THEN
                             BEGIN
                                New_DO:=Low_DO + (5 * sensitivity);
                                Exit;
                             END
                          ELSE
                             BEGIN
                                Writeln;
                                Writeln('Continuing with DO sampling');
                                Writeln;
                             END;
                        END;
                    END;
                END;
        Current:= Timer;
        If ( Initial > Current ) Then
           Current:= Current + 86400   { add 24 hours }
   Until (( Current - Initial ) >= Delay ) or skip;
END;

PROCEDURE Time_Delay( Delay : Real; Access : Boolean );
VAR Initial, Current : Real;
    Clear_buffer: Char;
BEGIN
   Initial:= Timer;
   Write(' In Time_Delay.');
   Writeln(' Access=',access,'. Delay = ',delay:4:1,'. alt F1 ');
   skip:=false;
   Repeat
      If Access = True
         THEN If Keypressed
            THEN If ReadKey = Chr(0)
```

```
                THEN If ReadKey = 'h'    {  alt F1  }
                   THEN
                      BEGIN
                        WHILE Keypressed DO    { Clears keyboard buffer so     }
                        Clear_buffer:= ReadKey;{ than no accidental characters }
                        Manual_Mode            { are passed to Manual_Mode     }
                      END;
       Current:= Timer;
       If ( Initial > Current ) Then
          Current:= Current + 86400  { add 24 hours }
   Until (( Current - Initial ) >= Delay ) or skip;
END;

PROCEDURE Fill_Dosing_Vessel( In_weight : Real);
{ In weight is the total reactor volume }
VAR
   Dummy_weight,Dosing_weight,Desired_weight:Double;
   Tare,Fudge:real;
   Delay:LongInt;
   Access:Boolean;
   Start_channel,End_channel,Conversions: Byte;
BEGIN
   Writeln(' In Fill_Dosing_Vessel.');
   Start_channel:=1;
   End_channel:=1;
   Conversions:=10;
   Set_AD_Sampling(Start_channel,End_Channel,Conversions);
   Tare:=Reset_Tare(Conversions);
   Dosing_weight:= In_weight / 2;

   Solenoid_0:= 0;
   Solenoid_1:= 16;
   Actuate_Valve( Solenoid_0, Solenoid_1 );

   { The fudge compensates for any additional weight, supported or
     added, that may affect the actual weight delivered }
   Fudge:= 0.0;
   Desired_weight:= Dosing_weight;
   Dummy_weight:=Vessel_Weight( Desired_weight, Tare, Conversions ,Fudge );

   Close_all_valves;
   Delay:=5;
   Access:= True;
   Time_Delay( Delay, Access );
END;

PROCEDURE Sample_Dissolved_Oxygen( VAR Low_DO: Real; Sensitivity: Real);
VAR Access: Boolean;
    Start_channel,End_channel: Byte;
    New_DO:Real;
    Delay:LongInt;
BEGIN
```

```
    Writeln(' In Sample_Dissolved_Oxygen.');
    Start_Channel:= 0;
    End_Channel:= 0;
    Conversions:= 100;
    Set_AD_Sampling(Start_Channel, End_Channel, Conversions);
    Low_DO:= 10;
    Repeat
    Begin
       New_DO:= Read_Data( Conversions );
       New_DO:= ( Int( New_DO * 1000 + 0.5 )) / 1000;

       If ( Abs( Low_DO - 10 ) < 0.1 ) OR
          ( New_DO < Low_DO ) THEN
       BEGIN
          Low_DO:= New_DO;
          Delay:= 120;
          Access:= True;
          Time_Delay_Manual( Delay, Access, Low_DO, New_DO )
       END

       ELSE If New_DO - Low_DO <= Sensitivity THEN
       BEGIN
          Delay:= 60;
          Time_Delay_Manual( Delay, Access, Low_DO, New_DO )
       END;

    END;
    UNTIL New_DO - Low_DO > Sensitivity;
END;

