FERMENTER STUDIES ON THE PRODUCTION OF PIMARICIN BY *STREPTOMYCES NATALENSIS*

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by

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I hereby declare that the research described within this thesis is based entirely upon my own work.

David Mahon

David Mahon

Date : 8/9/90

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(David Mahon, B.Sc.)

ABSTRACT

The production of the polyene antibiotic, pimaricin by the organism *Streptomyces natalensis* CBS 700.57 was studied using an apparatus comprising a 10 litre fermentation vessel, and facilities for temperature, pH, dissolved oxygen, vessel pressure and gas composition measurement.

The apparatus was connected via a Texas Instruments PM550 programmable logic controller to an IBM-compatible computer in order to record data from the fermentation and to control certain parameters.

Adjustment of medium composition, and the use of fed batch techniques did not produce any improvements in pimaricin yields. However, a crucial role for carbonate in stimulating the production of the antibiotic was demonstrated. Yields as high as 1 g/litre were obtained. Patterns in dissolved oxygen were observed which suggested that autolytic processes may occur towards the end of the fermentation due to glucose depletion. The production of the antibiotic was not shown to be dissociated from cell growth.

An assay method, using High Performance Liquid Chromatography, for routinely estimating the levels of pimaricin in fermentation broths was successfully developed.

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1. INTRODUCTION

Pimaricin (also known as natamycin) is an anti fungal antibiotic which is classified among the polyene group of antibiotics.

It is produced by fermentation methods using the organism *Streptomyces natalensis* (CBS 700.57) which was first isolated from soil near Pieter Maritzburg, in the Natal province of South Africa (Struyk et al, 1957).

1.1 STRUCTURE AND FUNCTION OF PIMARICIN

Polyenes are a subgroup of the class of macrolide antibiotics whose structures consist of macro cyclic rings closed by a lactone bond, with one or more sugars glycosidically bonded to the ring.

The polyene subgroup is typified by a macrocyclic ring of 26 to 38 atoms, a polyene chromophore comprising 4 to 7 alternating double bonds which is an integral part of the macrolide ring, and one amino sugar.

Pimaricin (figure 1.1) (Raab, 1972) consists of a 27 carbon ring which includes a free carboxylic acid side group, four alternating double bonds (hence it is classified as a tetraene) and a mycosamine sugar residue (Struyk et al, 1957). The empirical formula is $C_{34}H_{49}NO_{14}$ and the molecular weight is approximately 658. The antibiotic loses its activity at extremes of pH and at high temperatures (Struyk et al, 1957).

The four double bonds endow a section of the molecule with a rigid planar and hydrophobic character. Due to this, and the degree of hydroxylation in the rest of the molecule, pimaricin is amphipathic in nature, a characteristic which is reflected in its low water solubility, photolability, and other physicochemical properties.

The loss of anti-fungal activity due to exposure to sunlight and oxidation in air can be prevented by the addition of NaK-Chlorophyllin or of certain dyes (Dekker et al, 1959).

The amphipathic nature of pimaricin is also reflected in its biological activity whereby it is soluble in sterols and membranes. Pimaricin attacks the metabolism of fungi at

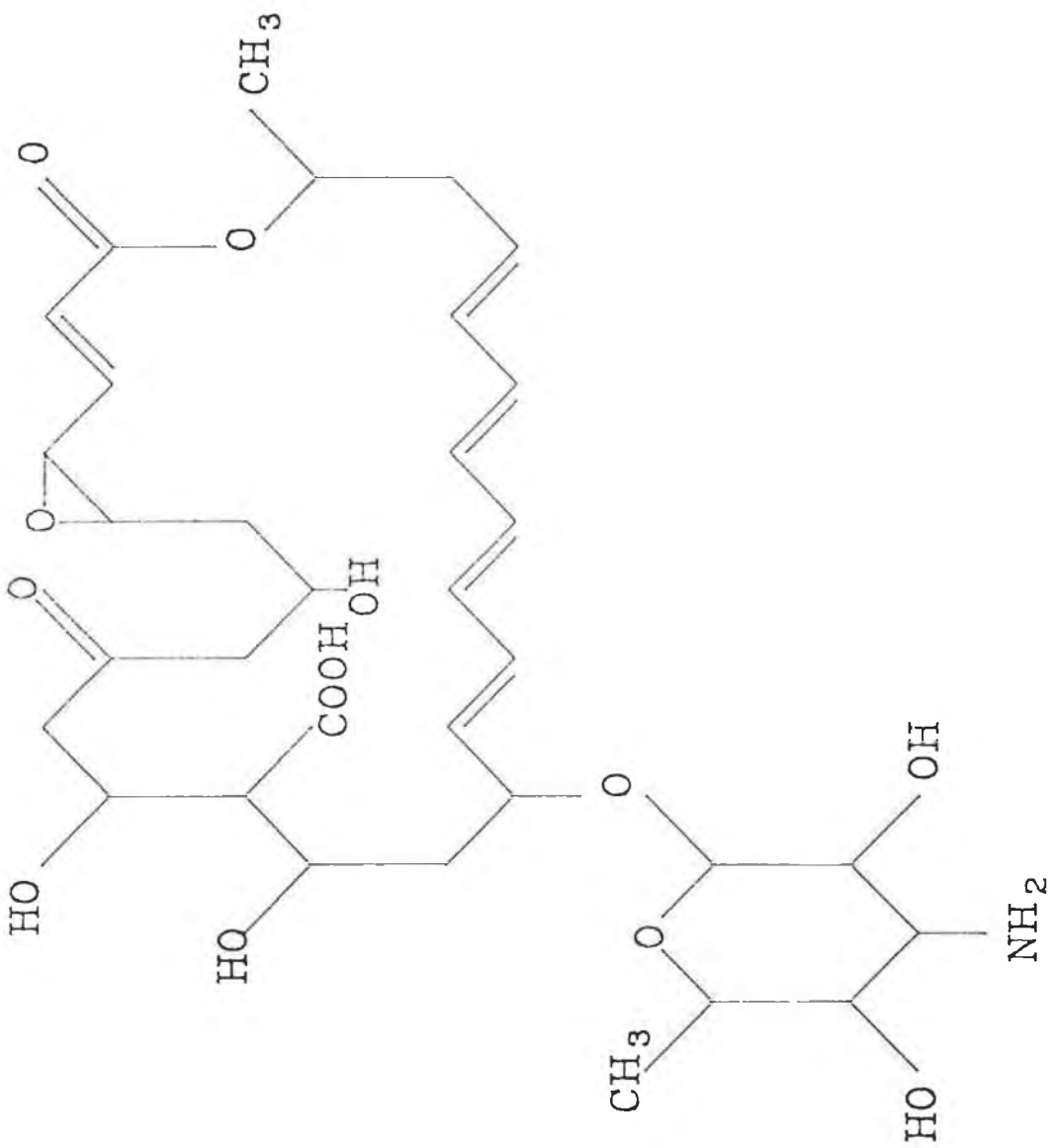


Figure 1.1 : Structure of Pimaricin

concentrations as low as 1-15 μg/ml. The antibiotic affects the permeability of cell membranes and is particularly active against replicating cells (Van Eek, 1968). It has been shown that ergosterol and steroids interfere with the anti fungal activity of pimaricin (Zygmunt et al, 1966).

Pimaricin is more soluble in C1-C6 alcohols than in water thus allowing it to be extracted from aqueous solutions by solvents which are immiscible in water such as n-butanol (Struyk et al, 1957).

The antibiotic can be recovered after solvent extraction by azeotropic evaporation (Struyk et al, 1957) or spray-drying (Bridger, 1968).

1.2 USES OF PIMARICIN

Since its discovery in 1957, many uses of the antibiotic have been proposed. It has found particular use in preserving food and beverages which are particularly prone to fungal contamination.

Amati et al (1980) have proposed the use of the antibiotic to reduce the amounts of sulphur dioxide required in wine production.

Pimaricin has also found use in protecting the surface of foods such as cheese (Lindgren et al, 1969), fruit (Smink, 1976) and salami (Holley, 1981) as well as preventing the degradation of corks used in beverage packaging (Fleischer, 1982).

Pimaricin can be made soluble for pharmaceutical uses by complexation with gamma-cyclodextrin (Szejtli et al, 1985). It has found use in treating candoses of the skin (Kejda et al, 1970), as a growth stimulant for poultry (Carter, 1985) and as a meat preservative (Kohler, 1962).

1.3 BIOSYNTHESIS OF PIMARICIN

In order to gauge the importance of the data obtained during this project, it is worth examining the biosynthesis of pimaricin. Although little is known directly about the synthesis of pimaricin, much can be inferred from the synthesis of other

related antibiotics.

The synthesis can be split into two sections:

1. The production of the macrolide ring
2. The production of the amino sugar.

1.3.1 Macrolide synthesis

Macrolide biosynthesis consists of three stages (Martin, 1977)

(1) Formation of Acetyl-CoA precursors from glucose

(2) Carboxylation of Acetyl-CoA and propionyl CoA to malonyl CoA and methylmalonyl CoA respectively

(3) Condensation of these precursors and cyclisation of the ring

Further "late transformations" of the ring also occur.

1.3.1.1 Formation of acetyl CoA

Acetyl-CoA is generated in microbial cells by the oxidation of pyruvate, the oxidative degradation of some amino acids and the beta-oxidation of some amino acids. However, evidence to date suggests that the first of these pathways is the only one of any importance in the synthesis of polyene antibiotics (Martin, 1977).

Glucose is converted to pyruvate by glycolysis and under aerobic conditions, will be converted to Acetyl-CoA.

The overall equation is:

$$\text{Glucose} + 2P_i + 2ADP + 2NAD^+ \longrightarrow 2\text{Pyruvate} + 2ATP + 2H_2O + 2NADH + 2H^+$$

Glycolysis has three key regulating points and these are the enzymes phosphofructokinase (the major rate-limiting step), hexokinase and pyruvate kinase. Phosphofructokinase catalyses the conversion of D-fructose 6-phosphate to D-fructose 1,6-diphosphate and uses ATP. It is inhibited by high concentrations of ATP, citrate and long-chain fatty acids but is stimulated by ADP or AMP. Mg^{2+} is also required (Lehninger, 1975).

Hexokinase is also a Mg^{2+} requiring enzyme which produces D-glucose 6-phosphate from D-glucose and ATP and is

inhibited by its own product (Lehninger, 1975).

Pyruvate kinase catalyses the transfer of phosphate from phosphoenolpyruvate to ADP producing ATP and pyruvate. The enzyme requires Mg^{2+} or Mn^{2+} with which it forms a complex before binding the substrate. Ca^{2+} competes to form an inactive complex. K^{+} is also required as a physiological activator (Lehninger, 1975).

Pyruvate is converted to acetyl CoA by oxidative decarboxylation involving the pyruvate dehydrogenase complex:

$$\text{Pyruvate} + NAD^{2+} + \text{CoA} \longrightarrow \text{acetyl-CoA} + \text{NADH} + H^{+} + CO_2$$

This reaction is important as there are many other pathways competing for pyruvate. Activation of the complex requires Ca^{2+} (Lehninger, 1975).

Acetyl-CoA and propionyl-CoA may also be synthesised directly from acetate and propionate by acetyl-CoA synthetase and propionyl-CoA synthetase (Martin, 1977).

Cellular pools of acetyl-CoA and propionyl-CoA are important in the synthesis of polyenes. The acyl-CoA pools are up to 30 times higher in high producers of polyenes compared to low producers (Martin, 1977).

1.3.1.2 Carboxylation reactions

Three carboxylating systems seem to be involved in polyene synthesis (Martin, 1977). These are:

(1) Acetyl-CoA and Propionyl-CoA Carboxylases

(2) Acetyl-CoA and Propionyl-CoA Carboxyltransferases

(3) Phosphoenolpyruvate carboxylase

The first enzyme class catalyses the following reactions:

$$\text{Acetyl-CoA} + \text{ATP} + CO_2 + H_2O \longrightarrow \text{malonyl-CoA} + \text{ADP} + P_i$$

$$\text{Propionyl-CoA} + \text{ATP} + CO_2 + H_2O \longrightarrow \text{methylmalonyl-CoA} + \text{ADP} + P_i$$

In many polyene producing organisms, the specific activities of these enzymes are twice as high in high-producing strains. Furthermore, the activities are at a maximum at the onset of antibiotic synthesis and decrease threefold during the production phase. This is thought to be due to the action of

other carboxylating systems (Martin, 1977). Among these are carboxylate both acetyl-CoA and propionyl-CoA) and catalyse the following reaction:

$$\text{Acetyl-CoA + oxaloacetate} \longrightarrow \text{malonyl-CoA + pyruvate}$$

$$\text{Propionyl-CoA + oxaloacetate} \longrightarrow \text{methylmalonyl-CoA + pyruvate}$$

The last enzyme involved is pyruvate carboxylase, which is involved in anaplerotic reactions catalysing the reaction:

$$\text{Pyruvate} + CO_2 + \text{ATP} + H_2O \longrightarrow \text{Oxaloacetate} + \text{ADP} + P_i$$

The replenishment of oxaloacetate is crucial for the action of the carboxyltransferases. It has been proposed that oxaloacetate is the key intermediate in polyene synthesis (Martin, 1977). It is suggested that acetyl-CoA carboxylase is associated with fatty acid synthesis, while the carboxyltransferase is part of a hypothetical oxaloacetate cycle which produces malonyl-CoA and methylmalonyl-CoA during antibiotic production (Martin, 1977).

1.3.1.3 Condensation of precursors

Comparison of pimaricin with a very similar polyene, etruscomycin, would indicate that pimaricin's macrolide ring arises from 2 methylmalonate units and 12 malonate units. The two antibiotics differ in that etruscomycin has an n-butyl side-chain whereas pimaricin has a methyl group. The structure of pimaricin is illustrated in figure 1.1. The chain is initiated with a malonate rather than a methylmalonate unit. The rings are synthesised via the polyketide pathway by repeated head-to tail condensation of malonate and methylmalonate units. The name of the pathway is derived from the fact that β-polyketo chains are formed by condensation of malonate units. The pathway is involved in the biosynthesis of plant and microbial metabolites. The pathway is similar to that for fatty acid synthesis. Evidence suggests that the $NADPH/NADP^+$ ratio determines whether polyene or fatty acid synthesis occurs (Martin, 1977).

The chromophore group is formed by the exclusive condensation of malonate units and, in the case of pimaricin, is

flanked on one side by a methylmalonate unit and on the other by the attachment site of mycosamine (Martin, 1977).

1.3.2 Synthesis of the sugar moiety

The amino sugar moiety of pimaricin is mycosamine which is synthesised exclusively from glucose. The pathway involves the use of nucleoside diphosphate derivatives of glucose which are formed using ATP, GTP, CTP and TTP. Although there has been very little work performed in this area, preliminary evidence suggests that TTP is involved in the synthesis of these moieties (Martin, 1977). Once the amino sugar has been formed, it must be attached to the macrolide ring.

This stage may be crucial in pimaricin synthesis as many macrolide rings are antibiotically inactive when the amino sugar is not present.

1.4 PRODUCTION OF PIMARICIN BY FERMENTATION

A literature search by computer was performed. Very few references regarding pimaricin were found. This is thought to be due to the production process being patented until quite recently.

No fermentation studies of the production of pimaricin were listed.

2. FERMENTATION EQUIPMENT AND INSTRUMENTATION

To study the fermentation process, a fermentation vessel was equipped with various devices to monitor and record parameters of interest. The vessel and its panel were mounted on a cast-iron frame constructed at Dublin City University (DCU).

To measure vital process parameters not monitored by the existing fermentation equipment, the equipment detailed in the following sections was mounted on the frame and connected to the vessel. Equipment was selected on the basis of

(i) its having a measuring range suited to the conditions during fermentation

(ii) it being sufficiently accurate

(iii) it having an electrical output signal to connect to a programmable logic controller (PLC).

This method of selecting individual pieces of equipment and constructing a fermentation rig was deemed better than purchasing a complete "off-the-shelf" package as it allowed a greater flexibility in choosing the correct equipment for the task in hand.

Figure 2.1 shows the overall layout of equipment. Sections 2.1-2.7 contain a more detailed explanation of the equipment used.

2.1 FERMENTATION VESSEL

The vessel used was a New Brunswick SF-116 fermenter. This consisted of a stainless steel pressure vessel with a capacity of 15 litres, though a working volume of 12 litres was rarely exceeded. The vessel diameter was 22 cm. The vessel was fitted with an agitator, an air sparger and temperature measurement and control equipment. The front of the fermenter also had a viewing port to examine the contents. There were a number of stoppered ports on the headplate and side of the vessel. The vessel was also equipped with a manual pressure regulator to set the operating pressure.

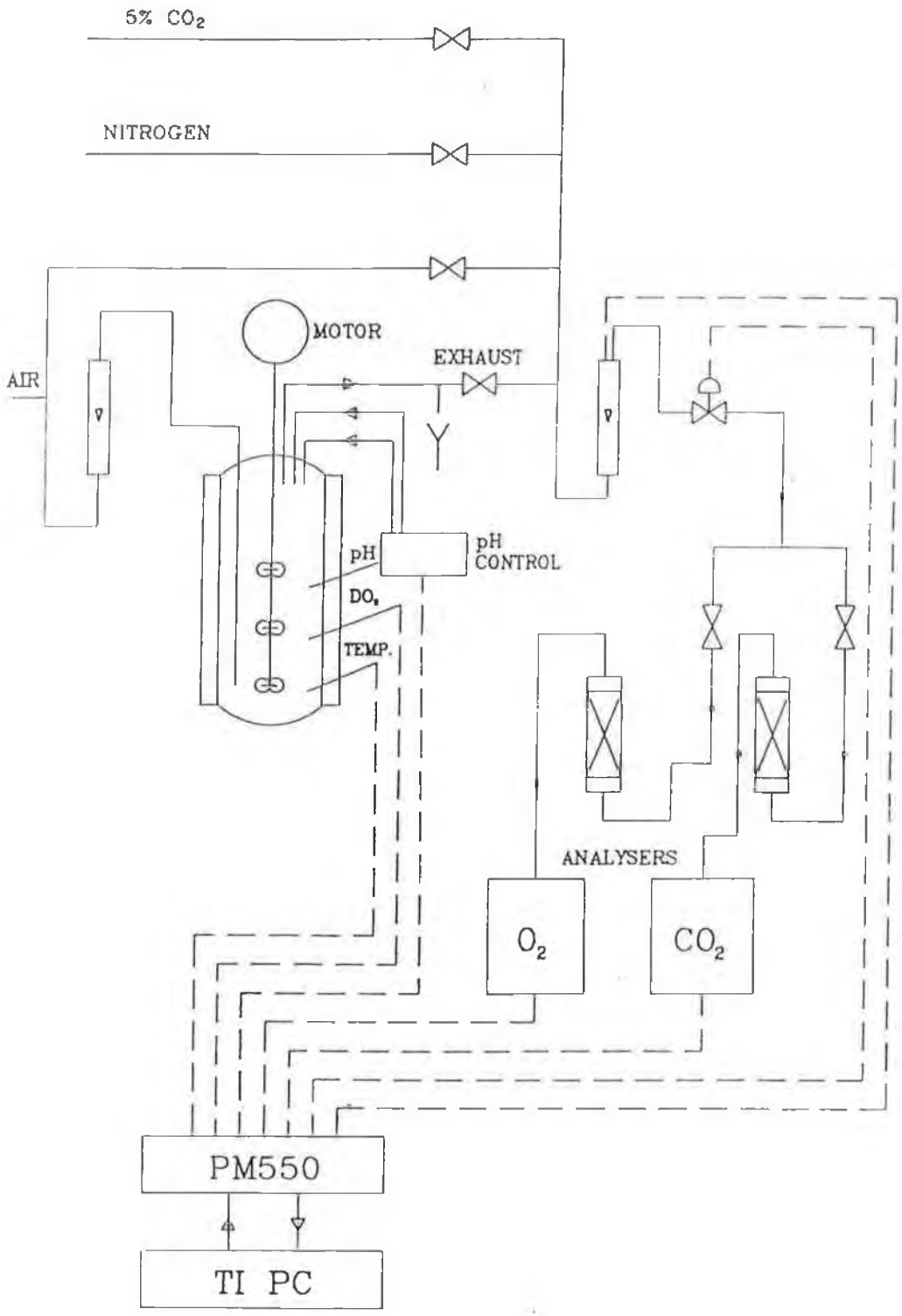


Figure 2.1 : Schematic Diagram of the Fermentation Rig constructed at D.C.U.

2.1.1 Sterilisation

The vessel was steam-sterilisable in situ. The sterilisation was controlled by a set of valves mounted on the front panel of the fermenter. Steam was permitted to pass into hollow baffles in the vessel, thus heating the contents. The sterilisation temperature was set using a potentiometer on the front panel and a built-in controller regulated the flow of steam as required. The vessel contents were typically sterilised at 121°C and 15 psig for 30 minutes. At the end of this exposure time the contents were cooled rapidly by setting the valves on the front panel to allow cold water to pass through the baffles.

2.1.2 Temperature measurement and control

To provide the required growth temperature for the culture the vessel was equipped with a water pump and heating element which were used to provide a flow of hot or cold water through the baffles to control the temperature. The temperature was monitored by a resistance thermometer and displayed on the front panel. The temperature setpoint was set with a potentiometer which was located on the front panel.

Since the output signal from the temperature measurement equipment on the vessel was incompatible with that required for connection to the PLC, a Bush Beach Engineering Ltd. resistance thermometer with a transmitter providing a 4-20 mA linear output signal was used. The transmitter required a stabilised 24 Vdc power supply for its operation. The thermometer had a measuring range of 0-200°C. It was coated with silicon grease and placed in a spare thermowell in the vessel.

2.1.3 Agitation

The vessel was equipped with a motor which could be set between 0 and 1200 R.P.M. by means of a multi-turn potentiometer on the front panel. The motor speed was also displayed on the front panel. During sterilisation the motor was set between 300 and 400 R.P.M..

The agitator consisted of a stainless steel shaft on which

was mounted three six-bladed turbines. Each turbine was 6.5 cm in diameter.

2.1.4 Aeration

Air from a central compressor was supplied to the vessel via a regulator at 20 psig. The supply to the vessel was controlled by a rotameter mounted on the front panel. The air was then filtered through a 0.22μm Dominick Hunter filter and entered the vessel via a sparger mounted directly under the agitator shaft.

2.1.5 Sampling

To allow sampling during fermentation, the vessel had a sampling port which could be steam-sterilised prior to taking the sample. The sample was forced out through the port by a combination of gravity and the slight excess of pressure maintained in the vessel during operation to insure asepsis. The sample was drawn out via a small ball valve located centrally at the base of the vessel.

2.2 MODIFICATIONS TO THE VESSEL

In order to fit additional probes and instruments to the vessel, several modifications to the headplate had to be made at DCU. There were no facilities to add acid, base or nutrient feeds, so appropriate fittings were added to the headplate. A fitting normally used to mount an antifoam probe was modified in order to use a pressure transducer.

2.3 pH MEASUREMENT AND CONTROL

The existing pH monitoring and control equipment associated with the SF-116 vessel proved unsuitable as it was inaccurate and had no electrical output. A Life Science Laboratories pH Controller was obtained. This consisted of a three-term controller and two peristaltic pumps to which acid and base reservoirs were connected. The reservoirs were 1000 ml glass bottles with outlets on the side of the bottles near the base. The top of the bottle was stoppered with a neoprene bung fitted

with a 0.45μm filter. A length of silicon tubing (internal diameter 4.5mm) was connected to each reservoir.

A display on the front panel of the controller allowed the required setpoint and control terms to be set.

An Ingold type 405 pH electrode was connected at the rear of the controller and was used to measure the pH of the vessel contents. The controller also had two potentiometers to allow calibration of the probe using standard solutions. The pH probe was inserted in a special port on the side of the vessel. The probe was contained in a stainless steel housing which allowed it to be pressurised during sterilisation. This was to insure sufficient flow of electrolyte through the diaphragm to avoid fouling, which can cause drift of pH readings.

The controller also provided a 1-5 Vdc linear output equivalent to 0 to 14 pH units.

2.4 DISSOLVED OXYGEN MEASUREMENT

An Ingold polarographic dissolved oxygen electrode was used to measure dissolved oxygen tension in the fermentation liquor. A Rosemount 1181 dissolved oxygen transmitter was used to polarise the anode and to provide a 4-20 mA output. The probe measures the amount of oxygen diffusing through an oxygen permeable membrane rather than the concentration of oxygen in the liquid. The transmitter had a number of switch-selectable operating ranges of which the 0-200 mmHg range was chosen as the most suitable since the maximum partial pressure of oxygen in air is approximately:

$$760 \text{ mmHg} \times 0.209 = 158.84 \text{ mmHg}$$

The probe was polarised overnight before use. Calibration was performed by passing nitrogen gas into the fermentation broth after sterilisation, until the reading on the meter stabilised near to zero. The transmitter display and output were then adjusted to zero if necessary. The liquid was then saturated with air for 10 minutes and the maximum transmitter output was taken to correspond to the saturation concentration of dissolved oxygen.

2.5 PRESSURE MEASUREMENT

Vessel pressure was monitored by a Sensym signal conditioned pressure transducer. This transducer was mounted in a zinc housing and was suitable for measuring in the range 0-30 psia. However, the electrical output of 2.5-12.5 Vdc was unsuitable for connection to the PLC.

A voltage divider was constructed to reduce the signal level to 1.25-6 Vdc which could be read by the PM550. Since an output to a LCD unit was also required, a second voltage divider was built to convert to the required signal level. The circuitry, a 15 Vdc power supply, and the LCDs themselves were all mounted in a box. Figure 2.2 shows details of the circuitry used.

The vessel pressure could be set manually by a needle valve. (See section 2.7.1.).

2.6 PERISTALTIC PUMP

In order to add nutrient feed a Watson-Marlow type 101U/R peristaltic pump was employed. This pump can deliver liquids at flow rates up to 53 ml/min (based on a tubing internal diameter of 4.8 mm).

The pump speed could be set manually via switches on the front panel or alternatively by providing the pump with a 0-10 Vdc signal.

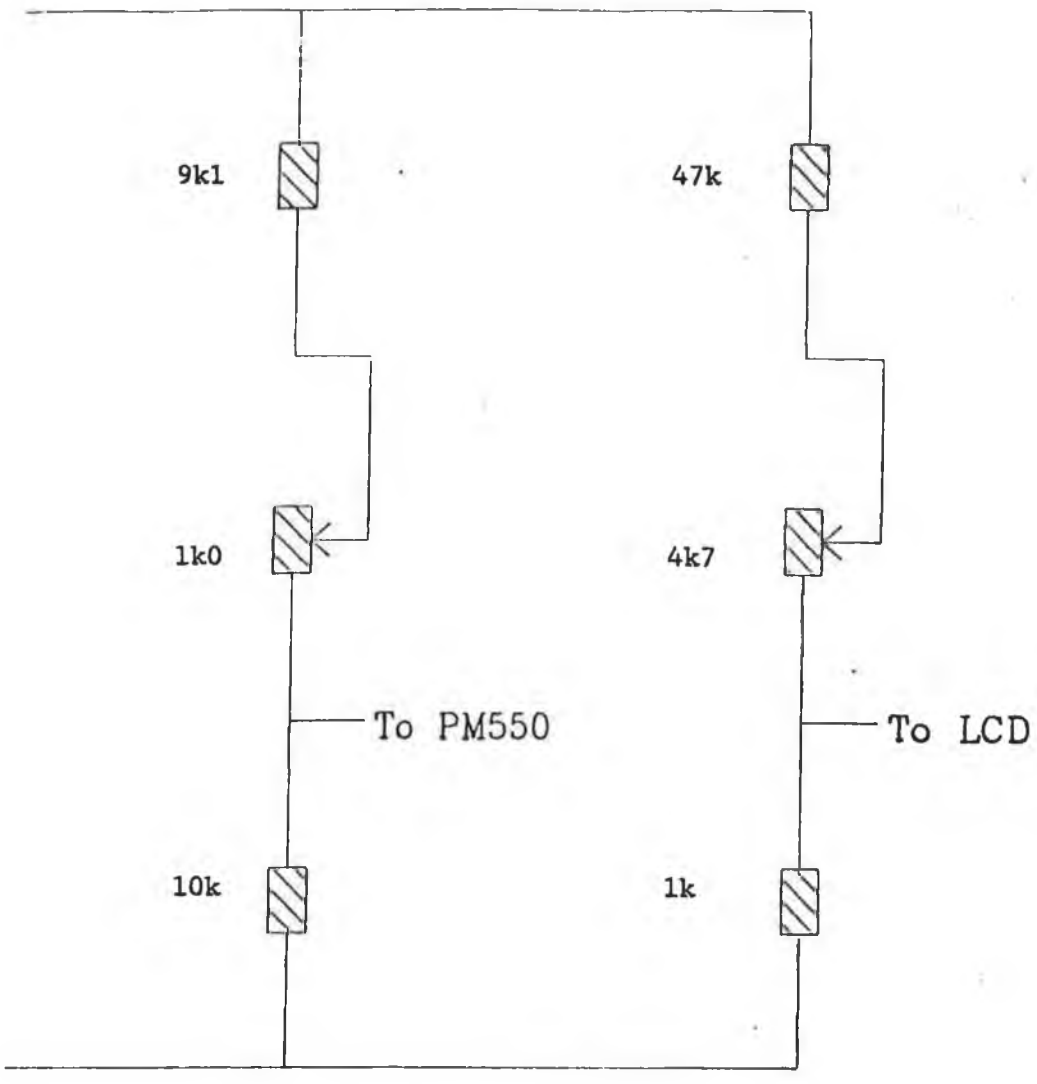
2.7 GAS ANALYSIS

Useful information on the progress of a fermentation can be obtained by measuring the rates of carbon dioxide evolution and oxygen uptake.

To this end, gas analysers, a flow meter, a control valve and a set of solenoid valves were set up in the configuration shown in figure 2.3.

