THE INFLUENCE OF DISSOLVED OXYGEN ON THE PRODUCTION OF DIFFICIDIN BY *BACILLUS* SP.

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by

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ABSTRACT

Difficidin and oxydifficidin are two related antibacterial antibiotics produced by the fermentation of *Bacillus subtilis* ATCC 39374. Difficidin was much more stable than oxydifficidin under alkaline conditions. Both antibiotics were very unstable at an extremely low pH level (pH $\leq$ 3.5). In the whole culture broth, most of the antibiotics were found to be bound to the cell mass and appeared to be more stable than the free compounds.

The influence of dissolved oxygen tension (DOT) on the fermentation was investigated using a 20-L fermenter coupled with a proportional-integral-derivative (PID) control system to control DOT at a constant level throughout the fermentation by simultaneously varying the air flow rate and agitator speed based on a constant nitrogen gas feed rate. Growth and antibiotics production during the fermentation were studied at different DOT levels. The critical DOT level for the maximum specific growth rate of the culture was found to be lower than 5% air saturation. The difficidin production was significantly affected by DOT in the fermentation broth while the oxydifficidin synthesis appeared to be unaffected. The DOT level of 20% air saturation was critical for difficidin synthesis, below which the volumetric production rates of difficidin were sharply decreased. These results demonstrate the distinct difference between the critical DOT values for difficidin production and specific growth rates.

Fermentations with cycling DOT with a periodicity of 1 min were performed to simulate the heterogeneity in DOT of a large scale fermenter. The antibiotics production was not significantly affected by cycling DOT above, below as well as around the critical level for difficidin production. However, an increase in the growth rates when DOT was cycled below and around the critical level caused a marked reduction in the specific production rates of the two antibiotics. In contrast, no effect on growth rates was observed when DOT was cycled above the critical level.

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CONTENTS

1. INTRODUCTION

1.1 General Aspects of Oxygen in Fermentation Processes

The importance of oxygen to microbial activity is well recognized. Like all other living systems, microorganisms utilize oxygen (molecular form or oxidizing compounds) and eliminate carbon dioxide through respiration. The response of cells to available oxygen is a complex phenomenon and no organism is known to be indifferent to oxygen, although they differ widely in their sensitivities to free oxygen. Thus, based on oxygen sensitivity microorganisms can be classified as aerobic, anaerobic and facultative.

Most microorganisms do not seem to derive oxygen directly from the gas phase but as dissolved oxygen and other oxygen-containing compounds from the aqueous phase. Therefore, in aerobic microbial reactions air is sparged into the fermentation liquid and oxygen in the dissolved state reaches the microorganisms. Depending on the availability of dissolved oxygen to the microbial culture their behaviour can be observed to vary widely. However, the availability of dissolved oxygen is, in turn, dependent on the oxygen demand of the cultures and the oxygen supply capacity of the fermentation systems.

1.1.1 Oxygen demand in fermentations

The nature of the interaction between oxygen and microbial cells is a prerequisite to an understanding of the oxygen demand of the cultures. Oxygen may play an important role in microorganisms (Harrison, 1972; Mukhopadhyay and Ghose, 1976) as (1) an electron acceptor, (2) a nutritional component, (3) a fermentative metabolism regulator, (4) a metabolic poison and mutagenic agent, (5) a redox potential controller, (6) an energy generator, i.e. energy-rich cellular chemical (ATP) synthesis and/or heat energy generated by biothermochemical reactions, and (7) a controller of cell membrane permeation. However, little is known about the oxygen requirements of living organisms under submerged conditions, and probably still less about the mechanism of transfer of oxygen from the air to the cells (Richards, 1961).

Many authors have stated that oxygen consumption is higher by growing than by resting cells and the rate of respiration is highest at the start of the logarithmic phase (Konno and Terui, 1961; Brighton, 1966).

The rate of oxygen uptake by a microbial culture has been shown to be independent of the concentration of dissolved oxygen provided that this concentration is above a certain critical level, C_{crit} (Finn, 1954, 1967; Arnold and Steel, 1958; Mukhopadhyay and Ghose, 1976; Mudgett, 1980; Kargi and Moo-Young, 1985). Below this critical level, the respiration rate is dependent on the concentration of dissolved oxygen, and the respiratory activity decreases in a hyperbolic manner as the concentration of dissolved oxygen decreases. Finn (1954) found that for unicellular organisms C_{crit} is extremely low, and quotes typical values. Usually, C_{crit} is in the range of 5 to 10% of air saturation dependent on the type and activity of the cells, the nature of the substrate (Kargi and Moo-Young, 1985), the age of culture (Kubicek *et al.*, 1980), and temperature (Winzler, 1941). Therefore, it is not necessary to keep an active culture completely saturated with air in order to maintain its maximum respiratory activity; it is only necessary to supply sufficient oxygen to exceed the critical level.

Arnold and Steel (1958) attempted to correlate respiratory activity and product formation. They concluded that there can be no simple relationship between product formation and the oxygen uptake rate of an organism, though a correlation between aeration efficiency and product synthesis has readily been observed. However, Shu (1953) divided this kind of relationship into three distinct cases (1) a type where the oxygen requirement for the fermentation was the same as the maximum oxygen demand of the organism employed, e.g. the ustilagic acid fermentation by *Ustilago zeae*, (2) a type where the optimum oxygen requirement of the fermentation was far below the maximum oxygen demand of the organism, e.g. the production of α-amylase by *Aspergillus niger*, and (3) a type where the oxygen supply rate necessary for maximum product formation was far in excess of that required to satisfy the respiratory activity of the organism, e.g. the citric acid fermentation using *Aspergillus niger*.

Calam *et al.* (1951) measured the activation energies for growth rate, respiration, and penicillin production using the Arrhenius equation, and found them to be entirely different. They deduced that the three reactions were substantially independent of each other. They did find, however, an apparent correlation between respiration rate and penicillin titre provided that the type of fermenter, medium, and strain were unchanged.

Oxygen requirement of a culture depends on the microbial species employed and the state of growth in the life cycle. Furthermore, the rate of oxygen demand is also affected by the following environmental factors (Finn, 1954)

(1) Concentration and nature of carbon source.
(2) Concentration and nature of nitrogen source.
(3) Concentration and nature of minerals and vitamins.
(4) Accumulation of toxic end products.
(5) Loss of volatile intermediates.
(6) Supply of oxygen.
(7) Other culture conditions such as temperature, pH.

In certain aerobic processes, insufficient oxygen may cause irreparable damage to respiring cells, or may shift the metabolism of the cells to production of undesirable compounds (Phillips and Johnson, 1961b; Phillips, 1969). Usually aerobic and facultative anaerobic microorganisms grow best at high dissolved oxygen tensions and form fewest fermentation products under these circumstances (Harrison and Pirt, 1967). However, high dissolved oxygen tensions can exert toxic effects (Wimpenny, 1969; Harrison, 1972; Pirt, 1975; Mukhopadhyay and Ghose, 1976) by (1) formation of hydrogen peroxide in catalase negative organisms, (2) inhibition of labile SH-groups on protein molecules, (3) repression or inhibition of specific enzyme synthesis, (4) oxidation of cytochromes, (5) oxidation of thiol-groups, lipid peroxidation and free radical accumulation, or (6) disturbing the redox equilibrium in the cell by directing reducing equivalents from vital biosysthetic reactions to the excess oxygen. For most aerobic organisms the inhibitory dissolved oxygen tension is hyperbaric, that is > 0.21 atm (>100% air saturation) (Pirt, 1975). He distinguishes aerobic and microaerobic organisms

on the basis of oxygen toxicity above and below partial pressure of 0.21 atm (≈ 100% air saturation), respectively.

1.1.2 Oxygen supply in agitated-aerated cultures

Oxygen supply, provided by agitation and aeration, has a great influence on growth and product formation of microorganisms cultivated under submerged conditions (Steel and Maxon, 1962; Tanaka *et al.*, 1975; Takei *et al.*, 1975).

Oxygen is usually supplied to the fermentation medium by sparging air bubbles underneath the impeller of an agitated fermenter. Oxygen from a rising air bubble is first dissolved in the liquid medium and then transferred to the oxygen utilization sites inside the cell. The process of oxygen transfer may be decribed in terms of individual mass transfer resistances by considering the film model. There are seven major mass transfer resistances in oxygen transfer to microbial cells (Bartholomew *et al.*, 1950a; Arnold and Steel, 1958; Richards, 1961; Finn, 1967) which can be summarized as follows : (1) gas film resistance between the bulk gas and gas-liquid interface, (2) interfacial resistance at the gas-liquid interface, (3) liquid film resistance between the interface and bulk liquid phase, (4) liquid phase resistance for the transfer of oxygen to the liquid film surrounding single microbial cells, (5) liquid film resistance around cells, (6) intracellular or intrapellet resistance (in the case of microbial flocs or mycelial pellets), and (7) resistance due to consumption of oxygen inside a microbial cell.

The overall oxygen transfer resistance is equal to the sum of the individual resistances. The relative magnitudes of the individual resistances depend on bubble and liquid phase hydrodynamics, the composition and rheological properties of the fermentation medium, the density and activity of microbial cells, and the gas-liquid interfacial phenomena (Kargi and Moo-Young, 1985). In gerneral, the gas film and the gas-liquid interfacial resistances are usually negligible compared to other resistances. Therefore, the major supply side resistance is the liquid film resistance around the gas bubbles and the rate of oxygen transfer is given by the following equation

$$\frac{dC}{dt} = k_L a\ (C^* - C_L)$$

where k_L is the liquid film oxygen transfer coefficient, a is the gas-liquid interfacial area per unit volume of liquid, C^* and C_L are the saturation and actual dissolved oxygen concentrations in the liquid medium, respectively.

In a well-mixed fermenter, the concentration of dissolved oxygen in the bulk liquid phase is constant and oxygen transfer resistance in the bulk liquid is assumed to be negligible. However, in non-Newtonian fermentation broth, i.e. in mycelial fermentation, there may be significant concentration gradients within the bulk liquid and therefore oxygen transfer resistance in the bulk liquid may not be negligible.

The liquid film resistance around a single cell, i.e. unicellular organisms such as bacteria, may be negligible due to the fact that microbial cells are sufficiently small that their surface area per unit volume is extremely large. However, the liquid film resistance around the cell clumps, i.e. in mycelial fermentation, may be significant since the pellets are much larger than single cells.

Intracellular oxygen transfer resistance is usually negligible compared to other resistances because of the small size of cells and the proximity of organelles inside cells. However, if microbial growth is in the form of pellets, then the intrapellet resistance may be important since oxygen has to diffuse through to be available to the interior cells.

The rate of oxygen consumption within the cells may be the limiting factor when the rate of oxygen transfer is higher than the rate of biological oxygen consumption.

Operating and design variables which affect oxygen transfer in a fermentation broth are:

(1) Aeration rate (Cooper *et al.*, 1944; Bartholomew *et al.*, 1950a; Phillips, 1969; Finn, 1967; Pirt, 1975).

(2) Type and intensity of agitation (Chain and Gualandi, 1954; Steel, 1959; Steel and Maxon, 1962, 1966a, 1966b; Blakebrough and Hamer, 1963; Wang and Fewkes, 1977).

(3) Type and position of sparger (Bartholomew *et al.*, 1950a; Finn, 1954; Flynn and Lilly, 1967; Martinez *et al.*, 1989).

(4) Vessel geometry and liquid volume (Cooper *et al.*, 1944; Chain *et al.*, 1966; Manfredini *et al.*, 1983).

(5) Solubility of oxygen (Finn, 1954; Phillips, 1969; Pirt, 1975; Arnold and Steel, 1958).

(6) Physical properties of fermentation broths (Chain *et al.*, 1966; Taguchi, 1971; Jarai *et al.*, 1969; Jarai, 1972; Yagi and Yoshida, 1974; Kargi and Moo-Young, 1985).

The interactions between these factors are complex and may affect either the driving force (C^*-C_L) or the transfer coefficient (k_La), therefore it is possible to enhance the rate of oxygen transfer by controlling the aforementioned factors at their optimal level.

1.1.3 Dissolved oxygen levels in submerged culture

The level of dissolved oxygen concentration in aerobic fermentation media has a profound effect on the rate of microbial metabolism and product formation. The dissolved oxygen concentration is determined by the relative rates of oxygen consumption and oxygen transfer in the fermentation broth. At steady state the rate of oxygen transfer into the fermentation broth is equal to the rate of oxygen demand by the microbes ($Q_{O_2}X$), that is

$$k_La\ (C^*-C_L) = Q_{O_2}X$$

or transforming,

$$C_L = C^* - \frac{Q_{O_2}X}{k_La}$$

where Q_{O_2} is the specific rate of oxygen consumption by microbial cells and X is the cell concentration (Aiba *et al.*, 1973; Kargi and Moo-Young, 1985). In order to eliminate oxygen limitations, the dissolved oxygen concentration in a fermentation broth should be above the critical value (i.e. $C_L > C_{crit}$). However, oxygen demand of the cultures ($Q_{O_2}X$) usually varies with the course of fermentation. Therefore the rate of oxygen supply should be increased during the high oxygen demand rate of the culture. This could be done by increasing either k_La or C^*.

When the temperature, medium composition and oxygen partial pressure in the gas phase are constant, the equilibrium DO level (C^*) is fixed and the only term which influences the DO level at a given oxygen demand ($Q_{O_2}X$) is the oxygen transfer coefficient (k_La). In such cases, the oxygen transfer coefficient, k_La, should be increased to a level where the DO level is satisfied by increasing the air flow rate and/or agitation speed in agitated fermenters.

One obvious means of maintaining the satisfactory DO level of a culture medium during stringent demand rates is to raise the solubility, C^*, by increasing the partial pressure of oxygen. This may be done either by using oxygen-enriched air or pure oxygen or by increasing the total pressure of air supplied to the fermenter. For further high oxygen tension high pressure pure oxygen can be used (Finn, 1954; Phillips, 1969; Kargi and Moo-Young, 1985). Except for (1) the sweeping effect of the nitrogen, (2) the concomitant increase in carbon dioxide solubility as pressure is raised, and (3) a possible failure to supply enough carbon dioxide along with the pure oxygen, these methods for increasing C^* are equivalent.

A combination of the aforementioned methods may be inevitable in some cases. As seen in the Cooney and Wang correlations, agitation requirements for oxygen transfer at atmospheric levels eventually reach a practical limit beyond which some degree of oxygen enrichment is necessary (Cooney and Wang, 1971). Phillips (1969) has pointed out that recycling of fermenter headgases is desirable to prevent oxygen waste in such processes.

Dissolved oxygen concentration is measured electrometrically in term of dissolved oxygen tension (DOT) by the dissolved oxygen electrode, the current from which is directly proportional to the oxygen tension. Thus the oxygen electrode measures the oxygen activity of a solution, which roughly equivalent to the partial pressure of oxygen in that solution, and not the concentration (Finn,1967; Pirt, 1975). An excellent review of theory, construction, operation, and application of dissolved oxygen electrodes has been presented by Lee and Tsao (1979).

Finn (1954) has emphasized that the only way to ensure the sufficient aeration in a particular set up is to measure the dissolved oxygen tension of the fermentation broth. If a microbial culture fails to respire more rapidly as C_L is increased, then it may be safely assumed that the critical oxygen concentration has been exceeded and all is well.

The dissolved oxygen electrode has been used successfully to measure and control DOT down to about 0.01 atm ($\simeq$ 5% air saturation), beyond which difficulty in measuring DOT is encountered (Kjaergaard, 1977; Hirose and Shibai, 1980). Unfortunately, the most interesting effects of oxygen on many aerobic microbial processes take place at DOT levels below the sensitivity of the commercial dissolved oxygen electrodes used. Moreover, the range of the oxygen-limited state is infinite. Therefore, it is important to have a tool that can monitor the degree of oxygen limitation (Harrison, 1972; Kjaergaard, 1977). Such a tool is the measurement of the redox potential in the culture broth.

Redox potential (E_h) measures the tendency of a solution to give or take up electrons.

The reactions determining the redox potential in microbial fermentation are quite complex and not fully understood at the present time. However, it has been shown that oxygen can control the E_h value of complex culture media (Chung and Lee, 1986). The E_h of an oxygen solution may be deduced from consideration of the reaction

$$\frac{1}{2}O_2 + 2H^+ + 2\bar{e} \rightharpoonup H_2O$$

For the above reaction, the redox potential can be calculated using the Nernst equation

$$E_h = E_0 + \frac{RT}{2F} \cdot \ln\frac{A_{O_2}^{0.5} A_{H^+}^2}{A_{H_2O}}$$

Where E_h is the potential referred to the normal hydrogen electrode, E_0 is the standard potential of the system at 25° C when the activities of all reactions are unity, R is the gas constant, T is the absolute temperature, F is the Faraday constant, and A is the activity of redox species.

And the relationship between DOT and E_h can be written as

$$\log (DOT) = a \cdot (E_h) + b$$

in which a and b are specific for each microbial system. An interesting point of this equation is that the logarithmic relationship gives an amplifying effect in the detection of low-level DOT, i.e. large changes in E_h will represent very small changes in DOT. However, at the DOT levels required in aerobic cultures, E_h measurements are much less sensitive to DOT than are measurements with the membrane-covered oxygen electrode (Wimpenny, 1969; Pirt, 1975). Therefore, one should use the oxygen electrode which is more specific and reliable than redox electrodes as a measure of dissolved oxygen (Harrison *et al.*, 1969; Harrison, 1972).

The electrometric measurement of E_h was discussed in detail by Kjaergaard (1977). The E_h reading is highly dependent on the preparation of the platinum electrode, for instance the degree of polishing of the electrode (Jacob, 1974).

It has been known that the redox potential by its nature is not solely dependent on DOT but also on pH and activities of other oxidative/reductive components in the media (Kjaergaard, 1977; Ishizaki *et al.*, 1974). Hence, redox potential might in principle be regulated by various control strategies : aeration-agitation, addition of chemicals, and controlled addition of substrate in a fed-batch system (Kjaergaard and Joergensen, 1980; Chung and Lee, 1986).