PROCEDURE Fill_Harvesting_Vessel( Harvest_time: Real );
VAR Access: Boolean;
    Delay:Real;
BEGIN
   Writeln(' In Fill_Harvesting_Vessel.');
   Solenoid_0:= 0;

   Solenoid_1:= 1;    {harvest valve only}
   Actuate_Valve( Solenoid_0, Solenoid_1 );

   Delay:= Harvest_time;
   Access:= true;
   Time_Delay(Delay,Access);

   Solenoid_0:= 0;
   Solenoid_1:= 9;    {harvest and air bypass valves}
   Actuate_Valve( Solenoid_0, Solenoid_1 );

   { Wait 20 seconds to allow vessels to equilibrate
     without air flow            }
   Delay:= 20;
   Access:= true;
```

```
   Time_Delay( Delay, Access );
   Close_all_valves;
END;

   PROCEDURE Syringe_addition( Volume:Real );
   VAR  Delay:Real;
        Access:Boolean;
   BEGIN
      Writeln(' In Syringe_addition');
      Valve_code:=16384;
      Open_Selected_Valves(Valve_code);
      Delay:= Volume;
      Access:= true;
      Time_Delay( Delay, Access );
      Close_all_valves;
   END;

PROCEDURE Add_Fresh_Medium_To_Reactor( VAR Cycle_start_time: Real);
{  Procedure to empty the contents of the dosing vessel into
   the reactor.  The time the cycle starts is passed back to the
   main program.}
VAR Delay : Real;
    Access:Boolean;
    Start_channel,End_channel,Conversions: Byte;
BEGIN
   Writeln(' In Add_Fresh_Medium_To_ Reactor.');
   Start_channel:=1;
   End_channel:=1;
   Conversions:=10;
   Set_AL_Sampling(Start_channel,End_channel,Conversions);
   If sample_volume = 100 then valve_code:=14 else (* drain *)
                               valve_code:=12;  (* no drain *)
   Open_selected_valves(valve_code);
   Delay:= replenish_time;
   Access:= True;
   Time_Delay( Delay, Access );
   Close_all_valves;
   Cycle_start_time:= Timer;
END;

PROCEDURE Fill_Collection_Jar( VAR Sample_jar : Byte );
VAR Delay:Real;
    Access:Boolean;
    Valve_code:integer;
BEGIN
   If (((Choice = 4) and (sample_volume < 100)) or
       ((Choice = 8) and (sample_volume > 0))) then
       Begin
          Writeln(' In Fill_Collection_Jar.');
          Valve_code:= 2;
          Open_Selected_Valves(Valve_code);
          if choice = 4 then delay:= (100-sample_volume)
```

```
            else if sample_volume=100 then delay:= 100 - replenish_time
                 else delay:= sample_volume;
            Access:= True;
            Time_Delay( Delay,Access);

            Close_all_valves;

            If ( altcount=1 ) then
               begin
                  If available_jars < 1 then sample_jar := 0;
                  CASE Sample_jar of
                     0 : Valve_code:= 0;
                     1 : Valve_code:= 32;
                     2 : Valve_code:= 64;
                     3 : Valve_code:= 128;
                     4 : Valve_code:= 256;
                  END;
                  Open_Selected_Valves(Valve_code);
                  Delay:= 40;
                  Access:= True;
                  Time_Delay( Delay, Access );

                  Close_all_valves;

   {  Increment jar number for sampling }
                  Available_jars:=Available_jars - 1;
                  Sample_jar:= Sample_jar + 1;
                  IF Sample_jar > 4 THEN Sample_jar:= 1

                  altcount:=alternate;   {alternate sampling}
               end    {If altcount = 1}
            else
               begin
                  altcount:= altcount - 1;
                  if (altcount < 1) then altcount := alternate;
                  Writeln('Alternating sample not collected');
               end;   {else alternate <> 1}
         End
         Else
           Writeln('Skipped Fill Collection Jar');
END;