2.7.1 Routing of the gas stream

Six one-quarter inch solenoid valves were used to direct the



N.B. Values of resistance in Ohms are given beside each resistor

Figure 2.2 : Diagram showing signal conditioning circuitry to output signals to PM550 and LCD display

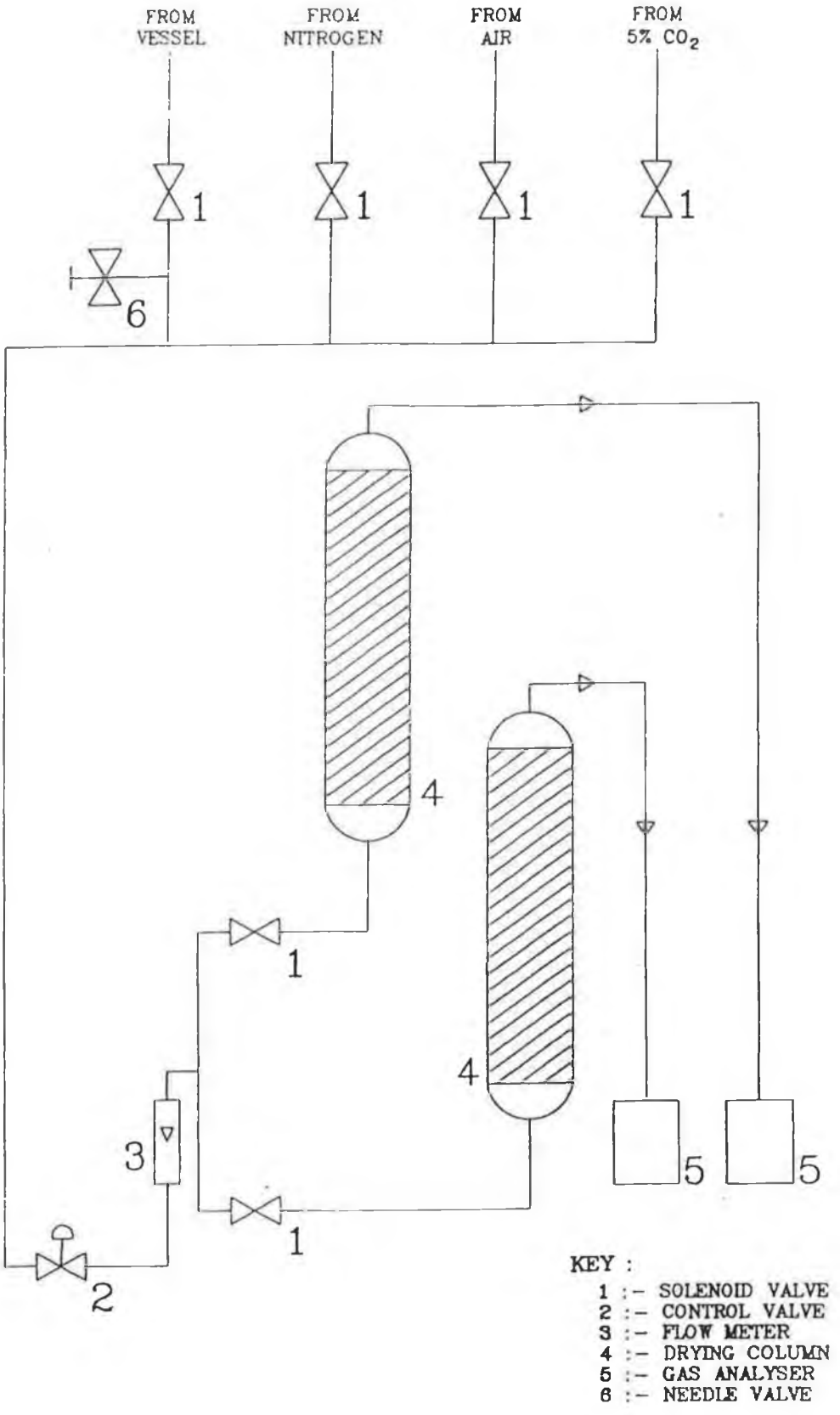


Figure 2.3 Gas Analysis Equipment

flow of gas to the analysers. The solenoids were split into two banks. The first bank controlled the flow of gases from different sources. These were

(i) Fermenter exhaust gases

(ii) Nitrogen gas to zero the dissolved oxygen probe and the oxygen gas analyser

(iii) Air to set the span on the oxygen analyser and to zero the carbon dioxide analyser

(iv) 5% carbon dioxide to set the span on the carbon dioxide analyser

The second bank of two solenoids was used to select which analyser the gas stream entered. The gases were routed through one-quarter inch Decabon tubing. A needle valve placed before the solenoid controlling gas flow from the vessel to the analysers ensured that gas could escape the vessel and also allowed regulation of vessel pressure.

A program was written in ladder logic (section 3) to sequence the solenoids, to allow the standard gases to enter each of the analysers to account for drift. The program also permitted exhaust gas from the fermenter to be analysed.

2.7.2 Regulation of gas flow rates

The measurement of gas composition using these CO_2 and O_2 analysers is dependent on the flow rate. Therefore, a flow meter and control valve were placed in between the two banks of solenoids.

The flow meter was a Platon SDF-2044 flow transducer and display unit which produced a 4-20 mA signal over the range 0-12 litres/min. This signal was fed back to the PLC, which controlled the position of a Platon control valve via a John Watson & Smith Ltd I/P transducer and a Moore Products Co. valve positioner. Details of this control loop will be given in section 3.

2.7.3 Drying of gas streams

The gas stream exiting the fermenter was quite moist so it was necessary to dry the gas stream.

A packed column was placed between the second bank of

solenoids and the analysers. For oxygen analysis the column was packed with self-indicating silica gel. For carbon dioxide analysis the column was packed with calcium chloride, as it was found by trial and error, that silica gel adsorbed carbon dioxide.

Both packing materials were dried overnight at 100°C prior to use. The columns were constructed at DCU from Perspex to allow the state of the packing to be observed.

2.7.4 Carbon dioxide analysis

An Analytical Development Company Ltd. Type SS200 analyser was used to measure CO_2 levels in the gas streams. This was a two range instrument with each range having its own analysis cell. The range 0-10% CO_2 was used during the project as the outlet CO_2 levels can typically reach 5% in aerobic fermentations (Carleysmith, 1986).

The gas stream entering the analysis cell absorbs energy from a hot-wire source, which was detected by an infra-red detector. The detector output was a function of the CO_2 concentration. An optical filter minimised interference from other IR absorbing gases which may have been in the sample. A linearisation circuit corrected for the non-linearity of CO_2 absorption.

The analyser was zeroed by selecting 'zero' on the front panel which directs a stream of air through a soda lime column thus freeing it of CO_2. This stream was then passed through both analysis cells. An alternative method was to zero the analyser using air, making the assumption that the levels in air are negligible. The analyser's span was checked with a 5% CO_2 gas. The output from the analyser was 0-5 Vdc for the range 0-10% CO_2.

2.7.5 Oxygen analysis

The paramagnetic susceptibility of oxygen was exploited by the Servomex 570A oxygen analyser to measure the concentration of oxygen in gas streams. Unlike oxygen, most common gases are diamagnetic and are thus repelled by magnetic fields. A magneto-dynamic type measuring cell in the analyser determined the magnetic susceptibility, and hence the oxygen concentration, of

a gas by measuring the force developed by a strong non-uniform magnetic field on a diamagnetic test body in the sample stream.

The analyser was calibrated by using oxygen-free nitrogen to set the zero point and then setting the span with air assuming a concentration of 20.9% oxygen.

The analyser provided a linear 0-1 Vdc output for the range 0-100% oxygen.

2.8 COMMISSIONING OF EQUIPMENT

Due to the nature of the equipment used to monitor the fermentation many revisions of the controlling program had to be made until it reached the form discussed in section 3.

2.8.1 Gas analysis

Originally, it was planned to use a 12 minute timer on the PLC to sequence the valves to take a zero reading, a span reading and finally a reading from the vessel exhaust for each analyser. This would have involved three sets of valve openings per analyser. Each set of valves remaining in position for two minutes. It was found that the oxygen gas analyser was insensitive to the changes in oxygen concentration between the inlet and outlet streams. Figure 2.4 shows a typical profile for the oxygen levels in the exhaust gas during a pimaricin fermentation.

It was noted during trial runs, that levels in the exhaust gas were virtually identical to those in the inlet stream. The initial drop appeared to be due to analyser drift. It can be seen that after recalibrating the analyser at around 65 hours the levels exhibited considerable drift rising to above 20.9%. It was decided to leave the valve sequencing program as it was, but data from the analyser was not written to disk.

The carbon dioxide analyser was found to operate satisfactorily if it was calibrated prior to the run and the zero set manually on a daily basis. The program was then rewritten to sample the vessel exhaust only.

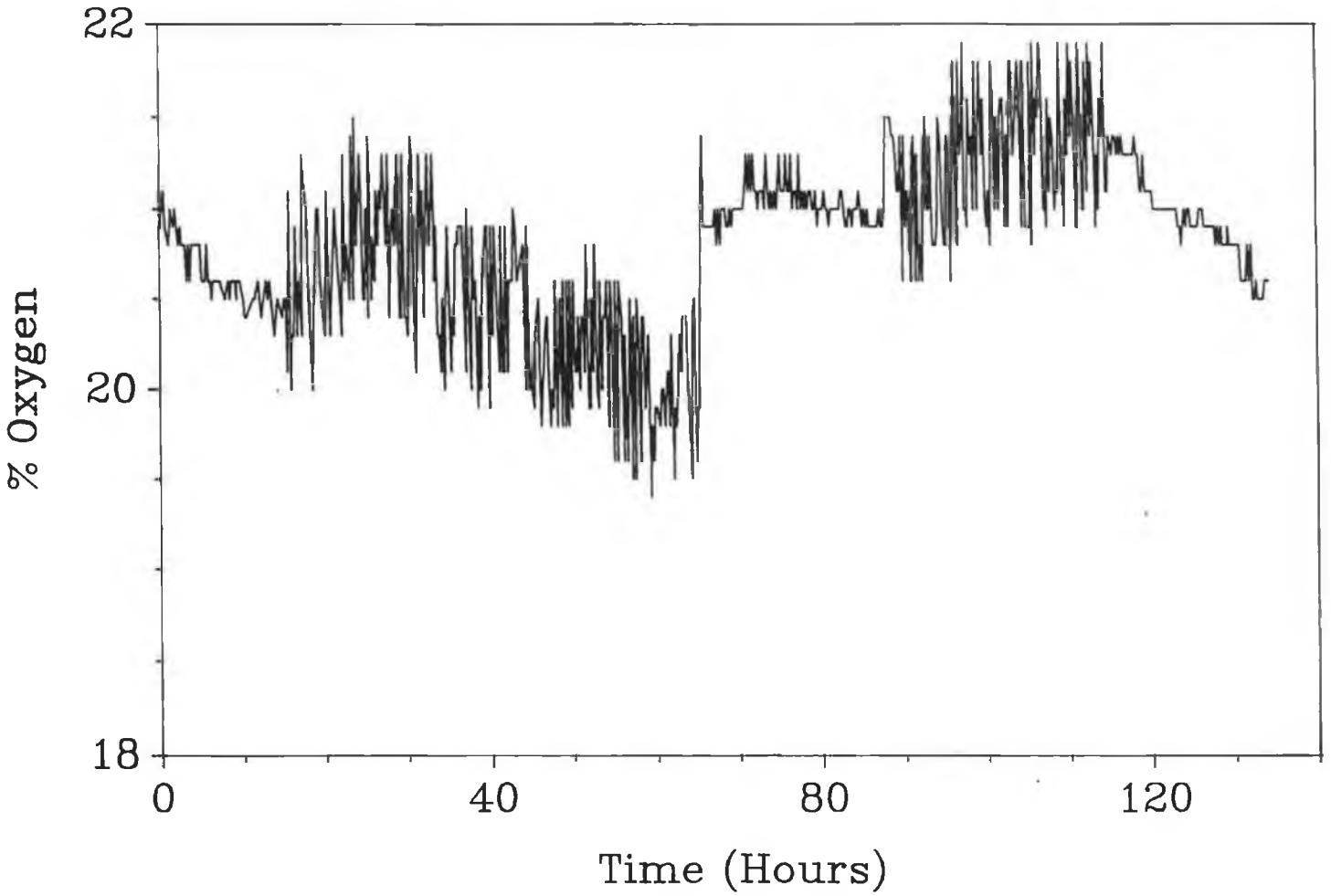


Figure 2.4 Concentration of Oxygen in the vent gas of a typical run.

2.8.2 Microgen SF-116 temperature control

The Microgen's built-in temperature control system was found to be adequate, controlling the temperature at the required setpoint to ±1°C. An example of a typical temperature profile during a fermentation run is shown in figure 2.5.

2.8.3 Pressure measurement

Atmospheric and vessel pressures were measured and recorded on disk for all the fermentation runs. This allowed variations in dissolved oxygen levels, due to changes in vessel pressure to be distinguished. A typical profile of vessel and atmospheric pressure is shown in figure 2.6.

2.8.4 pH measurement and control

Figure 2.7 shows a typical profile of pH data collected during a fermentation run. The tuning of the controller was such that this profile exhibited little variation between runs.

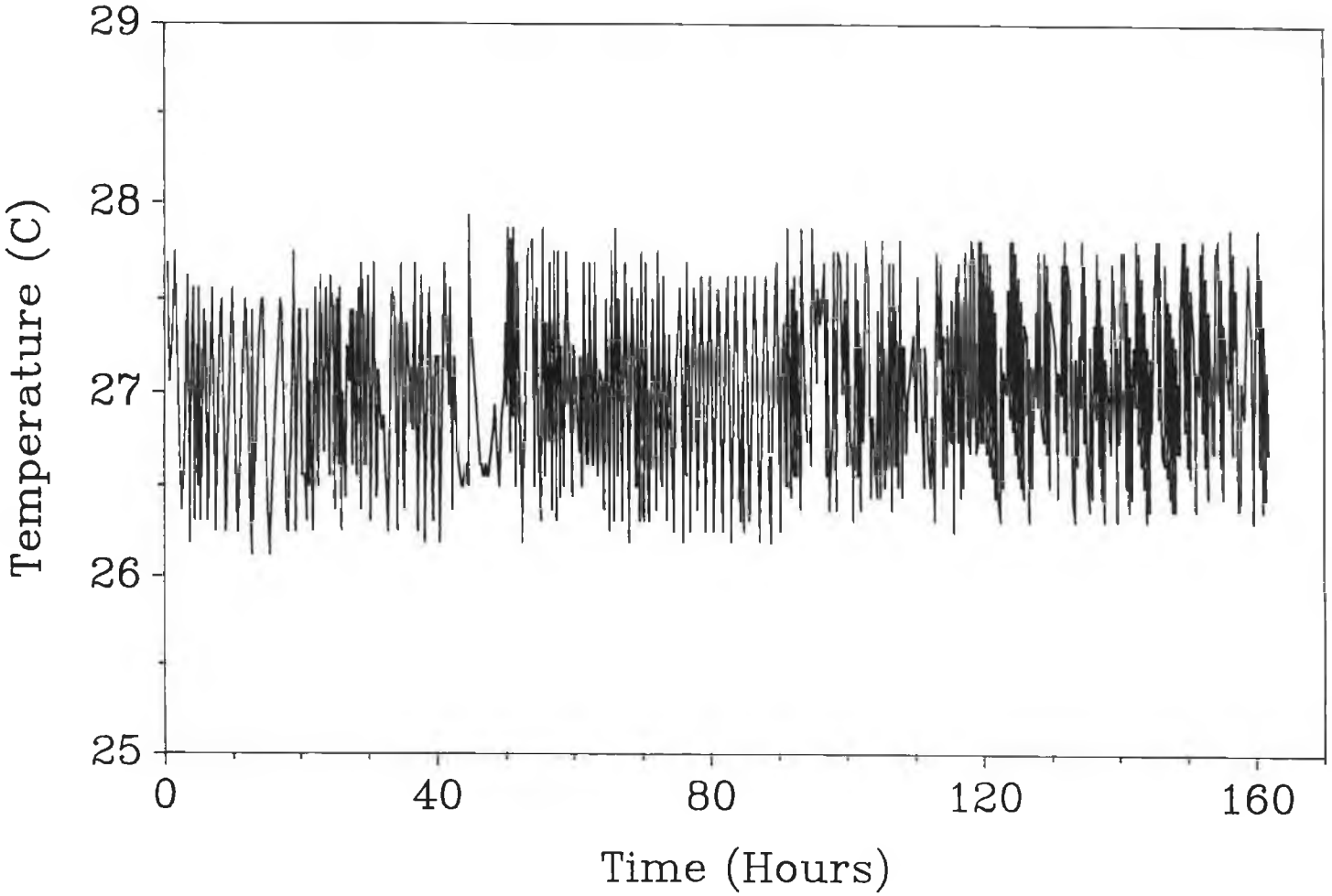


FIGURE 2.5 Temperature profile during a typical run.

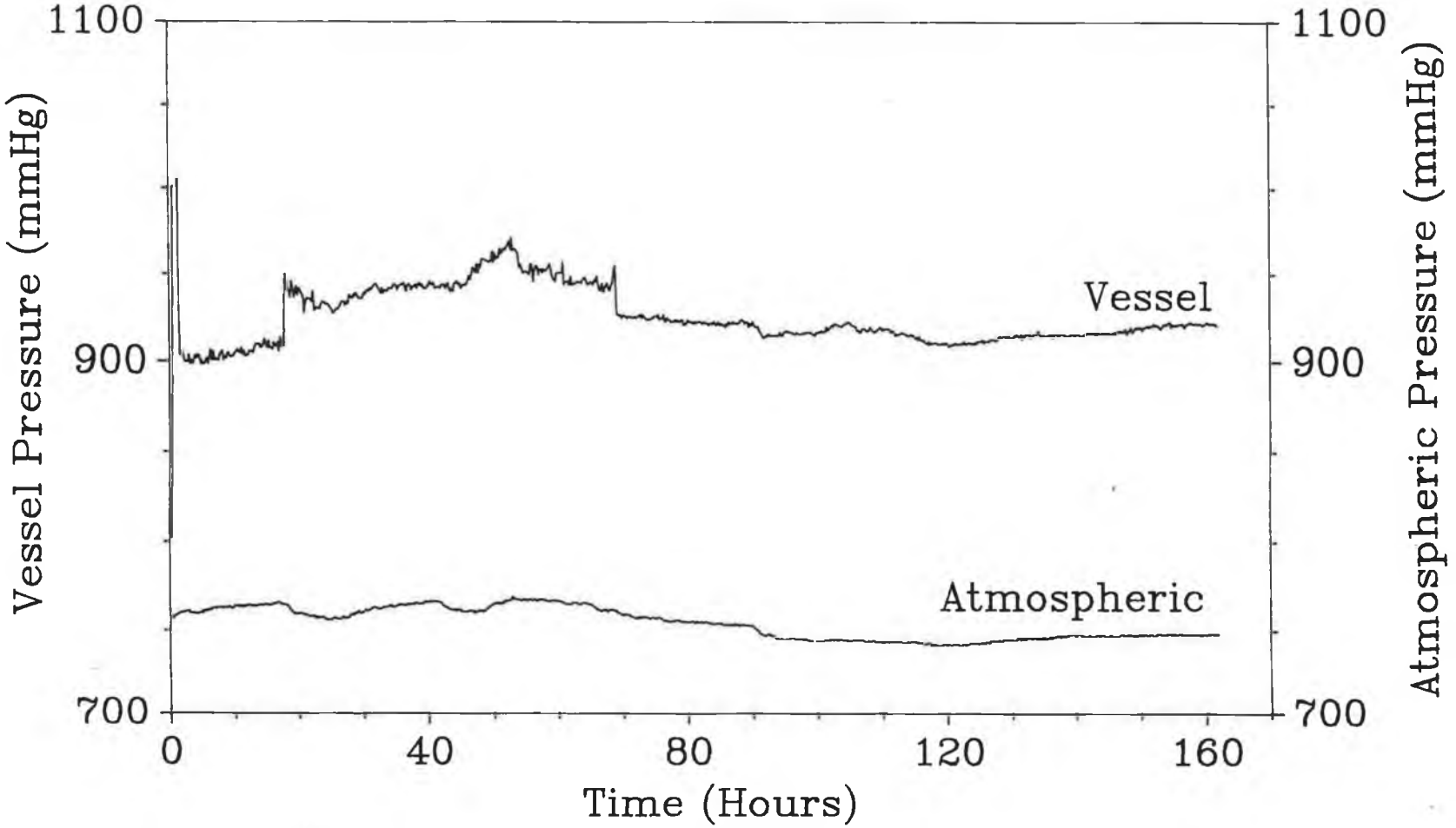


FIGURE 2.6 Vessel and atmospheric pressure during a typical run

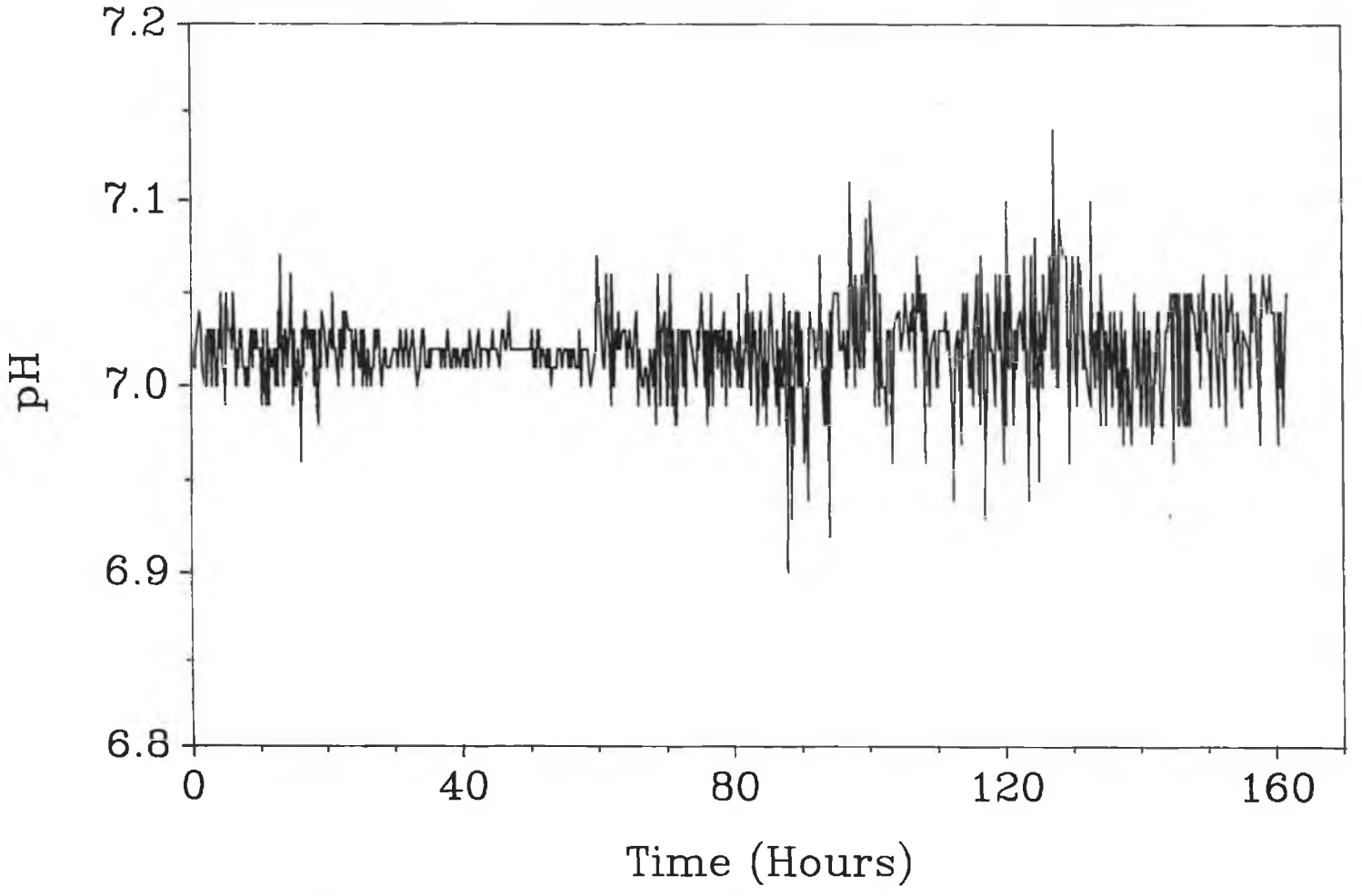


FIGURE 2.7 Typical pH profile during a fermentation run.

3. DATA ACQUISITION AND CONTROL

In order to gain a better insight into the fermentation process, the equipment discussed in section 2 was connected to a data logging device consisting of a Programmable Logic Controller (PLC) and a computer. This enabled data to be recorded on floppy or hard disks at intervals determined by the user.

In addition to data logging, there was also a need for the following functions to be performed:

(i) sequencing of solenoid valves to permit the flow of gases to the gas analysers at the required time

(ii) control of the flow rates of the above gases

(iii) control of the nutrient feed pump

(iv) monitoring of incoming signals and generation of alarms when low or high limits were exceeded

(v) conversion of signals between engineering units e.g. degrees C, mmHg etc., and voltage or current signals.

3.1 PM550 PROGRAMMABLE CONTROLLER

A programmable controller can be defined as

A digitally operating electronic apparatus employing a programmable memory for internal storage of instructions relating to implementation of specific control functions. These include logic, sequencing, timing, counting, and arithmetic functions, and may control machines or processes via digital or analog input or output modules. (NEMA Definition from GE manual GET-6911)

The Texas Instruments PM550 PLC consisted of a central control unit, which could be programmed with both logic and process control functions, and input and output modules which allowed analog and digital signals to be interfaced.

3.1.1 Central control unit (CCU)

The CCU was programmed by a Texas Instruments Read/Write programmer which is connected to the CCU via an RS-232 serial communications link. The user then has access to several areas of memory :

(i) Logic Memory (L)

This area contains 4096 storage locations for ladder logic programs.

(ii) Variable Memory (V)

The 1024 *variable* memory locations are used to store data which can be updated during program execution.

(iii) Constant Memory (C)

Constant data are stored in these 1024 locations.

(iv) Auxiliary Memory (A)

A maximum of 128 locations of this type are used for storing data from analog input and output signals.

These areas can be accessed by each of the two 16-bit microprocessors used for logic and control functions, which are contained in the CCU. They are termed the executive and logic processors respectively.

The logic processor was programmed with ladder logic statements to perform ladder logic, timer, counter, integer move, and integer compare functions. The logic processor was also programmed to request the second processor to perform special functions.

This second processor, which has executive control over the first, handles all communications, loop processing, and special function operations.

Both processors also have access to an image register which stores the on/off status of 1024 digital inputs(X) or digital outputs(Y). The image register also has 512 inputs/outputs which are purely internal. These are known as control relays and were used in ladder logic statements.

3.1.2 Input/output modules

3.1.2.1 6MT digital output modules

A set of Texas Instruments 6MT digital output modules were connected to the solenoid valves. Four of these modules were used and each contained four channels. The channels could be addressed from the ladder logic program via the image register as follows:

Module 1 - Y0,Y1,Y2,Y3

Module 2 - Y4,Y5,Y6,Y7

and so on, where Yn = A valid digital output reference used by the image register.

By switching on an output, power from an external 24 Vdc

supply was permitted to flow to the actuating coil of the required solenoid valve.

The assignment of output channels is shown in figure 3.1.

6MT Output Modules	
Y0	Solenoid Valve - Exhaust Gas
Y1	Solenoid Valve - Inlet Air
Y2	Solenoid Valve - Inlet Nitrogen
Y3	Solenoid Valve - Oxygen Analyser
Y4	Spare
Y5	Solenoid Valve - Inlet 5% Carbon Dioxide
Y6	Solenoid Valve - Carbon dioxide Analyser
Y7	Spare

Figure 3.1 Assignment of valves to the 6MT output modules

3.1.2.2 7MT analog input/output modules

7MT modules were used to convert incoming analog current or voltage signals into a 16-bit computer word. Each input module consisted of a four channel analog to digital converter. The signals ranges handled were 0-5 or 1-5 Vdc or 0-20 or 4-20 mA. Selection of voltage or current was performed by wiring the signal to different terminals on the front of the module.

Each channel was automatically assigned a reference in auxiliary memory depending on the position of the module on a mounting rack. Thus the channels in the first module were referenced as A0, A1, A2, A3. The channels in the next module were then referenced as A100, A101, A102, A103. A full listing of the assignments of each channel is shown in figure 3.2. The range of each signal is shown also.

7MT Input/Output Modules		
Address	Designation	Signal Range
A0	Pressure Transducer	1.25-5 V
A1	Unused	
A2	Pressure Transducer	1.25-5 V
A3	Vessel Temperature	4-20 mA
A100	pH Meter	1-5 V
A101	Unused	
A102	Unused	
A103	Dissolved Oxygen	4-20 mA
A200	Oxygen Analyser	0-1 V
A201	Carbon Diox. Analyser	0-5 V
A202	Unused	
A203	Unused	
A300	Unused	
A301	Unused	
A302	Air Flow Rate	4-20 mA
A303	Unused	
A600		
A601	Peristaltic Pump	4-20 mA
A602		
A603		
A700	Control Valve (Rig)	4-20 mA
A701		
A702		
A703		

Figure 3.2 Assignment of signals to the 7MT input and output modules

The result of the A/D conversion was read into the corresponding memory location as an integer between 0 and 32000 for a 0-5 Vdc or 0-20 mA signal. If a 20% offset existed in the signal, i.e. 1-5 Vdc or 4-20 mA, the signal was read as 6400 to 32000.

Similarly, the 7MT output modules consist of a four-channel D/A converter. The output modules generate a 0-10 Vdc or 0-20 mA signal, or, if required, a 2-10 Vdc or 4-20 mA signal.

The A/D and D/A converters had a 12-bit resolution, that is, 1 part in 4096.

3.2 PROGRAM DESIGN AND IMPLEMENTATION

The design and implementation of the program to perform the functions listed in the introduction to this chapter is detailed in the following section.

First, the input and outputs were assigned the locations shown in figures 3.1 and 3.2. Next, areas of V and C memory were allotted for various functions. These included storing the scaled values of incoming signals, storing the answers to calculations, storing timers and counters, storage of control loop data and storage of the programmed special functions.

The flowsheet shown in figure 3.3 illustrates the tasks to be executed by the PM550. From this flow sheet which was developed over several months, the ladder logic program was written.

The program contained the following features:

1. A control relay was used to switch the program on or off using an end of scan function.

2. A timer/counter arrangement was used to count the elapsed time of the fermentation in minutes. A special function request then converted this to hours.

3. To control the movements of solenoid valves a recycling drum sequencer counting from 0 to 12 minutes was programmed. The solenoids were opened at the required times during this cycle.

4. Special function requests were made to scale incoming signals into the required engineering units.

5. An integer compare function was used to generate alarm signals by checking if incoming signals are outside the defined high or low limits.