Many workers have supported the use of redox measurement for monitoring and controlling microbial cultures. Sukharevich *et al.* (1970) found that the redox potential influenced the biosynthesis of the antibiotics levorin A and B, which are produced simultaneously by *Streptomyces levoris*, but with the A compound preferably produced at high values of E_h and the B compound preferably produced at low redox potentials. Shibai *et al.* (1974) found that the metabolism of *Bacillus subtilis* in the batchwise production of inosine was strongly influenced by the redox potential, leading to production of different by-products, depending on the E_h value in the stationary phase: thus, there was no accumulation of inosine in the medium when $E_h \leq -160$ mV, but the accumulation of lactic acid in the culture was increased. However, Balakireva *et al.* (1974) considered a characteristic of a microbial culture and found that there is no thermodynamic steady equilibrium state. The measured E_h value that may be constant in a system is called a stationary potential and is considered a purely instrumental notion.

In conclusion, redox potential is a useful index in monitoring low-level dissolved oxygen during the oxygen-limited phase of the fermentation. However, it is not a parameter directly influencing the fermentation, only an indirect factor in the sense that it is related to dissolved oxygen tension.

1.2 Effect of Dissolved Oxygen Levels on Biosynthesis of Microbial Metabolites

The dissolved oxygen tension (DOT) is one of the principle controlling parameters either in aerobic or anaerobic microbial fermentation processes (Harrison *et al.*, 1969). However, the requirement of oxygen for maximal production vary widely among different microorganisms and products (Küenzi and Auden, 1983). Oxygen shows diversified effects on product formation in fermentation processes. There is evidence that the productivity of some fermentations depends critically upon maintaining a sufficiently high dissolved oxygen level to satisfy the optimal oxygen demand of the microorganism for product formation. On the other hand, some fermentation products are found to be maximal only at barely detectable dissolved oxygen levels. Therefore, in order to obtain the best results, strict control of oxygen supply is indispensable based on the knowledge of the cell's behavior to oxygen. Unfortunately, the effects of dissolved oxygen tension on the product formation in microbial processes, not much has been published. Onken and Liefke (1989) have recently presented a comprehensive review of the effects of oxygen on growth and product formation in a wide range of microbial fermentation processes.

1.2.1 Effect of dissolved oxygen tension on enzyme synthesis

Oxygen is essentially required in a growing aerobic culture and often the achievement of a fermentative enzyme production depends on a sufficient supply of oxygen (Frost and Moss, 1987). However, this conclusion might be oversimplified (Lilly, 1979) because the inhibitory effect of the satisfactory dissolved oxygen levels has readily been found in some cases of enzyme production. Table 1.1 summarized the reports on the effects of oxygen on enzyme production.

In batch fermentation of α-amylase production by *Bacillus subtilis*, Mazza and Ertola (1970) have shown that an enzyme production can be maintained without aeration after reaching the stationary phase of growth if pH is controlled so that it follows the pH pattern of a continuously aerated cultivation. Moreover, Markkanen and Bailey (1975)

Table 1.1 : Reports of effects of dissolved oxygen tension on enzyme synthesis.

Enzyme	Producing organism	References
α-Amylase	*Bacillus subtilis*	Mazza and Ertola, 1970
		Markkanen and Bailey, 1975
	Schwanniomyces castellii	Boze *et al.*, 1987
Glucoamylase	*Endomycopsis* sp.	Gracheva *et al.*, 1973
Glucose isomerase	*Bacillus coagulans*	Diers, 1976
		Skøt, 1978
Glucose oxidase	*Aspergillus niger*	Zetelaki and Vas, 1968
		Zetelaki, 1970
	Penicillium vitale	Lavrishcheva *et al.*, 1969
	Penicillium sp.	Nakamatsu *et al.*, 1975
β-Galactosidase	*Escherichia coli*	Gray *et al.*, 1972
L-Asparaginase	*Erwinia aroideae*	Peterson and Ciegler, 1969
		Liu and Zajic, 1973
	Escherichia coli	Boeck *et al.*, 1970
		Netrval, 1980
	Serratia marcescens	Heinemann *et al.*, 1970
	Citrobacter	Bascomb *et al.*, 1975
Protease	*Bacillus pumilus*	Fabian, 1970
	Streptomyces spheroides	Krivova *et al.*, 1977
	Bacillus subtilis	Kole *et al.*, 1988
Lipase	*Rhizopus delemar*	Giuseppin, 1984
Pectolytic enzymes	*Aspergillus niger*	Zetelaki-Horvath and Vas, 1981
Cellulase	*Aspergillus fumigatus*	Wase *et al.*, 1985
Penicillin acylase	*Escherichia coli*	Kaufmann and Bauer, 1964
		Devcic and Divjak, 1968
		Kleiner and Lopatnev, 1973
		Gebauer *et al.*, 1987
		Lee and Chang, 1988

Table 1.1 - *Continued*

Enzyme	Producing organism	References
Catalase	*Rhodopseudomonas spheroides*	Gallili and Mateles, 1977
Phosphatase	*Staphylococcus aureus*	Arvidson *et al.*, 1976
11α-Hydroxylation enzyme	*Rhizopus nigricans*	Hanisch *et al.*, 1980
11β- and 19-Hydroxy--lation enzymes	*Pellicularia filamentosa*	Clark *et al.*, 1982
Cholesterol oxidase	*Nocardia rhodocrous*	Buckland *et al.*, 1976
Catechol 1,2-dioxygenase	*Pseudomonas aeruginosa*	Bird and Cain, 1968
	Pseudomonas putida	Alonso, 1982
Cytochrome P-450	*Saccharomyces cerevisiae*	Rogers and Stewart, 1973
		Blatiak *et al.*, 1985
Flavocytochrome b2	*Hansenula anomala*	Hours, 1982, 1983
D-Amino acid oxidase	*Trigonopsis variabilis*	Wei *et al.*, 1989

have reported a marked increase in α-amylase yields by reduction of aeration to one-fifth of its original value after the exponential growth phase under pH uncontrolled conditions. However, no mention of actual dissolved oxygen levels in the culture liquid was made in these experiments. On the other hand, in continuous culture of *Schwanniomyces castellii*, Boze *et al.* (1987) have found that α-amylase production is increased with increasing the dissolved oxygen concentration of the culture liquid.

In an examination of the influence of aeration on glucoamylase biosynthesis by *Endomycopsis* sp., Gracheva *et al.* (1973) have concluded that an increase in degree of oxygen solution, determined by sulfite oxidation rate, caused an improvement in enzyme biosynthesis as well as a reduction in process duration.

Diers (1976) and Skøt (1978) have reported in agreement that glucose isomerase

production by *B. coagulans* was enhanced under oxygen-limited conditions, when the dissolved oxygen tension fell to zero in glucose-limited cultures.

The effectiveness of aeration and agitation in the production of glucose oxidase by *A. niger* has been studied in both laboratory (Zetelaki and Vas, 1968) and pilot-scale fermenters (Zetelaki, 1970). In laboratory fermenters the growth rate and glucose oxidase activity increased considerably with the increase of agitation speed up to 700 rpm, decrease again as higher agitation rates were used. When pure oxygen was used rather than air the glucose oxidase activity was approximately doubled. In 500 L fermenters, similar effects of agitation speed were observed. Doubling the head pressure in the fermenters gave higher initial growth rates and a more rapid enzyme synthesis. However, the excess oxygen content in culture liquid of *Penicillium vitale* has been reported to suppress mycelium growth and inactivate glucose oxidase (Lavrishcheva *et al.*, 1969). Moreover, the effects of aeration on glucose oxidase production were remarkably different among various species of *Penicillium* (Nakamatsu *et al.*, 1975). Comparing between effects of aeration and agitation of the culture medium, Nakamutsu *et al.* (1975) found that the more significant effects on enzyme production caused by variation of aeration.

Gray *et al.* (1972) have shown that β-galactosidase production by *E. coli* was unaffected by a reduction of maximum oxygen transfer rate from 250 mmol/L.h to 150 mmol/L.h. A further decrease in oxygen transfer rate to 100 mmol/L.h, however, resulted in notably decrease in enzyme production, possibly due to a longer peroid of oxygen limitation occurring in the latter fermentation.

The prerequisite for a high synthesis of L-asparaginase by *Erwinia aroideae* is reported to be a low aeration condition (Peterson and Ciegler, 1969). At a high aeration condition cell growth was promoted whereas an enzyme production was suppressed. Heinemann *et al.* (1970) confirmed that the maximum yield of L-asparaginase of *Serratia marcescens* accumulated only at undetectable levels of dissolved oxygen. The similar results have also been observed in a large scale (3000

L) fermentation by a strain of *Citrobacter* (Bascomb *et al.*, 1975). Liu and Zajic (1973) reported that the maximum L-asparaginase from *Erwinia aroideae* was obtained under moderate aeration condition where the dissolved oxygen level became zero at 12.5 h of cultivation. Boeck *et al.* (1970) suggested that enzyme production could be markedly improved by employing high aeration for cell growth and subsequently reduced oxygen tension for enzyme synthesis. The composition of culture medium used for enzyme production has also shown some effect on the oxygen sensitivity of microorganism in biosynthesis of L-asparaginase (Netrval, 1980).

In an investigation of the influence of oxygen on the biosynthesis of protease by *B. pumilus*, Fabian (1970) has found that both the growth rate and protease formation were inhibited under low oxygen concentrations. The similar results have been reported by Krivova *et al.* (1977) and Kole *et al.* (1988), who found that the biomass and protease accumulations were stimulated by high dissolved oxygen levels in the medium.

In the fermentative production of lipase by *Rhizopus delemar*, there existed a critical level of dissolved oxygen concentration at 47 μmol/L (≈ 20% air saturation) below which a sharp decrease in the amount of lipase was observed (Giuseppin, 1984). At an oxygen concentration higher than this critical value, the biomass production and enzyme activity were highest and independent of dissolved oxygen levels in the fermentation broth.

The effects of oxygen are particularly complicate when the desired product is a mixture of enzymes. Zetelaki-Horvath and Vas (1981) have shown that the maximum production of the various components of the pectolytic enzyme complex, produced by *A. niger*, occurred at different oxygen transfer rates.

Increasing the oxygen transfer rate by increasing the agitation speed may result also in increased shear stress in the culture system. Wase *et al.* (1985) found that the

yield of cellulase, produced by *Aspergillus fumigatus* in the agitated vessels, was decreased with increasing the agitation rate. Furthermore, in an air-lift fermenter, where shear effects were much less than in the disc-turbine-agitated vessel, yields of cellulase were, by comparison, increased by about 20%.

Data on the effect of oxygen on the production of the enzyme penicillin acylase are rather discordant. Kaufmann and Bauer (1964) emphasized the requirement of intensive aeration in the production of penicillin acylase by *Escherichia coli.* Other workers (Devcic and Divjak, 1968; Kleiner and Lopatnev, 1973), however, found that the penicillin acylase activity of *E. coli* fell with increasing aeration. Gebauer *et al.* (1987) have reported that the optimum dissolved oxygen tension for an enzyme synthesis was below 10% saturation in a stirred-tank reactor and at 35% in an airlift tower-loop reactor. Nevertheless, the enzyme productivity was inhibited when dissolved oxygen level dropped to zero (Lee and Chang, 1988).

The effect of the agitation speed on the production of catalase by *Rhodopseudomonas spheroides* was studied in 3.5 L fermenter (Gallili and Mateles, 1977). Low agitation speed (200 rpm) resulted in a relatively rapid decrease of the dissolved oxygen tension in the medium and a prolonged period of fermentation with a constant catalase activity. Increase agitation speed up to 500 rpm shortened the fermentation time by half without affecting the enzyme synthesis. An extremely high agitation rate (800 rpm) caused a rapid growth and catalase production, followed by a drastic drop in the enzyme activity when the culture reached the stationary phase, probably due to active destruction of the enzyme.

In continuous culture of *Staphylococcus aureus*, Arvidson *et al.* (1976) have found that the phosphatase production was enhanced under the oxygen limited conditions when the dissolved oxygen tension fell to zero. Increasing the oxygen tension in the culture resulted in an inactivation of phosphatase synthesis.

The effect of DOT on the synthesis of an enzyme catalyzing an oxygen-requiring reaction has only been studied in detail in a few cases (Lilly, 1979). Hanisch *et al.* (1980) have demonstrated that the optimum induction of the enzyme progesterone 11α-hydroxylase by *Rhizopus nigricans* in a fermentation occurred at a reletively low dissolved oxygen tension of around 10% saturation, whereas the optimum expression of this enzyme lay at a much higher DOT. Similar results have also been reported by Clark *et al.* (1982) for the 11β- and 19-hydroxylation enzymes of *Pellicularia filamentosa*. For both hydroxylations, these authors found an optimum DOT for induction at 15% saturation, while the optimum for expression was at 30% saturation. Furthermore, the ratio of the two compounds was invariably constant, at 1.0 : 0.84 19-: 11β-, at different DOT levels, so that their relative production rates could not be manipulated by variation of DOT alone. In conclusion, the data have shown that maximum rates of hydroxylation could be achieved when induction was performed at low DOT followed by the elevation of the DOT to enhance enzyme expression.

In a kinetic study of cholesterol oxidase biosynthesis by *Nacardia rhodocrous*, Buckland *et al.* (1976) observed that the maximum enzyme production was obtained in the range of DOT values 30 - 40% air saturation. At DOT values below 30% saturation, the enzyme synthesis decreased by at least 20%. On the other hand, at DOT values higher than 60% there was a drop in the maximum cholesterol oxidase production together with an increase in foaming and morphology changes in the culture, i.e. aggregation of cells.

Oxygen has been shown to be one of the important factors in the production of catechol 1,2-dioxigenase by *Pseudomonas* species. The enzyme activity in the anaerobically induced cultures was extremely low compared with those obtained under aerobic induction (Bird and Cain, 1968). Alonso (1982) has confirmed that the emzyme production rate increased with increasing DOT. The influence of oxygen was more pronounced at DOT values above 50% saturation.

There is agreement in the literature that the optimum production of cytochrome P-450 in *Saccharomyces cerevisiae* occurred at a relatively low dissolved oxygen concentration. Rogers and Stewart (1973) have reported that cytochrome P-450 synthesis was maximal at a dissolved oxygen concentration of 0.25 - 0.5 μmol/L (≈ 0.1 - 0.2% air saturation). However, under strictly anaerobic condition, no detectable cytochrome P-450 was produced. Blatiak *et al.* (1985) found that the biosynthesis of cytochrome P-450 was inhibited without altering growth rate when an anaerobic condition was made during the exponential growth phase of an aerobically growing culture of *S. cerevisiae* in 20% D-glucose medium. This result leads to the conclusion that oxygen might play an important role as a substrate inducer of yeast cytochrome P-450.

The correlation between the content of flavocytochrome b2 of *Hansenula anomala* and the dissolved oxygen concentration of the medium has been studied by Hours (1982, 1983), who found that the enzyme production commenced when dissolved oxygen tension reached 80% air saturation, and increased as a linear function of the dissolved oxygen tension up to 180% air saturation beyond which yeast cells were damaged. Yeast growth was maximum at minimum flavocytochrome b2 concentration.

Wei *et al.* (1989) found that the activity of D-amino acid oxidase in *Trigonopsis variabilis* in continuous culture decreased as the dissolved oxygen level in the culture increased. The reason for low enzyme production under high oxygen tension might be due to the oxidative inactivation of the enzyme by hydrogen peroxide formed during the course of fermentation and/or by oxygen itself. However, the enzyme synthesis was adversely affected under a strictly anaerobic condition. The highest productivity was obtained at dissolved oxygen level about 10% air saturation.

1.2.2 Influence of DOT on synthesis of amino acids, organic acids and other metabolites

A list of reports on effects of oxygen on the synthesis of amino acids, organic acids and other metabolites is shown in Table 1.2. Cell growth and amino acids biosynthesis are aerobic processes in which large quantities of oxygen are essentially required in the fermentive production of these compounds (Enei *et al.*, 1982). However, high oxygen tension sometimes plays an inhibitory role even in aerobic fermentation (Akashi *et al.*, 1979a). Hirose and Shibai (1980) investigated the effects of the dissolved oxygen concentration on the production of a range of amino acids which had closely related metabolic pathways. These workers demonstrated the limitation of the conventional membrane-coated oxygen electrode in measuring dissolved oxygen in oxygen deficient aerobic fermentation and considered the extent of oxygen supply to the culture in terms of the "degree of oxygen satisfaction", r_{ab}/KrM, that is, the respiration rate of the culture expressed as a fraction of the maximum respiration rate. Thus, a value of oxygen satisfaction below unity implied that the dissolved oxygen concentration was below the critical level. In relation to the mode and the extent of the influence of oxygen supply, amino acids fermentation was classified into three groups. The first group included L-glutamic acid, L-glutamine, L-arginine and L-proline fermentation in which the maximum production was observed under sufficient oxygen supply ($r_{ab}/KrM > 1.0$) and accumulation of amino acids was strongly inhibited by oxygen deficiency. The second group included L-lysine, L-isoleucine and L-threonine fermentation in which accumulation was maximum under sufficient oxygen supply, and decreased only slightly with oxygen shortage. Finally, the third group included L-leucine, L-valine and L-phenylalanine fermentation where product formation was inhibited under sufficient oxygen supply and product accumulation was maximum when cell respiration was inhibited by oxygen limitation ($r_{ab}/KrM < 1.0$). The differing behavior of the cells to oxygen was related to the different biosynthesis pathways of each amino acid. The amino acids of the first and second groups were all produced from tricarboxylic acid (TCA) cycle intermediates, whereas those of the third group were produced from the glycolysis intermediate, pyruvate and phosphoenol pyruvate.

Table 1.2 : Reports of effects of DOT on amino acids, organic acids and other metabolites.