FUNCTION  Elapsed_Time( Cycle_time, Old_cycle_time: Real ):Real;
BEGIN
   IF Cycle_time < Old_cycle_time
   THEN Cycle_time := Cycle_time + 86400;
   Elapsed_Time:= Cycle_time - Old_cycle_time;
END;

PROCEDURE Convert_Time( Cycle_Length: Real;
                        VAR Hour, Minute, Second : Word );
BEGIN
```

```
   Hour:=Trunc( Cycle_length/3600 );
   Minute:=Trunc( ( Cycle_length - Hour*3600 )/ 60 );
   Second:=Trunc( Cycle_length - Hour*3600 - Minute*60 );
END;


PROCEDURE Get_User_Input(VAR End_cycle:Byte;
                         VAR In_weight: Real;
                         VAR Volume: Real);
BEGIN
   Write('What experiment is this? ');
   Readln(Title);
   Writeln;
   Write('How many cycles are desired? ');
   Readln(End_Cycle);
   Writeln;
   Write('What reactor weight is desired? ( GRAMS ) ');
   Readln(In_weight);
   Writeln;
   Write('What syringe pump running time is desired? ( SECONDS ) ');
   Readln(Volume);
   Rewrite(disk);
   Writeln(disk, Title);
   Writeln(disk, End_cycle);
   Writeln(disk, In_weight);
   Writeln(disk, Volume);
   Close(Disk);
END;

PROCEDURE Get_User_Data(VAR End_cycle:byte;
                        VAR In_weight:real;
                        VAR Volume: Real;
                        VAR New_run:boolean;
                        VAR Cycle: Byte);
BEGIN
   Assign(disk, 'PHASING.PRM');
   Write('Is this a new experiment?  ( Y or N ) ');
   Readln(Reply);
   Writeln;
   If (reply = 'N') OR (reply = 'n')
      THEN
      BEGIN
         New_run:= False;
         Writeln;
         Write('What cycle is this? ');
         Readln(Cycle);
         Writeln;
         Writeln;
         Write('Which SAMPLE JAR do you want?   ( 1 -> 4 ) ');
         Readln(Sample_jar);
         Writeln;
         Reset(disk);
```

```
            Readln(disk, Title);
            Readln(disk, End_Cycle);
            Readln(disk, In_weight);
            Readln(disk, Volume);
            Writeln(Title);
            Writeln;
            Writeln('The maximum number of cycles is ',End_Cycle);
            Writeln('Reactor weight is ',In_weight:5:1);
            Writeln('The syringe pump running time is ',Volume:2:1);
            Writeln;
            Write('Do you want to use these settings? ( Y or N ) ');
            Readln(Reply);
            Writeln;
            If (Reply = 'N') or (Reply = 'n')
               THEN
               BEGIN
                  Get_User_Input(End_cycle,In_weight,Volume);
               END;
         END
      ELSE
      BEGIN
         New_run:= True;
         Cycle:=1;
         Sample_jar:=1;
         Get_User_Input(End_cycle,In_weight,Volume);
      END;
   END;


PROCEDURE Start_Up_Reactor(Inweight:real; Volume: Real; Var Start_Time: Real);
VAR Start_Up_Weight: Real;
    OK_erase:char;
BEGIN
   Writeln(' In Start_up_Reactor.');
   Writeln(' Do you want to rewrite your run file?');
   write(' Y or N -->');
   Readln(OK_erase);
   If (OK_erase = 'Y') or (OK_erase = 'y') then
      rewrite(run_data);
   Start_Up_Weight:= Inweight * 0.99;  { allow for 1% innoculum }
   Fill_Dosing_Vessel(Start_Up_Weight);
   Add_Fresh_Medium_To_Reactor(Start_Time);
   Fill_Dosing_Vessel(Start_Up_Weight);
   Add_Fresh_Medium_To_Reactor(Start_Time);
   Start_Time:= Timer;
   Syringe_addition(2*Volume);
END;
```