6. The final section of the program controlled the action of a nutrient pump. It was found that the calcium carbonate present in the feed inhibited the action of the pump at low speeds. To overcome this, it was decided to give the pump a pulsing action, switching it on at a high speed for a short time followed by a longer off-time. Over a long period, this high speed pulsing would be equivalent to a continuous flow at low flow rates. Two timers were used to regulate the on/off status of a control relay. This status was then used to activate two integer-move functions. If the control relay was off, zero

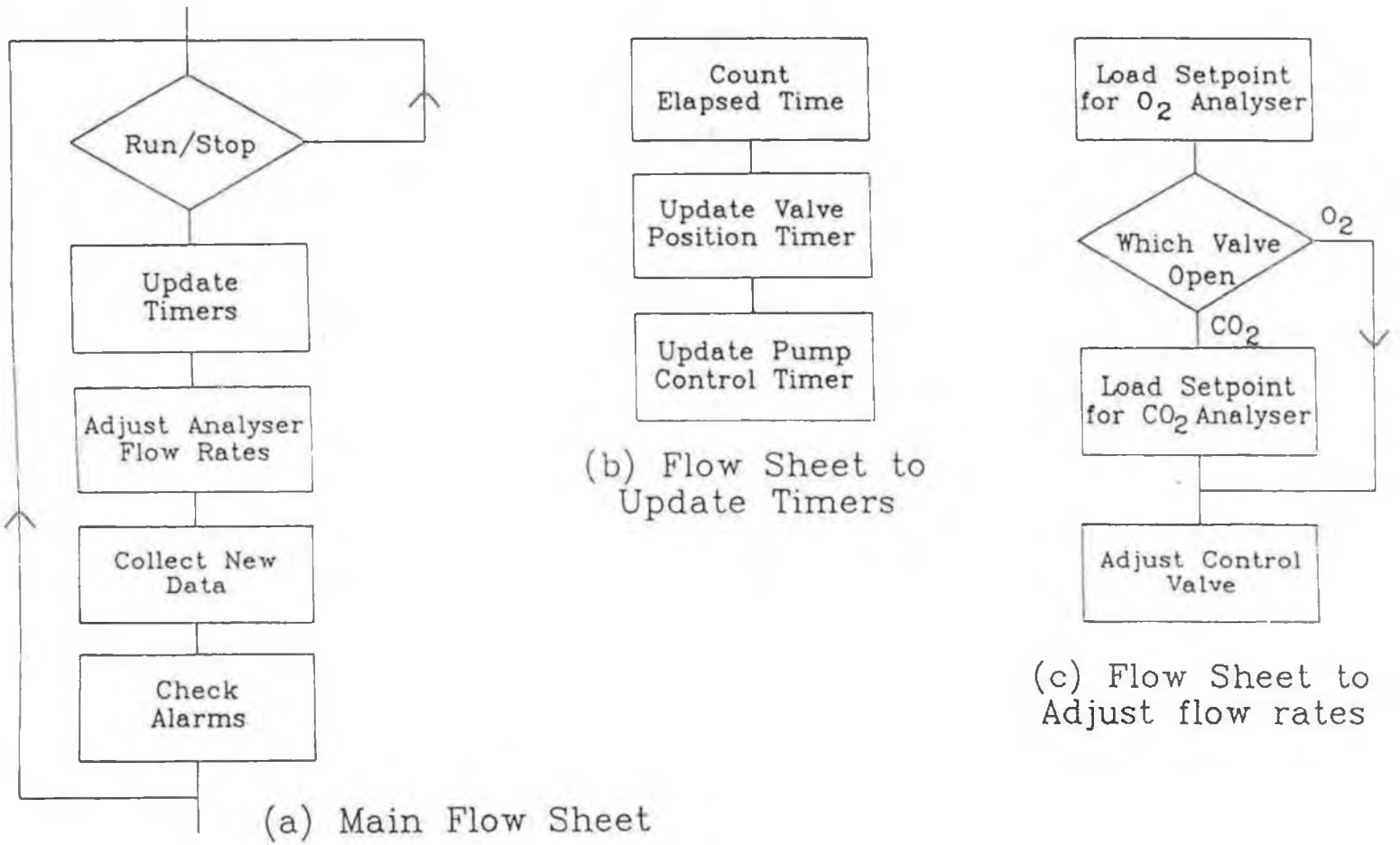


FIGURE 3-3 : Flow Sheet of Ladder Logic Program

was moved to the auxiliary memory location assigned to controlling the pump's speed. When on, the control relay activated the pump by moving a value from C memory to A memory.

Several programming features of the PM550 were found to be of particular use and are discussed in sections 3.2.1-3.2.3 .

3.2.1 Special functions

Special functions requested from the logic were stored in V memory. Several special functions could be 'chained' together so that they were all executed from the one request statement.

Although there are 18 different special functions available only two were used. These were numbers 3 and 8.

Special function 3, scales incoming signals between two user-definable limits, compensates for a 20% offset, if required, and stores the answer in user memory.

Special function 8, allows maths calculations to be performed using values stored in V and C memory locations.

3.2.2 Integer manipulations

In addition to the special functions, integers in V, C and A memory can be moved and compared. The latter was of use in sequencing valve movements and in detecting alarm conditions in incoming signals.

3.2.3 Proportional-integral-derivative (PID) control loops

The PM550 contains 8 PID loops, one of which was used to control the flow rate of gas to the analysers. The entry of the required details was performed via a menu system of prompts. Locations in V memory were assigned to store the tuning parameters, i.e. values for the proportional, integral and derivative constants, and the loop setpoint.

The equipment used for monitoring and controlling the flow rate is detailed in section 2 and the loop tuning in section 3.4. However, an additional piece of ladder logic was required. Since the analysers were in operation at different times and each required different flow rates, the ladder logic program contained

instructions to access the loop and change the setpoint as required. The setpoints were 0.8 litres/min for the CO_2 analyser and 1.2 litres/min for the O_2 analyser.

3.3 DATA LOGGING

A Texas Instruments Professional Computer was connected to the PM550 via an RS-422 linkage. This PC is an IBM XT compatible computer and is equipped with 512K RAM, a 10 Mbyte winchester disk a floppy disk drive and a colour monitor.

Using CVU 6209 software from Texas Instruments the computer was programmed to read PM550 memory locations and to store readings on a floppy or hard disk at regular intervals. The memory addresses in the PM550 of data to be stored, were entered in a database in the CVU 6209. This database was also used to store alarm text messages which could be activated by the status of control relays as described in section 3.2..

The entry numbers of database entries to be recorded on diskette were then entered on a report page. The contents of a report page are shown below:

```
/$O1E:R1.PRN|
{  73  }{  33  }{ 75  }{  40  }{  46  }{  81  }{  83  }
```

Figure 3.4 Database entry numbers entered on report

In another section of the software, a control relay address in the PLC was assigned to this report. Thus, when this control relay was switched on in the PM550, the report was stored on disk in the file using the filename contained in the string at the start of the report shown above i.e. in file "R1.PRN" on drive E:. A section was added to the ladder logic program to activate this control relay when the cycle controlling the solenoid movements was completed.

In this manner data was collected at 12 minute intervals throughout the fermentation. The data collected was examined after the fermentation and was found to contain the filename string. A FORTRAN program was used to remove this string. An example of the sorted data is shown in Figure 3.5.

This data was then imported into LOTUS 1-2-3. The

worksheet was exported in the *.DIF* format to SIGMAPLOT to generate the graphs used in this thesis.

Hrs	Temp.	pH	D.O.	% CO2	Pv	Patm
0.4	27.69	7.01	103.2	0.035	802	757.5
0.6	27.06	7.02	129.6	0.145	1001	758.6
1.2	27.31	7.04	128.2	0.190	1001	759.8
1.4	27.62	7.03	129.3	0.172	1005	760.6
1.6	27.75	7.01	117.8	0.170	904	760.6
2.0	27.00	7.00	117.9	0.188	906	761.0
2.2	26.62	7.00	117.1	0.167	903	761.0
2.4	26.37	7.03	116.4	0.175	900	761.4
2.8	26.56	7.03	115.4	0.160	900	761.8
3.0	26.81	7.00	116.1	0.157	904	760.2
3.2	27.31	7.03	115.9	0.157	899	760.6
3.4	27.62	7.03	117.7	0.147	901	760.2

Figure 3.5 A sample of sorted data collected from a fermentation run using CVU 6209 software

3.4 ADDITIONAL USES OF THE COMPUTER

The CVU 6209 also allowed on-line generation of graphs to allow the user to follow the progress of the fermentation on the computer screen. This was achieved by placing database entries on special graph pages. Text lines from the database were also used on display pages to indicate alarm conditions of incoming signals, if the control relays indicating the appropriate alarm conditions were switched on.

The CVU 6209 also contained the facility for loop-tuning which was used to set up the tuning parameters for the control loop to regulate the air flow rate to the gas analysers.

The tuning page contained a graph showing the levels of the loop process variable, setpoint and output. This enabled the response of the loop to changes in tuning parameters, to be measured and any necessary adjustments to be made. Thus the response to a value of gain could be visualised real-time on the screen.

The loop gain was adjusted by trial and error, until a satisfactory response to a step change was noted on the above screen. It was found that proportional-integral control was sufficient to control the flow rate at the required setpoint.

The tuned parameters obtained by the above method were:

gain = 0.5 %/% (proportional term)

reset = 0.1 minutes (integral term)

4. MATERIALS AND METHODS

This section reviews the materials and methods employed for:

1. culture maintenance
2. inoculum production
3. batch and fed-batch fermentations
4. biomass and glucose assays
5. detection of pimaricin levels.

4.1 FERMENTATION MATERIALS AND METHODS

4.1.1 The organism

The organism used was the actinomycete *Streptomyces natalensis*(CBS 700.57). This was obtained in a freeze-dried vial from Centralbureau Voor Schimmelcultures in Holland. The freeze-dried spores were suspended in nutrient broth and pure colonies were isolated on oatmeal agar.

4.1.1.1 Culture maintenance medium

Oatmeal agar (Booth, 1971) was prepared as follows:

Oatmeal	30 g/l
Agar (Technical No.3)	20 g/l

Oatmeal was ground in a centrifugal mill to produce a coarse powder. The required amount of powder was suspended in distilled water and brought to the boil. After simmering for one hour, the liquid was filtered through cheesecloth to remove any remaining large pieces of oatmeal. The liquid was made up to the required volume and the agar added. The mixture was then brought back to the boil to dissolve the agar. The medium was then autoclaved for 15 minutes at 121°C and 15 psig. and dispensed into plates or flasks as required.

4.1.1.2 Culture maintenance techniques

The cultures were routinely maintained on agar plates. The incubation temperature was typically 27°C. For further subculturing, sterile loops could be used to remove portions of the culture from plates.

The culture was also maintained in 250ml flasks with approximately 50ml agar at the bottom. A solution containing 0.1% Triton X-100 and glass beads could be used to remove the spores.

An attempt to maintain the culture on small agar slopes was unsuccessful. This was thought to be due to aeration limitations.

It was found that subculturing on agar plates provided a simple and very effective way of maintaining the culture. It was decided to abandon the more cumbersome agar flask technique.

4.1.1.3 Culture morphology

On agar, the organism formed single definite colonies which were slightly elevated in the centre. Colonies appeared after two days, and maximum growth was achieved after four to seven days. The colonies initially appeared white or cream but gradually developed a greyish-brown tint with time. There was a noticeable earthy aroma from the colonies.

In liquid culture, the organism was found to exhibit several types of morphologies, which may be dependent on growth conditions. In the most common form, the hyphae tended to form loose nebulous clumps with no distinct core. The second form of morphology was exhibited as distinct compact clumps of mycelia with a few hyphae protruding from the central core. These clumps were clearly visible to the naked eye.

4.1.2 Inoculum preparation

To provide enough cells to inoculate the SF-116 fermenter the cells were transferred from plates to flasks.

4.1.2.1 Inoculum production medium

The following medium was used based on the medium developed by Struyk et al (1957). Corn steep powder was added to this medium rather than corn steep liquor. An amount of corn steep powder equivalent to the w/v% of the liquor used by Struyk et al was found to provide adequate growth in shake flask studies.

The medium consisted of:

Peptone	5 g/l
Corn Steep Powder	3 g/l
Glucose	10 g/l
Sodium Chloride	10 g/l

After the mixture was prepared, the pH was adjusted to 7.0 with sodium hydroxide. The mixture was dispensed in 50 ml volumes into 250 ml flasks. The flasks were then autoclaved at 121°C and 15 psig for 15 minutes.

4.1.2.2 Inoculum production

Due to the nature of the culture's morphology, it was found that spore numbers were impossible to quantify . It was thus decided to inoculate the flasks with the spores taken from one-quarter of the surface of an agar plate.

The flasks were then placed on an orbital shaker for four days at 27°C and at a speed of 150-200 rpm. The flasks which were observed to have the best growth were transferred to 1000 ml conical flasks containing 350 ml of similar medium. These flasks were then incubated under identical conditions for two days.

4.1.3 **Fermentation methods**

The standard fermentation process used during this project was a traditional batch method. As will be discussed later, a fed-batch variation was also used.

One day prior to the start of the batch, all pre-run work was completed. This included calibration of the two gas analysers, preparation of the drying columns and polarisation of the dissolved oxygen electrode, as detailed in section 2.

At this stage, the feed reservoirs of acid and base were

prepared. The reservoirs contained 1000 ml of 2M H_2SO_4 and 2M NaOH respectively. An extra 1000 ml of each solution was prepared and kept for replenishing the reservoirs. These solutions were autoclaved at 121°C and 15 psig for 20 minutes.

4.1.3.1 Production medium

The required volume of the following medium was prepared:

Glucose	30 g/l
Corn Steep Powder	1 g/l
Ammonium Sulphate	5 g/l
Potassium Chloride	4 g/l
Potassium Dihydrogen Phosphate	0.2 g/l
Calcium Carbonate	8 g/l

This medium is based on that of Struyk et al (1957), except that corn steep liquor was replaced by corn steep powder.

The medium was then placed in the vessel and the pH was manually adjusted to 7.0 with sodium hydroxide. Antifoam was added at a dosage level of 1 ml antifoam per litre of medium. The vessel was then heated to 121°C for 30 minutes.

Upon cooling, all monitoring devices were connected, if this had not already been done. This included the two feed reservoirs, the pressure transducer and the vessel exhaust, which was connected to the solenoid bank.

The vessel was then inoculated with 800 ml of culture. The inoculum was poured in through a removable port on the headplate of the vessel. A bunsen burner was used to aid aseptic transfer. The programs for data logging and control of the rig were then enabled by a single keystroke.

4.1.3.2 Fermentation conditions

The following conditions were used during the batches:

Temperature - 27°C

pH - 7.00

Air Flow Rate - 12 l/min (1.5 v/v/min)

Sampling was performed every three hours using the following procedure. The sampling port on the fermenter was steam sterilised for approximately five minutes. A small amount of

fluid (a mixture of culture broth and condensate) was allowed to flow out of the port and was collected in a volumetric cylinder. A 25-30 ml sample was then removed into a sterile universal and this was stored at 4°C for analysis. An equivalent amount of distilled water was placed in a separate universal.

The volume of sample removed was calculated by adding the water in the universal to the liquid in the volumetric cylinder. This allowed a reasonable estimate of the volume to be made in the absence of any sensor for volume measurement. The volumes of base, acid and nutrient feed additions were also noted. If required, nutrient feeds were added after 2-3 days.

At the end of the run, the vessel contents were sterilised and discarded.

4.2 ANALYTICAL MATERIALS AND METHODS

Samples taken were analysed for biomass, glucose and pimaricin levels.

4.2.1 Biomass determination

As mentioned in section 4.1.2.2, the morphology of the organism precludes easy determination of biomass levels. It was decided to use a wet weight technique to estimate biomass levels. A dry weight estimation technique was attempted, but the constituents of the medium were found to contribute a high background to the readings. These constituents also contribute to problems with the wet weight assay as will be discussed in section 5.

The method eventually chosen was that of determining the wet weight of a pellet after centrifugation. A 5 ml sample was spun at 5,500-6,000 rpm for 60 minutes. The pellet weight was determined as the difference in weight of the centrifuge tube with and without the pellet. The assay also had the added advantage that the supernatant could be used for sugar determination.

Duplicates of each sample were assayed.

4.2.2 Estimation of reducing sugars (DNS method)

DNS reagent was prepared using the following method of Miller (1957).

The following components were weighed out:

30g Potassium Sodium Tartarate

50ml Distilled Water

16ml 10% Sodium Hydroxide

These were warmed to 60°C to dissolve, and 1g 3,5 Dinitro Salicyclic Acid was added. The mixture was then cooled and made up to 100 ml with distilled water.

A stock solution of 1% glucose was prepared in a volumetric flask and solutions of 0.1% (1 mg/ml) were prepared from this as required.

Dilutions of samples were prepared using distilled water and clean, dry glassware.

A set of glucose standards in the range 0-1 mg/ml was also prepared.

2 ml of sample or standard was placed in a tube and 2 ml of DNS reagent added. 2 ml of distilled water was used as a blank. All solutions were assayed in duplicate.

The tubes were covered with tinfoil and placed in a boiling water bath for exactly 10 minutes. They were then removed and put in cold water. 10 ml distilled water was then added to all tubes. The absorbance of the sample solutions at 540 nm was then measured with a Pye Unicam SP6-550 spectrophotometer.

A graph was drawn using the values obtained from the range of standards. This was then used to calculate the glucose concentration of the samples.

4.2.3 Detection of pimaricin

Three methods of pimaricin detection were considered during the project. These were:

1. Plate Assay
2. Isolation of Pimaricin Crystals
3. High Performance Liquid Chromatography (HPLC)

4.2.3.1 Plate assay

A plate bioassay involving the measurement of the sensitivity of *Saccharomyces cerevisiae* to the antibiotic was proposed. However, work at Dublin City University suggested that this assay would have relatively low accuracy (White, 1988).

4.2.3.2 Recovery of crystals

Recovery of crystals of pimaricin was attempted and the resultant concentrations determined. The procedure employed is based on the technique of Struyk et al, 1957 and is summarised below:

Stage 1: Adjustment of broth pH to 10.00

This stage causes cell lysis and releases any pimaricin contained within the mycelia. The pH was adjusted to 10.00 with 2M NaOH.

Stage 2: Filtration/Centrifugation

The mycelial debris is removed at this step by either of the above methods. The filter aid kieselgühr was used to increase efficiency

Stage 3: Adjustment of broth pH to 3.00

Concentrated phosphoric acid was used to adjust the pH of the broth to pH 3.0 which is the pk_a of the antibiotic and thus encourages its precipitation. However, other compounds are also precipitated.

Stage 4: Solvent Extraction

The antibiotic can then be selectively isolated by exploiting the fact that pimaricin is more readily soluble in C1-C6 alcohols than in water. To this end, butan-1-ol was used for the extraction. Several additions of butan-1-ol were attempted. Typically, 1 litre of the solvent was used to extract the entire broth contents.

Stage 5: Washing of Extract

To remove any remaining water soluble components, a 2% borax (sodium tetraborate) solution was employed.

Stage 6: Concentration

Concentration of the antibiotic solution was performed in a rotary evaporator at 45°C under vacuum.

In practice this assay was cumbersome, time consuming, material intensive and did not lend itself to analysing a large number of samples. It was thus decided to develop an assay for pimaricin using a technique which would be quick, accurate and also suitable for the routine handling of large numbers of samples.

4.2.3.3 HPLC detection of pimaricin

The detection of pimaricin in cheese samples using HPLC had already been demonstrated by Engel et al (1983). It was found that this method was suitable for detecting pimaricin in a pure sample purchased from Sigma Chemicals. However, analysis of fermentation samples proved somewhat more difficult. The following method of sample pretreatment was thus developed to improve HPLC analysis of fermentation samples. Complete details of the difficulties encountered and the subsequent results obtained are given in section 5.

METHOD:

2 ml of sample was placed in a tube and 2 ml of 2M NaOH was added. The samples were vortexed and left overnight to leach pimaricin from the mycelia. The pH of each sample was then adjusted to approximately pH 3.0 with 0.5 ml concentrated phosphoric acid. The sample was then immediately extracted with 5 ml of butan-1-ol.

A Waters High Performance Liquid Chromatograph consisting of a Model 510 pump and a Model 481 spectrophotometer was used for analysis. A chart recorder was attached to the spectrophotometer to record the peaks obtained during analysis. The chart speed was set at 10 mm/min.

The following parameters were set on the detector:

Wavelength : 303 nm

Sensitivity : 0.1-0.5 AUFS

The sensitivity was adjusted as required depending on the concentration of the samples.

The following parameters were set on the pump.

Flow Rate : 1.5 ml/min

Pressure Cut-Off : 2000 psi

A guard column packed with Technoprep silica C8 packing was

used to protect the analysis column. This was a LiChrosorb 10RP8 column obtained from HPLC Technology. The column dimensions were 25 cm x 4.6 mm.

The mobile phase used was a 65:35 mixture of HPLC grade methanol and potassium dihydrogen phosphate (3.03 g/l). This solution was filtered through 0.45 μm Millipore filters and degassed prior to use.

10 μl of sample was loaded into a Waters UK6 sample injection port using a Hamilton syringe. The trace from the chart recorder was used to estimate pimaricin levels. The area of the peak obtained by injecting a 0.05 g/l sample, was used to quantify the concentration in the injected fermentation samples.

5. RESULTS AND DISCUSSION

This chapter presents the results obtained from several trial runs and 9 fermentation runs. A full discussion is included at the end of the chapter.

5.1 TRIAL FERMENTATIONS

5.1.1 Trial fermentation no.1

A trial fermentation run was performed on a smaller than normal scale to investigate the effectiveness of the techniques employed.

5.1.1.1 Fermentation

280 ml of fermented broth from shake flasks was used to inoculate 4 litres of production medium. Growth was then allowed to continue for 48 hours under the following conditions:

Temperature : 28°C

Motor Speed : 500 rpm

Air Flow Rate : 10-12 litres/min

It can be seen from figure 5.1 that the dissolved oxygen decreased, as expected, even at maximum aeration and agitation levels during the initial growth phase. It then levelled off at a lower value but rose dramatically towards the end of the fermentation. This was thought to have been due to a decrease in metabolic activity. Since the secondary phase of microbes coincides with decreased metabolic rate, it was decided to extend the duration of future runs to examine this later stage more closely.

5.1.1.2 Extraction of pimaricin from fermentation broth

The final broth volume of 4.5 litres from this trial run was used to investigate the feasibility of extracting the antibiotic by the method outlined in section 4.2.3.2.

After fermentation, 2M NaOH was used to adjust the pH of the broth to pH 10.0. 90g kieselgühr was added to the broth and an

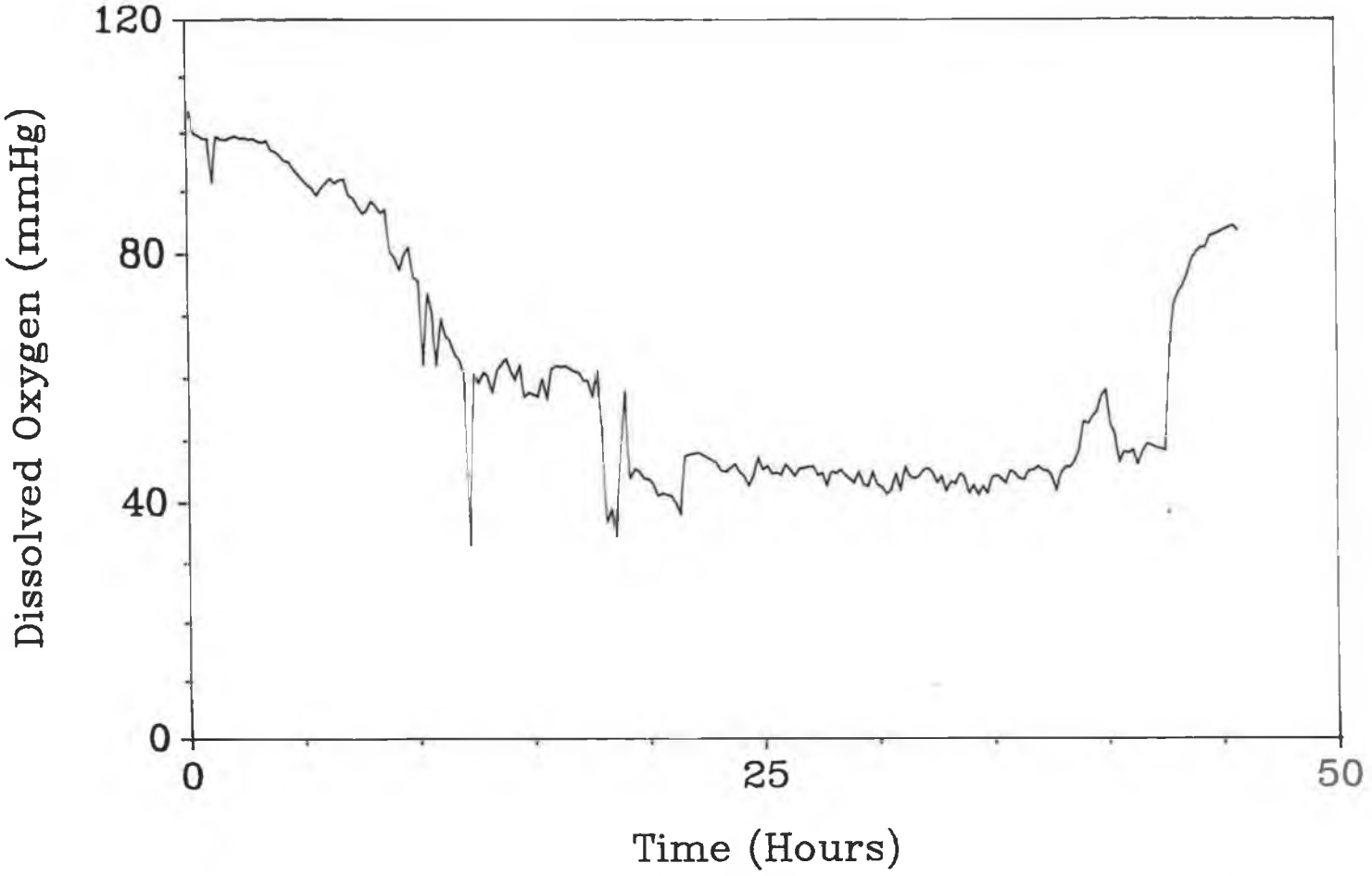


FIGURE 5.1 Dissolved oxygen profile for trial run 1

attempt was made to clarify the solution with Whatman No.1 filter paper. The filter paper clogged easily and made further filtration impossible. A Sharples tubular bowl centrifuge was successfully used to separate the cell debris and kieselgühr. 314.1g of solids were collected from the centrifuge. Thus, allowing for 90g kieselgühr, a final wet weight of 224.1g of cell debris was estimated.

Phosphoric acid was added to the clarified broth to bring the pH to 3.0. The broth was then extracted in a 5 litre separating funnel with 750, 350 and 300 ml of 1-Butanol. The light phases were pooled and washed with 360 ml of a 4% Borax solution. At this stage three phases were seen:

* a heavy borax phase,
* a phase containing some insoluble compounds
* and the butanol phase.

The butanol extract was kept and was further washed with 200 ml water.

The butanol extract was evaporated in vacuo at 44°C using a rotary evaporator. Crystals formed in the extract when approximately 200 ml had been removed under vacuum. When dried, these had a dark brown colour. 1.2 litres of butanol still remained and could not be evaporated with the equipment available at DCU.

A sample of the crystals was analysed by infra-red (I.R.) spectroscopy but the results obtained were inconclusive and are not presented.

The amount of butanol (1.4 litres) used was very ineffective and resulted in the yield of crystals being too low to estimate accurately. It was decided to abandon this method for estimating the yield of pimaricin.

5.1.2 Trial fermentation no.2

The purpose of this experiment was to evaluate a larger scale fermentation, and to investigate whether HPLC could be effectively used to quantify pimaricin production.

5.1.2.1 Fermentation

250 ml of inoculum was used to inoculate a fermenter containing 6 litres of production medium. The fermentation was allowed to run for 94 hours (see section 5.1.1.1) under the following conditions:

Temperature : 27°C

Motor Speed : 600 RPM

Air Flow Rate : 5 litres/min

Samples were taken during this fermentation and analysed for glucose and biomass. The results obtained are shown in figure 5.2. There was a noticeably rapid uptake of glucose during the first 24 hours. However, biomass levels increased steadily until around 70 hours.

It was noted that the removal of samples and addition of acid and base changed the volume of the broth considerably over the course of a fermentation. Therefore, it was decided that the measurement of volume would be incorporated in future runs. Levels of glucose, biomass and pimaricin would be represented as total amounts (g) rather than concentration (g/l).

No data on dissolved oxygen, carbon dioxide etc. was collected from the computer during this run.

5.1.2.2 HPLC analysis

Samples of pure pimaricin and centrifuged broth were injected for analysis and some typical chromatograms produced are shown in figure 5.3. As can be seen chromatogram (b) is quite "noisy" with two large peaks, both of which varied in size, becoming larger as the fermentation progressed. The larger peak had a retention time close, but not equal, to that of a pure sample of pimaricin (figure 5.3 (a)). It was decided that an extraction procedure to isolate the antibiotic from the broth would be developed to investigate if this would produce just a single "clean" peak.