Product	Producing organism	References
Amino acids		Hirose and Shibai, 1980
		Hirose, 1986
L-Glutamic acid	*Brevibacterium lactofermentum*	Okada and Tsunoda, 1965
		Hirose *et al.*, 1966
		Hirose,1972
	Corynebacterium hydrocarboclastus	Kobayashi *et al.*, 1972
L-Proline, L-Glutamine and L-Arginine	*Brevibacterium flavum*	Akashi *et al.*, 1979b
L-Arginine and L-Histidine	*Brevibacterium flavum*	Akashi *et al.*, 1979a
L-Serine	*Protomonas extorquens*	Sirirote *et al.*, 1986
L-Lysine	*Brevibacterium* sp.	Beker *et al.*, 1973
L-Isoleucine	*Serratia marcescens*	Chibata *et al.*, 1962
L-Valine	*Aerobacter aerogenes*	Takamura *et al.*, 1962
		Hongo and Uyeda, 1972
	Brevibacterium lactofermentum	Akashi *et al.*, 1977
L-Leucine	*Brevibacterium lactofermentum*	Akashi *et al.*, 1978
L-Phenylalanine	*Brevibacterium lactofermentum*	Akashi *et al.*, 1979b
L-Tryptophan	*Hansenula anomala*	Terui *et al.*, 1961
	Candida utilis	Beker *et al.*, 1971
Inosine	*Bacillus subtilis*	Shibai *et al.*, 1973
		Enei *et al.*, 1982
Citric acid	*Aspergillus niger*	Martin and Waters, 1952
		Buelow and Johnson, 1952
		Clark and Lentz, 1961
		Khan and Ghose, 1973
		Kovats and Gackowska, 1976
		Kubicek *et al.*, 1980
		Kubicek and Röhr, 1986
		Dawson *et al.*, 1986, 1988

Table 1.2 - *Continued*

Product	Producing organism	References
Citric acid	*Aspergillus foetidus*	Kristiansen and Sinclair, 1978, 1979
	Candida tropicalis	Okoshi *et al.*, 1987
Gluconic acid	*Pseudomonas ovalis*	Humphrey and Reilly, 1965
	Gluconobacter oxydans	Oosterhuis *et al.*, 1985a
	Aspergillus niger	Lee *et al.*, 1987
		Sakurai *et al.*, 1989
Itaconic acid	*Aspergillus terreus*	Pfeifer *et al.*, 1952
		Nakamura and Kobayashi, 1969
Acetic acid	*Acetobacter aceti*	Hromatka *et al.*, 1951
		Hromatka, 1952
		Levonenmunoz and Cabezudo, 1981
		Muraoka *et al.*, 1982, 1983
		Park *et al.*, 1989
Polyhydroxy alcohols	Osmophilic yeasts	Spencer and Sallans, 1956
	Saccharomyces rouxii	Spencer and Shu, 1957
	Pichia miso	Onishi and Saito, 1962
	Yeastlike fungus	Hajny *et al.*, 1964
	Pichia farinosa	Vijaikishore and Karanth, 1984, 1986
2,3-Butanediol	*Aerobacter aerogenes*	Sablayrolles and Goma, 1984
	Klebsiella oxytoca	Jansen *et al.*, 1984
		Qureshi and Cheryan, 1989
	Bacillus subtilis	Moes *et al.*, 1985
Exopolysaccharide	*Rhizobium trifolii*	Thompson and Leps, 1986
Mosquito larval toxin	*Bacillus sphaericus*	Yousten *et al.*, 1984

The maximum productivity of glutamic acid in submerged culture using sugar as carbon source was obtained at oxygen transfer rate greater than 7×10^{-7} mol/mL.min (≃ 42 mmol/L.h) (Hirose *et al.*, 1966; Hirose, 1972) whereas oxygen transfer rate of more than 14.3×10^{-7} mol/mL.min (≃ 86 mmol/L.h) was required in glutamic

acid fermentation from hydrocarbon (Kobayashi *et al.*, 1972). At low oxygen supply glutamic acid formation was depressed and accumulation of lactic acid and succinic acid occurred instead (Okada and Tsunoda, 1965; Hirose, 1972). However, extremely high oxygen supply led to a significant reduction in cell growth, sugar consumption and glutamic acid formation, although the dissolved oxygen level was well above critical value and the cellular oxygen demand was satisfied (Hirose, 1972). This damaging effect was observed at a very early stage (in the growth phase) which had a continued influence on the production phase, i.e. the cells grown under high oxygen tension had poor ability to produce glutamic acid. The inhibitory effect of high oxygen tension on the cell growth and product formation was also observed in arginine and histidine fermentations (Akashi *et al.*, 1979a).

In proline, glutamine and arginine fermentations, the maximum amount of the products was produced during a sufficient supply of oxygen at the r_{ab}/KrM value of 1.0, while lactic acid accumulated in extremely oxygen deficient culture (Akashi *et al.*, 1979b)

In the fermentative production of L-serine by *Protomonas extorquens*, oxygen supply is an important factor. L-serine was not produced when oxygen was not supplied. The maximum productivity was obtained at the dissolved oxygen concentration 0.5 ppm (≃ 7% air saturation), above which L-serine production was decreased (Sirirote *et al.*, 1986).

In L-lysine fermentation by *Brevibacterium* sp., Beker *et al.* (1973) found that the decrease of the dissolved oxygen level from 0.10 atm to 0.01 atm (≃ 48% to 5% air saturation) resulted in a decrease of the biomass and an increase of the product concentrations in continuous culture.

Investigations on the effects of dissolved oxygen in L-isoleucine fermentation by Chibata *et al.* (1962) showed that the increase in aeration rate caused an improvement of both growth and isoleucine formation.

Takamura *et al.* (1962) studied the relationship between L-valine accumulation and liquid volume in shaken flasks and observed more product formation in anaerobic cultures than in aerobic cultures. By contrast, Hongo and Uyeda (1972) showed that aerobic condition favored product formation in experiments using the redox potential of the medium as an index. This inconsistency might result from the lack of adequate quantitative standards for both the analysis and control of oxygen (Akashi *et al.*, 1977). However, it was clarified that maximum production of L-valine occurred when the cell's oxygen demand was slightly inhibited at a value of r_{ab}/KrM 0.5 - 0.7 (Akashi *et al.*, 1977). When the oxygen supply was extremely limited (r_{ab}/KrM < 0.3), product formation was markedly inhibited and lactic acid was excreated as a dominant product. These results are similar to those obtained in the L-leucine fermentation (Akashi *et al.*, 1978). The similar stimulating effects of limited oxygen supply were also observed in L-leucine and L-phenylalanine fermentations where the maximum product produced at r_{ab}/KrM 0.8 - 0.9 in L-leucine fermentation (Akashi *et al.*, 1978) and at r_{ab}/KrM 0.45 - 0.65 in L-phenylalanine fermentation (Akashi *et al.*, 1979b). There existed a close relationship between the excretion of other amino acids as by-product and the condition of oxygen supply (Akashi *et al.*, 1978; Hongo and Uyeda, 1972).

The effect of oxygen on L-tryptophan formation by yeasts was also studied (Terui *et al.*, 1961). The specific rate of product formation was at a maximum when the aeration rate is 0.17 - 0.10 vvm and the dissolved oxygen level is 4×10^{-5} M (≃ 17% air saturation) or a little lower. At higher levels of oxygen supply, high cell growth was observed but productivity was low; at lower levels of oxygen supply, both cell growth and productivity were decreased. At the optimum aeration level, higher productivity was maintained until the latest stage of the fermentation process. Beker *et al.* (1971) reported that the optimum oxygen transfer rate for L-tryptophan fermentation ranged from 70 to 100 mg/L.min (≃ 131 to 188 mmol/L.h) in a special column fermenter.

In inosine fermentation employing an inosine producing mutant derived from *B.*

subtilis, a clear correlation between the extent of product formation and the degree of oxygen supply was observed (Shibai *et al.*, 1973). The maximum accumulation of inosine occurred at the oxygen transfer rate more than 5×10^{-7} mol/mL.min (≈ 30 mmol/L.h). At the condition of insufficient oxygen supply, on the other hand, inosine production was markedly inhibited and the by-products such as acetoin or 2,3-butyleneglycol excreted as a main metabolite (Enei *et al.*, 1982).

Aeration of the liquid medium has been shown to be a critical factor in the submerged citric acid fermentation (Kubicek and Röhr, 1986). Citric acid production was stimulated by increasing aeration rates (Buelow and Johnson, 1952; Khan and Ghose, 1973; Dawson *et al.*, 1988), and it has also been shown that using pure oxygen rather than air leads to an increase in product formation (Kristiansen and Sinclair 1978, 1979). The growth and production rates were increased without effecting the final yields in a batch sparged with pure oxygen (Kristiansen and Sinclair, 1978) whereas a greater citric acid yield and a constant growth rate were obtained in continuous culture (Kristiansen and Sinclair, 1979). On the other hand, the use of large amount of pure oxygen seems to be a commercial disadvantage. Clark and Lentz (1961) and Kristiansen and Sinclair (1978), however, have indicated that the gas phase of citric acid production could be recycled provided the carbon dioxide gas was trapped. Kubicek *et al.* (1980) reported that critical dissolved oxygen tension (DOT) values for growth and production phases were 9 - 10% air saturation and 12 - 13% air saturation, respectively and that production of citric acid steadily increased between DOT values of 20 and 75% air saturation. Moreover, Clark and Lentz (1961) and Okoshi *et al.* (1987) found that the maximum citric acid production was obtained at a dissolved oxygen concentration of around 60 ppm (≈ 800% air saturation) by using pure oxygen gas at 1.7 atm; above and below which the production declined. Martin and Waters (1952) reported that an interruption to aeration during batch fermentation adversely affected citric acid production. This effect depended on both the duration of the interruption and the stage of fermentation (Kovats and Gackowska, 1976). Kobicek *et al.* (1980) reported that a 20 min interruption to aeration during idiophase did not reduce the viability of the organism,

but irreversibly destroyed its ability to produce and accumulate citric acid. In contrast, Dawson *et al.* (1986) showed that a 120 min interruption to the air supply retarded citric acid production, but the effect was not permanent and subsequent recovery could occur.

Gluconic acid fermentation is an oxidative process which is strongly affected by the dissolved oxygen concentration. The growth and acid production rates were enhanced with increasing dissolved oxygen concentration up to 40% saturation in the *Pseudomonas ovalis* culture (Humphrey and Reilly, 1965) and up to 60% saturation in the *Gluconobacter oxydans* culture (Oosterhuis *et al.*, 1985a), above which the growth as well as the product formation were diminished. In addition, the dissolved oxygen concentration as high as 150 ppm (≃ 2000% air saturation) obtained by supplying oxygen gas under 6 atmospheric pressure has been reported to give the maximum rate of gluconate production by *Aspergillus niger* grown at 36 ppm dissolved oxygen concentration (≃ 480% air saturation) (Lee *et al.*, 1987; Sakurai *et al.*, 1989). Lee *et al.* (1987) have also reported that the mycelial activity for gluconate production was increased in mycelial grown at higher dissolved oxygen concentrations, though the mycelial growth decreased with increasing dissolved oxygen concentration.

In the fermentative itaconic acid production by *A. terreus*, the production rate was generally increased as the rate of aeration or agitation was increased (Pfeifer *et al.*, 1952). The maximum specific production rate was observed to be in the ranges of 0.06 to 0.2 atm oxygen partial pressure (≃ 29 to 95% air saturation) (Nakamura and Kobayashi, 1969). In addition, the interruption of the air supply for peroids of from 15 to 60 min resulted in complete inhibition of itaconic acid production, but this could be restarted, at a lower rate, after adding more nutrients and allowing the mould to proliferate (Pfeifer *et al.*, 1952).

An oxidative microbial acetic acid production has long been recognized as a highly aerobic process where the production rate is significantly affected by the dissolved

oxygen levels in fermentation broth (Hromatka, 1952; Levonenmunoz and Cabezudo, 1981; Ghose and Bhadra, 1985; Park *et al.*, 1989). It has been shown that an increase in oxygen transfer rate gives rise to better growth and production rates (Levonenmunoz and Cabezudo, 1981). Moreover, Park *et al.* (1989) stated that the optimum dissolved oxygen concentration for acetic acid production in continuous culture was around 1 - 3 ppm (≈ 13 - 40% air saturation). Hromatka *et al.* (1951) studied the influence of complete interruption of aeration on the rate of fermentation and found that complete stoppage of aeration for only 30 s caused a rapid decline in acid production. However, the extent of the damage to acid production due to interruption of aeration was a function of the acetic acid concentration and duration of aeration suspension. Muraoka *et al.* (1982) reported that in *Acetobacter aceti* submerged fermentation, when the acid concentration reached 6%, if aeration and agitation were interrupted for only 10 s, complete inhibition of the subsequent acid production occurred, whereas there was no damage even under conditions of oxygen dificiency for 720 s at an acid concentration of 4%. The impairment of acid production in submerged culture by oxygen deficiency was found to be caused by damage to bacterial cells. The oxygen uptake rate with ethanol and the activity of alcohol dehydrogenase of the cells were found to decrease by 20% and 50%, respectively after damage by oxygen deficiency (Muraoka *et al.*, 1983).

Early experiments on the effect of aeration on production of polyhydric alcohols by osmophilic yeasts indicated that high levels of aeration reduced the yield of ethanol but increased the yield of glycerol, without affecting the D-arabitol yield (Spencer and Sallans, 1956). In contrast, subsequent experiments provided some evidence that D-arabitol yields also increased as aeration levels were raised (Spencer and Shu, 1957). Hajny *et al.* (1964) also obtained yields of erythritol with an optimum level of aeration somewhere between 40 and 60 mmol O_2/L.h, above which yields of erythritol slightly decreased. Onishi and Saito (1962) obtained further confirmation of the effect, using *Pichia miso*. Glycerol production was lowered as the aeration level was decreased. The experiments using a Waldhof fermenter demonstrated convincingly that yields were a function of the degree of dispersion of the air in the

medium rather than of the air flow rate. More recently, Vijaikishore and Karanth (1986) reported that aeration and agitation should be maintained above a critical value to give a dissolved oxygen of 40% saturation for enhanced glycerol and negligible ethanol.

The oxygen supply rate has been shown to have a profound effect on the microbial production of 2,3-butanediol where the high oxygen transfer rate enhanced the cell respiration, resulting in a high cell yield and a low butanediol yield (Jansen *et al.*, 1984). On the other hand, the butanediol production increased whereas the cell mass declined at the low oxygen supply rate. Similar results were reported by Sablayrolles and Goma (1984), who also found that the optimum oxygen transfer coefficient (k_La) for butanediol production was in the range of 50 - 100 h^{-1}. Qureshi and Cheryan (1989) pointed out that the improvement of butanediol yield under the optimal aeration and agitation conditions resulted from the suppression of inhibitory by-products fromation, i.e. ethanol and lactic acid which occurred under insufficient aeration and acetic acid which was produced in the excess aeration conditions. Acetoin which is a metabolic intermediate in the butanediol pathway is synthesized under high aeration condition instead of butanediol. However, there exist reversible conversions of these two products which are, in turn, dependent on the oxygen level of the medium. In a *Bacillus subtilis* culture, the product concentration ratio of acetoin and butanediol changed rapidly in the range of dissolved oxygen concentration between 80 - 90 ppb (≈ 1.1 - 1.2% air saturation) (Moes *et al.*, 1985). In general, the acetoin/butanediol ratio increased with oxygen availability and decreased with decreasing dissolved oxygen. These workers also suggested this product ratio which was a function of dissolved oxygen concentration as a simple means of characterizing oxygen transport and mixing in a bioreactor.

It is beneficial to minimize the exopolysaccharide produced in the *Rhizobium* biomass production by submerged culture. The simple and economical method using dissolved oxygen as control parameter was proposed by Thompson and Leps (1986), who found that the exopolysaccharide production increased by 19% as the dissolved

oxygen decreased from 20 to 4% saturation and further increased up to higher levels at extremely low dissolved oxygen (< 1%). Therefore, to obtain the optimum biomass production with negligible amount of exopolymer produced the dissolved oxygen tension should be maintained above approximately 1 - 4% saturation.

In a fermentative production of mosquito larval toxin by *Bacillus sphaericus* on submerged fermentation, Yousten *et al.* (1984) found that bacterial sporulation was inhibited whereas toxin formation was not affected as the aeration was shift from air to pure oxygen in the fermenter. The toxin production might be limited under deficient oxygen levels, however, no experimental data was presented under these conditions by these authors.

1.2.3 Effect of DOT on antibiotic production

The antibiotic-producing microorganisms are normally strictly aerobic (Calam, 1987). Oxygen is required as energy source for (1) cell production (2) cell maintenance and (3) antibiotic production and other secondary metabolism. It has been found (Ryu and Hospodka, 1980) that 1 g of penicillin requires 2.2 L of oxygen. Table 1.3 lists a number of reports on effects of DOT on antibiotic production.

The effects of aeration and agitation on penicillin production in submerged fermentations were extensively studied by many earlier groups of workers. The penicillin yields were improved by increasing air flow rate through the fermenters (Stefaniak *et al.*, 1946a; Bartholomew *et al.*, 1950b; Brown and Peterson, 1950). There existed the optimum air flow rate beyond which no improvement or even decrease in antibiotic production was observed. The favorable effects of increasing degree of agitation on penicillin production were also reported by Bartholomew *et al.* (1950b), Brown and Peterson (1950), Calam *et al.* (1951) and Wegrich and Shurter (1953). However, the effects of aeration and agitation rates on antibiotic production were shown to be interdependent, i.e. the degree of mechanical agitation considerably affects the air flow requirements of the system.

Table 1.3 : Reports of effects of dissolved oxygen on antibiotic production.