5.2 FERMENTATION RUN 1

Fermentation run no. 1 investigated a fermentation incorporating techniques optimised during the trial runs (Section

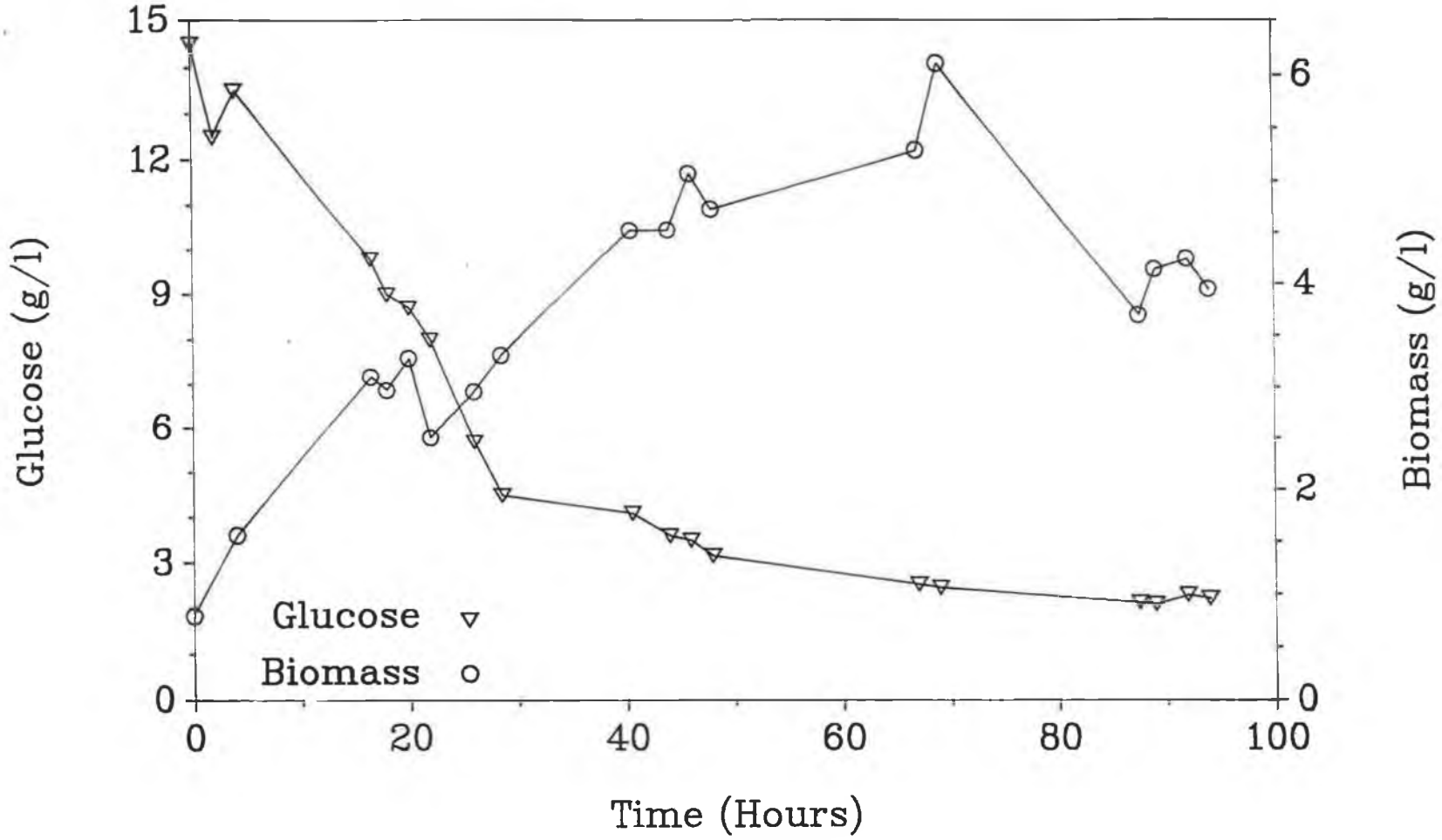
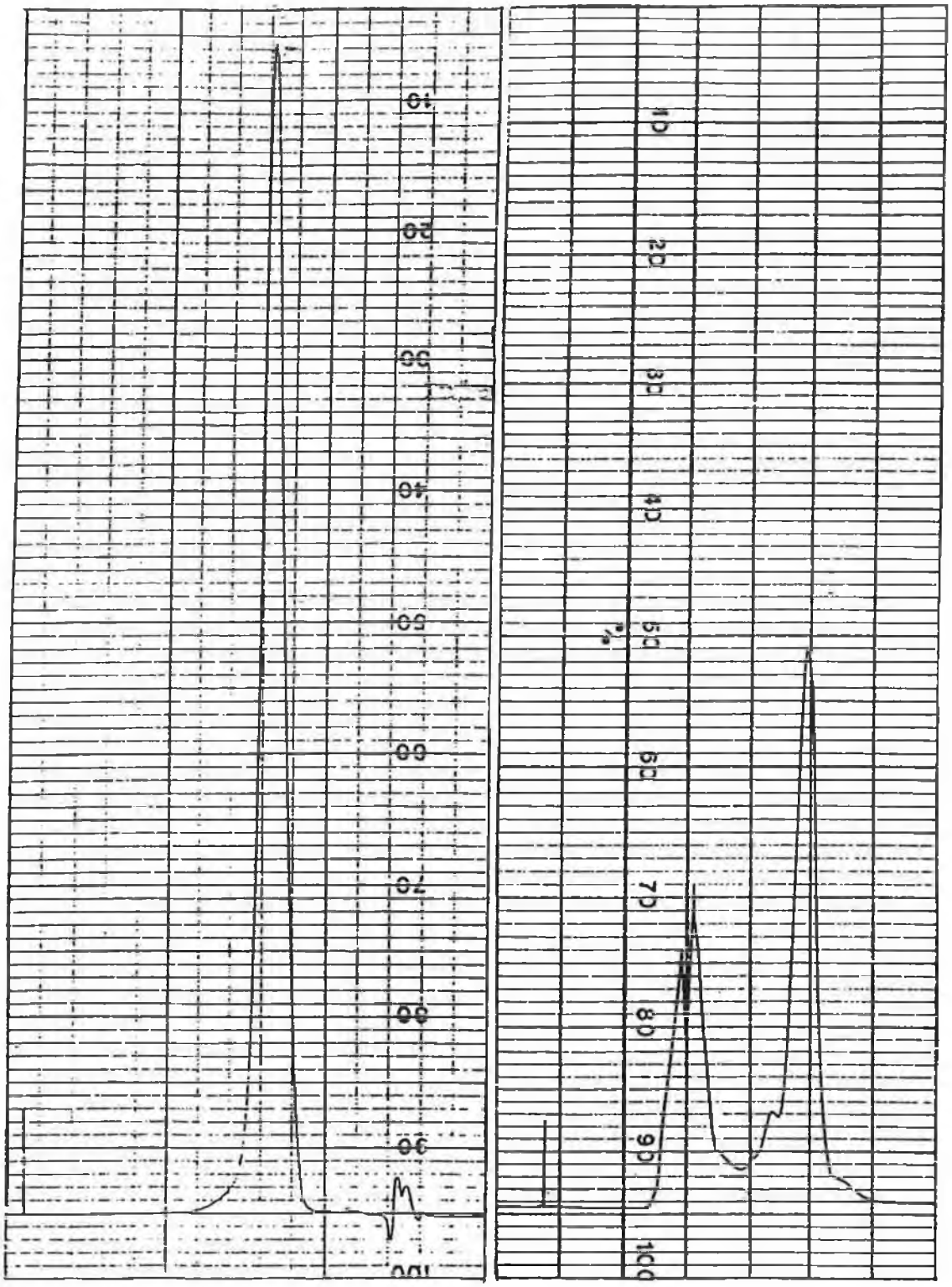


FIGURE 5.2 Concentrations of glucose and biomass during trial run 2



(a) (b)

FIGURE 5.3 : Chromatograms of
(a) pure pimaricin
(b) fermentation broth

5.1). This run endeavoured to optimise the HPLC analysis by employing an extraction procedure on samples prior to analysis. A comparison between dry and wet weight techniques for estimating biomass was also made.

5.2.1 Fermentation

Figure 5.4 shows the profiles of exhaust gas carbon dioxide composition and fermentation broth dissolved oxygen generated from data logged by the computer. This figure reveals several points of interest.

As in trial run 1, an initial linear decrease in levels of dissolved oxygen was observed. However, in this run the decrease was extended until 63 hours. Two rapid increases each followed by a longer "plateau" level of dissolved oxygen then occurred. It was noted that a third large increase was due to an excess of vessel pressure caused by a valve malfunction.

The level of carbon dioxide decreases initially, but increases steadily in the period between 50 and 100 hours. There then occurred a steady decrease towards the end of the run.

This period of static dissolved oxygen and rising CO_2 occurred while biomass was increasing and glucose decreasing (figure 5.5).

A lag of ≃ 15 hours after inoculation was also observed in biomass production and glucose consumption . The initial decrease in carbon dioxide mentioned above could also be attributed to this lag.

It was thought that the wet weight technique for estimating biomass would be prone to errors due to the presence of solids such as calcium carbonate . Previous runs had revealed discrepancies between duplicates. For example, pipetting the sample into the centrifuge tube was thought to be a primary source of error due to the morphology of the culture.

Samples from this run were also assayed for dry weight to examine the feasibility of using this method for quantifying biomass. A 5 ml aliquot of each sample was placed in a drying dish. The dishes were placed in a drying oven for 1 hour at 120°C.

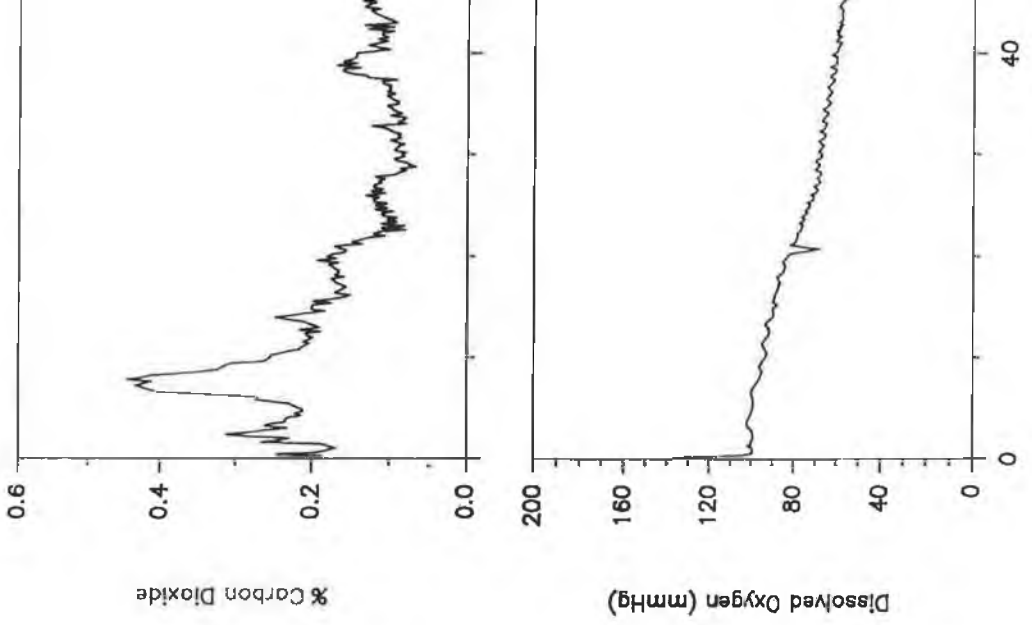
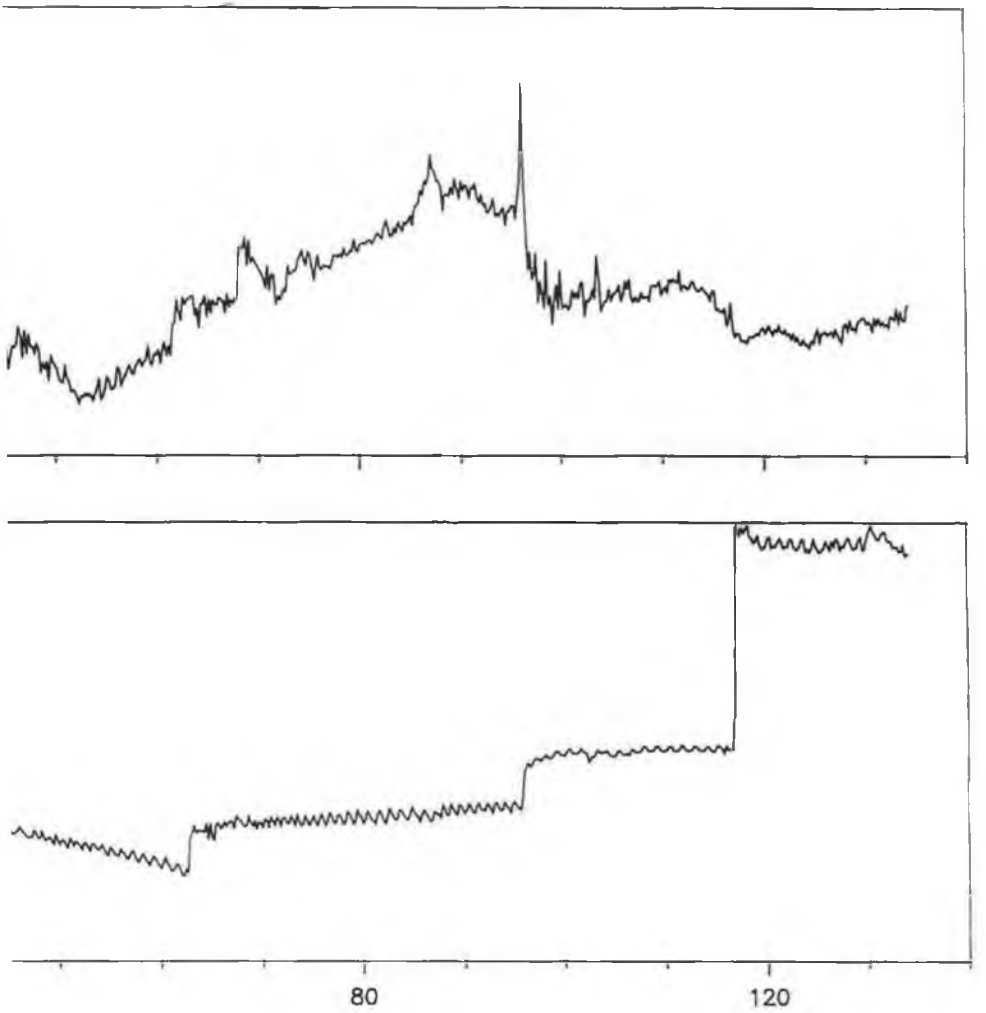


FIGURE 5.4 : D.O. and carbon dioxide profiles for run 1


80
120
Time (Hours)

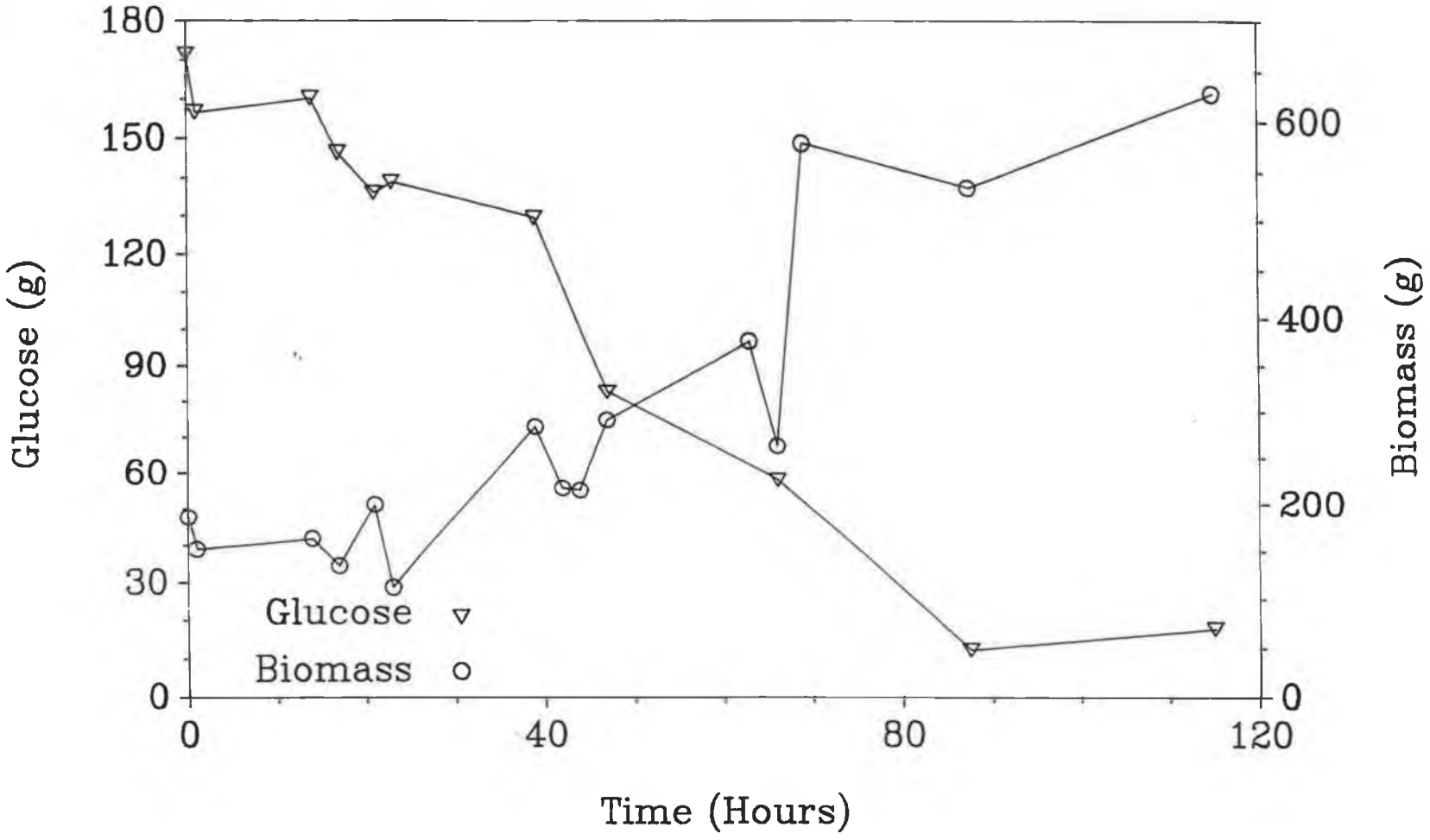


FIGURE 5.5 Glucose and biomass profiles for run 1

It was found that the components of the medium contributed a high background level to the readings making it difficult to quantify increases in biomass, particularly in the initial growth stages.

It was decided to maintain the wet weight method as the method of choice for biomass determination.

5.2.2 Extraction and HPLC analysis

The levels of pimaricin shown in figure 5.6 were determined by HPLC. Fermentation samples were extracted using the method described in section 4.2.3.3. Samples of pure pimaricin and extracted fermentation broth were injected and the traces obtained are shown in figure 5.7 (a) and (b). In both cases the traces show a single large peak but the retention times vary by over two minutes. The other peaks were confirmed to be methanol, in figure 5.7(a), and butanol, in figure 5.7(b), by injection of each of these solvents and measurement of the retention times of the detected peaks.

To confirm the identity of the large peak obtained in the fermentation sample (figure 5.7(b)), a Waters photodiode-array detector was used to analyse samples of pure pimaricin and extracted broth eluted from the column. The detector was set to scan wavelengths between 200 and 400 nm. The profiles of wavelength versus absorption obtained are shown in figures 5.8 (pure pimaricin) and 5.9 (extracted sample). The ultra-violet (u.v.) absorption spectrum of the sample was identical to that of pure pimaricin. These peaks are caused by the double bonds of the ring structure.

It is thought that the difference in retention times may have been due to the pimaricin in the broth being chemically modified during the extraction procedure in such a way that its uv spectrum was unaltered but that one of its peripheral groups was more attracted to the HPLC column and thus had a longer retention time.

The effectiveness of the extraction procedure is shown in figures 5.10 and 5.11. It can be seen that the extracted sample (figure 5.11) produces a peak almost four times the height of the untreated sample (figure 5.10) when the dilution during

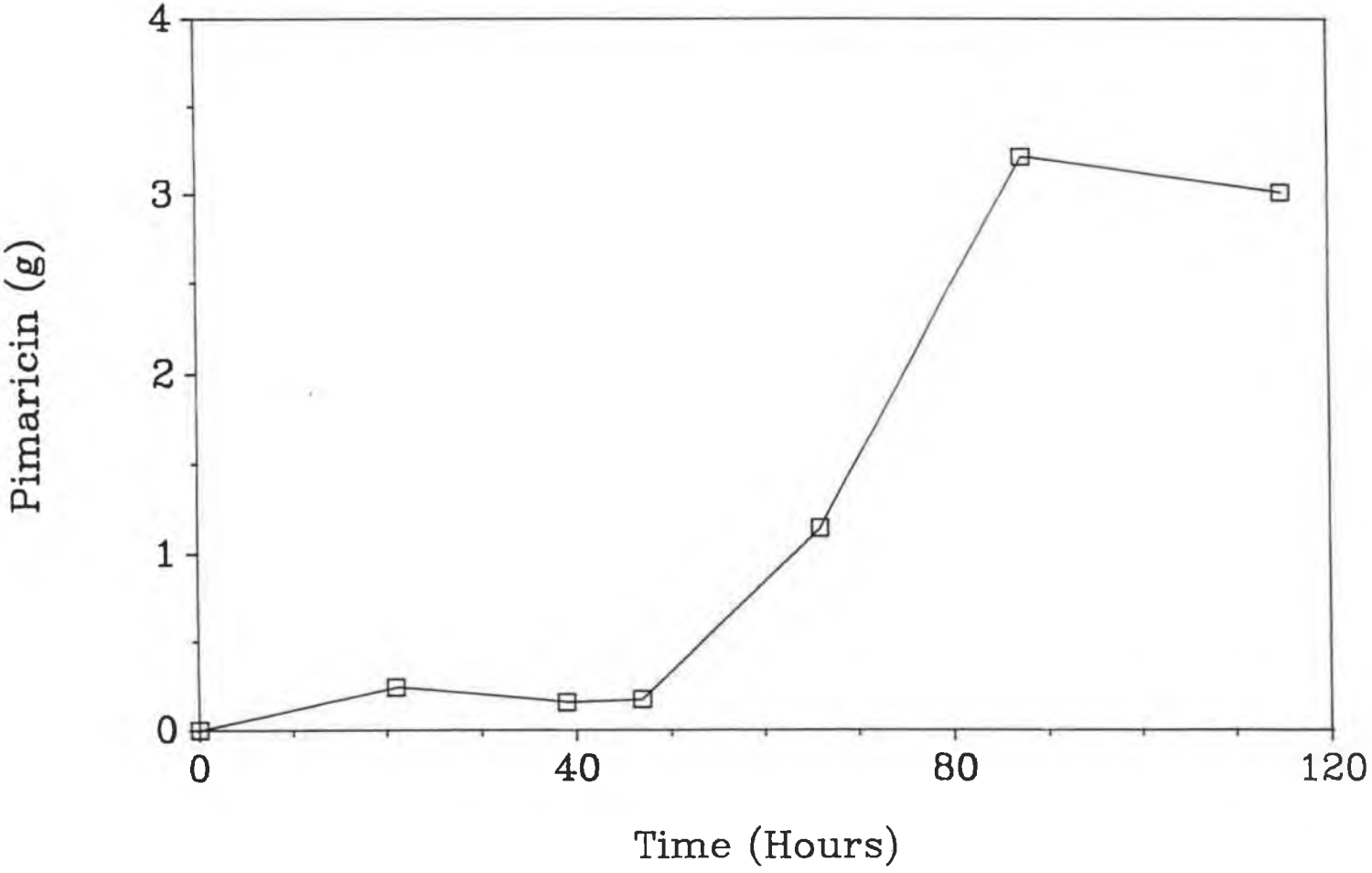


FIGURE 5.6 Pimaricin profile for run 1

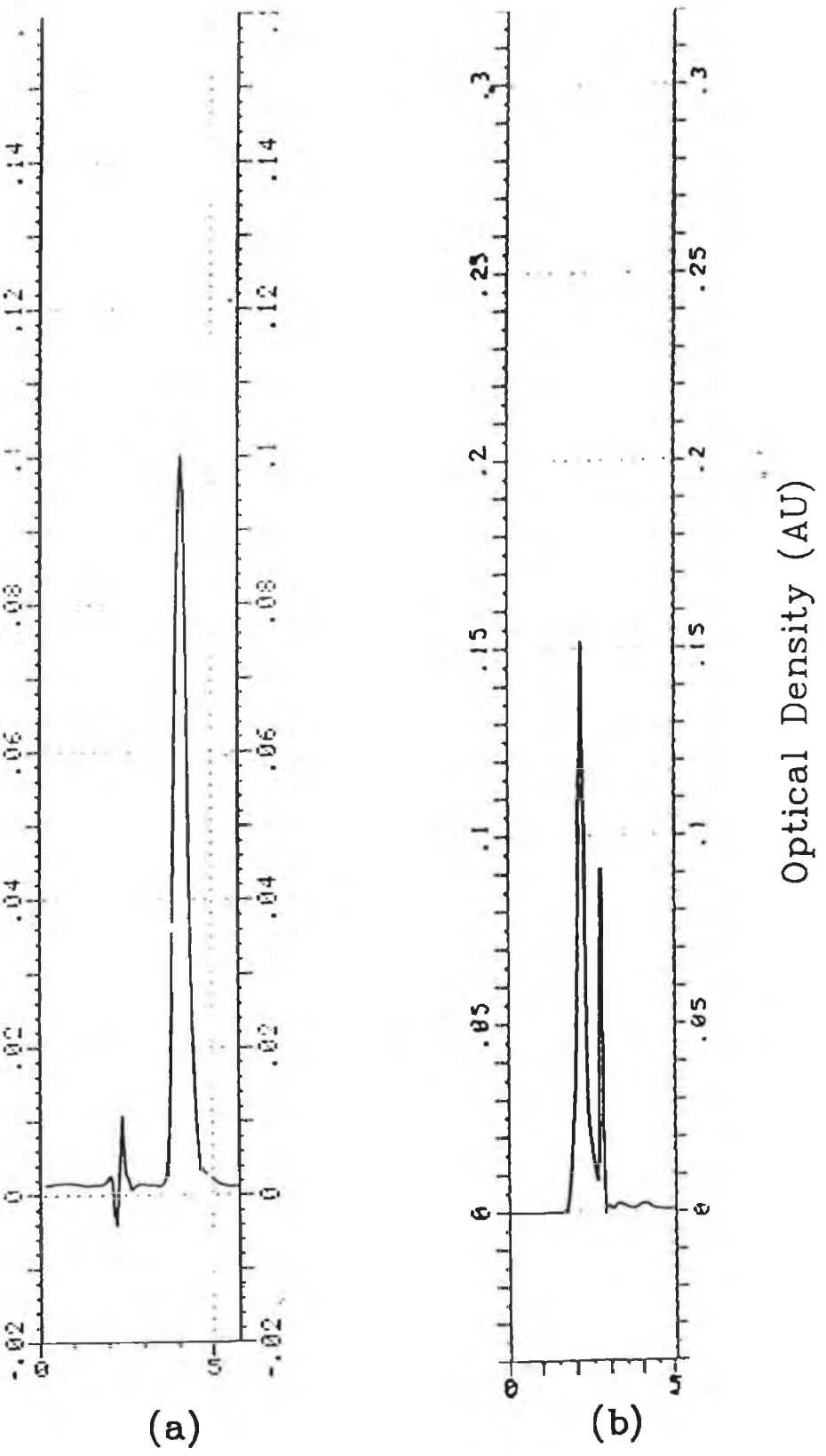


FIGURE 5.7 : Chromatograms of (a) pimaricin and (b) extracted sample

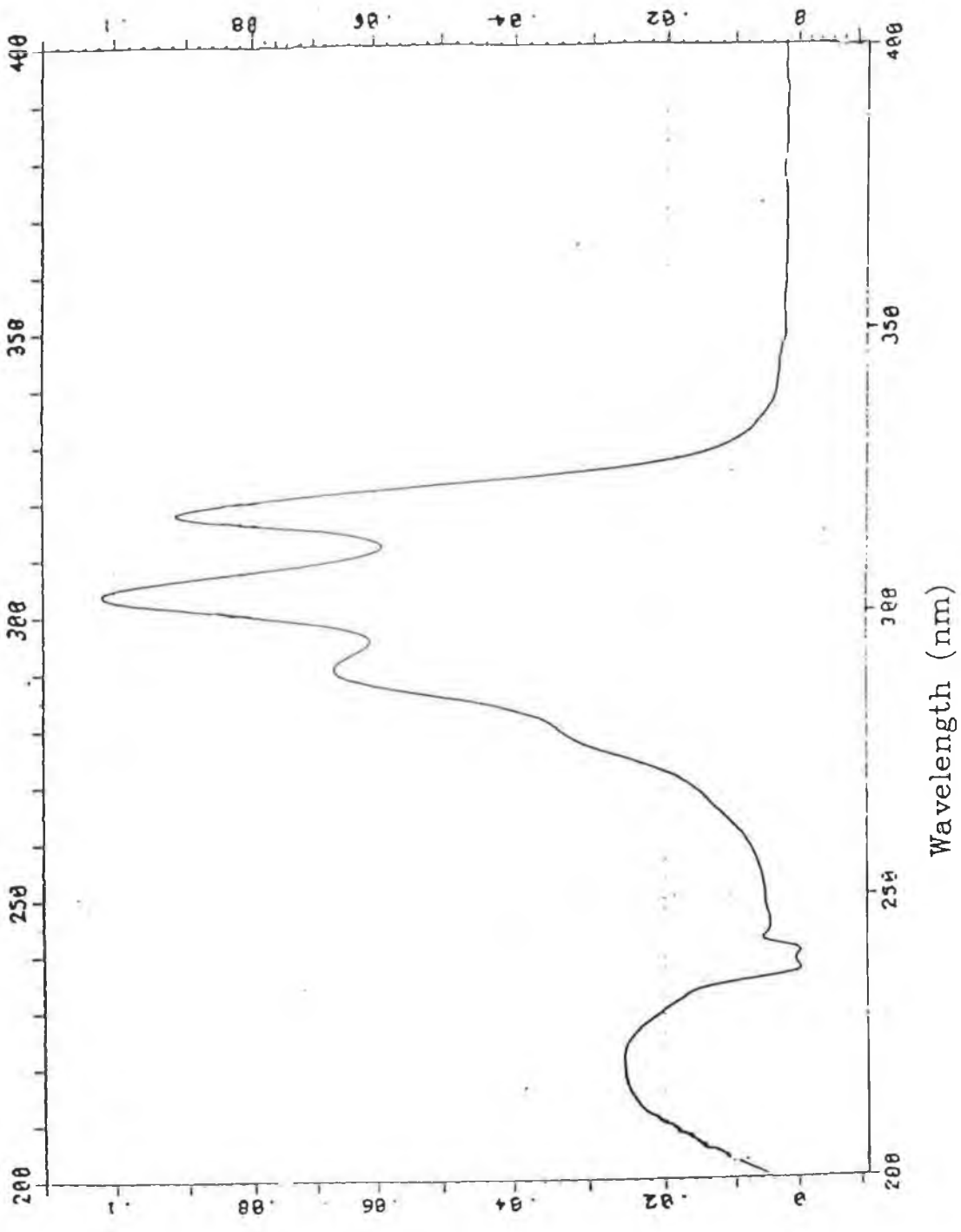


FIGURE 5.8 : U.V. absorption spectrum of peak in figure 5.7(a)

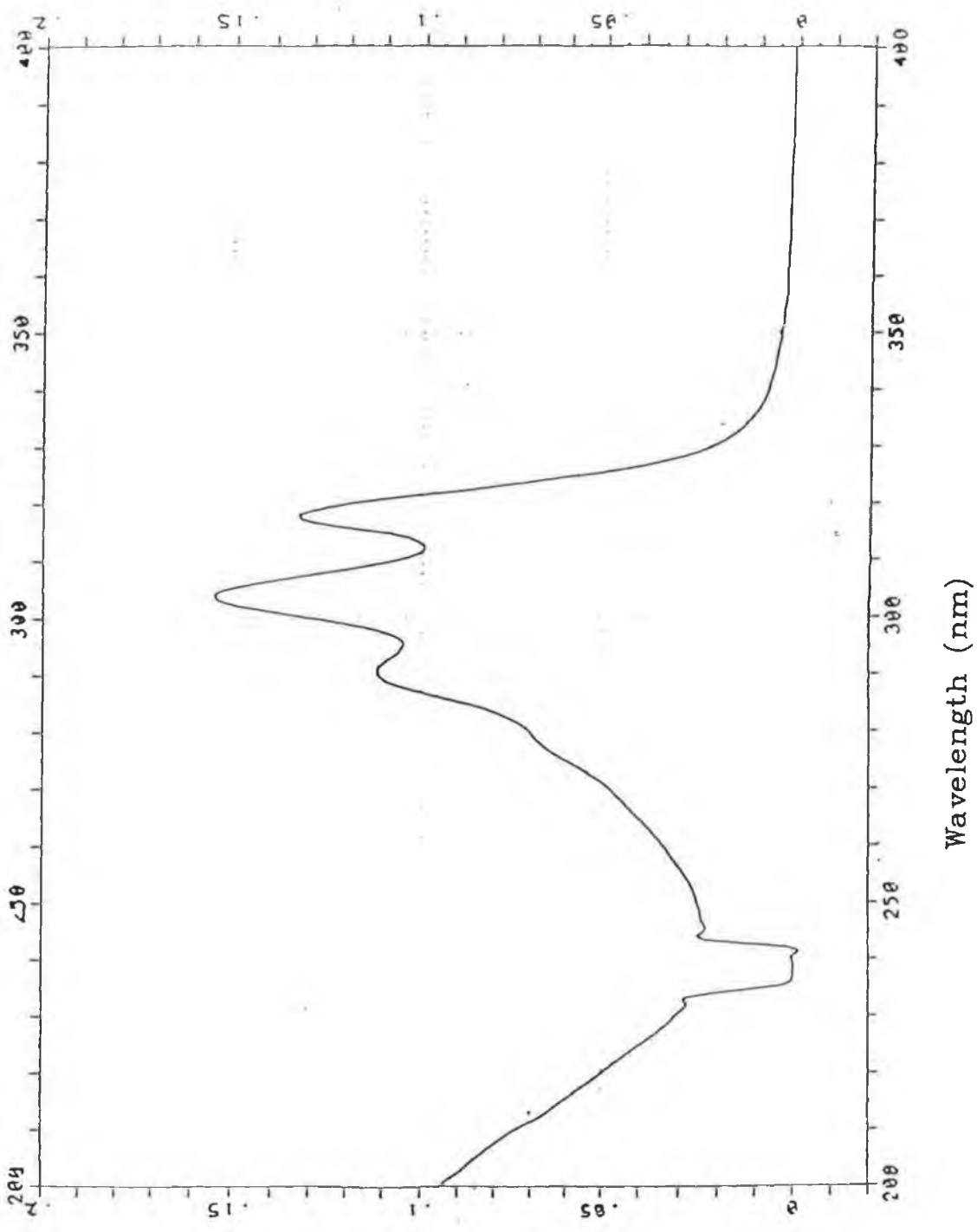


FIGURE 5.9 : U.V. absorption spectrun of peak in figure 5.7(b)

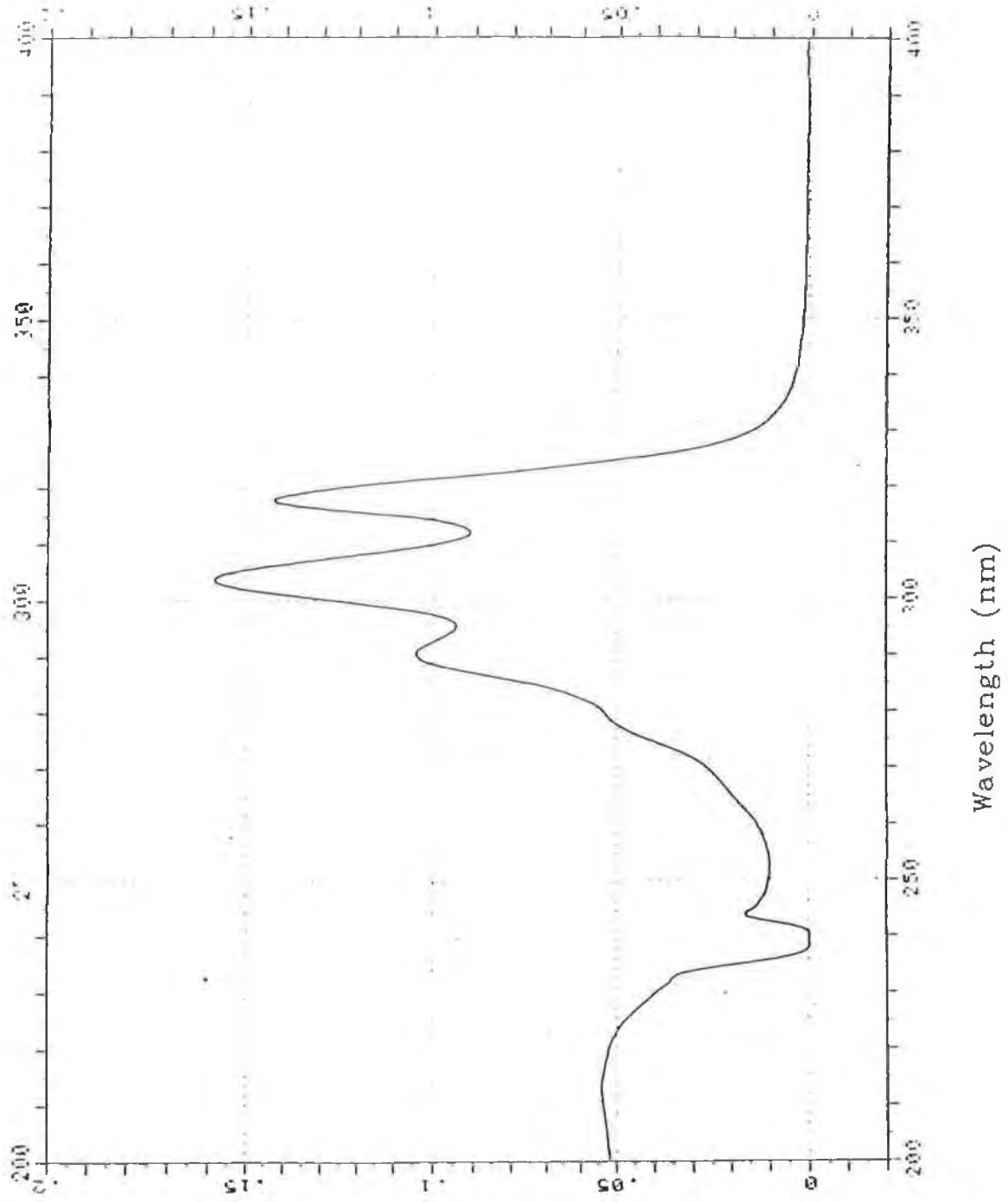


FIGURE 5.10 : U.V. absorption spectrum of "raw" fermentation broth

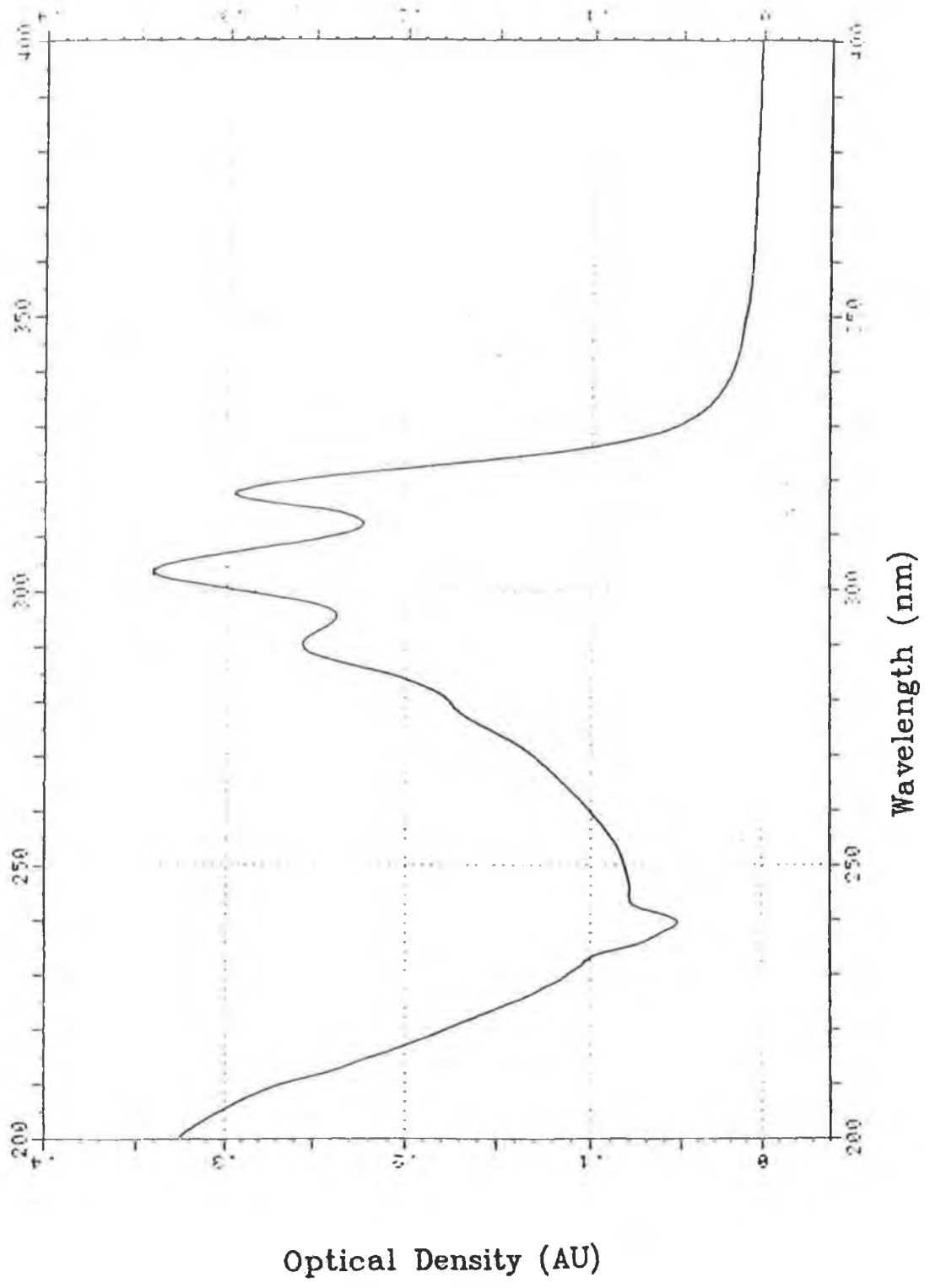


FIGURE 5.11 : U.V. absorption spectrum of extracted broth

extraction is taken into account.

5.3 FERMENTATION RUN 2

This run was a repeat of Run 1 with slightly differing conditions. The motor speed was reduced to investigate if any decrease in mechanical shear on the pronounced filamentous morphology of the organism would have a role in increased pimaricin production. Aeration was increased to investigate if growth could be stimulated to minimise the initial lag observed in fermentation run 1.

5.3.1 Fermentation

The following settings were employed:

Motor Speed : 400 RPM

Air Flow Rate : 12 litres/min

500 ml of inoculum was used to inoculate 6800 ml of production medium. These conditions provided slightly higher levels of inoculum and aeration.

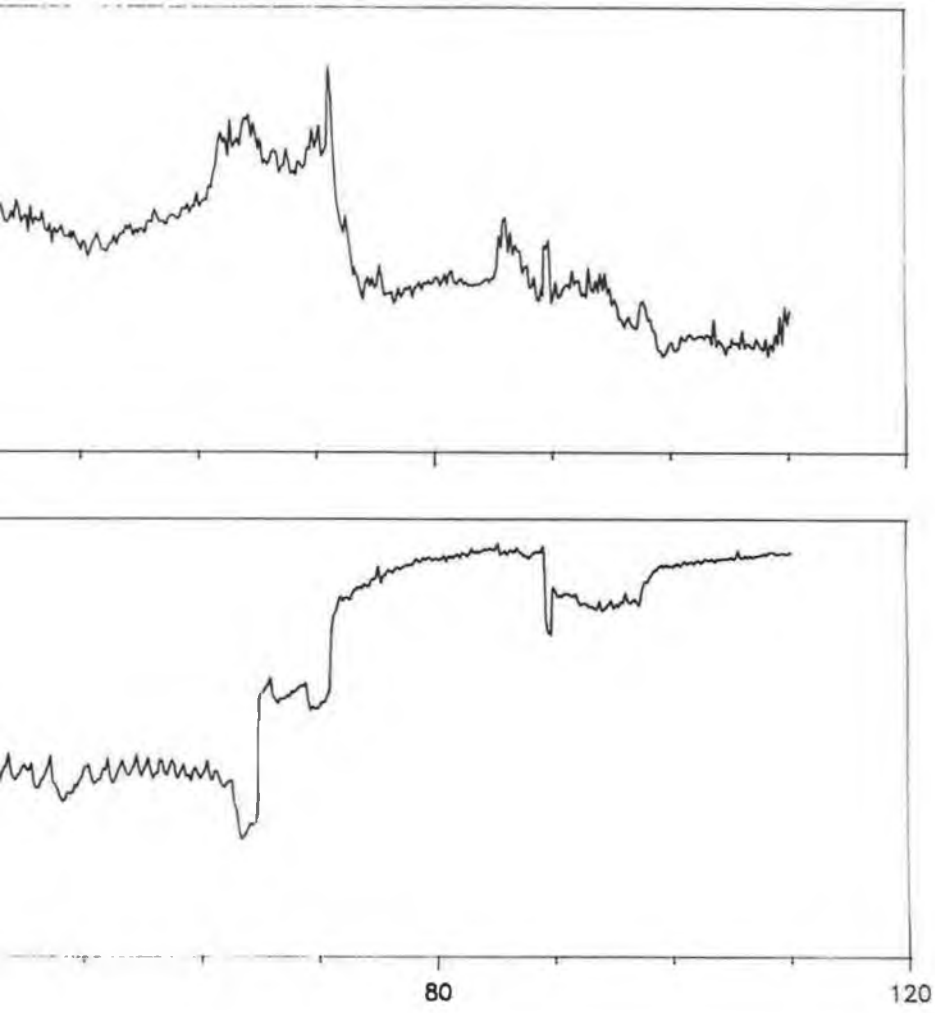
Figures 5.12 and 5.13 show that similar results to run 1 were obtained. The culture appeared to be static for the first 24 hours. As in run 1, the increase in biomass and glucose uptake rate thereafter coincided with increases in carbon dioxide evolved. Furthermore, the initial fall in dissolved oxygen levels were followed by a series of rapid rises and plateaus, also observed in run 1.

It was also noted that the levels of white carbonate deposit in the centrifuged pellets disappeared gradually over 1-2 days. Any increase in biomass may then have been masked by the decreasing carbonate level.

5.3.2 HPLC analysis

No pimaricin assay was possible this time, however, due to an equipment failure.

FIGURE 5.12 : D.O. and carbon dioxide profiles for run 2


80
120
Time (Hours)

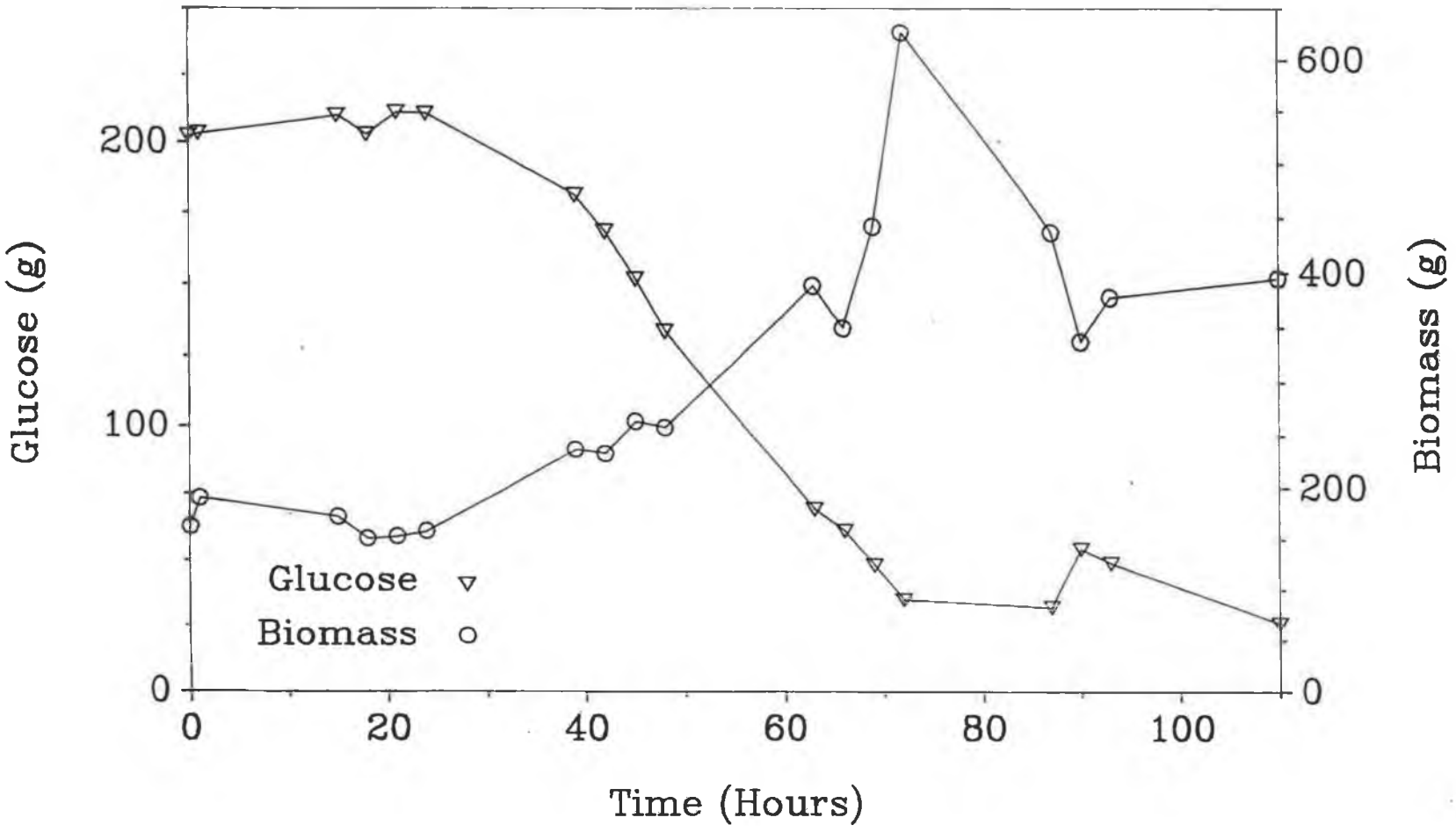


FIGURE 5.13 Glucose and biomass profiles for run 2

5.4 FERMENTATION RUN 3

The purpose of this run was to investigate the effects of fed-batch techniques on pimaricin production. It had been noted in run 1 that glucose supplies were depleted towards the end of the run and that pimaricin production had accordingly levelled off. (Figures 5.5 and 5.6).

It was also decided to increase the inoculum level to 10% and adjust the aeration rate to 1.5 v/v/min in order to minimise the initial lag phase of the fermentation observed in runs 1 and 2.

5.4.1 Fermentation

7.2 litres of production medium was inoculated with 800 ml of inoculum and the aeration rate was set to 12 l/min. It was later discovered that the amount of glucose added was incorrect. (29 g/l was added instead of 33 g/l).

The nutrient feed solution added was basically the same as the production medium with the exceptions that a higher level of glucose, 10% was used, and that calcium carbonate was omitted.

Feed Solution Composition (Made up to 1 litre)

Glucose	100 g/l
Corn Steep Powder	1 g/l
$(NH_4)_2SO_4$	5 g/l
KCl	4 g/l
KH_2PO_4	0.2 g/l

Feed was added each time DNS assays indicated that the total sugar level had dropped below 50 grams, as pimaricin production did not seem to occur below this level in run 1. The feed was added in the following amounts:

69 hours - 220 ml
90.5 hours - 100 ml
99 hours - 200 ml

Figures 5.14, 5.15 and 5.16 show the results obtained from this run. It can be seen that the initial lag highlighted in previous runs had almost been eliminated by the techniques used. (See glucose profile, figure 5.15). The trends in dissolved oxygen and carbon dioxide evolved followed the same general pattern observed in runs 1 and 2. The effect of feeding the

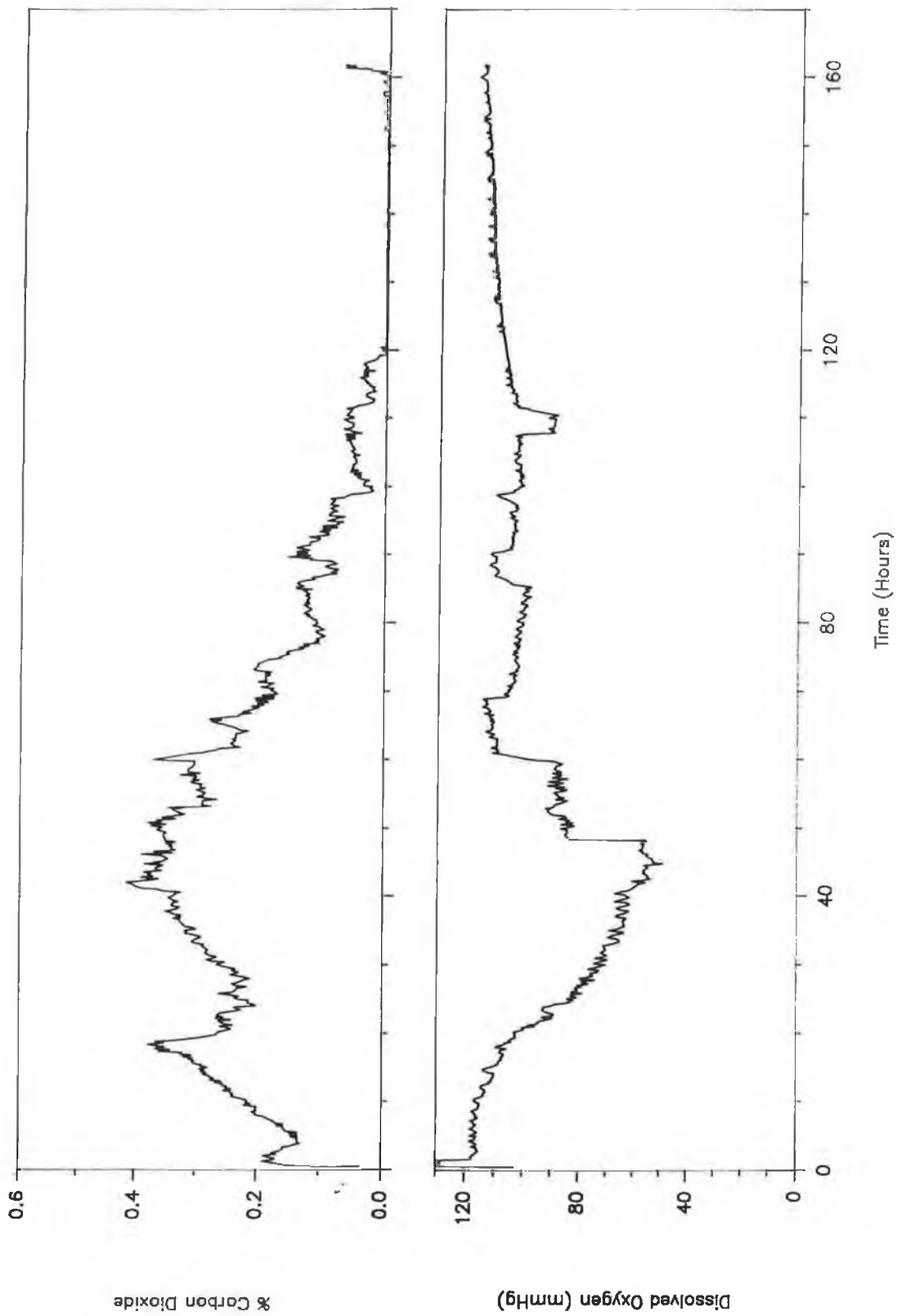


FIGURE 5.14 : D.O. and carbon dioxide profiles for run 3

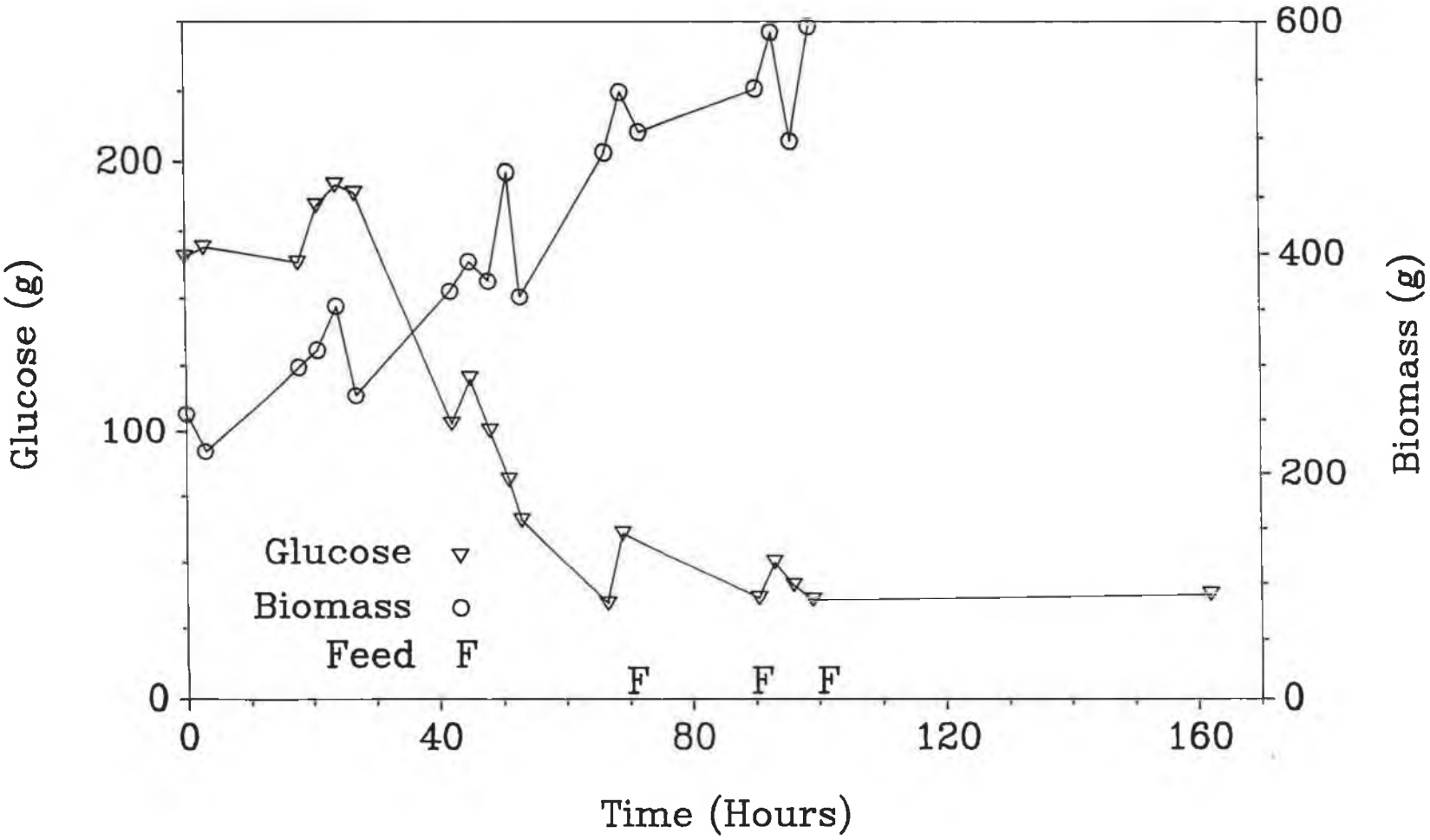


FIGURE 5.15 Glucose and biomass profiles for run 3

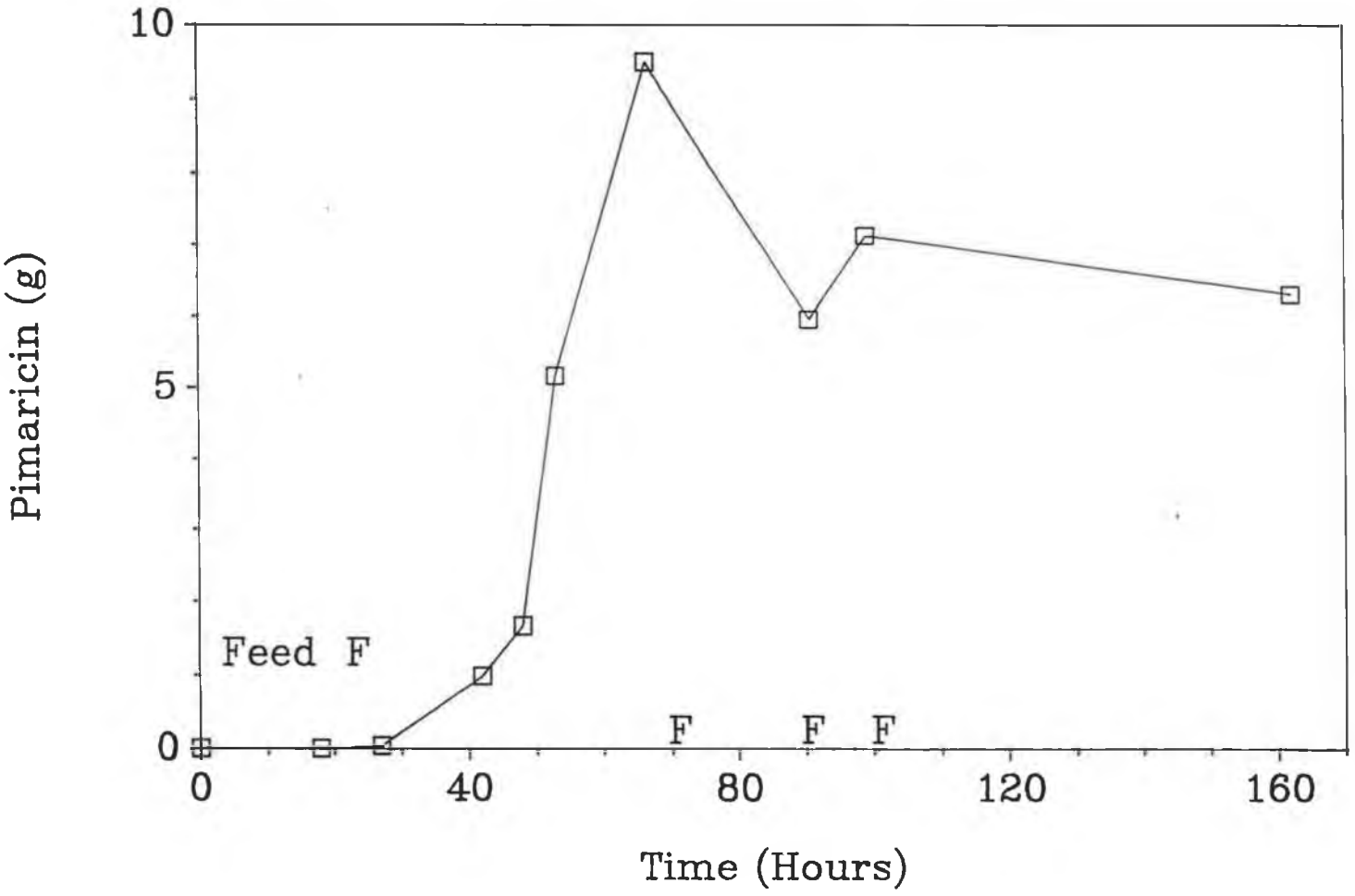


FIGURE 5.16 Pimaricin profile for run 3

above solution seemed to be to increase biomass. No increase in pimaricin production was obtained after feeding.