Antibiotic	Producing organism	References
Penicillin	*Penicillium chrysogenum*	Stefaniak *et al.*, 1946a
		Bartholomew *et al.*, 1950b
		Brown and peterson, 1950
		Calam *et al.*, 1951
		Wegrich and Shurter, 1953
		Squires, 1972
		Giona *et al.*, 1976a, 1976b
		König *et al.*, 1981
		Bernard and Cooney, 1981
		Vardar and Lilly, 1982b
Cephalosporin C	*Cephalosporium* sp.	Feren and Squires, 1969
		Smith, 1985
		Maksimova *et al.*, 1985
		Hilgendorf *et al.*, 1987
Cephamycin C	*Streptomyces clavuligerus*	Rollins *et al.*, 1988a, 1989
		Yegneswaran *et al.*, 1991a
Gramicidin S	*Bacillus brevis*	Koupletskaya, 1965
		Udalova *et al.*, 1972, 1986
		Vandamme *et al.*, 1981
Tyrothricin	*Bacillus brevis*	Seddon and Fynn, 1973
		Fynn and Davison, 1976
Bacitracin	*Bacillus licheniformis*	Flickinger and Perlman, 1979
Oxytetracycline	*Streptomyces rimosus*	Orlova and Verkhovtseva, 1957
		Oblozhko and Orlova, 1975, 1977
		Oblozhko *et al.*, 1977
Chlortetracycline	*Streptomyces aureofaciens*	Orlova and Verkhovtseva, 1957
		Matelová *et al.*, 1955
		Hošťálek, 1964
		Plakunova and Kiseleva, 1965
		Plakunova, 1966
		Cherkasova *et al.*, 1978

Table 1.3 - *Continued*

Antibiotic	Producing organism	References
Erythromycin	*Streptomyces erythreus*	Brinberg, 1959
		Stark and Smith, 1961
		Lobanova *et al.*, 1970
Leucomycin	*Streptomyces kitasatoensis*	Kodama *et al.*, 1968
Rifamycin	*Streptomyces mediterranei*	Virgilio *et al.*, 1964
		Sensi and Thiemann, 1967
Amphotericin B	*Streptomyces nodosus*	Vekshin and Malkov, 1989
Candidin	*Streptomyces viridoflavus*	McDaniel and Bailey, 1969
		Martin and McDaniel, 1974
Candicidin	*Streptomyces griseus*	Martin and McDaniel, 1975
		Liu *et al.*, 1975
Candihexin	*Streptomyces viridoflavus*	Martin and McDaniel, 1974
Nystatin	*Streptomyces noursei*	Lopatnev *et al.*, 1973
Streptomycin	*Streptomyces griseus*	Kempf and Sayles, 1946
		Bartholomew *et al.*, 1950b
		Hockenhull, 1963
Neomycin	*Streptomyces fradiae*	Flickinger and Perlman, 1980
Kanamycin	*Streptomyces kanamyceticus*	Brinberg *et al.*, 1970
Levorins	*Streptomyces levoris*	Sukharevich *et al.*, 1970
Carminomycin	*Actinomadura carminata*	Kochetkova *et al.*, 1976
A40104A	*Clitopilus pseudo-pinsitus*	Boeck *et al.*, 1980
Actaplanin	*Actinoplanes missouriensis*	Boeck and Stark, 1984

Wise (1951) and Bartholomew *et al.* (1950b) determined the yields of penicillin and streptomycin from broths under different conditions of aeration and found that the titres increased with increasing efficiency of aeration, up to a point where the rate of solution of oxygen satisfied the rate of demand of the culture. Thereafter the titre was independent of the level of aeration. However, the dissolved oxygen levels in culture broth were not presented in most of these earlier papers.

More recently, Giona *et al.* (1976a, b) reported the correlation between specific rate of penicillin production (q_{pen}) and dissolved oxygen concentration (C_L) in an industrial semicontinuous glucose-fed fermentation (Figure 1.1).

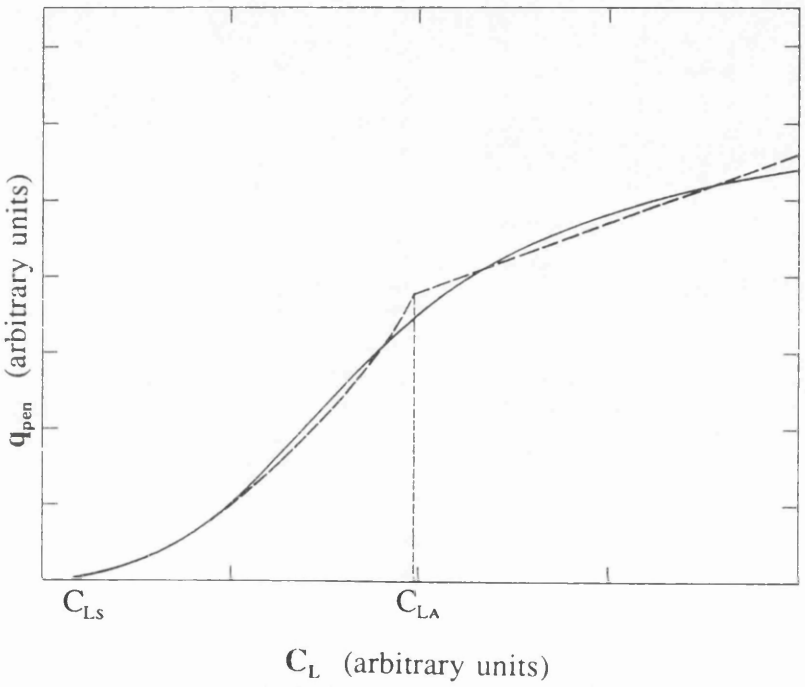


Figure 1.1 : The dependence of the specific production rate of penicillin on the oxygen concentration in the broth (From Giona *et al.*, 1976a).

The data formed a sigmoidal curve which could be represented mathematically as two functions, one parabolic for concentrations lower than a value C_{LA} and one linear for $C_L > C_{LA}$. The increasing trend of q_{pen} with C_L was demonstrated up to a C_L value of 0.4 mol/m^3 (≈ 171% air saturation), which approached the equilibrium concentration of oxygen obtained in broth in the absence of oxygen demand. Penicillin synthesis was irreversibly damaged at C_L value below 0.022 mol/m^3 (≈ 9% air saturation); this value agrees with that suggested by Finn (1954). In some related reports, the irreversible loss of q_{pen} occurred at DOT below 5 to 10% saturation in a fed-batch fermentation (Squires, 1972) and at DOT below 10% in a batch culture (Vardar and Lilly, 1982b). König *et al.* (1981) and Bernard and Cooney (1981) also found 8 - 10% air saturation might be

critical for penicillin production. Vardar and Lilly (1982b) reported the maximum DOT for maximum q_{pen} at 30% while an artificially cycling of DOT above and below this value resulted in a considerable decrease in q_{pen} probably due to the metabolic disturbance.

Working on cephalosporin and capreomycin synthesis by *Cephalosporium* species, Feren and Squires (1969) demonstrated that the critical dissolved oxygen concentration (C_{crit}) for the cephalosporin-producing strain was between 0 and 7% saturation and that for the capreomycin-producing strain was between 13 and 23%. However, the dissolved oxygen levels below which inhibition of antibiotic synthesis occurred were 10 to 20% for cephalosporin and 8% for capreomycin. Similar results were also reported by Vardar and Lilly (1982b) on the penicillin production. The critical dissolved oxygen concentration for the penicillin producing strain was 7% whereas the C_{crit} for penicillin production was 30%. Thus, in the case of cephalosporin and penicillin production the dissolved oxygen concentration should be considerably higher than C_{crit} to ensure optimum production, and for capreomycin production it should be lower than C_{crit}. The increase in cephalosporin productivity with raising dissolved oxygen tension have also been confirmed by other workers (Maksimova *et al.*, 1985; Smith, 1985; Hilgendorf *et al.*, 1987).

The elevation of dissolved oxygen levels during the rapid growth phase of *Streptomyces clavuligerus* in complex medium led to a marked increase in specific cephamycin C production but did not significantly alter the specific growth rate of the culture (Rollins *et al.*, 1988a). Controlling the DOT at either 50% or 100% throughout the fermentation raised the final antibiotic levels two- and three-fold of those found under uncontrolled conditions respectively. In a further study (Rollins *et al.*, 1990), the specific activities of two enzymes in the antibiotic biosynthetic pathway, deacetoxycephalosporin C synthase (DAOCS) and isopenicillin N synthase (IPNS), were found to increase 2.3- and 1.3-fold, respectively, when the DOT was controlled at 100% saturation throughout the fermentations. Yegneswaran *et al.* (1991a) found a 2.4-fold increase in the final cephamycin C yield when the DOT was controlled at 100% saturation only during the

growth phase when the biosynthetic enzymes were probably synthesized, compared to experiments without DOT control. However, the detrimental effect of maintaining dissolved oxygen at saturation levels have been observed with this strain when grown in a chemically defined (glycerol-asparagine) medium (Rollins *et al.*, 1989). The marked reduction of specific cephamycin C levels was noted under these conditions. The authors suggested that the composition of the fermentation medium itself may regulate the effect of oxygen on antibiotic biosynthesis.

Recently the effect of aeration on the production of the peptide antibiotic gramicidin S (GS) by *Bacillus brevis* has been studied in a batch fermentation (Vandamme *et al.*, 1981). These workers observed that the optimum conditions for antibiotic production were correlated with low DOT levels during growth, and since high oxygen concentrations are known to accelerate the inactivation of GS synthetase (Friebel and Demain, 1977; Agathos and Demain, 1986), the low DOT levels probably improved the efficiency of antibiotic production through an increase in GS synthetase stability. Koupletskaya (1965) and Udalova *et al.* (1972) have also reported that there existed a certain narrow range of aeration appropriate for gramicidin S biosynthesis. Below and above this range antibiotic production undergoes a drastic reduction. The influence of medium composition on the sensitivity of the antibiotic biosynthetic pathway to the level of dissolved oxygen in the culture was also stated (Vandamme *et al.*, 1981; Koupletskaya, 1965; Udalova *et al.*, 1972). A much lower aeration rate was required to produce high specific gramicidin S levels in a chemically defined medium, compared to fermentations in a complex medium. The temperature effect of dissolved oxygen level in fermentation broth was investigated by Udalova *et al.* (1986). At the lower temperature and under oxygen limiting conditions of the submerged culture, the biomass synthesis and cell respiration was low but the solubility of oxygen in the liquid was increased which resulted in the longer period of antibiotic production thereby a 30 - 40% increase in the gramicidin S yield was observed. The effect of DOT levels in submerged culture on the tyrothricin production was studied in a scaled culture flask (Seddon and Fynn, 1973; Fynn and Davison, 1976). Exponential growth on chemically defined (glycerol asparagine) media was diphasic, the onset of a slower growth rate and

of tyrothricin production occurred when the DOT values of the culture approached zero. In the contrary, the antibiotic production was suppressed in the continuously aerated culture in which the organisms grew rapidly. However not all peptide antibiotic fermentations are improved by maintaining DOT at relatively low levels. A fourfold increase in the bacitracin production rate by *Bacillus licheniformis* was achieved by raising the dissolved oxygen tension from 0.01 to 0.05 atm (≈ 5 to 24% air saturation) using oxygen-enriched air (Flickinger and Perlman, 1979). The effect of the increased oxygen supply was to reduce the carbohydrate uptake and the maximum specific growth rate, thus simulating an analogous situation to that which would occur if the carbon source were fed slowly. No effect of DOT levels on the ratio of bacitracins A and B was observed in these experiments.

The effects of dissolved oxygen on tetracycline antibiotic production were investigated by many workers. Orlova and Verkhovtseva (1957) found that *Streptomyces aureofaciens* required a more intensive aeration than *S. rimosus*. However, all tetracycline producers were reported to be very sensitive to reduction in adequate air supply (Goodman, 1985). The short interruptions in aeration of a submerged culture of *S. aureofaciens* resulted in the decrease of chlortetracycline biosynthesis (Matelová *et al.*, 1955). This effect was more pronounced between 6 and 12 h of cultivation, during which a single ten-minute pause resulted in a 50% yield reduction while six ten-minute pauses resulted in a greater than 75% yield reduction (Hošťálek, 1964). At low levels of aeration Cherkasova *et al.* (1978) found an increase in the pyruvic and acetic acid in the medium and a decrease in tetracycline production. The medium composition has been reported to affect the dissolved oxygen requirements of *S. rimosus* (Oblozhko and Orlova, 1975, 1977; Oblozhko *et al.*, 1977). On a rich complex medium, biosynthesis of the antibiotic was most intensive at an oxygen-solution rate in the fermentation medium equal to 14 - 25 mg/L.min (≈ 26 - 47 mmol/L.h). A drop in this rate led to retardation of growth and nutrient consumption, to changes in mycelial micromorphology, and to lower oxytetracycline biosynthesis accompanied by accumulation of pyruvate and acetate. The dependence on aeration was less pronounced during cultivation on synthetic media. However, there is general agreement that critical

dissolved oxygen levels do exist. Excessive oxygen has also been reported to result in decreased chlortetracycline yields due to a destruction of cellular metabolites (Plakunova and Kiseleva, 1965; Plakunova, 1966).

Brinberg (1959) found that production of erythromycin in a nutritionally weaker medium was unaffected by the aeration rate, while in an enriched medium it was enhanced by an increased air supply. The author deduced that in the first case the aeration was adequate for metabolism of the nutrients present, while in the presence of additional nutrients the air supply was limiting. Thus an increase in aeration was effective in raising the amount of erythromycin produced. These results were attributed either to a general effect of increasing cell mass, or to a specific action in supplying a greater amount of a specific intermediate.

Studies on the effects of aeration and agitation in leucomycin fermentation with two different scales of fermenters, i.e. 15 L and 2000 L fermenters, Kodama *et al.* (1968) observed good correlations both for the optimum oxygen transfer rate, i.e. 1 kg mol $O_2/h.m^3$ ($\simeq$ 1000 mmol/L.h) and for the optimum power requirement per unit volume of liquid, i.e. 50 $kg.m/s.m^3$ ($\simeq$ 0.50 W/L) which were required for maximum antibiotic yield. However, a shift to higher air flow rate was also observed in greater liquid depth of a fermenter.

The effect of aeration and agitation as well as the influence of the mechanical and geometrical characteristics of the fermenter design on rifamycin production was the subject of a detailed study by Virgilio *et al.* (1964). Their results, summarized by Sensi and Thiemann (1967), were as follows. There was a linear correlation between rifamycin production and turbine diameter within the range of absorbed mechanical power (expressed in watts per litre of broth) between 1.25 - 2.75 W/L, i.e. the smaller the impeller diameter the higher were the antibiotic yields. Below 1.25 W/L and above 2.75 W/L the rifamycin production was independent of the power absorbed and the turbine size used. It was also shown that the higher antibiotic yields occurred when the oxygen demand of the culture was satisfied and an excess of dissolved oxygen was still present

during a critical period of the fermentation between 50 and 80 h. To meet this requirement a power input of about 3.0 W/L and an air flow ranging from 0.8 to 1.5 vvm were found to be necessary.

The polyene macrolide antibiotic fermentations are well established as strongly aerobic processes (Martin, 1979). Studies have been carried out in shake flasks and fermenters. In flasks the requirement of baffles has been reported for optimal production of candidin (McDaniel and Bailey, 1969). For maximum candicidin synthesis, the oxygen transfer rate has to be above 0.8 mmol/L.min (≃ 48 mmol/L.h) (Liu *et al.*, 1975) determined by sulphite oxidation rates. In fermenters, candicidin production decreased at a dissolved oxygen tension below 20% (Martin, 1979). At 40% saturation and above, the yields were constant. The optimal conditions of amphotericin B biosynthesis have been observed at the oxygen transfer rate of 40 to 110 mg/L.min (≃ 75 to 206 mmol/L.h) and an agitation rate of 450 to 800 rpm (Vekshin and Malkov, 1989). Below and above this range the inhibitory effects were observed. An agitator speed of 300 rpm has been reported in the fermentation of nystatin (Lopatnev *et al.*, 1973). A minimal agitation rate of 300 rpm has to be used to prevent the dissolved oxygen content from falling below critical levels in the candidin and candihexin fermentations (Martin and McDaniel, 1974) whereas an agitation rate of 400 to 420 rpm has been used to maintain dissolved oxygen tension above 50% saturation in the candicidin fermentation (Martin and McDaniel, 1975). Maximal oxygen uptake rates were about 0.7 mmol/L.min (≃ 42 mmol/L.h) in the candidin and candihexin fermentations, using a soybean meal medium, and 0.5 mmol/L.min (≃ 30 mmol/L.h) in the candicidin production using a soya peptone medium. This strong requirement for oxygen occurred only during the growth phase and the transition phase, decreasing during the antibiotic production phase.

The production of streptomycin has been observed to be a highly aerobic fermentation process in which the value of oxygen uptake rate might reach 120 µL/h.mL of culture (≃ 5 mmol/L.h) (Hockenhull, 1963). The titre of streptomycin increased with increasing the aeration rate above 2 vvm in deep culture (Kempf and Sayles, 1946). Bartholomew *et al.* (1950b) demonstrated that the production of streptomycin increased with

increasing power expenditure for agitation until a limiting value was reached when it remained constant. At very much higher impeller speeds, productivity declined. A similar pattern was obtained on increasing airflow, though the position of the optimal range was also dependent upon agitation.

Flickinger and Perlman (1980) showed an increase of 2- to 3-fold in neomycin production rate by *Streptomyces fradiae* as a result of maintaining the DOT at a level of 0.05 atm (≈ 24% air saturation) with oxygen enrichment of the aeration during the period of maximum oxygen demand.

In kanamycin fermentation by *S. kanamyceticus*, Brinberg *et al.* (1970) found that the antibiotic formation was unaffected by increasing the air flow rate from 1 to 3 vvm under strong agitation while it slightly increased under low levels of agitation. However, a decrease in the degree of agitation markedly reduced the antibiotic production.

With shake flask cultivation, Sukharevich *et al.* (1970) studied the influence of dissolved oxygen which was determined by the redox potential (E_h) of the medium on the production ratio of antibiotic levorins A and B by *Streptomyces levoris*. The authors found that an increase in the redox potential by increasing the effectiveness of aeration (changing the volume of medium) resulted in an increase in the fraction of levorin A in the antibiotic complex, whereas when the redox potential was lowered the fraction of levorin A decreased. Moreover, the total amount of antibiotic produced has been found to be much higher at the high value of the redox potential than those produced at the lower E_h of the medium.

Boeck *et al.* (1980) reported that increasing the oxygen level from 10 to 80 % saturation gave a 12-fold higher production of A40104A, a pleuromutilin glycoside antibiotic and about 6-fold increase in the ratio of A40104A to pleuromutilin. Aeration dependence was also evident in the carminomycin (Kochetkova *et al.*, 1976) and actaplanin (Boeck and Stark, 1984) fermentations where both subcritical and supraoptimal levels of dissolved oxygen inhibited the biosynthesis of the antibiotics.

1.3 Influence of Dissolved Carbon Dioxide on Biosynthesis of Microbial Metabolites

Carbon dioxide is one of the principal metabolic end products of microorganisms as well as a raw material for its fixation in aerobic fermentation (Hirose, 1986; Ho *et al.*, 1987). In a culture system, carbon dioxide excreted by cells is in the following four forms in equilibrium : carbon dioxide in gas- and liquid-phase, bicarbonate and carbonate ions. Therefore the concentration of these forms can be calculated by measuring gas-phase carbon dioxide. The presence of carbonate ions, one-thousandth of bicarbonate ions in concentration, is negligible (Ishizaki *et al.*, 1971a). Moreover, the partial pressure of carbon dioxide in the liquid phase is practically the same as that in the gas phase of a fermenter (Ishizaki *et al.*, 1971b).

Carbon dioxide may influence a submerged fermentation process in various manners. For instance, carbon dioxide and its ionic form might affect pH, neutralization, buffer capacity and acid-base equilibria of culture system (Ishizaki *et al.*, 1973b). Its physiological effects are therefore very complicated and its regulatory role is perhaps less clear than that of oxygen in microbial metabolism. The behavior of microorganisms in the presence of carbon dioxide in aerobic fermentation depends on the strains employed, the composition of the medium and the nature of the products (Jones and Greenfield, 1982; Hirose, 1986). For example, it has been observed that the inhibitory effect of carbon dioxide is much more pronounced in penicillin fermentation (Nyiri and Lengyel, 1965; Pirt and Mancini, 1975) than in glutamic acid (Hirose *et al.*, 1968) and inosine fermentations (Shibai *et al.*, 1973).

A few studies have been made on the effect of carbon dioxide in the fermentation processes (Gandhi and Kjaergaard, 1975). Nevertheless, the importance of carbon dioxide in microbial fermentations has been widely recognized, and the detrimental effects of elevated levels of carbon dioxide on growth and metabolism has been discussed in recent review articles (Jones and Greenfield, 1982; Ho *et al.*, 1987; Onken and Liefke, 1989).

1.3.1 Effect of dissolved carbon dioxide on antibiotic production

Carbon dioxide is released during the catabolism of various organic substrates under aerobic conditions. Antibiotics are commonly produced in highly aerated submerged culture on complex organic substrates. Such conditions encourage formation of metabolic carbon dioxide which may influence the production of the antibiotic. There exists few reports on the effects of carbon dioxide on antibiotic production (Table 1.4).

Initial investigation on penicillin production in submerged cultures, Stefaniak *et al.* (1946b) found that when 2 - 3 times the usual amount of carbon dioxide was added to the air in the fermenter there was no change in penicillin yield. Similarly, Foster (1949) reported that some carbon dioxide in the atmosphere was essential for penicillin production and that excess (> 1.0%) had an inhibitory effect. However, the dissolved oxygen level of the culture was not mentioned in these investigations. The experiments of Nyiri and Lengyel (1965) and Lengyel and Nyiri (1966) pointed out that a rise of carbon dioxide up to 4% or more in the effluent gas, caused by a reduction in an air flow rate through an industrial fermenter, adversely influenced the respiration and sugar uptake rate and penicillin biosynthesis, even though the dissolved oxygen concentration was far above its critical value (0.022 mol/m^3) (≈ 9% air saturation). Pirt and Mancini (1975) also showed that carbon dioxide strongly inhibited penicillin production. Carbon dioxide at a partial pressure of 0.08 atm (≈ 8%) in the effluent gas caused a 50% decrease in penicillin production rate in chemostat culture. However, no inhibition was observed at 0.006 atm (≈ 0.6%). Similar results have been reported in batch fermentations by Ho and Smith (1986). These authors found that penicillin production rate was reduced by 50% at 12.6% carbon dioxide in the influent gas stream and dropped to nearly nil at 20% carbon dioxide. Upon exposure to influent gases of 3 and 5% carbon dioxide, no pronounced inhibitory effects were noted.

Production of tetracyclines is also affected by the concentration of the carbon dioxide in the fermentation broth which is, in turn, determined by the respiration intensity of the culture and aeration and agitation conditions, i.e. by the rate of disappearance of carbon

Table 1.4 : Reports of effects of dissolved CO_2 on antibiotic production.

Antibiotic	Producing organism	References
Penicillin	*Penicillium chrysogenum*	Stefaniak *et al.*, 1946b
		Foster, 1949
		Nyiri and Lengyel, 1965
		Lengyel and Nyiri, 1966
		Pirt and Mancini, 1975
		Ho and Smith, 1986
Tetracycline	*Streptomyces aureofaciens*	Nikitina *et al.*, 1974
		Sherstobitova *et al.*, 1976
		Chagin and Biryukov, 1980
Erythromycin	*Streptomyces erythreus*	Nash, 1974
Streptomycin	*Streptomyces griseus*	Bylinkina and Birukov, 1972
		Bylinkina *et al.*, 1973
		Nikitina and Cherkasova, 1974

dioxide from the system. The range of the optimum carbon dioxide concentration found with *S. aureofaciens* was 2 to 8 mL carbon dioxide per 100 mL fermentation medium (Sherstobitova *et al.*, 1976). A level greater than 15 mL/100 mL decreased the rate of respiration of various tetracycline producers by 40 - 50% (Bylinkina and Birukov, 1972; Bylinkina *et al.*, 1973; Nikitina *et al.*, 1974). Chagin and Biryukov (1980) found that changes in carbon dioxide concentration paralleled changes in tetracycline production by *S. aureofaciens*. Slowing the agitation rate, which increased carbon dioxide concentration in the culture broth, increased the antibiotic production rate by 25 - 30%.

The possible effects of carbon dioxide on erythromycin synthesis have been investigated by adding 11% carbon dioxide into the inlet air. The supplement of carbon dioxide did not affect pH and growth, but inhibited synthesis of erythromycin

by 40%. The latter effect might result from feed-back inhibition of methylmalonic acid formation (Nash, 1974).

In the course of streptomycin fermentation, the concentration of dissolved carbon dioxide exhibited a certain effect upon the respiration and antibiotic production. It has been demonstrated that the detrimental effect of increased dissolved carbon dioxide concentration was especially pronounced during the growth and development period, i.e. the first 40 h of cultivation, resulted in the inhibition of respiration and a marked decrease in the final antibiotic production (Bylinkina *et al.*, 1973). In shake flasks, Bylinkina and Birukov (1972) found inhibition in streptomycin synthesis at levels greater than 8%. At 14% carbon dioxide, the antibiotic synthesis was inhibited 50%.

In conclusion, it can be stated that aeration is necessary to a biosynthetic process such as antibiotic fermentation in two important ways : to maintain the dissolved oxygen level above the critical value and to assure ventilation by which the carbon dioxide level may be kept below its critical inhibitory value (Nyiri and Lengyel, 1965).

1.3.2 Influence of dissolved carbon dioxide on biosynthesis of amino acids and other metabolites

The effect of carbon dioxide tension on amino acids and other metabolites fermentation has been studied by many investigators (Table 1.5). Hirose *et al.* (1968) found only a slightly inhibitory effect of increasing carbon dioxide level in a glutamic acid fermentation under controlled pressure of dissolved oxygen at satisfactory level (0.21 atm) (≃ 100% air saturation). This suggests strongly that carbon dioxide is far less influential in L-glutamic acid fermentation than oxygen. Akashi *et al.* (1979a) reported that carbon dioxide inhibited L-histidine accumulation. On the other hand, L-arginine fermentation had the optimum level of carbon dioxide tension around 0.12 atm (≃ 12%) for maximum productivity.

Table 1.5 : Reports of effects of CO_2 on biosynthesis of amino acids and other metabolites.

Product	Producing organism	References
L-Glutamic acid	*Brevibacterium lactofermentum*	Hirose *et al.*, 1968
		Hirose, 1972
L-Arginine and	*Brevibacterium flavum*	Akashi *et al.*, 1979a
L-Histidine		
Inosine	*Bacillus subtilis*	Shibai *et al.*, 1973
		Ishizaki *et al.*, 1973a, 1973b
Itaconic acid	*Aspergillus terreus*	Nakamura and Kobayashi, 1969
L-Malate	*Schizophyllum commune*	Tachibana and Murakami, 1974
Fumarate	*Rhizopus nigricans*	Foster and Davis, 1949
α-Amylase	*Bacillus subtilis*	Zajic and Liu, 1969
		Gandhi and Kjaergaard, 1975
		Ishizaki *et al.*, 1986

In inosine fermentation, a low rate of air flow resulted in the inhibition of the product formation even if the oxygen demand of the cell was satisfied (Shibai *et al.*, 1973). This was due to the inhibitory effect of carbon dioxide accumulated in the culture system. Therefore, the problems of agitation and aeration in inosine fermentation could be discussed from the following standpoints : first, there should be sufficient oxygen supply to satisfy the oxygen demand of the cell, and the second, there should be adequate ventilation to maintain the partial pressure of carbon dioxide below the inhibitory critical level, i.e. 0.05 atm ($\simeq$ 5%) (cf Ishizaki *et al.*, 1973b). In addition, Ishizaki *et al.* (1973b) proposed the chemical absorbtion method to eliminate carbon dioxide in gas phase in the culture system which resulted in an improvement of product formation. Although carbon dioxide may be dissolved in various ionic forms, depending on pH, inosine yields were reported to be inhibited

by carbon dioxide concentrations in equilibrium with the gas phase and not by high bicarbonate ion levels (Ishizaki *et al.*, 1973a).

Nakamura and kobayashi (1969) pointed out that the specific production rate of itaconic acid was maximized in the ranges of carbon dioxide pressure from 0.018 to 0.20 atm (≃ 1.8 to 20%) and an excess aeration resulted in marked decrease in product yield, was not due to a high oxygen pressure in the fermenting broth, but due to a low carbon dioxide pressure.

The positive effects of carbon dioxide were also observed in the fermentative production of L-malate from ethanol by *Schizophyllum commune* (Tachibana and Murakami, 1974). The production of L-malate was increased by 30% using carbon dioxide gas bubbled into the $CaCO_3$-free medium.

In anaerobic production of fumaric acid from glucose by *Rhizopus nigricans*, on the other hand, high carbon dioxide tension exhibited suppression on product formation. However, this inhibition was reversible and the production could be restored when the carbon dioxide was removed (Foster and Davis, 1949).

Finally, the influence of carbon dioxide on the production of α-amylase by *Bacillus subtilis* was also studied by a number of investigators. Zajic and Liu (1969) observed that 2.5 - 13% carbon dioxide inhibited the α-amylase production during the first 48 h, but 4 - 5 days later a stimulating effect was observed with highest yield at 9% carbon dioxide. Gandhi and Kjaergaard (1975) found that the maximum enzyme production was obtained using 6% carbon dioxide in batch cultures and 8% carbon dioxide in chemostat cultures. The fact that carbon dioxide may influence the pH of the culture system was also investigated by Ishizaki *et al.* (1986) who found that amylase production and cell mass activity significantly increased under low aeration conditions in uncontrolled pH cultures. This was due to the high carbon dioxide accumulated in the culture system under low ventilation which, in turn, resulted in a decrease of the pH of the culture liquid to a favourable level for enzyme synthesis.

1.4 Methodology of Controlling DOT in Fermentations

The interest in controlling the dissolved oxygen tension (DOT) lies in the fact that both growth rate and metabolism of microorganisms change at different oxygen tension (Brookes, 1969). Therefore, measurement and control of DOT in stirred tank fermenters is of paramount importance for all fermentation processes. There exist numerous DOT control strategies cited in the literature which can be classified into 3 main categories as described below.

1.4.1 Constant DOT throughout the fermentation periods

Maintaining the dissolved oxygen tension (DOT) in a fermenter at a specific value has been found to be useful in evaluating the influence of dissolved oxygen on the metabolism or product formation by growing microbial systems (Brookes, 1969; Fuchs and Wang, 1974) and in scaling up and designing any aerobic fermentation system (Hubbard *et al.*, 1988). Several aspects of fermenter design and operation have been shown to affect the dissolved oxygen level in the culture broth. Because of these various parameters, it is possible to design control systems based on a selection of different manipulated variables. The various techniques which have been employed for the measurement and control of DOT have been reviewed and evaluated by several authors (Brookes, 1969; Brown, 1970; Vincent, 1974). A summary of some control system installations are listed in Table 1.6. In all control systems the value of the parameter (i.e. in most case, DOT) is measured by a sensor and then compared with a set position; any deviation is used through amplifiers and converters to drive some unit which will maintain the desired value of DOT. However, each particular control system has its inherent advantages and disadvantages which have to be considered to achieve a satisfactory application.

Table 1.6 : Dissolved oxygen control systems.

Controlled variable	Opera--tion scale† (litres)	Controlled system	References
Stirrer speed	1	Closed loop servo control of D.C. motor/generator	Herbert *et al.*, 1965
	8	Electropneumatic transducer to piston actuator to Zero-max continuously variable gear	Moss and Bush, 1967
	2	Proportional-integral (PI) controller on the D.C. motor speed	Clark *et al.*, 1985
	14	Discrete signum integral controller on rotation speed	Hilgendorf *et al.*, 1987
	2	Continuous regulation of the agitation speed by electronic DOT controller	Du Preez and Hugo, 1989
Air flow rate	8	Proportional valve on air inlet	Lengyel and Nyiri, 1965
	2	On-off valve on air inlet	Clark *et al.*, 1982
	§	Adaptive control of air flow rate	Lubentsov *et al.*, 1987
Gas composition	7	Mixing valve supplied with oxygen and nitrogen	Siegell and Gaden, 1962
	2	Nitrogen steady; electropneumatic converter to proportional valve on air	MacLennan and Pirt, 1966 Rowley, 1970
	14	Timer operated solenoid valves supplied with either oxygen or nitrogen	Hughes and Wimpenny, 1969
	5	On-off electromagnetic valve on oxygen inlet	Kilburn and Webb, 1968
	2	Microswitch activated solenoid valve on O_2 inlet; recirculation of head space gas	Fuchs and Wang, 1974
	7.5	Electropneumetic transducer to proportional valve on air; mass flow transducer on sparge line to control valve on N_2 + CO_2 inlet line	Chen *et al.*, 1985

Table 1.6 - *Continued*

Controlled variable	Opera-tion scale† (litres)	Controlled system	References
Gas composition	2	Computer controlled on-off valves on pure O_2, pure N_2 and a mixture of 20% CO_2 in N_2	Smith, J.M., *et al.*, 1990
Stirrer speed and aeration rate	1‡	Synchronous motor controlled stirrer speed, servomotor driven control valve on air inlet, manual operation on oxygen feed rate	Yano *et al.*, 1979
	1‡	Motor speed controller and air and oxygen mass flow controllers interfaced with microcomputer	Kobayashi *et al.*, 1980 Yano *et al.*, 1981
	§	Computerized DOT control through an interactive regulation of agitation speed and air flow rate	Nyiri *et al.*, 1974
	§	Cascaded control of DOT upon air flow rate and agitator speed of a computer-coupled fermenter	Mohler *et al.*, 1979
	2	CO_2 analyzer to proportional-differential (PD) controller equipped with microcomputer	Suzuki *et al.*, 1986
Geometry	5	Nitrogen steady; air steady through inlet moved with respect to impeller by rack and pinion	Flynn and Lilly, 1967
Nutrient feed	3.5	On-off valve on nutrient feed line	Hospodka, 1966
	2300	Proportional valve on nutrient feed line	Squires, 1972
	10	On-off action of nutrient feed pump	Rollins *et al.*, 1988b
	2	On-off action of inlet nutrient pump	De la Broise and Durand, 1989

† Total volume of fermenters unless otherwise stated.

‡ Working volume of fermenters.

§ No information.

Attempts to control dissolved oxygen levels by varying agitator speed have been reported by many workers. The most effective method amongst various existing agitator speed control systems is the control of motor speed (Vincent, 1974). Herbert *et al.* (1965) and Moss and Bush (1967) briefly described an apparatus for automatic controlling the dissolved oxygen tension in continuous microbial cultures by regulation of stirrer speed. These authors stated that reasonably good control was achieved over a wide range of dissolved oxygen concentrations, but the slow response of oxygen electrodes limited precision. More recently, the control technique based on proportional-integral (PI) control of fermenter agitation speed has been successfully employed for the control of DOT in both batch and continuous fungal fermentation (Clark *et al.*, 1985), whereas, the discrete signum integral controller has been quoted for the best control action in a batch cephalosporin C fermentation (Hilgendorf *et al.*, 1987). With these control techniques, controlling of DOT within ±2% of the saturation scale has been reported. Du Preez and Hugo (1989) designed the electronic DOT controller by means of a continuous speed control of the D.C. motor driving the fermenter agitator. The electronic circuit elements of the control system have been described in detial. The DOT control system by stirring method has its advantage that only one gas, usually air, is required (MacLennan and Pirt, 1966). The disadvantages, however, are (1) it could not be applied to large scale equipment because of some technical and economical problems (Siegell and Gaden, 1962; MacLennan and Pirt, 1966; Brown, 1970) and (2) it might cause significant changes in the mixing characteristics of the culture vessel (MacLennan and Pirt, 1966; Flynn and Lilly, 1967).