Note that the biomass level at 160 hours could not be determined as the pellet obtained after centrifugation disintegrated when removing the supernatant.

The glucose level at this point was roughly equal to that before the feed was added at 99 hours. It is thought that the cells may have been using glucose for maintenance purposes only as the carbon dioxide levels fell to zero towards the end of the run, and the dissolved oxygen gradually returned to the initial saturation level.

5.4.2 HPLC analysis

It is worth highlighting the exceptionally high yields of pimaricin obtained during this run. The yield of >9g pimaricin was obtained on only one sample. These yields were never matched during the remainder of the project. A full comparison of the yields from each run is included in section 5.11.

5.5 FERMENTATION RUN 4

It was thought that the rich nature of the nutrient feed used in the previous run was such that biomass production was stimulated and antibiotic production depressed. Therefore, the purpose of this run was to investigate the effects of feeding a nutrient solution containing glucose only.

Antibiotic production is also known to be inhibited by high levels of inorganic phosphate which may interfere with synthesis of the macrolide sugar (Omura & Tanaka, 1986). It was decided to omit phosphate from future nutrient feed solutions, but that the levels in the basic production medium would remain unchanged in order to stimulate cell growth in the initial stages.

As can be seen from figure 5.16, the production of pimaricin did not appear to occur after 100 hours of the fermentation. For this reason, it was decided that further runs would be for 90 hours only.

The fermentation was set up as in section 5.4 and 1 litre of 100 g/l glucose was used as a feed.

The first feed (100 ml) was added when the dissolved oxygen was observed to fall to its lowest level, since most of the pimaricin in the previous runs appeared to be formed after this point had been reached.

Subsequently, more feed was added at:

66 hours - 100 ml

71 hours - 200 ml

The feed points are shown with the letter F on figure 5.18. Figures 5.17 and 5.19 show the other results obtained. No appreciable increase in pimaricin production (figure 5.19) was obtained with this feeding regime.

Further problems were encountered in this run due to the fact that the thermometer on the SF-116 temperature controller broke. To rectify this, a water circulator was attached to the hollow vessel baffles normally used to heat the vessel. The PM550 was re-programmed to switch the circulator on when the temperature dropped below 27°C. Figure 5.20 shows the temperature profile during this run. Comparing this with figure 5.17 it can be seen that the temperature rises exactly match the dips in dissolved oxygen thus indicating periods of intense metabolic activity.

It is worth noting that this temperature rise could have been used as a better "real-time" indication of metabolic activity than wet weight estimation of biomass. Although the control of temperature was poor, the temperature could still have been used as an indicator of metabolic activity in more controlled situations, by measuring duration of addition of cooling water.

5.6 FERMENTATION RUN 5

Before this run was performed it was necessary to obtain a new freeze-dried vial of culture as the entire stock of *S.natalensis* was destroyed in a laboratory accident.

Since a new culture was beirg used, it was deemed necessary that this run would be a repeat of the initial simple batch run 1.

Again the temperature controller was out of action so the

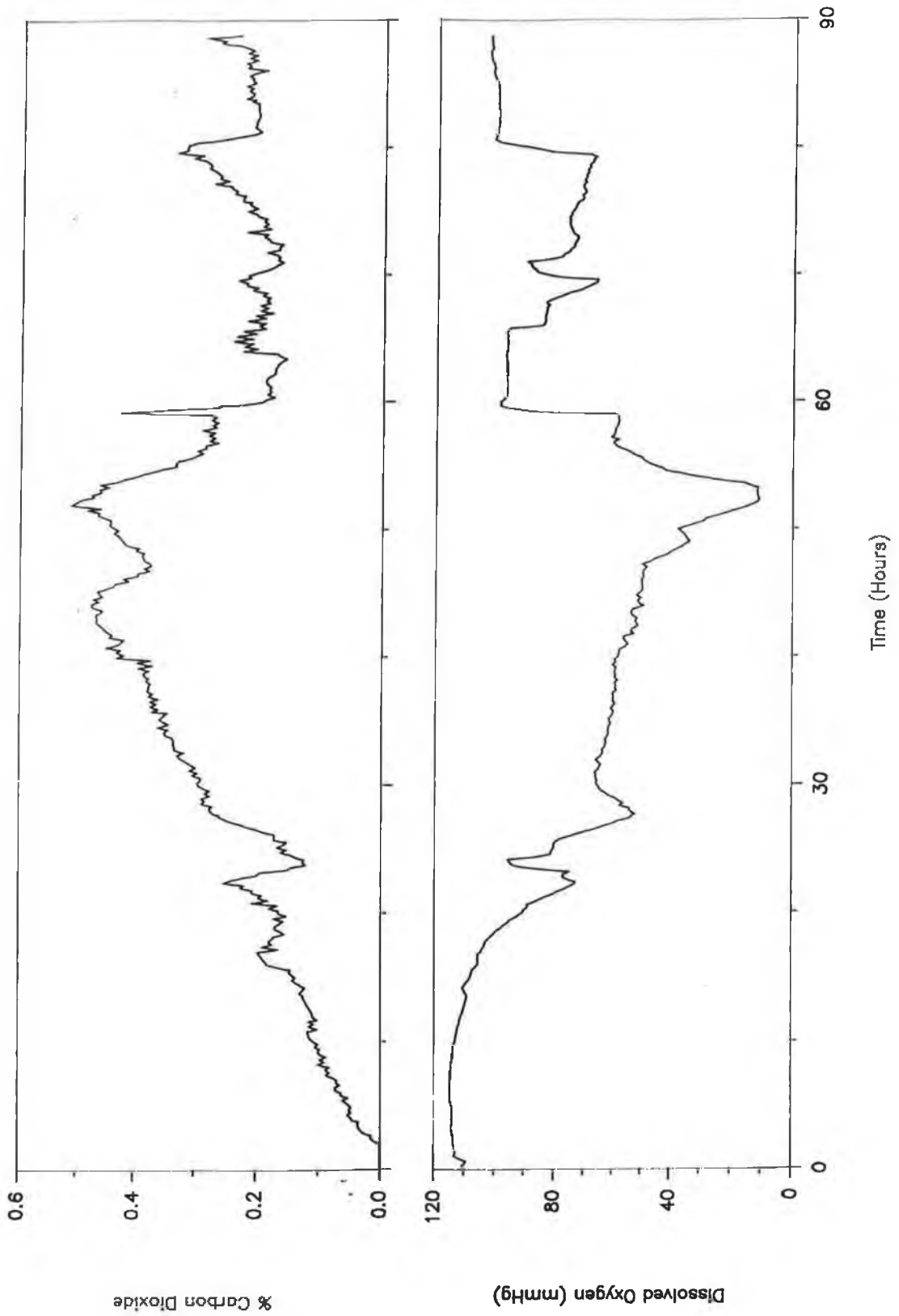


FIGURE 5.17 : D.O. and carbon dioxide profiles for run 4

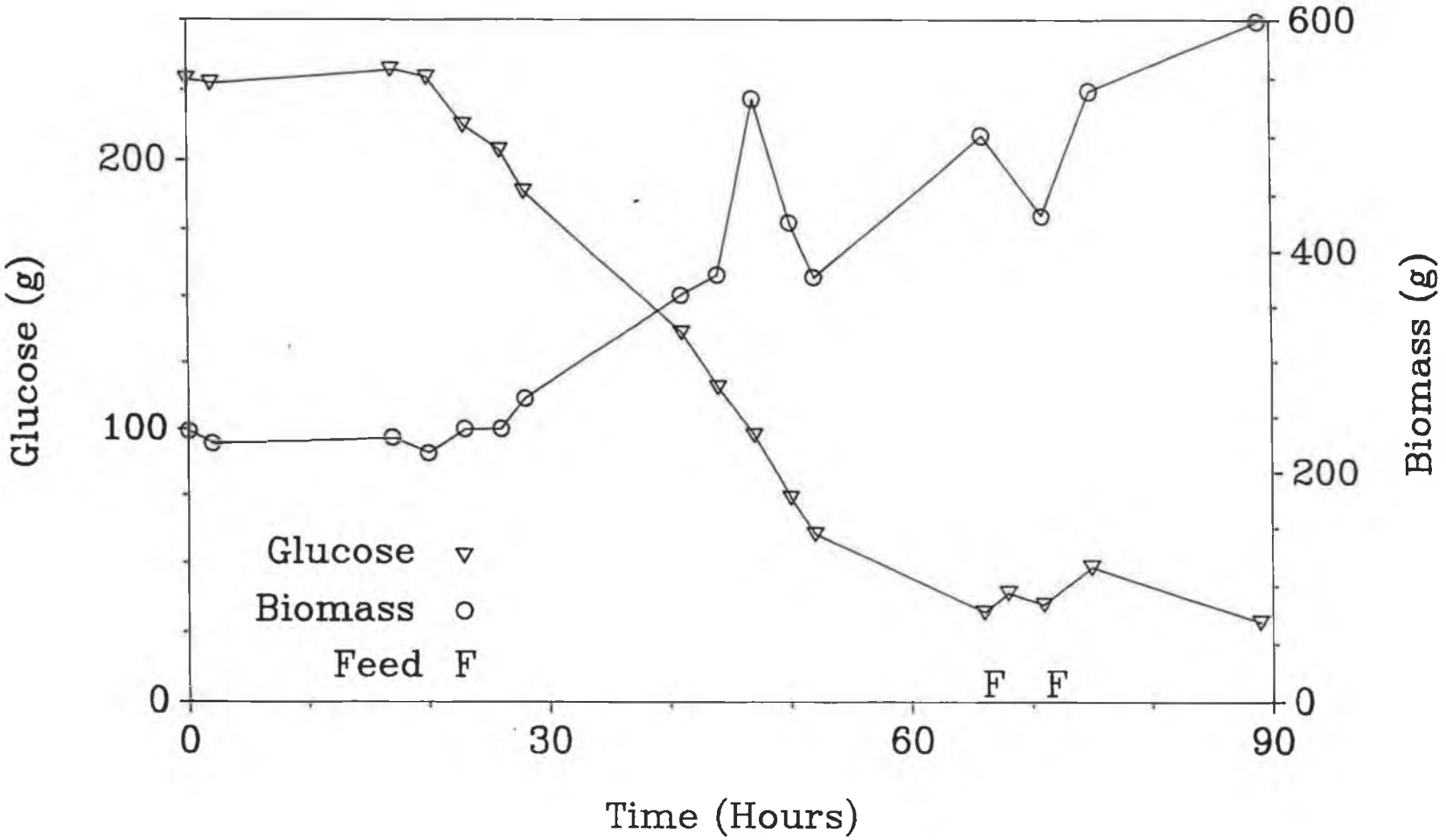


FIGURE 5.18 Glucose and biomass profiles for run 4

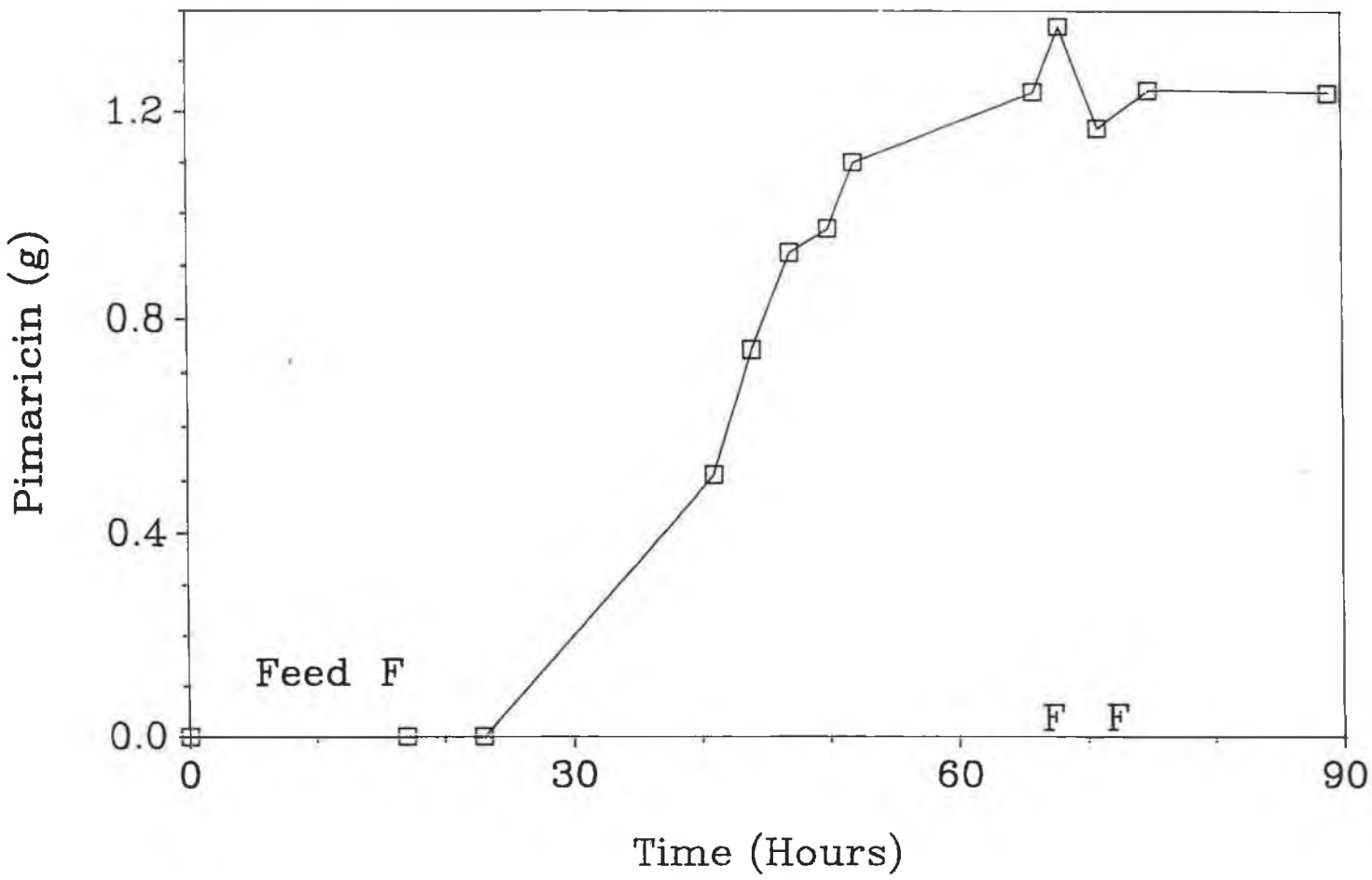


FIGURE 5.19 Pimaricin profile for run 4

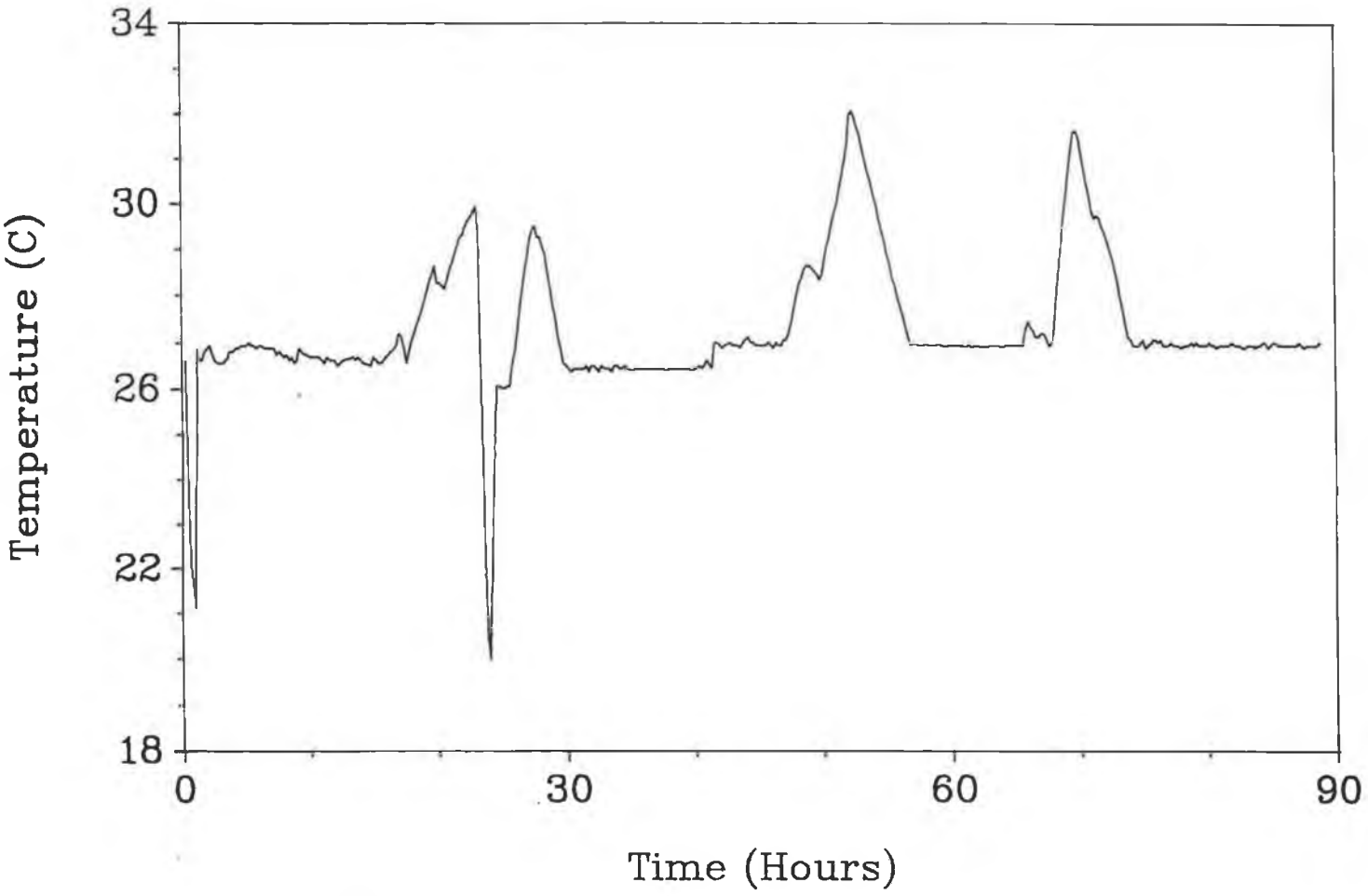


FIGURE 5.20 Temperature profile of broth in run 4

water circulator was employed. The temperature profile obtained is shown in figure 5.21.

Figures 5.22, 5.23 and 5.24 show the other profiles collected during the fermentation run. Although it may appear at first glance that the carbon dioxide and dissolved oxygen profiles (figure 5.22) are different to those previously obtained, it must be noted that these profiles are for 48 hours only and as such, compare favourably with the initial stages of previous runs.

The run was cut short once it was shown that the culture would grow adequately and produce pimaricin in reasonable amounts. It was noted that similar amounts of biomass were produced in this run, (figure 5.23) compared to run 1 (figures 5.5).

It can be seen that the results obtained with the new culture compare with those of the first culture in section 5.2.

5.7 FERMENTATION RUN 6

A new thermometer was obtained for the SF-116, so the temperature control system was reconnected.

In previous runs it was noted that the calcium carbonate in the medium interfered with the biomass readings during the initial stages of the fermentation (see section 5.3). it was noted, again however, that the white carbonate deposit visible in the pellet after centrifugation gradually disappeared during the first 20-24 hours.

It was thought if the pH of the medium was held constant,(as in this case by the addition of acid/base) then the role of carbonate as a pH buffer would be superfluous. However, the levels of carbonate would be expected to decrease slowly throughout the fermentation. The previous observation indicated, however that carbonate appeared to be consumed rapidly in the early stages of the fermentation.

This run investigated the effect of omitting calcium carbonate in the initial production medium.

It was noted in previous runs that there had been a fault in the pump head which allowed the slight pressure in the vessel to escape to the reservoir if the pump was on low speeds. Thus, the feed had been added at timed intervals with the pump at full speed. However, this fault was rectified and in this run the

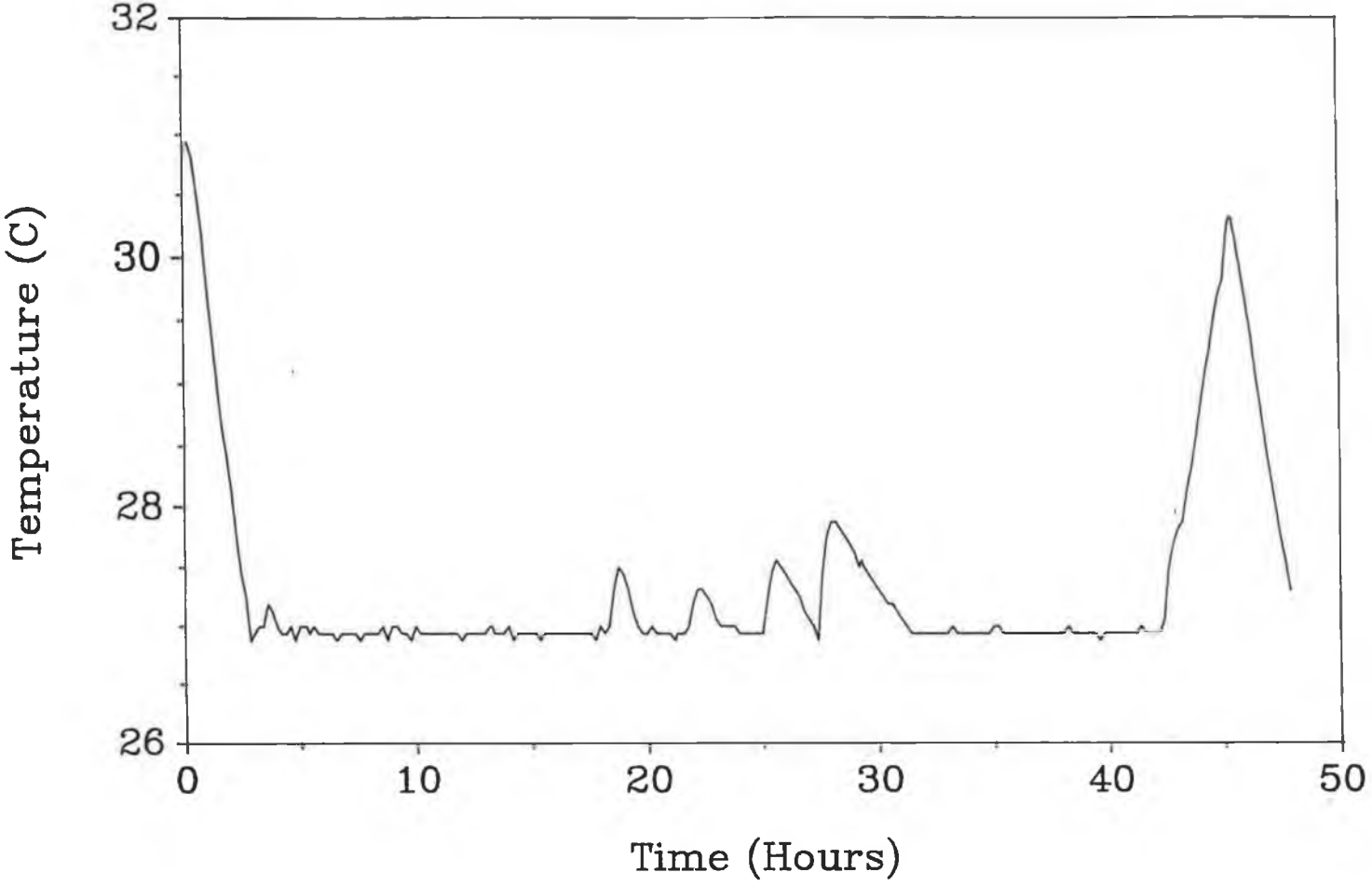


FIGURE 5.21 Temperature profile of broth in run 5

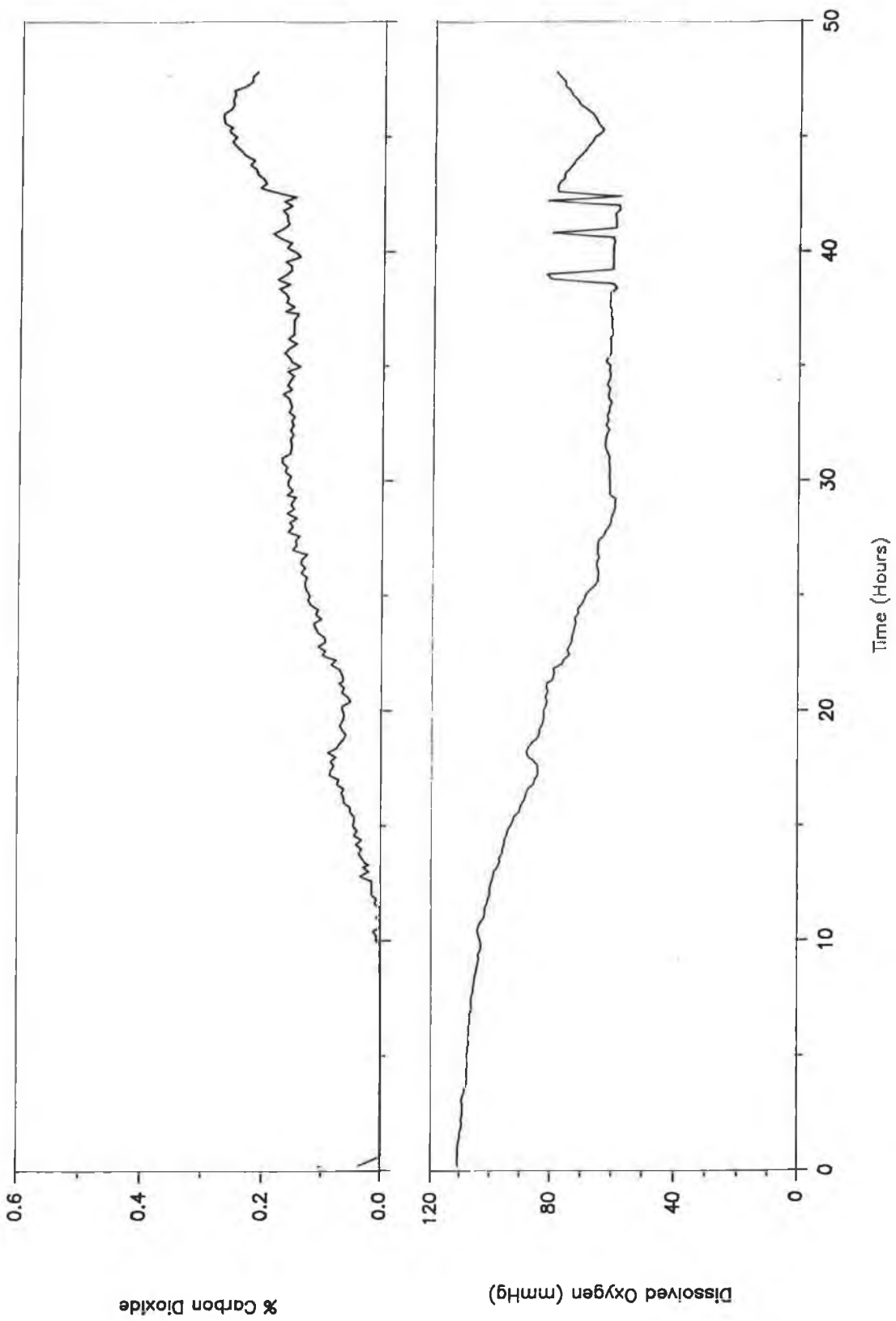


FIGURE 5.22 : D.O. and carbon dioxide profiles during run 5

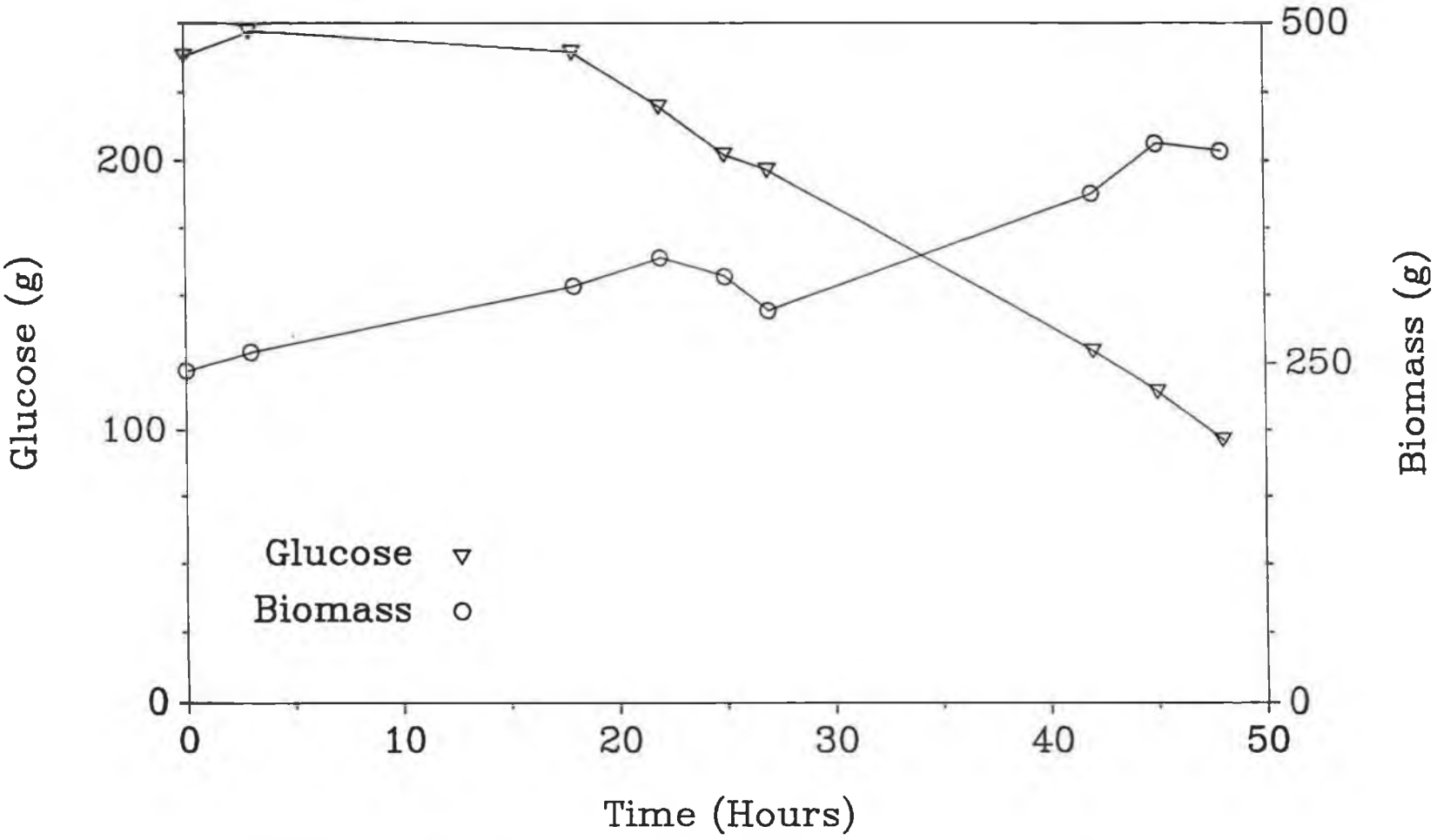


FIGURE 5.23 Glucose and biomass profiles for run 5

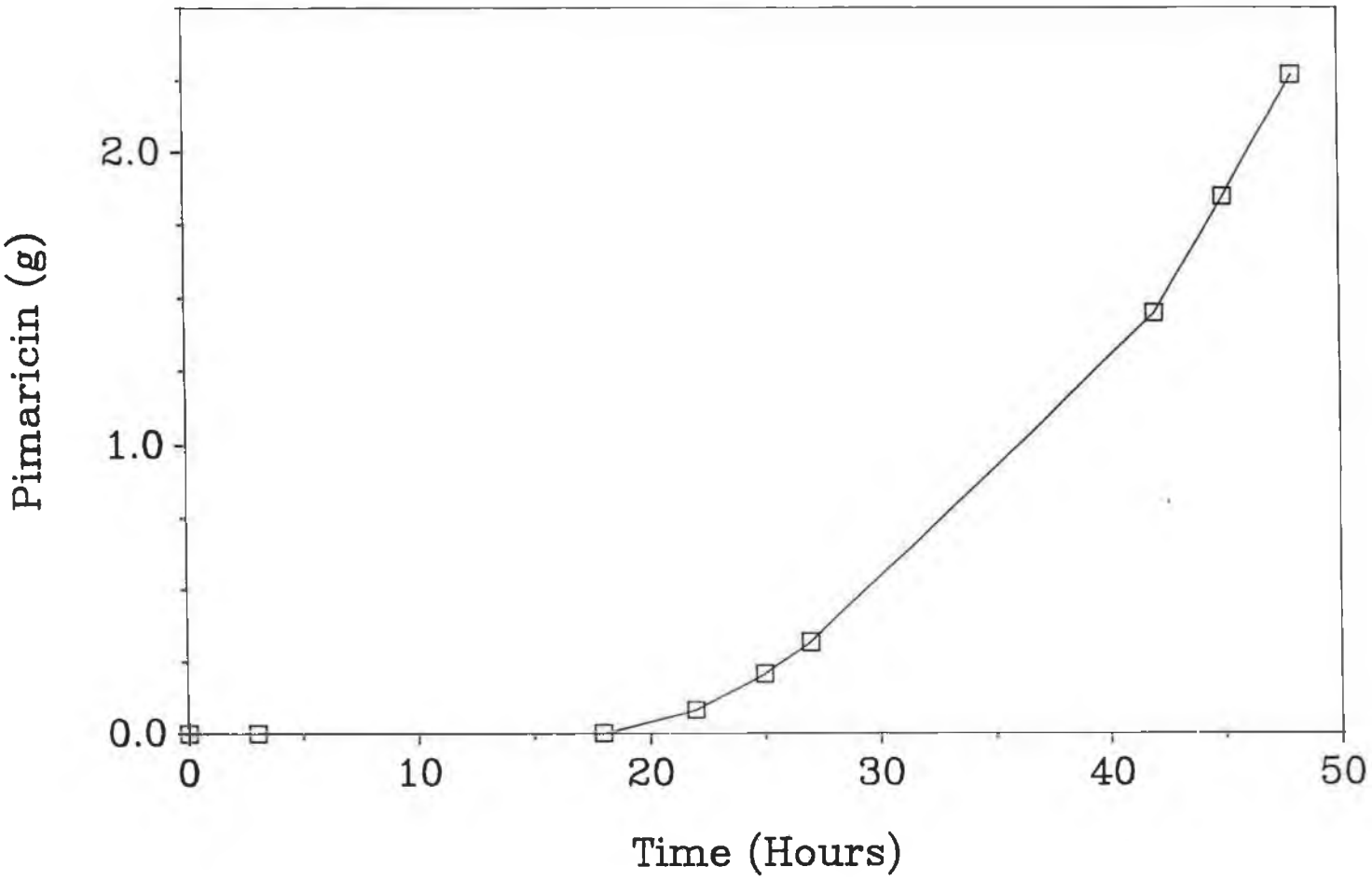


FIGURE 5.24 Pimaricin profile during run 5

feed was added in a continuous fashion.