The DOT control systems by altering the air flow rate to the culture have been mentioned in the works of Terui *et al.* (1960) and Clark *et al.* (1982); however, no detail of the control equipment and none of the performance is given. Lengyel and Nyiri (1965) described an automatic DOT control system using the redox potential (E_h), though the real relation between DOT and E_h is unknown, to control the air inlet so that for a too-low E_h value the inlet rate of air was increased. No experimental results were presented, however, about the calibration of the electrode,

or the accuracy and stability of the system. The article published by Lubentsov *et al.* (1987) discussed in detail about the dynamic response of an adaptive control system by means of regulation of air flow rate in antibiotic fermentation processes. Again the experimental data have not been reported. Variation in air flow as a means for controlling DOT frequently results in (1) excess foaming (Siegell and Gaden, 1962; Vincent, 1974; Fuchs and Wang, 1974), (2) loading of compressors (Siegell and Gaden, 1962), (3) complicating the pH controlling system due to different degrees of carbon dioxide removal (Flynn and Lilly, 1967; Fuchs and Wang, 1974), and (4) difficulty in the interpretation of the effluent gas concentrations (Flynn and Lilly, 1967; Rowley, 1970).

Control by variation of the inlet gas composition, and hence the partial pressure of oxygen in the gas mixture, can be achieved in various ways. Siegell and Gaden (1962) described an apparatus in which oxygen and nitrogen are fed to a mixing valve capable of varying the proportions of the two gases while maintaining a constant total gas flow rate. By including oxygen analysers before and after the fermenter, oxygen absorption and uptake rates can be determined. MacLennan and Pirt (1966) and Rowley (1970) developed the control system which is very similar to that of Siegell and Gaden in operating principle, the main difference being that the proportionally varying air stream is directed into a constant flow stream of nitrogen gas. Modification of these systems to include timing devices to allow for more adequate gas mixing have been presented by Hughes and Wimpenny (1969). Kilburn and Webb (1968) described a simple on-off control system which regulated DOT by periodically supplying oxygen to a fermenter operated as a closed system, i.e. no effluent gas. Consequently, the pressure in the culture vessel rose during the course of a batch fermentation. Fuchs and Wang (1974) also described the automatic control equipment using oxygen enriched air in a semiclosed loop fermentation system, where most of the air sparged through the fermenter was recycled. Chen *et al.* (1985) designed a system for control of low dissolved oxygen concentrations, used for the determination of critical oxygen concentrations of some microorganisms. Their system is similar to that of Siegell and Gaden, but instead of using pure nitrogen and

oxygen these authors used nitrogen with 1% carbon dioxide and air as components of gas mixtures. More recently, J.M. Smith *et al.* (1990) developed a control system for simultaneous control of the dissolved concentrations of both oxygen and carbon dioxide by altering the inlet gas composition, while maintaining a constant agitation rate and a total inlet gas flow rate. Although the use of variation of gas compositions as a control variable allows very convenient techniques for DOT control on a laboratory scale, it might not be a feasible approach for industrial processes, where the economics of obtaining or manufacturing large quantities of the separated gases would be prohibitive (Brown, 1970; Vincent, 1974). However, these problems might be overcome by using enclosed fermentation systems as described previously by Kilburn and Webb (1968) and Fuchs and Wang (1974), in which the high efficient utilization of gases can be achieved.

Amongst various control techniques, the variation of agitation speed in combination with aeration rate seems to be the most popular, with several recent publications describing its application (Clark *et al.*, 1985). There is, however, little information regarding the technical details of achieving this type of control. Yano *et al.* (1979) developed an apparatus called 'DO-stat' for controlling DOT during fed-batch cultivation. These workers have found that the better controllability of this system could be achieved by simultaneously changing agitation speed and aeration rate rather than by alternately changing of these two control variables. However, this system required manual operations to change the preset values for maximum and minimum agitation speeds and aeration rates during cultivation. To solve this problem, a microcomputer has been introduced for DOT control system by the same group of workers (Kobayashi *et al.*, 1980; Yano *et al.*, 1981). With this control system, an agitation speed and air flow rate were automatically varied according to instructions stored in the microcomputer. Another advantage is that the calculation of the optimum agitation speed and aeration rate based on minimum operating cost was possible while keeping the DOT constant. Nyiri *et al.* (1974) and Mohler *et al.* (1979) have also described computerized DOT control systems without any detail information on the hardware performance. The system designed by Mohler *et al.*

(1979) using cascaded control upon air flow rate and agitator speed was claimed to be more stable than a conventional feedback control introduced by Nyiri *et al.* (1974). Suzuki *et al.* (1986) developed an automatic DOT control system in which agitation speed and oxygen partial pressure of the inlet gas were changed stepwise in accordance with carbon dioxide concentration of the exhaust gas. With this system, the satisfactory performance has been quoted whether agitation speed and oxygen partial pressure were changed simultaneously or independently.

Flynn and Lilly (1967) described a simple control system in which gas of constant composition and flow rate entered the fermenter through an automatically raising or lowering sparge pipe. The variable entry point of the gas into the vessel resulted in a correspondingly variable gas dispersion and gas-liquid contact time. As the sparge pipe descended into the culture the rate of oxygen solution increased and conversely as the pipe was raised so less oxygen dissolved. The details of operation and performance of this control unit have also been discussed by the authors.

Another interesting method of control is by variation of the feed rate of the growth-limiting substrate, and thereby the oxygen uptake rate of the culture, as described by Hospodka, (1966). This system consisted of an on-off solenoid valve actuated by the signal from the dissolved oxygen electrode to control the nutrient feed rate into the fermenter. In this way the assimilation of the growth-limiting nutrient (usually an energy source) is controlled to match the oxygen transfer characteristics of the vessel. Hospodka (1966) used this system to match the rate of molasses feed in a yeast production system to the rate of oxygen supply. He claimed lower ethanol formation and better conversion of substrate into yeast dry matter as well as maximal utilization of the aeration system capacity of the fermenter. Similar control systems have also been described by Squires (1972) in a penicillin fermentation using the glucose feed rate as a control variable and, more recently, by Rollins *et al.* (1988b) who used the maltose feed rate for controlling DOT in a batch fermentation of cephamycin C. With some modifications of this type of control system, De la Broise and Durand (1989) developed a dissolved oxygen controlled turbidostat for studying

oxygen effects on the growth rate of a fungus, *Fusarium oxysporum.* In general, the feedback control system based on this principle is attractive from the practical point of view but there are some situations where it is not clear to be the best method (Küenzi and Auden, 1983). The drawbacks of this control system could be summarized as follow : (1) the difficulty in setting up due to the slow response rate of the culture to changing substrate concentration and the tendency of overshoot when substrate is added in discrete doses (Vincent, 1974), (2) the initial substrate concentration is very critical, i.e. if too low it may be difficult to introduce the substrate at the correct control rate, or if too high the DOT will drop below the desired control level (Brookes, 1969), and (3) the failure when apply to the culture, particularly the fungal fermentation, in which microorganisms show pronounced morphological change and hence the viscosity of the broth, i.e. the DOT of the culture is not only depended on the oxygen consumption rate but also the viscosity of the broth (Küenzi and Auden, 1983).

1.4.2 Variation of DOT during the course of fermentation

During the course of a batch fermentation, on which the most traditional industrial processes are based, the properties of the cells and of their environment are continuously changing. Therefore, it is unlikely that maintaining DOT level constant throughout the fermentation will give the best results. Moreover, it would be a waste of energy and could be detrimental to the cells (Küenzi and Auden, 1983). High agitation rates can lead to a prolonged lag phase as has been shown in the rifamycin SV fermentation (Küenzi, 1978), while high aeration rates may prevent a freshly inoculated culture from building up the carbon dioxide level necessary for growth.

As Phillips and Johnson (1961a) pointed out, an optimum level or levels of dissolved oxygen could be specified for various stages of a fermentation. Some stages are more critical than others. The critical periods are between 6 and 12 h in chlortetracycline (Matelová *et al.*, 1955; Hošťálek, 1964), 50 and 80 h in rifamycin (Virgilio *et al.*, 1964) and 18 and 40 h in inosine (Ishizaki *et al.*, 1973b) fermentations, during which

the influence of DOT on the productivity of these products are more pronounced.In an enzyme production, Konno and Terui (1961) established that *Bacillus amylosolvens* demands high oxygen tension in the proliferating phase and much lower levels of oxygen suffice for the production of hydrolases whereas Hanisch *et al.* (1980) and Clark *et al.* (1982) found different DOT optima for induction and expression of the hydroxylation enzymes.

From the examples given above, it might prove advantageous to maintain the dissolved oxygen tension in cultures at economically optimal levels instead of at a constant level resulting from the use of the constant DOT control strategy. However, to find the optimal conditions for any given moment would clearly be too difficult. Moreover, there is little information regarding the technical details of achieving this type of control strategy. Stepwise changes of parameters such as the agitation and aeration rates can be beneficial and at the same time reduce the operation costs.

1.4.3 Cyclic variation of DOT during the course of fermentation

In large scale fermentations, due to inefficient bulk mixing and changes in hydrostatic pressure, microorganisms are exposed to a continually changing environment as they pass through different zones in a fermenter (Vardar, 1983). This may result in a decrease in the yield of biomass or desired metabolites, an increase in the formation of by-products, or loss of viability (Sweere *et al.*, 1988a). However, as has been reviewed recently by Onken and Liefke (1989), it can be safely concluded that cyclic variation of DOT may have positive or negative effects, depending on microbial strain, culturing conditions, range of DOT and frequency of cyclical changes. These effects should be accounted for during scale up, development and optimization of an industrial fermentation process as well as during strain selection programme.

In order to simulate the cyclic variation of DOT taking place in industrial scale

fermenters, a few different experimental installations using a laboratory scale fermenter have been employed. A survey of published data on system installations is given in Table 1.7. There are three possible methods to achieve this kind of simulation : (1) a gas of constant composition, i.e. air, is used for aeration and total pressure is varied, (2) total pressure is kept constant and composition of aerating gas is changed, e.g. by alternately supplying of air or oxygen and nitrogen gas, and (3) culture liquid is circulated through different parts of fermentation system which maintain constant DOT at different levels. The first method seems to be the most realistic in term of simulating the effects of local changes of DOT as well as hydrostatic pressure on microbial cultures in industrial scale fermenters (Onken and Liefke, 1989). However, as pointed out by many workers that neither the total pressure itself nor the pure oxygen gas, in the practical range of fermentation processes, but only the DOT seems to have influences on the microbial systems (Clark and Lentz, 1961; Kataoka *et al.*, 1986; Okoshi *et al.*, 1987; Onken and Liefke, 1989). Hence, the second method can be reasonably used as a simulation model of an imperfect mixing and a pressure fluctuation in a large scale fermenter. In the first two methods all cells are exposed to the same condition. Therefore, these methods seem very appropriate for physiological studies. At large scale, however, the individual cells experience the conditions which depend on the residence time distribution of the cells over the different zones in the fermenter. Consequently, to simulate these conditions at a laboratory scale, the third method should be chosen (Oosterhuis *et al.*, 1985b; Sweere *et al.*, 1988b).

For the first method, i.e. varying total pressure, Vardar and Lilly (1982b), who employed this method on penicillin production, reported excessive foaming of their culture particularly at the venting stage, when they applied sinusoidal oscillations of total pressure with periods of 2 min. Kataoka *et al.* (1986) apparently did not have such difficulties in their investigation of *Pseudomonas aeruginosa* with oscillating pressure in the form of a triangular wave. Both research groups used a system of control valves for generating the pressure cycling and hence DOT cyclic variation, whereas Takamatsu *et al.* (1980) employed a moving piston installed at the top of a

Table 1.7 : System installations for cyclic variation of DOT.

Cyclic variable	Operation scale† (litres)	System	References
Total pressure	6	Moving piston installed at the top of a stirred tank reactor	Takamatsu *et al.*, 1980
	7	Cam drive operated orifice tubes on exit gas valves	Vardar and Lilly, 1982b
	1.3	Electrical pressure control system on gas outlet	Kataoka *et al.*, 1986
Gas composition	5	Electronic timer operated two solenoid valves on oxygen and nitrogen feed lines	Soni and Ghose, 1974
	1‡, 2‡	Electropneumatic device controlled alternately feeding of air and nitrogen gas	Sokolov *et al.*, 1983a
	2, 5	Time switches and valves on the inlet air and nitrogen gas streams	Sweere *et al.*, 1988a
	10	Computer control of the oxygen and nitrogen gas feed rates	Träger *et al.*, 1991
	2	On-off control of the air supply to the fermenter	Yegneswaran *et al.*, 1991b
Geometry	2	Two fermenters, one sparged with air and one with nitrogen gas, with an exchange flow of liquid controlled by a pump and overflow	Sweere *et al.*, 1988b Oosterhuis *et al.*, 1983, 1985b
	20	Two compartments fermenter, one well mixed aerobic part and one minor anaerobic part, with an liquid circulation controlled by pumps	Larsson and Enfors, 1988
	8.5‡	Tubular loop reactor with draught tube as downcomer and annular space as riser, an air injector at the top of downcomer	Katinger, 1976

† Total volume of fermenter unless otherwise stated

‡ Working volume of fermenter

stirred tank reactor in order to simulate a deep-tank process for aerobic waste water treatment. On effects of cycling DOT on microbial processes, Vardar and Lilly (1982b) reported a reduction of approximately 30% in specific penicillin production rate when DOT was cycling between 23% and 37% air saturation with periods of 2 min. Kataoka *et al.* (1986) found a minor decrease in cellular yield and a slightly increase in specific growth rates caused by cyclic variation of DOT between 9 and 64 ppm (≈ 120 and 850% air saturation) at rather short periods of about 1 - 2 min only. For longer cycles cellular yields and specific growth rates were the same as for steady state operation at low DOT of about 7 ppm (≈ 93% air saturation). Similar results have been reported by Takamatsu *et al.* (1980), who obtained slightly decreased cellular yields and distinctly higher specific growth rates by applying cyclic changes of total pressure between 1 and 9 atm (abs.), though with DOT far from saturation.

In the experimental set-up of Gow *et al.* (1975) for process development of single cell protein from methanol, permanent cycling of pressure and of concentrations of oxygen, carbon dioxide and methanol could be established; however, a description of this bioreactor has not been given. Dissolved oxygen tension was oscillated between zero and several upper limits with 665 mbar (≈ 310% air saturation) as the maximum. Above 535 mbar oxygen (≈ 250% air saturation) as upper limit, carbon-to-cell conversion decreased, whereas the specific growth rate was not affected by the oscillation.

For the second method of applying oscillating oxygen pressures by changing the oxygen content of the aerating gas, Soni and Ghose (1974) described a simplified experimental set-up in which the aerating gas was supplied alternately from two gas cylinders containing gases at different oxygen content. They used this arrangement to disturb the steady state in a continuous culture of *Saccharomyces cerevisiae* by introducing periodic square oxygen input waves, whilst keeping other environmental conditions constant. A periodic variation in cell population has been observed when nitrogen was passed through the culture for 2, 4, 5 and 6 min; for 1 and 3 min no

variation appeared. Metabolic oscillations have not been searched by these workers. However, these latter effects have been recently studied in detail by Sweere *et al.* (1988a) who imposed block wave DOT on a continuous culture of baker's yeast (*S. cerevisiae* CBS 8066) by switching the inlet gas between air and nitrogen. With their experimental set-up, Sweere *et al.* (1988a) also showed that the actual DOT wave in culture broth deviated from the block wave created in the inlet oxygen concentration due to mass transfer resistance, gas holdup and electrode response. This effect was more pronounce at high oscillation frequencies. For the effects on baker's yeast production, it has been found that relatively fast fluctuations in DOT with a frequency of 1 or 2 min^{-1} resulted in a reduction of the biomass concentration and an increase in the formation of metabolites (i.e. ethanol, acetic acid and glycerol) and the respiratory activity of the cells, compared to those measured in an oxygen-unlimited culture. However, little effect has been observed on the cell composition.

Sokolov *et al.* (1983a) developed a control system, without giving a detail description and its performance, using an electropneumatic device to control stepwise changes of DOT, pH, temperature and agitator speed in minute time intervals. With this control system, Sokolov *et al.* (1983b) investigated the effect of cyclic changes in DOT and pH on three yeasts (*C. utilis* on ethanol, *S. cerevisiae* and *C. scottii* on glucose). Dissolved oxygen was cycling between 70% and 0% saturation by alternately aerated with air and nitrogen for periods ranging from 0.2 to 12 min. In general, the anaerobic period (gassing with nitrogen) effected growth negatively with increasing length; in one case (*S. cerevisiae*) short anaerobic periods (2 min) had no effect. In a subsequent study Lirova *et al.* (1986) tried to obtain information on the morphological state of cells in chemostat cultures of *C. utilis*, when oxygen supply and pH were varied periodically, and to correlate these cyclic changes of operating parameters with morphological changes of the cells; but their findings did not yield detailed conclusions on mechanisms of growth behaviour.

Vasey *et al.* (1972) cultivated *Pseudomonas methylotropha* ASI on methanol in a continuous culture where the DOT was oscillated between 0 - 500 mm Hg (≈ 0 -

310% air saturation) by changing aeration between nitrogen and oxygen with a period of 3 min. They reported decreased carbon to cell conversion efficiency and in extreme cases decreased growth rates.

In the recent reports, Träger *et al.* (1991) developed a cycling device to cycle DOT in a laboratory airlift fermenter using a computer control (based on a PID algorithm) of the oxygen and nitrogen gas feed rates to vary the oxygen content in the inlet gas stream. The authors demonstrated a successful application in batch fermentations of gluconic acid by *Aspergillus niger*. The DOT cycling was performed between 60 and 140% air saturation and between 10 and 60% air saturation with a cycling period of 90 s in both cases. The effect of cycling DOT on the performance of these fermentations, however, has not yet been published. In order to simulate DOT fluctuations in large scale fermenters, Yegneswaran *et al.* (1991b) proposed two methods of cycling DOT achieved by on-off cycling of air supply to a 2 L fermenter: (1) a lognormal distribution of air supply (Monte Carlo method) consisted of air supply for 5 s followed by no aeration which ranged from 8 to 44 s, with a mean time of 20 s. (2) periodic cycling of air supply with the air was turned on for 5 s and turned off for 20 s. A suppression of the biosynthesis of cephamycin C and its precursor, penicillin N, by *Streptomyces clavuligerus* was observed with the two methods of cycling, as compared to the experiment with continuous air supply. However, the effect was more pronounced in the fermentation with Monte Carlo controlled aeration.

Finally, the third method has been described by Sweere *et al.* (1988b) who investigated the influence of DOT fluctuations on the growth and metabolite production of baker's yeast in a continuous culture. The fermenter system consisted of two fermenters, one sparged with air and one with nitrogen gas, and a pump and an overflow for circulating the fermentation broth between these fermenters. The DOT in the air sparged fermenter was controlled at 30% air saturation by the stirrer speed. The performance of this system could be characterized by a circulation time distribution of the culture liquid. If the circulation time was very short, i.e.

approached zero the culture would be similar to an oxygen-unlimited culture. These authors reported no effect on the yeast at circulation times less than 30 s, however, at circulation time more than 30 s, the biomass yield decreased and the metabolite formation increased with rising circulation time. The dramatic increase in the respiration quotient and the biomass yield on oxygen could be observed only at circulation times greater than 170 s. Oosterhuis *et al.* (1983, 1985b) used this reactor configuration to simulate DOT profiles which were observed in the large scale fermenters used for the production of gluconic acid by *Gluconobacter oxydans*. From small scale experiments, it was concluded that the reduction in gluconic acid production corresponded to the time the cells were exposed to oxygen-limited conditions. However, the potential capacity of the cells for gluconic acid production was not influenced

Similar in principle to the system described by Sweere *et al.* (1988b), Larsson and Enfors (1988) developed a two compartment fermenter consisting of one well mixed aerobic part (CSTR) and one minor anaerobic part (CPFR) for studying the effect of oxygen limitation on the respiration rate of *Penicillium chrysogenum*. They observed the irreversible inhibition of the respiration at circulation times of 5 and 10 min in an anaerobic plug flow reactor with a volume of 6% of the aerobic stirred tank reactor volume. However, no irreversible effects on the respiratory capacity was observed at circulation times of 1 and 2 min with an anaerobic zone of 1% of the stirred tank reactor volume.

An installation designed for the purpose of simulating of periodically changing environmental conditions in large scale recycle bioreactors has been described by Katinger (1976). It was a tubular loop reactor with a draught tube as downcomer and annular space as riser; an air injector was located at the top of the downcomer. Recycle times were about 80 s and mixing times in the order of 5 min. With the continuous culture of *Candida tropicalis* on n-alkanes as the test system, the author observed two types of oscillation in respiratory activity with periodicities of 15 to 20 min and 3 to 8 min and DOT's of 35 to 60% saturation and 2 to 10% saturation at the positions of maximum and minimum DOT's in the fermenter.

1.5 Purpose of the Work

Many of the published papers on mycelial fermentations describe the effects of agitation and aeration on antibiotic production. However, as dissolved oxygen levels and biomass concentrations were often not measured, it is difficult to distinguish between direct effects of dissolved oxygen on growth and product formation, and indirect effects caused by changes in morphology.

Since the objective of this work was to study the influence of dissolved oxygen on antibiotic synthesis, a bacterial fermentation was chosen to eliminate any indirect effects due to changes in morphology. However, as Table 1.3 illustrates *Bacillus* sp. are the most common bacteria for antibiotic production and the possibility of sporulation during fermentations has to be accepted.

Additionally, most *Bacillus* sp. frequently produce multicomponent antibiotics, for example difficidin and oxydifficidin from the strain used in the present study. The knowledge of whether the synthesis of the different components of the antibiotic complex can be influenced by the dissolved oxygen tension (DOT) in the fermentation broth will give a possibility to direct the antibiotic synthesis towards any desired direction, e.g. difficidin could be produced in a greater proportion than oxydifficidin.

Finally, the heterogeneity in DOT, a typical characteristic of large scale fermenters caused by imperfect mixing and inadequate mass transfer, will be simulated by cycling of DOT in the fermentation broth in a small scale fermenter. The response of the culture under these conditions could then be accounted for during scale up and optimization stages of this fermentation, if the productivity of the small scale fermenter has to be achieved in the large scale fermenters.

2. MATERIALS AND METHODS

2.1 Materials

2.1.1 Organism

The culture used throughout this study was a strain of *Bacillus subtilis* ATCC 39374 obtained from the American Type Culture Collection, Rockville, Maryland, U.S.A. This organism was originally isolated and identified by Merck & Co., Inc., Rahway, New Jersey, U.S.A. as a high producing strain, which is capable of synthesizing greater proportions of difficidin than oxydifficidin (Zimmerman *et al.*, 1985).

2.1.2 Chemicals

All laboratory chemicals were analytical grade and obtained from either BDH Chemicals Ltd., Poole, Dorset, U.K. or FSA Laboratory Supplies, Loughborough, U.K. unless otherwise stated. Dextrin Type III from corn was bought in bulk from Sigma Chemical Company Ltd., St. Louis, U.S.A. Pharmamedia was obtained from the Buckeye Oilseed Products Company, Fort Worth, Texas, U.S.A. Technical agar No. 3 and malt extract were bought from Oxoid Ltd., Basingstoke, Hampshire, U.K. The other medium ingredients were purchased from Sigma Chemical Company Ltd. NOPCO TDB-1 antifoam was a gift from Smithkline Beecham Pharmaceuticals, Worthing, Sussex, U.K. Oxygen free nitrogen gas was obtained from BOC Ltd., Guildford, Surrey, U.K.

Standard compounds of difficidin and oxydifficidin were kindly provided by Merck & Co., Inc., Rahway, New Jersey, U.S.A. These compounds were supplied in the form of methanolic solution with a concentration of 51.7 mg/mL for difficidin standard and 65 mg/mL for oxydifficidin standard. The solutions were stored at -70°C.

2.1.3 Equipment

2.1.3.1 Fermenter and instrumentation

The fermenter used was a 20-L LH fermenter 2000 Series I (L.H. Fermentation Ltd., Reading, Berks, U.K.) with a working volume of 12 L. The fermenter contained three, six-bladed disc-turbine impellers (70 mm diameter) and four baffles.

The instrumentation was a microprocessor based 8-loop process controller Model TCS 6358 (Turnbull Control System Ltd., Worthing, Sussex, U.K.) which is capable of monitoring and controlling up to 8 environmental parameters within the fermentation vessel. It was connected to a computer system (a MICRO/PDP-11 computer) for data acquisition, reduction and Digital Set-Point Control (DSC). This controller has only one set-point which has a PID (Proportional, Integral and Derivative) control action. Figure 2.1 shows the fermentation and process control system.

The stirrer speed was monitored and controlled in the range of 0 - 1500 rpm by the 8-loop controller through a small belt-driven DC tachogenerator attached to the motor shaft.

Temperature was measured by a resistance thermometer and controlled by a 500W cartridge heater and cooling water supplied through the heat exchanger, either of which can be on or off depending on whether heating or cooling is required.

Foam was detected and controlled by a foam probe inserted through the top plate of the fermenter and set at a defined level above the broth surface. When the foam rises and touches the probe, a circuit is completed with the foam acting as an electrolyte and the vessel acting as an earth. A peristaltic pump is actuated to release antifoam into the fermenter. Process timers were included in the circuit to prevent overcharging of the antifoam agent.

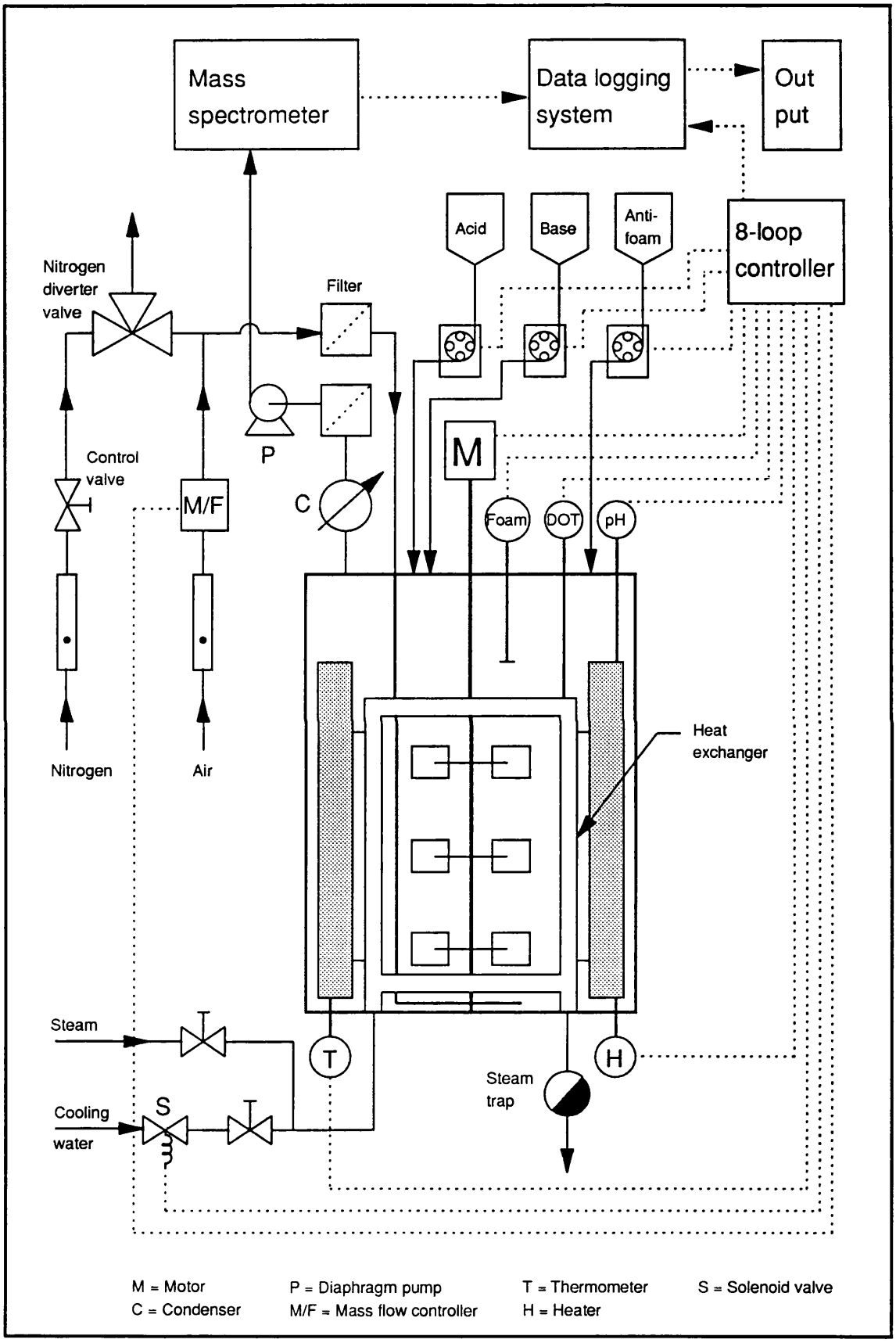


Figure 2.1 : Schematic diagram of 20-L LH fermenter equipped with instruments and automatic control systems.

pH was measured by a steam sterilizable pH electrode (Ingold Messtechnik AG, Industrie Nord, Urdorf, Switzerland) connected to the controller. The controller in turn activated peristaltic pumps which feed aqueous acid or base solutions from the respective reservoirs into the fermenter if the pH changed from a set point.

The dissolved oxygen tension (DOT) of medium was measured by a steam sterilizable polarographic dissolved oxygen electrode (Ingold Messtechnik AG, Industrie Nord, Urdorf, Switzerland).

The air flow into the fermenter was controlled by a HI-TEC series F-200 thermal mass flow meter/controller (Bronkhorst High Tech B.V., Ruurlo, The Netherlands) which was mounted in the air line that led to the vessel sparger.

The nitrogen gas flow rate was controlled by a flow control valve (Flostat Type MN, G.A. Platon Ltd., Basingstoke, Hampshire, U.K.). The feed of N_2 gas into the vessel was achieved by means of a N_2 gas diverter valve which could direct the flow of N_2 either through a quick release connection or into the air sparge line after the air flow control system. In the latter case the N_2 gas was filtered by the inlet air filter before fed into the fermenter vessel via the air sparger (see Figure 2.1).

The exit gas as well as the inlet gas was filtered by means of Pall filter cartridges (Pall Europe Ltd., Portsmouth, U.K.). The exit line had a water cooled condenser before the filter.

Sampling, inoculation, air input, acid, alkaline and antifoam additions were carried out by means of the ports on the top plate as well as DOT, pH, pressure and foam measurements.

2.1.3.2 Exit gas analysis

A VG MM8-80 mass spectrometer (VG Gas Analysis Systems Ltd., Middlewich,

Cheshire, U.K.) was used for on-line analysis of the fermenter off-gas for N_2, O_2, CO_2 and Ar. The exit gas was pumped with a small diaphragm air pump at a flow rate of about 150 mL/min to the mass spectrometer and analysed. The inlet air composition was also measured.

2.1.3.3 Data acquisition and computer system

The BIO-i software package (Biotechnology Computer Systems Ltd. (BCS), Maidenhead, Berkshire, U.K.) running on a MICRO/PDP-11 computer system (Digital Equipment Corporation, Maynard, Massachusetts, U.S.A.) was used for collection, storage, processing and presentation of on-line, off-line and derived data from the measurement equipment, i.e. the fermentation system and mass spectrometer. The BIO-i system included the algorithms for the on-line calculation of CER, OUR and RQ from input gas analysis values derived from the mass spectrometer. The on-line measurements were recorded every 3 min.

2.1.3.4 DOT cycling device

This instrument was designed and constructed to provide an on-off control on the air fed to the fermenter which resulted in a cycling of DOT in the culture broth. The on-off control was achieved by means of a solenoid valve operated by a timer (Cyclic Timer Relay, RS Components Ltd., Corby, Northants, U.K.) which was capable of setting an on period and an off period by the ON control knob and OFF control knob,respectively. The timing range of this timer was 0 - 200 s. A needle valve was also positioned on the air line after the solenoid valve for adjusting the air flow rate manually during the cycling experiments. All the three components were mounted in the same box which was, in turn, connected to the air inlet line parallel to the mass flow controller as shown in Figure 2.2. With this configulation of the gas system, a switch from a PID control of air during the constant DOT experiments to an on-off control for cycling of DOT and vice versa was easily attained.

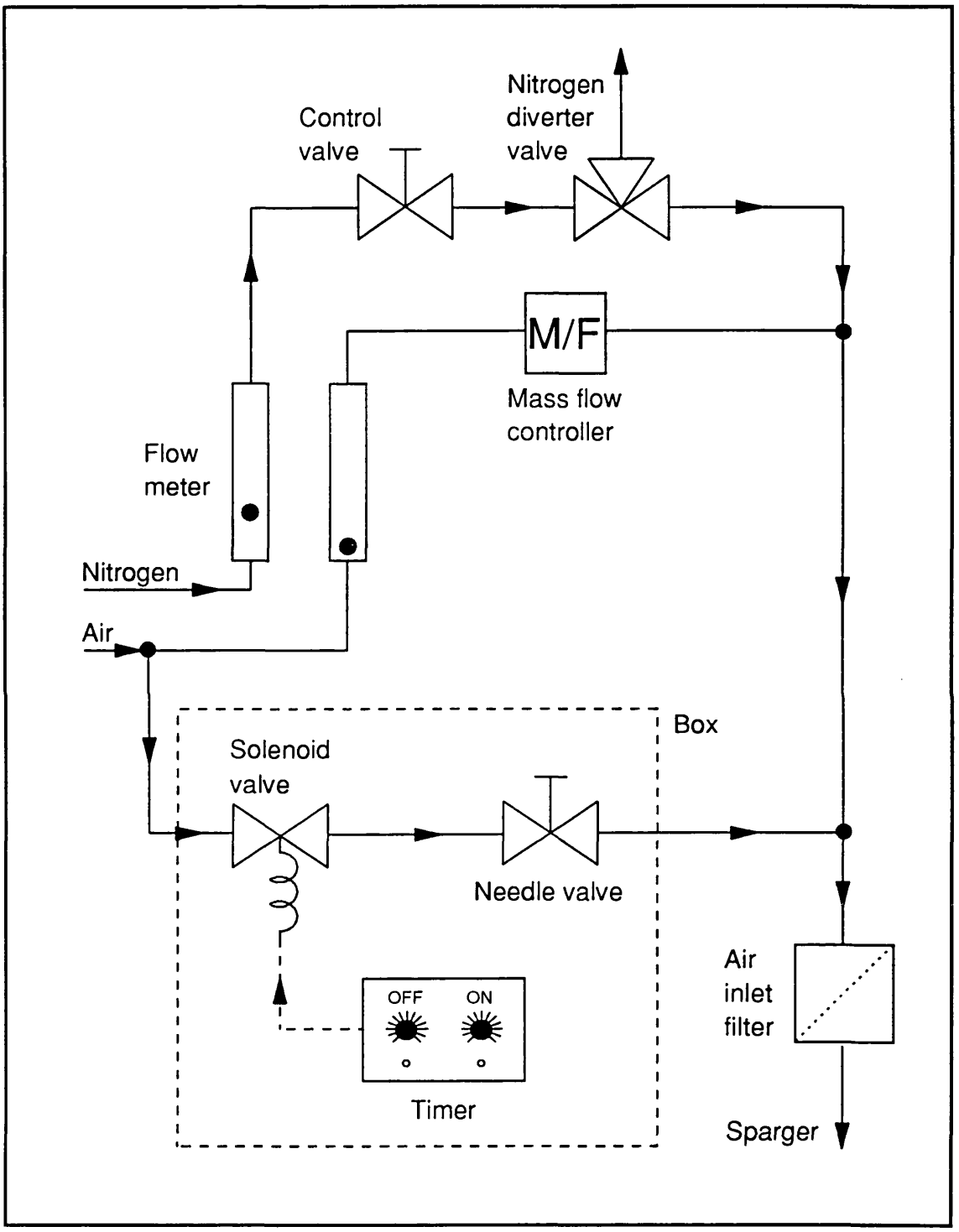


Figure 2.2 : Schematic diagram of the DOT cycling device and gas system.