The feed solution contained trace salts to investigate their role, if any, in pimaricin production. Magnesium and calcium were chosen for their roles as co-factors in cell metabolism. Solutions containing these trace elements used for fungal cultures have been shown to stimulate antibiotic production (Liu et al, 1975).

The feed solution was 1 litre of:

Glucose - 100 g/l
$(NH_4)_2SO_4$ - 5 g/l
$MgSO_4$ - 0.2 g/l
$CaCl_2$ - 0.2 g/l
$FeSO_4$ - 0.2 g/l

The speed of the addition pump was set manually and was adjusted during the course of the fermentation as the initial rates were considered too fast. The additions were:

67 - 70 hours - 125 ml
70 - 73 hours - 85 ml
73 - 88.5 hours - 500 ml

The commencement of feeding is marked with the letter F on figure 5.26. It is thus possible to decouple the effects of this feed solution from the investigation into the run without carbonate. Figures 5.25, 5.26 and 5.27 show the results obtained during the run.

Figure 5.25 shows that very high levels of carbon dioxide were produced under these conditions. During this phase of high CO_2 production the dissolved oxygen fell to a very low value and remained there for a considerable length of time indicating a period of intense metabolic activity.

This period coincided with the production of large amounts of biomass and a rapid uptake of glucose (figure 5.26). It can be seen that lower initial levels of wet weight are obtained by omitting calcium carbonate from the medium. It also shows that despite using a concentrated glucose feed, the levels of glucose remained around 50g in the vessel.

Furthermore, the high biomass production is matched by low pimaricin production. The production seemed to be stimulated

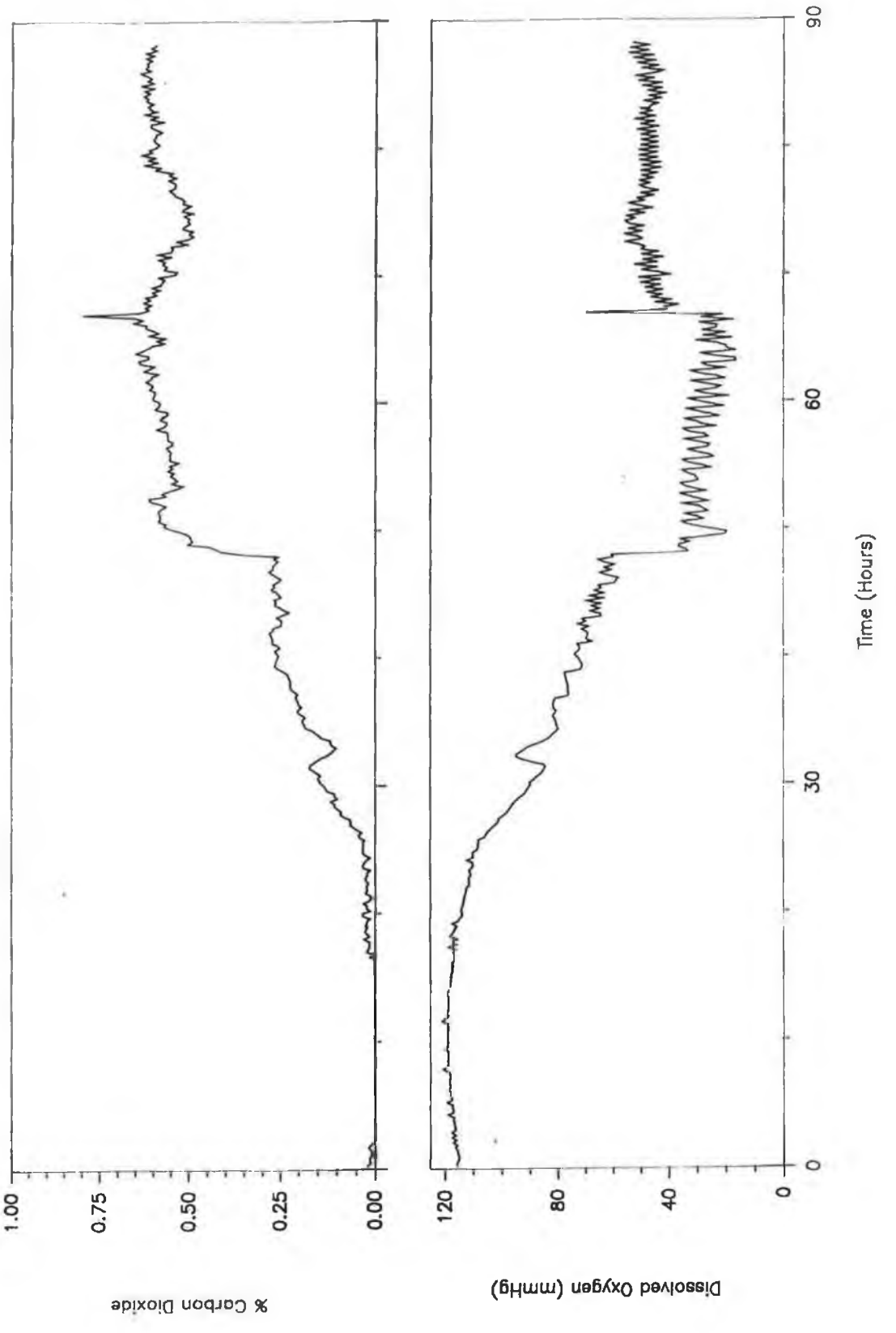


FIGURE 5.25 : D.O. and carbon dioxide profiles during run 6

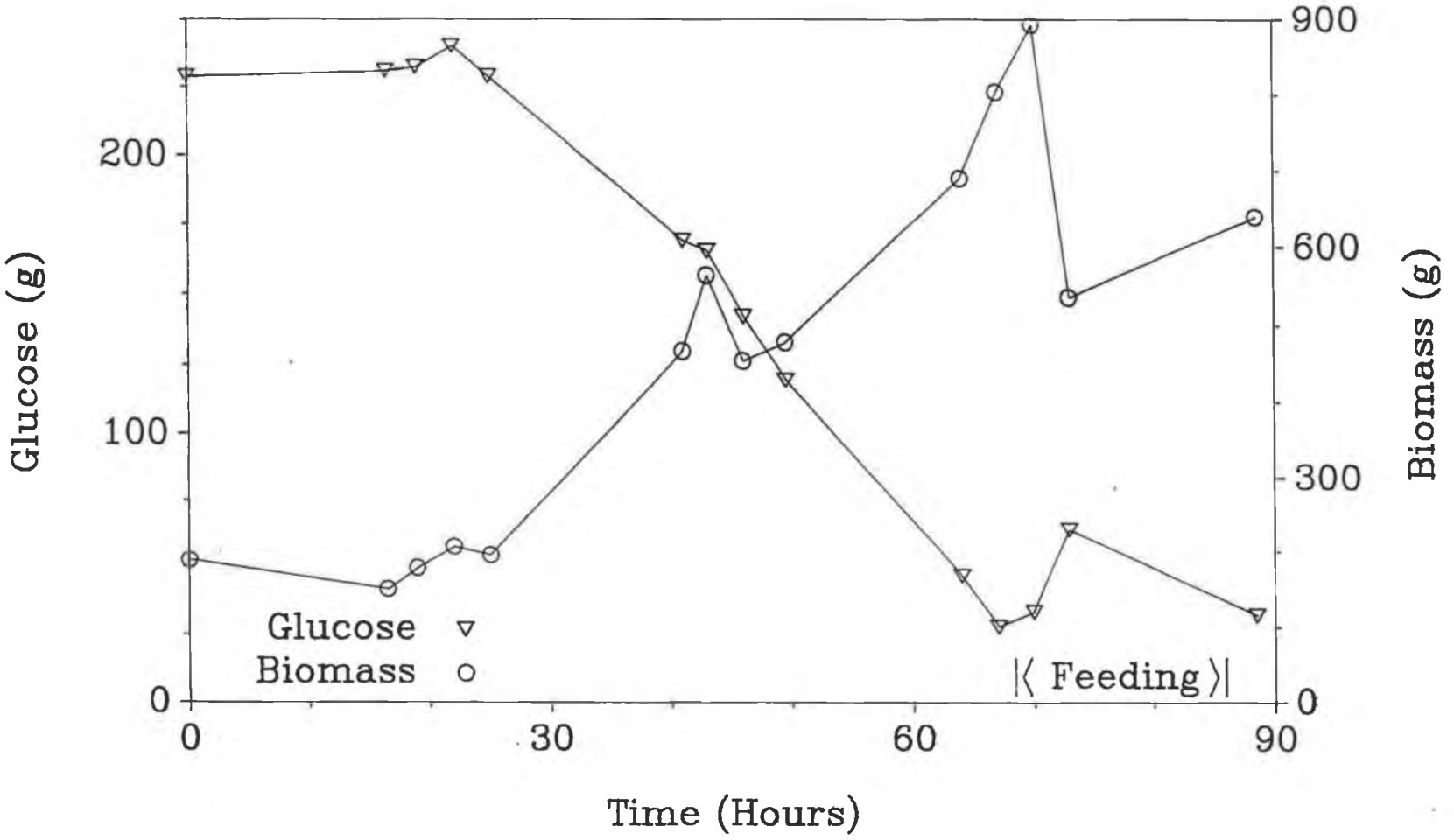


FIGURE 5.26 Glucose and biomass profiles for run 6

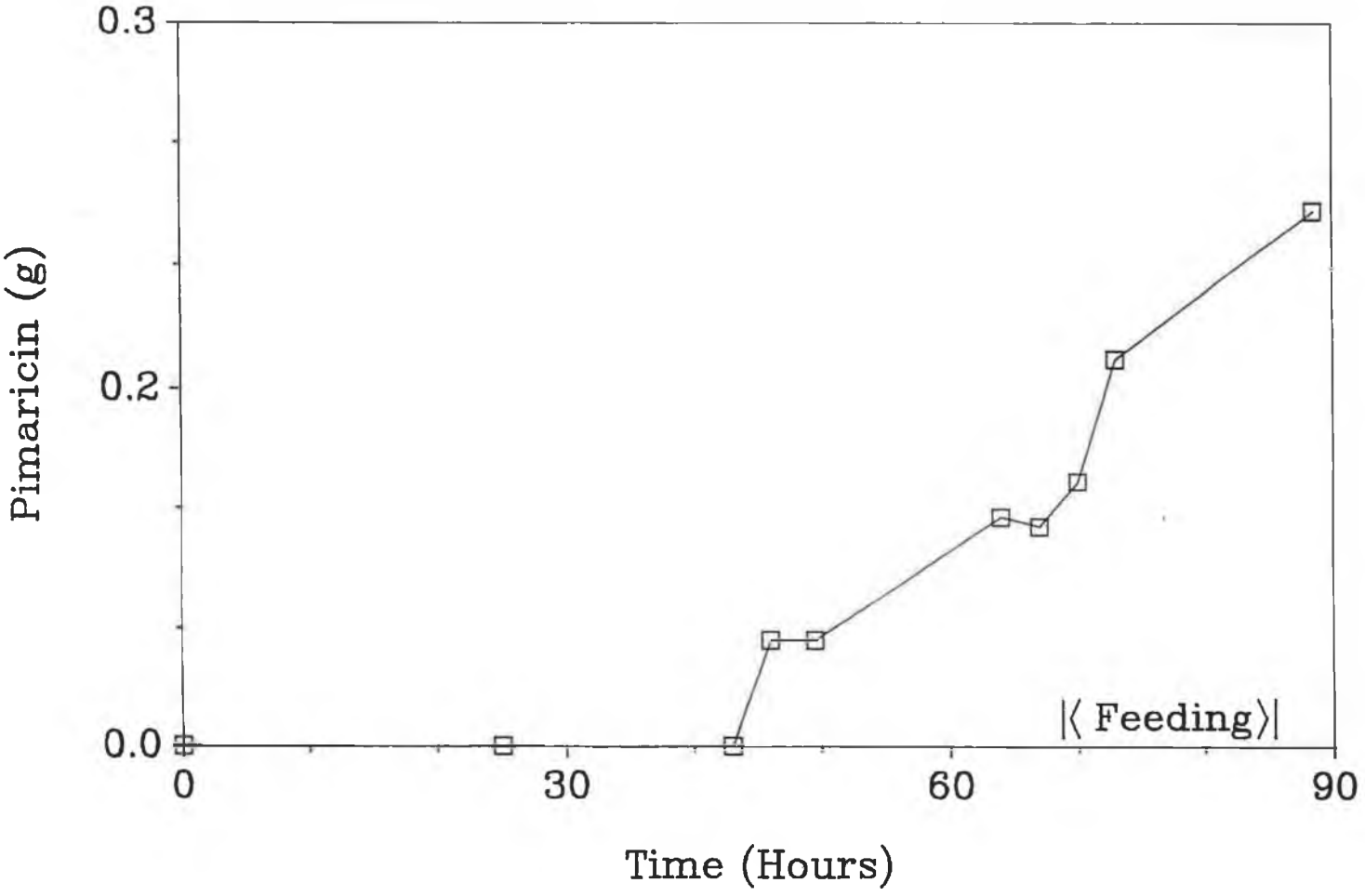


FIGURE 5.27 Pimaricin profile for run 6

after the addition of feed solution.

In previous runs, the antibiotic production did not usually increase after the initial growth phase, whereas, in this case the production seemed to be increasing towards the end of the run. This may have been due to the addition of feed. A rapid increase in pimaricin production can be seen in figure 5.27 after feed has been added.

5.8 FERMENTATION RUN 7

Thus far it had been observed that calcium carbonate had a role to play in the production of pimaricin, and also that carbonate was consumed during the fermentation. It was decided to investigate whether the effects were due to the presence of either carbonate or calcium.

Production medium was prepared and 12 g/l calcium chloride was added instead of carbonate. Figure 5.28 shows the data collected from DNS and wet weight assays. Overall biomass production and glucose uptake appeared to be lower than usual. It was noted that calcium chloride can precipitate other salts from the medium and this could account for some of the results observed.

HPLC analysis showed that pimaricin levels were too low to be detected and are not shown.

This suggested the role of carbonate, rather than calcium in the production of the antibiotic.

The run was terminated after only 63 hours due to the low pimaricin levels.

5.9 FERMENTATION RUN 8

Since a requirement for calcium carbonate had been suggested, this run attempted to investigate the effect of using a nutrient feed containing calcium carbonate and glucose. The latter was at levels lower than had been used previously to minimise any stimulation of biomass production during feeding.

The feed solution would provide sufficient materials for cell maintenance and would allow any effects of adding extra calcium carbonate to be observed.

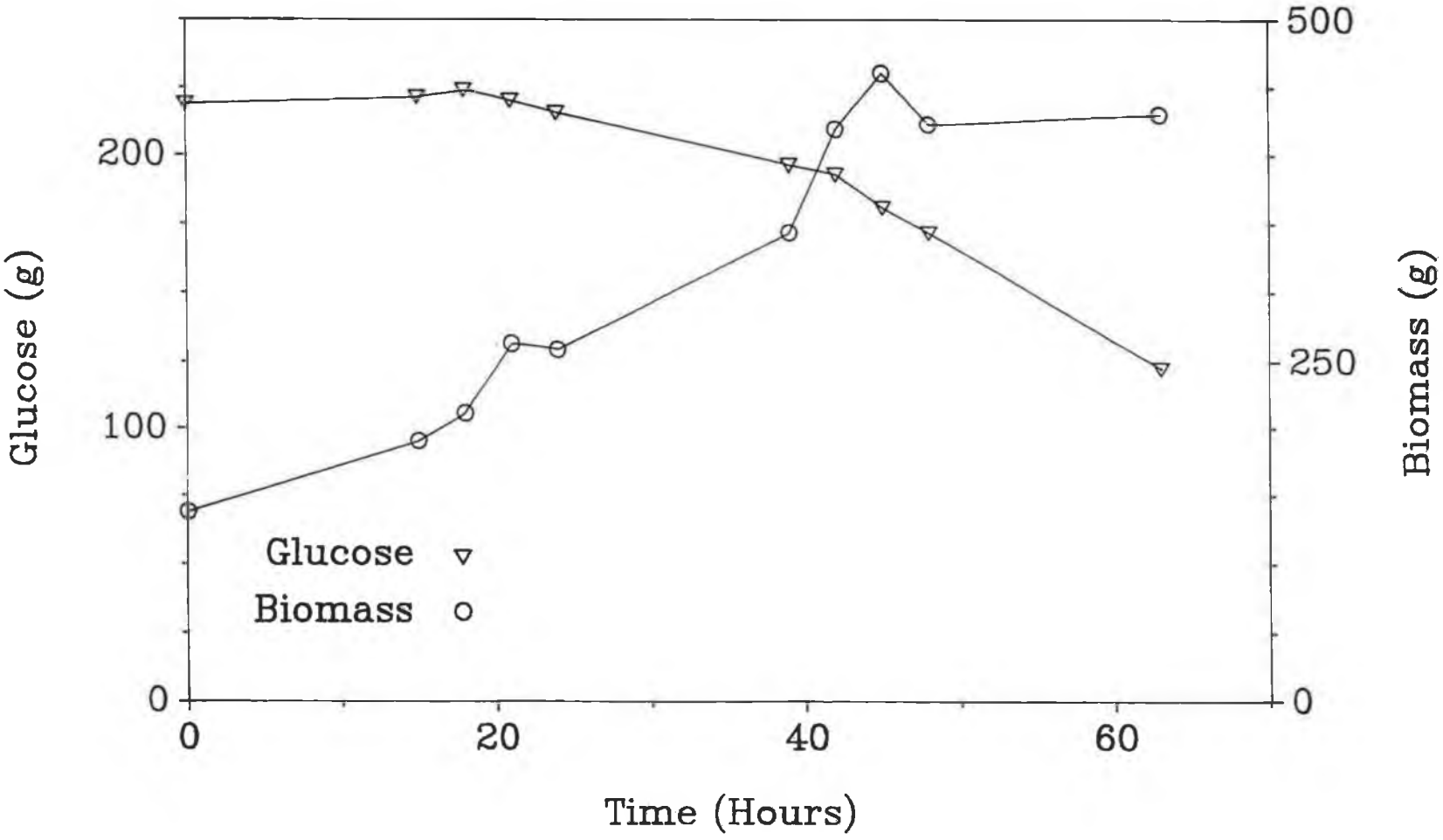


FIGURE 5.28 Glusose and biomass profiles for run 7

The composition was:

Glucose - 50 g/l

$CaCO_3$ - 30 g/l

$(NH_4)_2SO_4$ - 5 g/l

The solution was fed in by a pump under the control of the PM550 as described in section 4.

480 ml of feed was added between 45 and 48 hours and the remainder was added between 48 and 63 hours. The feeding time is clearly marked on figure 5.30.

The production medium also included 0.05% magnesium sulphate to investigate the effect of trace metals on pimaricin production. It had been noticed in the previous run that feeding a solution containing glucose, a nitrogen source and trace metals had a stimulatory effect on pimaricin production.

It can be seen from figure 5.29 that the dissolved oxygen did not drop as low during this run as it did in previous runs.

Pimaricin production was noted to be depressed overall relative to runs 5 and 6.

It was noted that during feeding the levels of biomass and pimaricin increased (Figures 5.30 and 5.31). Glucose levels continued to decrease despite constant feeding.

5.10 FERMENTATION RUN 9

This run was basically a repeat of number 8 except magnesium was excluded and the feed was added more slowly. The following additions of feed were noted:

48 - 50 hours - 100 ml

50 - 63 hours - 350 ml

63 - 66 hours - 70 ml

66 - 69 hours - 90 ml

69 - 72 hours - 100 ml

72 - 74 hours - 90 ml

A steady increase in biomass right to the end of the fermentation was noted (figure 5.33) along with a lower production of pimaricin (figure 5.34) although once again the feeding of carbonate appeared to have a stimulating effect on the production. Figure 5.32 shows that the dissolved oxygen

FIGURE 5.29 : D.O. and carbon dioxide profiles during run 8

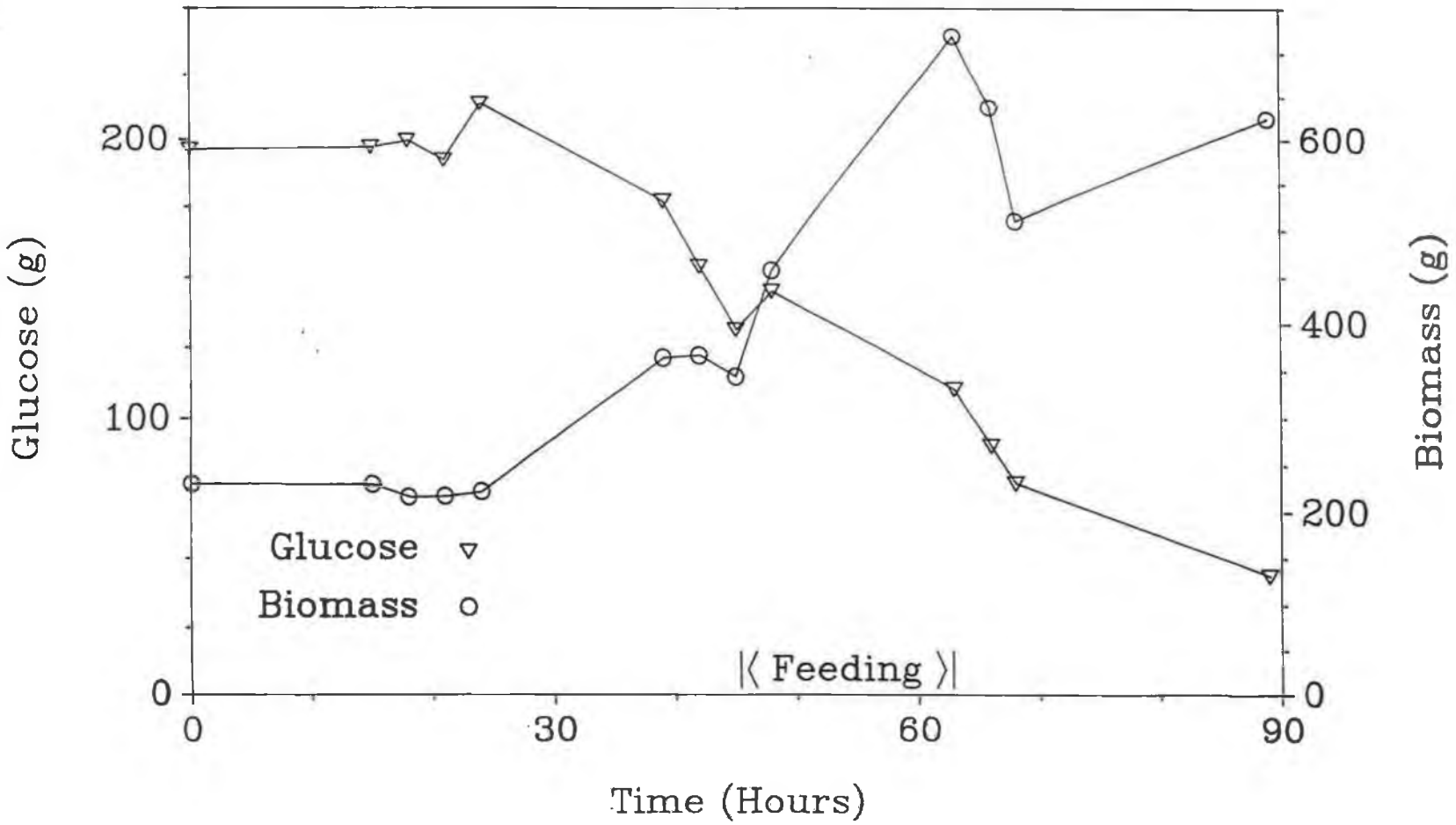


FIGURE 5.30 Glucose and biomass profiles for run 8

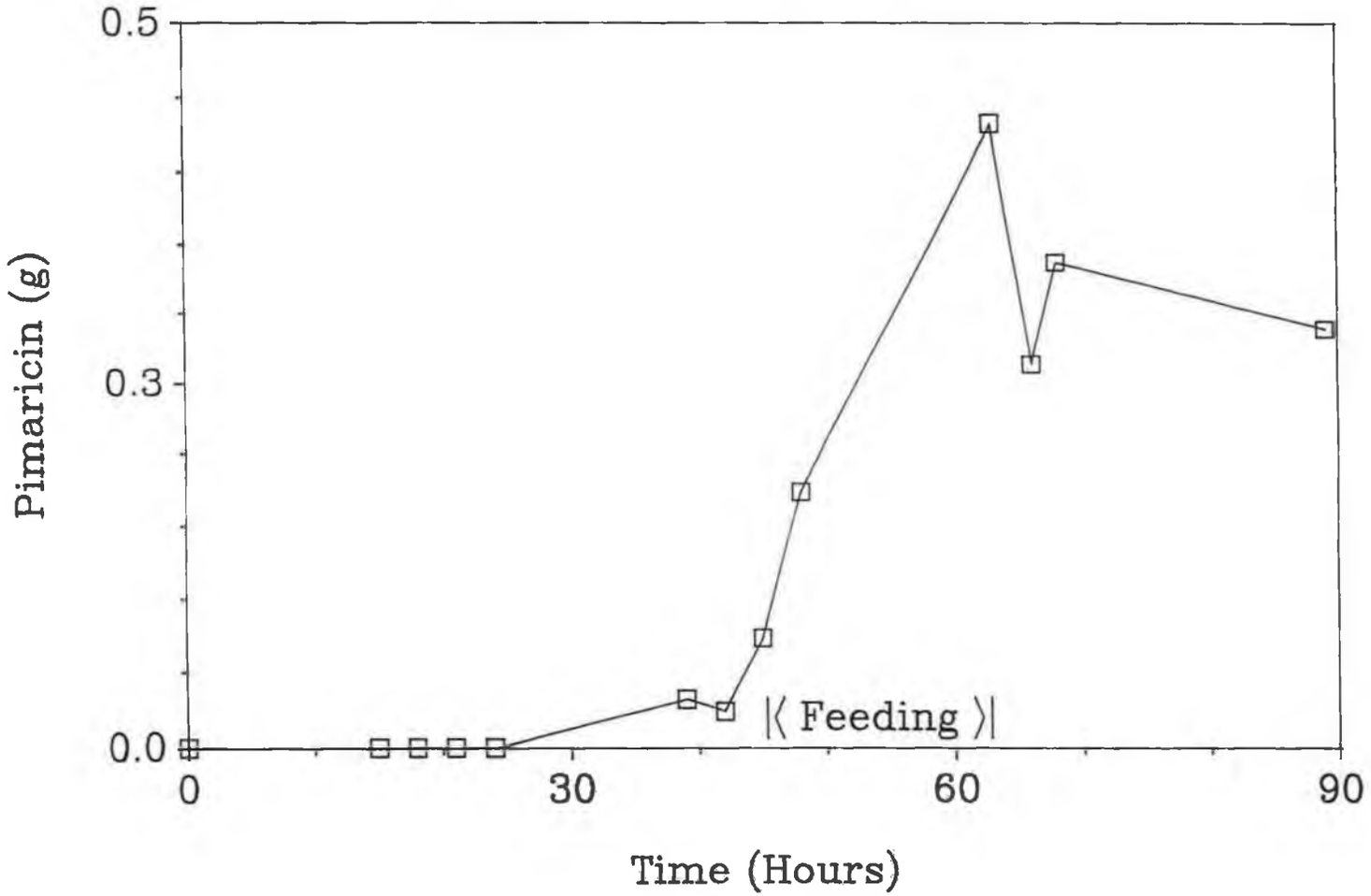


FIGURE 5.31 Pimaricin profile for run 8

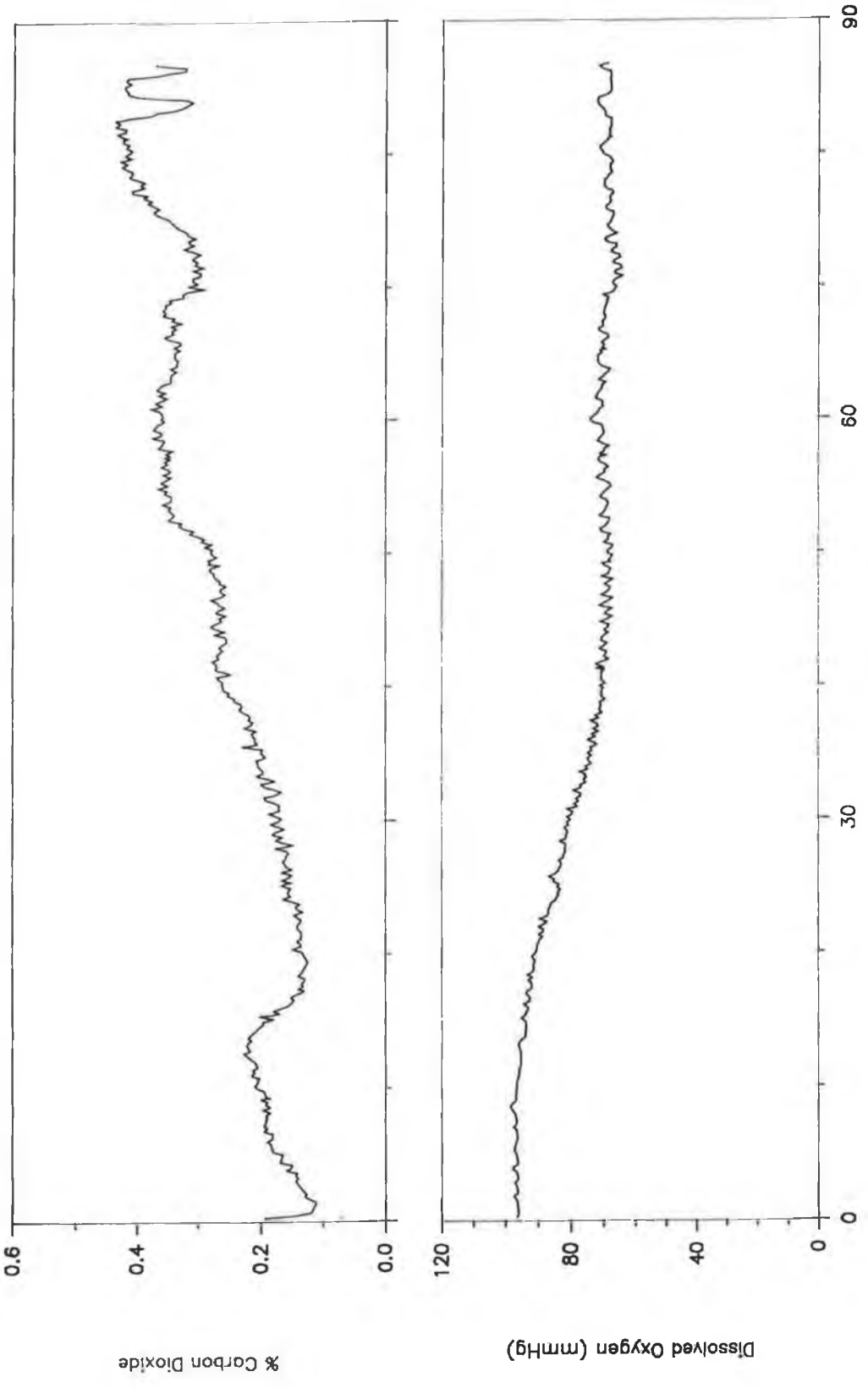


FIGURE 5.32 : D.O. and carbon dioxide profiles during run 9

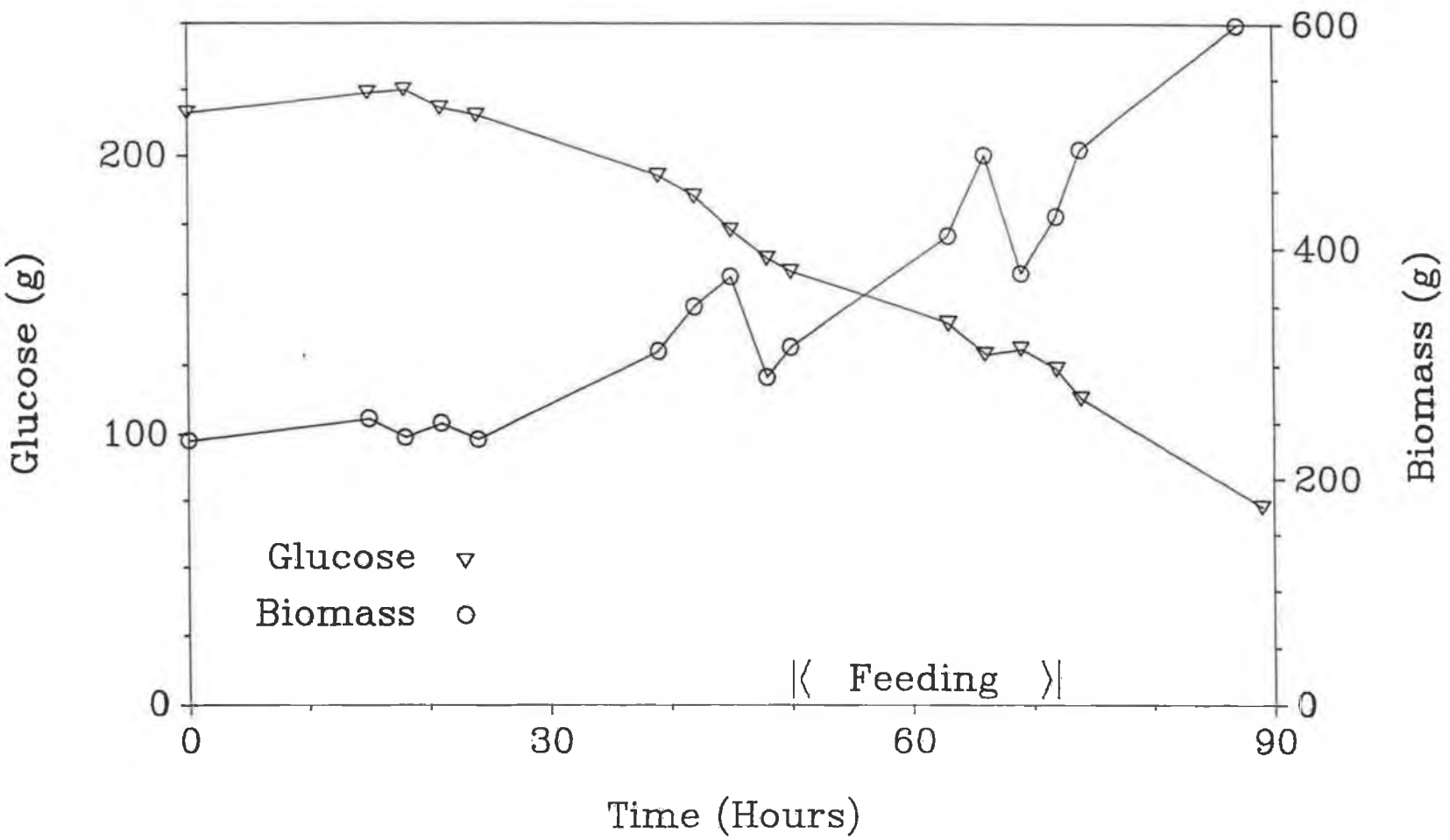


FIGURE 5.33 Glucose and biomass profiles for run 9

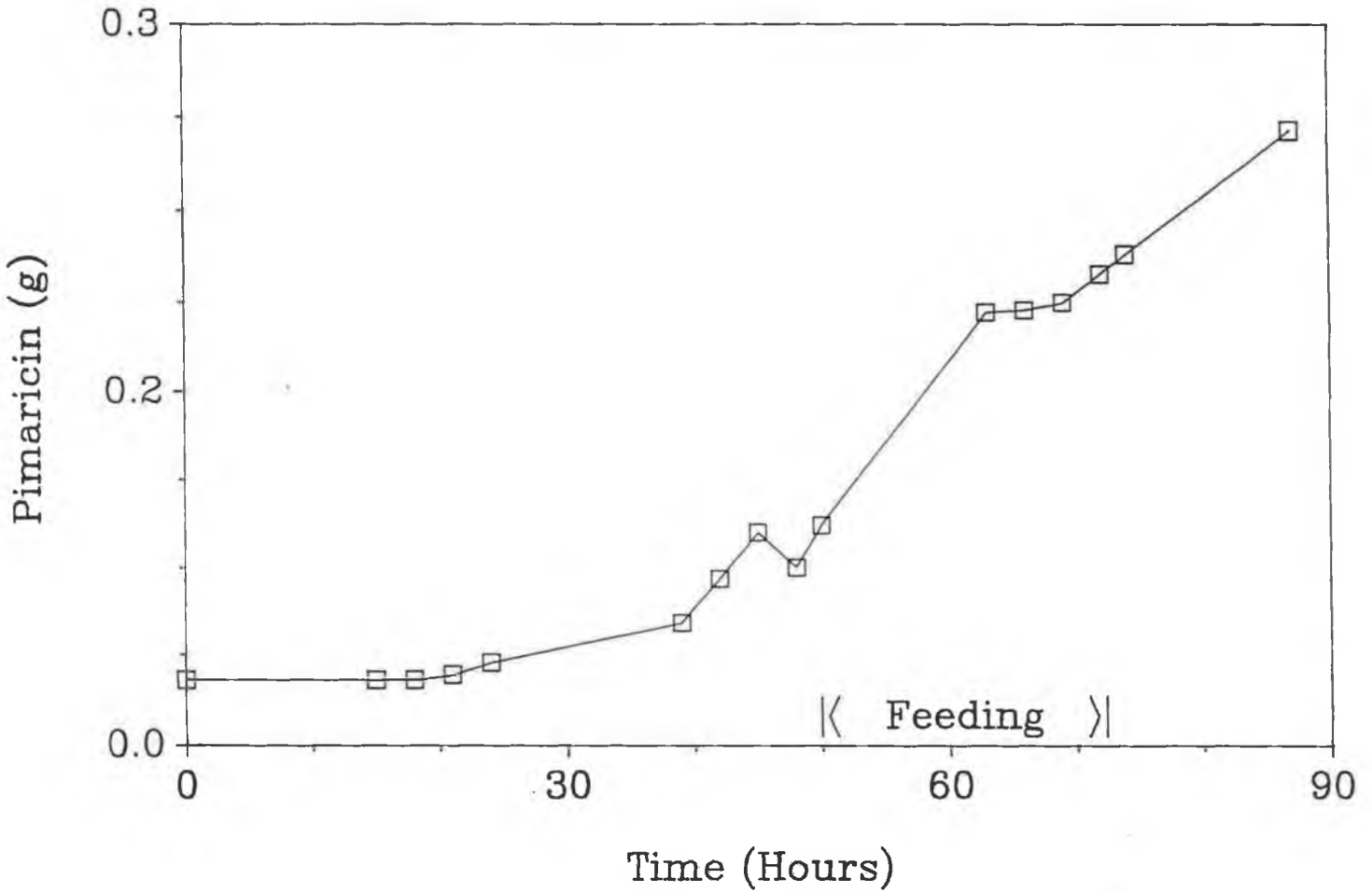


FIGURE 5.34 Pimaricin profile for run 9

level did not drop as low as usual and the carbon dioxide levels exhibited a steady increase right up to the end of the fermentation.

As in run 8, levels of pimaricin were lower than had been previously obtained so it is difficult to assess the roles, if any, of magnesium or carbonate as a stimulant to pimaricin production.

5.11 A REVIEW OF THE RESULTS

A table showing a summary of the results of the 9 fermentation runs is shown in figure 5.35.

5.11.1 Fermentation process

A large amount of time was spent at the beginning of the project in setting up the rig. Several vessel types were investigated. The control of parameters such as motor speed and inlet air flow rate were never fully implemented.

The main aims of building the rig were achieved. Flexibility of operation was provided by the PLC. Copious amounts of data could be collected simply and exported to other software for analysis or plot generation. Measurement of crucial fermentation parameters was also possible.

Although the rig would have been suitable to study most aerobic fermentation processes it was decided to study the production of a secondary metabolite as much work had been done in computer modelling control of primary metabolites such as ethanol or amino acids.

To this end, the most critical parameters to monitor would be dissolved oxygen, CO_2 evolved, O_2 uptake, biomass production and glucose uptake rate. Real-time estimation of the first two parameters was achieved. The measurement of the third parameter should also have been possible, however, the instrument used was not successful. Due to the morphological characteristics of the organism, it was impossible to measure the amount of biomass accurately offline (section 5.11.4), and real-time estimation techniques, which would have been desirable in order to control antibiotic production, were unusable.

RUN	*TIME*	*PIMARICIN*	*BIOMASS*	*FEED*	*COMMENTS*
1	134h	>3g	>620g	n/a	■ Batch run ■ High motor speed
2	111h	n/a	>600g	n/a	■ Batch Run ■ Decreased agitation ■ Decreased biomass
3	162h	>9g	≃600g	520 ml	■ Batch addition of feed ■ Feed composition same as production medium but with 10% glucose ■ High pimaricin
4	89h	1.2g	≃600g	500 ml	■ Feed glucose only ■ Batch addition of feed ■ Low pimaricin ■ Faulty temperature control
5	48h	>2g	≃400g	n/a	■ Repeat of run 1 with new culture ■ Short 50 hour run ■ Batch run ■ Faulty temperature control
6	89h	≃0.2g	≃900g	710 ml	■ Continuous feed at ≃ 30ml/hr ■ Feed contained trace elements ■ Medium without carbonate stunts pimaricin production
7	63h	none	≃450g	n/a	■ Medium made with calcium chloride rather than carbonate ■ No pimaricin detected
8	89h	≃ 0.45g	≃700g	1000ml	■ Continuous feed at 35ml/hour ■ Feed contained lower glucose but carbonate added ■ Magnesium in feed
9	89h	≃ 0.25g	≃600g	900ml	■ Feed at 30ml/hour ■ No magnesium in feed

Figure 5.35 Summary of the results from nine fermentation runs

The production of pimaricin was chosen as it was of commercial interest to an Irish company.

5.11.2 Pimaricin Production and Detection

Comparison of pimaricin production between runs shows that the antibiotic is produced while the culture is actively growing. Little or none is produced after idiophase was reached. Similar observations were made by Martin and McDaniel (1975), studying the production of candicidin by *Streptomyces griseus*. In that case, attempts were made to prolong the time taken to reach cell maturation in order to increase yields. Such a strategy may have had an application in pimaricin production since alteration in medium composition and fed-batch operation effected no increase in yields.

The HPLC assay developed had the advantage that large numbers of samples could be processed relatively quickly. For example, a plate assay to test the sensitivity of *Saccharomyces cerevisiae* to the antibiotic, was evaluated at D.C.U. (White, 1988). Although the test measures active pimaricin, it involved culturing the organism for 72 hours on malt extract agar.

The apparently lower levels of pimaricin produced in the last runs merit some comment. It was noticed that there was a large variation in peak area of standard samples between runs. Some typical absorbance values are shown in figure 5.36.

It can be seen that the overall trend in peak height is increasing towards the end of the project. This may have tended to minimise the estimation of pimaricin levels in later runs. It is not known whether this was due to a deterioration in the standard solution, or in the HPLC column.

It is also worth pointing out the exceptionally low reading obtained in run 3. This run was noted particularly for its high pimaricin production.

RUN NO.	ABSORBANCE
1	0.100
3	0.053
4	0.140
5	0.105
6	0.141
7	0.169
8	0.186
9	0.140

Figure 5.36 Variance of the absorbance at 303 nm when 10 μl of a 0.05 g/l standard solution of pimaricin was analysed by HPLC

It is thought that some percentage of the error could be attributed to dilution of the standard solution.

It can be seen from the relatively large variation in the readings that some future work could concentrate on defining a more rigorous technique for estimating the absorbance of the standard solution with particular emphasis to be placed on creating a standard curve.

5.11.3 Analysis of carbon dioxide profiles

From the metabolic pathways discussed in section 1 it can be seen that CO_2 has a key role to play in the formation of the glycone ring precursors. These can be summarised as:

* CO_2 Producing Reactions:

Pyruvate ⟶ Acetyl CoA

* CO_2 Consuming Reactions:

Acetyl CoA ⟶ Malonyl CoA

Propionyl CoA ⟶ Methylmalonyl CoA

Pyruvate ⟶ Oxaloacetate

The production of acetyl CoA is the most common of these reactions as it is the key intermediate in many of the cell's metabolic pathways. In the initial stages of the fermentation the culture should be growing and accumulating the components required for antibiotic production. The levels of carbon dioxide in the vent gas should increase in these stages since there is a high rate of aerobic metabolism.

The CO_2 consuming reactions which also occur during fatty acid synthesis, should increase during the latter stages of the fermentation and thus the CO_2 levels in the exhaust should decrease.

It can be seen from profiles of exhaust CO_2 and pimaricin shown in figure 5.37 that levels of exhaust CO_2 do decline towards the end of the runs. However, while pimaricin is being produced, the levels of CO_2 are high or increasing. It was observed that pimaricin production was at its highest during the first 80 hours of the fermentation and is impossible to decouple increases/decreases in CO_2 production from aerobic metabolism.

It has already been observed that CO_2 levels increase as biomass increases. It is worth highlighting the differences in the profiles of CO_2 evolved and biomass, when carbonate is present, and when it has been excluded. Figure 5.38 shows such a comparison.

With carbonate present, the antibiotic is produced. The CO_2 evolved is higher during the early stages, though does not attain as high a level overall, and is observed to decrease towards the end of the run.

With carbonate excluded, little antibiotic is produced. CO_2 evolved and biomass increase steadily right to the end of the run.

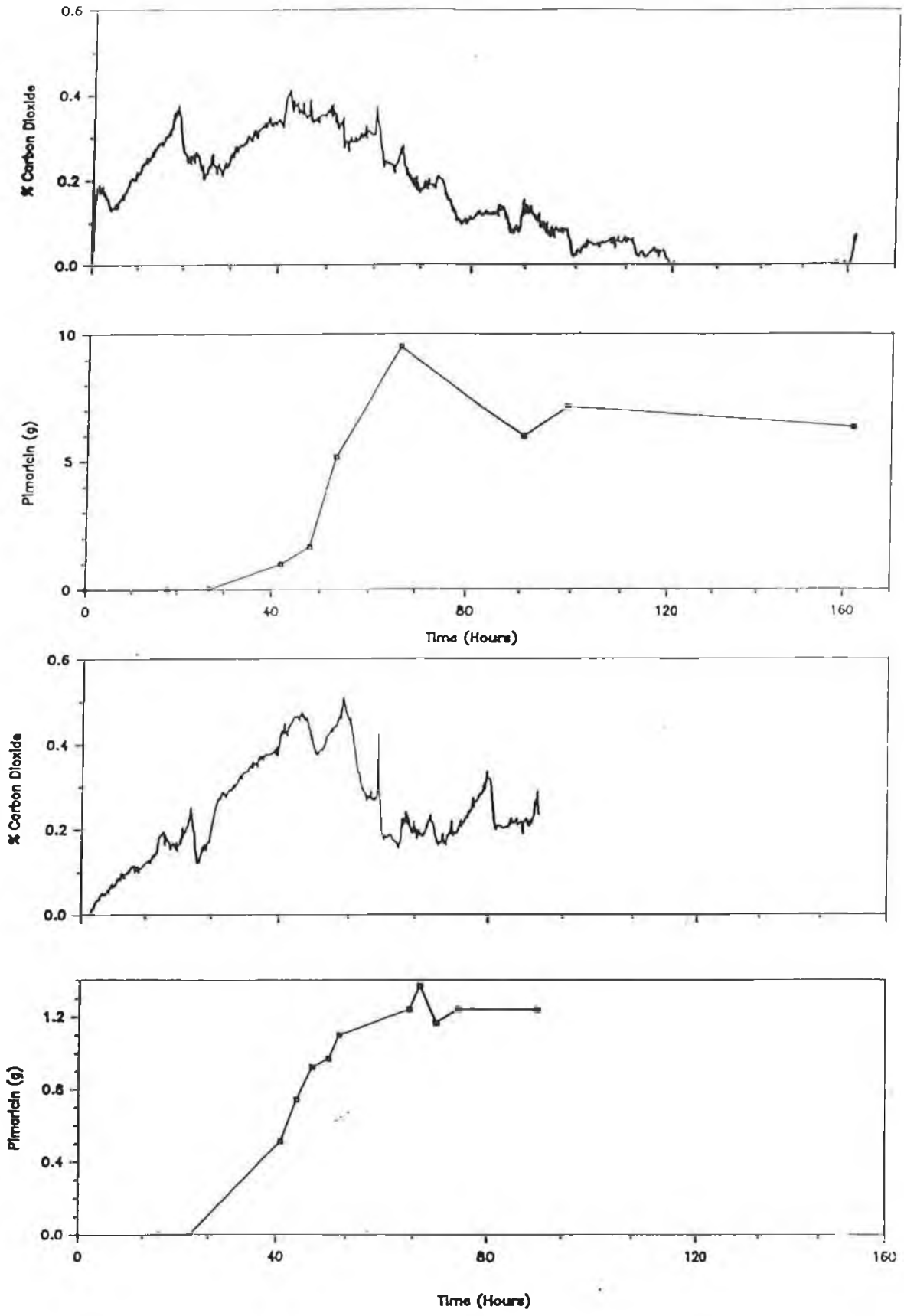


FIGURE 5.37 : Exhaust carbon dioxide and pimaricin profiles for runs 3 & 4

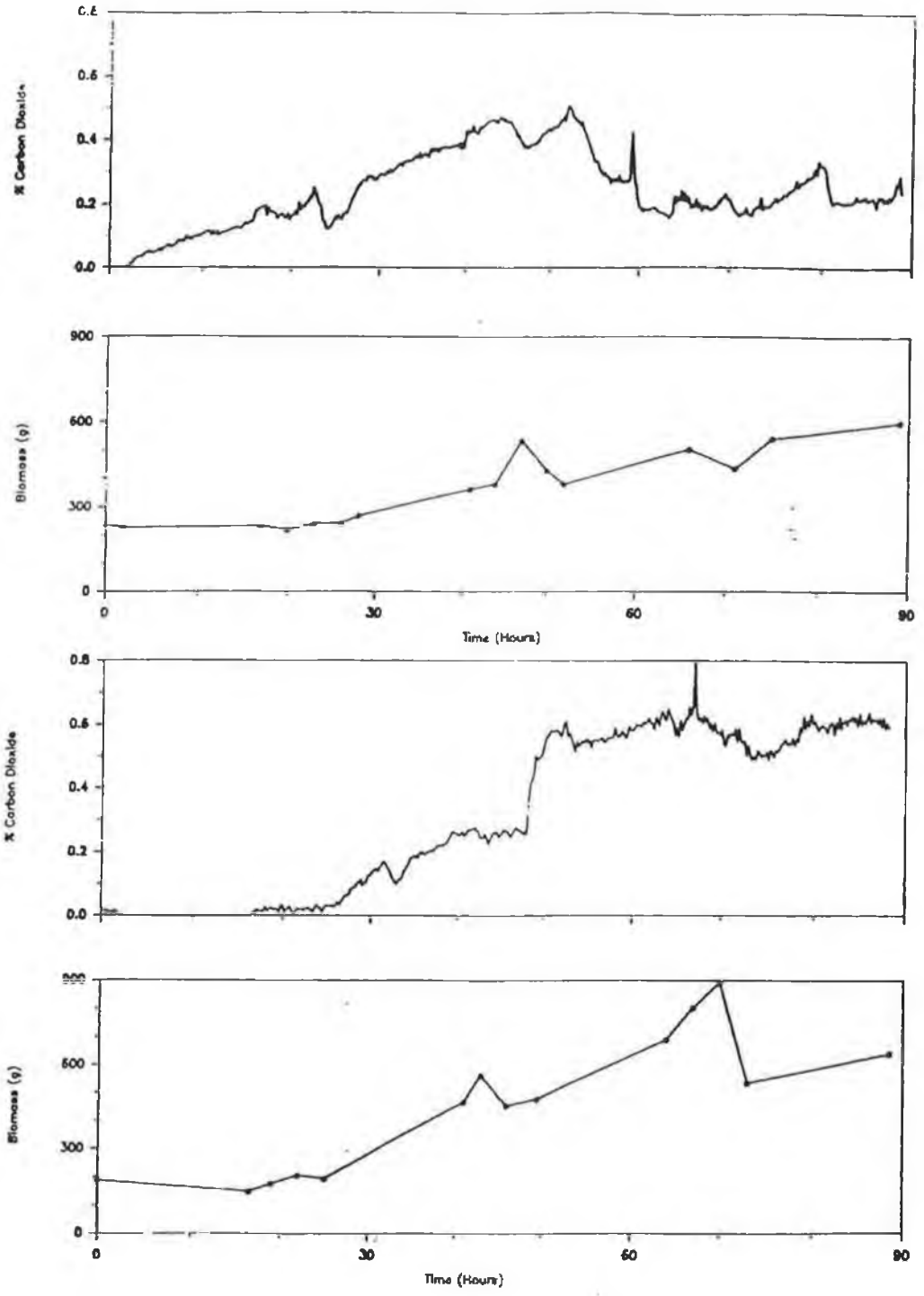


FIGURE 5.38 : Exhaust carbon dioxide and biomass profiles for run with carbonate (top) and without (bottom).

5.11.4 Biomass production

The profiles of wet weight shown previously reveal the marked variation in readings obtained using this assay. This is due to the pelleted nature of the organism which causes the culture to sediment naturally to the bottom of the sample container. This meant that it was sometimes difficult to ensure that each aliquot of the sample assayed would be representative of the sample as a whole. Since both dry weight and wet weight techniques were thought innaccurate further studies could investigate using packed cell volume as an estimation technique. Other workers using *Streptomyces* have applied this technique with some success (Omura and Tanaka, (1986).

It was noted that the organism exhibited different growth patterns between fermentations. Some of the patterns relate to the presence of carbonate in the medium. Without carbonate, the culture grew more vigorously than when carbonate was included (figure 5.38).

Other patterns include a varying lag time, which was primarily due to the difficulty in estimating and thus standardising initial inoculum size and activity. Smith and Calam (1980), noted that variations in the properties of inocula, such as biochemical activity, were as important as morphology in determining antibiotic production. Due to the morphology of the organism, such measurements were extremely difficult to evaluate.

It is worth pointing out some of the erratic data on graphs of biomass production. It can be seen that, in some cases, single points can vary dramatically from the rest of the curve. In figure 5.33, biomass production in run 9, two points at 48 and 66 hours exhibit such properties. This is due to the problems encountered in performing this assay discussed in section 5.2.1. Variances between samples taken at the same time could be ± 20%.

Some of the graphs show biomass decreasing towards the end of the run. This has been shown to occur in other *Streptomyces* fermentations (Vining et al, 1987).

5.11.5 Dissolved Oxygen Profiles

During several fermentations, it was noted that jumps occured in the dissolved oxygen profiles. Biomass was noted to decrease towards the end of fermentation runs also. Similar patterns attributed to autolytic processes have been shown to occur in the production of candicidin by *Streptomyces griseus* (Martin and McDaniel, 1975). Jumps in dissolved oxygen have also been observed during the production of thiostrepton by *Streptomyces laurentii* (Suzuki et al, 1987).

The jumps were noted to occur at low glucose concentrations suggesting that glucose depletion could be the trigger for autolysis. Further work could quantify the level at which autolysis occurs and adjust feeding levels accordingly. in addition, real time measurement of glucose could be used to control feeding on line.

6. CONCLUSIONS

6.1 FERMENTATION APPARATUS

A custom-built apparatus was designed and commissioned at DCU.

Equipment for measuring fermentation parameters such as dissolved oxygen, temperature, pH and vent gas composition was connected to a PLC and data from fermentation runs was successfully recorded on a personal computer.

Fermentations involving *Streptomyces natalensis* were successfully performed in this rig. The apparatus proved an excellent tool in collecting and analysing data during fermentations.

Problems were encountered in measuring the oxygen concentration in the vent gas. The PLC could in future be used to perform many more functions such as control of dissolved oxygen via agitation and/or inlet air flow or to have direct control over parameters such as temperature and pH which had their own controllers external to the PLC. More use could be made of the PLC's ability to monitor, analyse and control parameters in real-time. The apparatus could easily be used for studies on other organisms.

6.2 FERMENTATION STUDIES

Batch and fed-batch studies were performed. It was not conclusively shown that fed-batch techniques were a significant improvement over batch.

Using batch techniques a yield of over 1 g/l was obtained on one occasion.

Variations in the performance of the organism were noted and further work could be done to quantify a procedure for inoculum preparation.

The production of pimaricin was shown to increase as the fermentation progressed, and was demonstrated to be produced within 2-3 days of inoculation while biomass levels were still increasing.

A strict requirement for calcium carbonate in pimaricin production was demonstrated.

Graphs of dissolved oxygen and carbon dioxide evolution were shown in section 5. The profiles of dissolved oxygen revealed some peculiar "jumps" to new plateaus towards the end of fermentations. These jumps were attributed to autolysis.

6.3 HPLC ANALYSIS

A technique for the preparation and analysis of samples was successfully developed. The extraction procedure yielded samples which exhibited a single large peak at the wavelength used. This peak was shown to be pimaricin by photodiode array spectroscopy.

Some further work would need to be performed in order to improve the quantification of standard solutions. This could, for example, take the form of standard curves.

The HPLC analysis method detects the total amount of pimaricin and does not distinguish between active and inactive antibiotic. Future work could compare bioassay results to those obtained by HPLC.

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