2.1.3.5 High performance liquid chromatography (HPLC)

The HPLC system used for quantitative determination of difficidin and oxydifficidin is schematically shown in Figure 2.3. The system consisted of a Perkin - Elmer Liquid Chromatograph Pump model Series 10 (Perkin - Elmer Corporation, Norwalk, Connecticut, U.S.A.), a Perkin - Elmer Type ISS-100 sampling system, a spectroMonitor III variable wavelength detector (L.D.C. Milton Roy, Florida, U.S.A.) and a Perkin - Elmer LCI-100 laboratory computing integrator and recorder. A Whatman Partisil 5 PAC column (Whatman Inc., Clifton, New Jersey, U.S.A.) of 250 × 4.6 mm i.d. with a 70 × 2.0 mm i.d. stainless steel guard column packed with a pellicular PAC was used. The column temperature was controlled at a constant level using a temperature control unit (Jones Chromatography, Wales, U.K.).

2.2 Methods

2.2.1 Fermentation

2.2.1.1 Culture maintenance

The culture was maintained on agar slants. Inoculated slants were incubated at 28° C for 96 h, then refrigerated. The culture was transferred onto new agar slants every 3 - 4 months and also frozen in 60% glycerol, and stored at -20° C. The composition of the agar slants as recommended by Merck & Co., Inc. (Zimmerman *et al.*, 1985) was:

Constituent	Concentration (g/L)
Agar	20
Malt extract	10
D-Glucose	4
Yeast extract	4
pH 7.0	

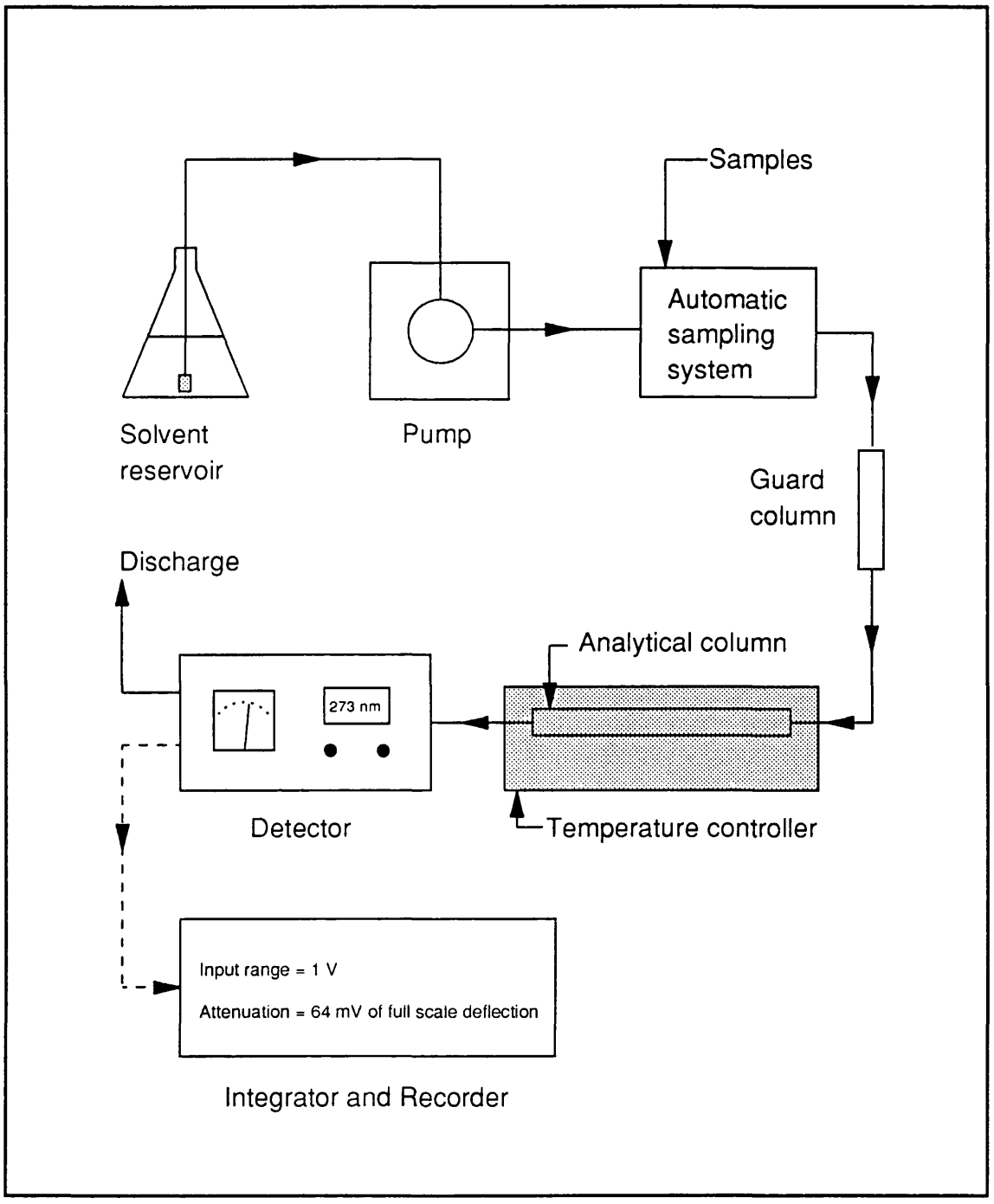


Figure 2.3 : Schematic diagram of the high performance liquid chromatography (HPLC).

2.2.1.2 Inoculum preparation

The inoculum medium was slightly modified from that recommended by Merck & Co., Inc. (Zimmerman *et al.*, 1985, 1987). This medium was prepared by suspending the ingredients listed below in deionized water.

Constituent	Concentration (g/L)
D-Glucose	1
Soluble starch	10
Beef extract	3
Yeast extract	10
$MgSO_4.7H_2O$	0.05
1.34 M Phosphate buffer (KH_2PO_4 91 g/L, Na_2HPO_4 95 g/L)	0.2 mL/L
$CaCO_3$ (After pH adjustment to 7.0 - 7.2)	0.5

Fifty mL of this suspension was dispensed into each of three 250 mL four-baffled shake flasks and 500 mL into each of two 2000 mL four-baffled shake flasks. The flasks were plugged with foam bungs and autoclaved 30 min at 121° C, 15 psig.

A loopful of the organism from the agar slant culture was aseptically transfered into each of three 250 mL four-baffled shake flasks of inoculum medium. These three flasks were incubated at 28° C for 12 h on a rotary shaker (Controlled Environment Incubator Shaker, New Brunswick Scientific Co. Inc., Edison, New Jersey, U.S.A.) running at 220 rpm. After 12 h, the three flasks were pooled and each of 15 mL of this first stage seed culture were used to inoculate each of two 2000 mL shake flasks of inoculum medium which were also incubated at 220 rpm, 28° C, for 12 h. This second stage seed culture was used to inoculate the fermenter containing sterile fermentation medium.

2.2.1.3 Fermentation procedure

The fermentation medium was a modification of that recommended by Merck & Co.,Inc. The actual compositions of the medium are listed below :

Constituent	Concentration (g/L)
Dextrin	40
Pharmamedia	20
Lactic acid (85% concentration)	1.8
$CoCl_2.6H_2O$	0.1
NOPCO TDB-1 antifoam	1 mL/L

This medium was prepared with deionized water. The pre-sterile pH was adjusted to 7.3 with 50% (w/v) NaOH. The medium was then sterilized *in situ* for 30 min at 121° C, 15 psig, by passing steam at about 20 psig through the heat exchanger rings. The post-sterile pH of medium was around 6.4 - 6.5.

The ancillaries, i.e. filters, sampling device and sample bottles, acid and base containers, inoculum flask including their addition lines and needles were sterilized in an autoclave at 121° C, 15 psig for 50 min. Acid, base and antifoam were sterilized separately at 121° C, 15 psig for 20 min.

The fermentation medium was cooled down to 28° C using cooling water and then aseptically inoculated with 1 L inoculum. Addition of NOPCO TDB-1 antifoam was controlled by a foam probe and control system to prevent the formation of foam during fermentation. Temperature was maintained at 28° C. pH was controlled at 6.8 by addition of 2M H_3PO_4 and 2M NaOH.

2.2.1.4 Medium for correlating dry cell weights to DNA content

One fermentation and several shake flask experiments were performed using a

chemically defined medium in order to obtain measurements for the DNA content and dry cell weight correlation. The medium components are listed below :

Constituent	Concentration (g/L)
Glucose	40
NH_4Cl	15
$CaCl_2.6H_2O$	0.219
$MgSO_4.7H_2O$	0.45
KH_2PO_4	1
Na_2HPO_4	0.426
$FeSO_4.7H_2O$	0.012
$ZnSO_4.7H_2O$	0.004
$CuSO_4.5H_2O$	0.004
$MnCl_2.4H_2O$	0.004
NaEDTA	0.6

Glucose was sterilized as a concentrated solution separate from the rest of the medium.

2.2.2 Determination of growth

2.2.2.1 Optical density

For a quick and convenient purpose to follow the growth of seed culture, optical density (absorbance at 600 nm) was measured with a Pye Unicam PU 8600 UV/VIS spectrophotometer (Pye Unicam Ltd., Cambridge, U.K.) using sterile medium as a blank. Dense samples were diluted with sterile medium to obtain OD in the linear range (0.05 - 0.50 OD).

2.2.2.2 Dry weight

Dry weight measurement was applied to determine growth in a chemically defined medium. Ten mL of the fermentation broth were filtered through a preweighed and dried

membrane filter of 0.2 μm pore size (Whatman International Ltd., Maidstone, Kent, U.K.), washed with the equal volume of deionized water and then dried to a constant weight at 80° C.

2.2.2.3 DNA analysis

Biomass concentrations in a very complex fermentation medium with high insoluble solids content were estimated using the correlation of dry cell weight and DNA content of the culture broth. The diphenylamine method of Burton (1956) was used in accord with Gürtler and Schulein's (1980) modifications for DNA determinations.

To carry out the determination, 5 mL of the refrigerated culture broth were diluted with an equal volume of ice-cold deionized water and centrifuged in a MSE Highspeed 18 Refrigerator Centrifuge (Measuring & Scientific Equipment Ltd., Manor Royal, Crawley, Sussex, U.K.) for 20 min at 6000 rpm and 4° C. Six mL of ice-cold 0.25 N perchloric acid ($HClO_4$) were added to the precipitate after the supernatant had been decanted. The precipitate was broken up with a glass rod, stirred and placed in an ice-water bath for 45 min, with occasional shaking and then centrifuged for 20 min at 6000 rpm and 4°C. The precipitate was re-extracted in the same way for another 15 min and centrifuged at 6000 rpm at 4° C. After the supernatant was decanted, the precipitate was reacted with 6 mL of 0.5 N perchloric acid at 70° C for 1 h and left overnight at room temperature. The mixed suspension was finally centrifuged for 20 min at 6000 rpm at room temperature. The supernatant from this step was kept for DNA determination. One mL of 0.5 N perchloric acid was added to 1 mL of the supernatant followed by the addition of 2 mL of diphenylamine reagent. A blank was produced similarly by adding 2 mL of 0.5 N perchloric acid which had been placed in the 70° C water bath for 15 min, to 2 mL of the diphenylamine reagent.

Diphenylamine reagent was prepared by dissolving 15 g of diphenylamine in 1000 mL glacial acetic acid and 15 mL concentrated sulphuric acid and kept in the dark. Immediately before use, 0.1 mL of a 16 mg/mL aqueous acetaldehyde solution was added for each 20 mL of reagent required.

DNA standards were prepared by dissolving 20 mg DNA - Na salt from calf thymus (Sigma Chemical Company Ltd.) in 100 mL 5 mM NaOH solution. This solution was diluted with an equal volume of 1 N perchloric acid immediately before use, giving a stock solution of 0.1 g DNA/L. Several different dilutions of this stock solution were prepared by using 0.5 N perchloric acid. Two mL of each dilution was heated at 70°C for 15 min before the addition of 2 mL diphenylamine reagent.

The solutions, i.e. the samples, the standards and the blank were thoroughly mixed after the addition of diphenylamine reagent and placed in the dark overnight (16 - 20 h) at room temperature. After this, the optical densities at 600 nm were measured against the blank.

The standard curve obtained from the DNA standard solutions is shown in Figure 2.4 and also the correlation of dry cell weight and DNA content obtained from the culture grown in a chemically defined medium is presented in Figure 2.5. Finally, the standard curve of optical density versus cell concentration was prepared (Figure 2.6) and then used in determination of the biomass concentration in the complex fermentation medium.

2.2.3 HPLC determination of difficidin and oxydifficidin

2.2.3.1 Sample preparation and storage

An appropriate quantity (1 mL), accurately measured, of the fermentation broth taken from the fermenter was pipetted into a centrifuge tube. Two mL of HPLC grade methanol were added. The mixture was shaken vigorously for 1 min on a vortex mixer (Whirlimixer, Fisons Scientific Equipment, Loughborough, U.K.) to extract difficidin and its related compounds, and centrifuged for 15 min at 5000 rpm and 4° C. One mL of the clear supernatant obtained after centrifugation was transferred into an Eppendorf tube, stored at -70° C and assayed within a week.

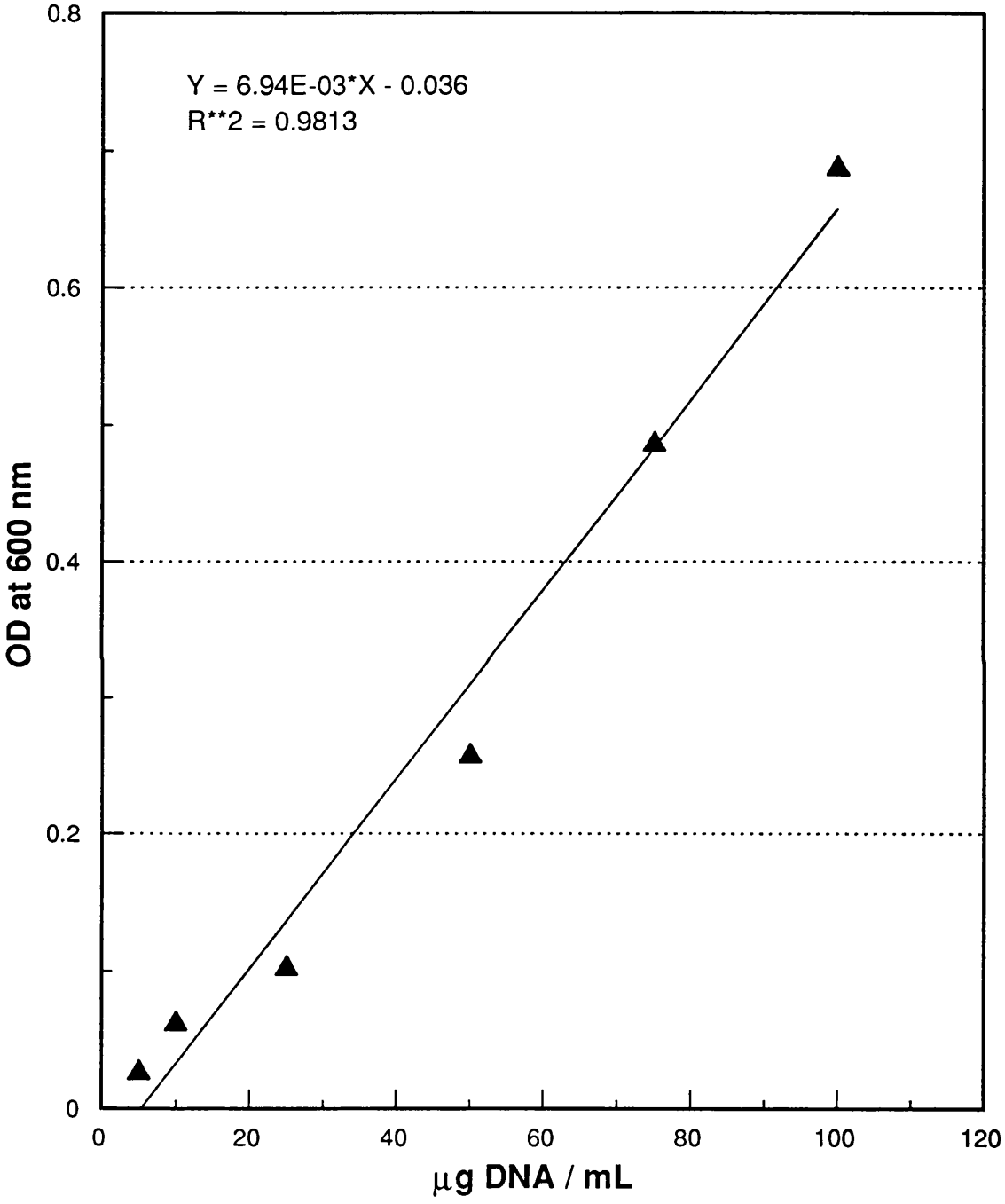


Figure 2.4 : Standard curve for DNA measurements.

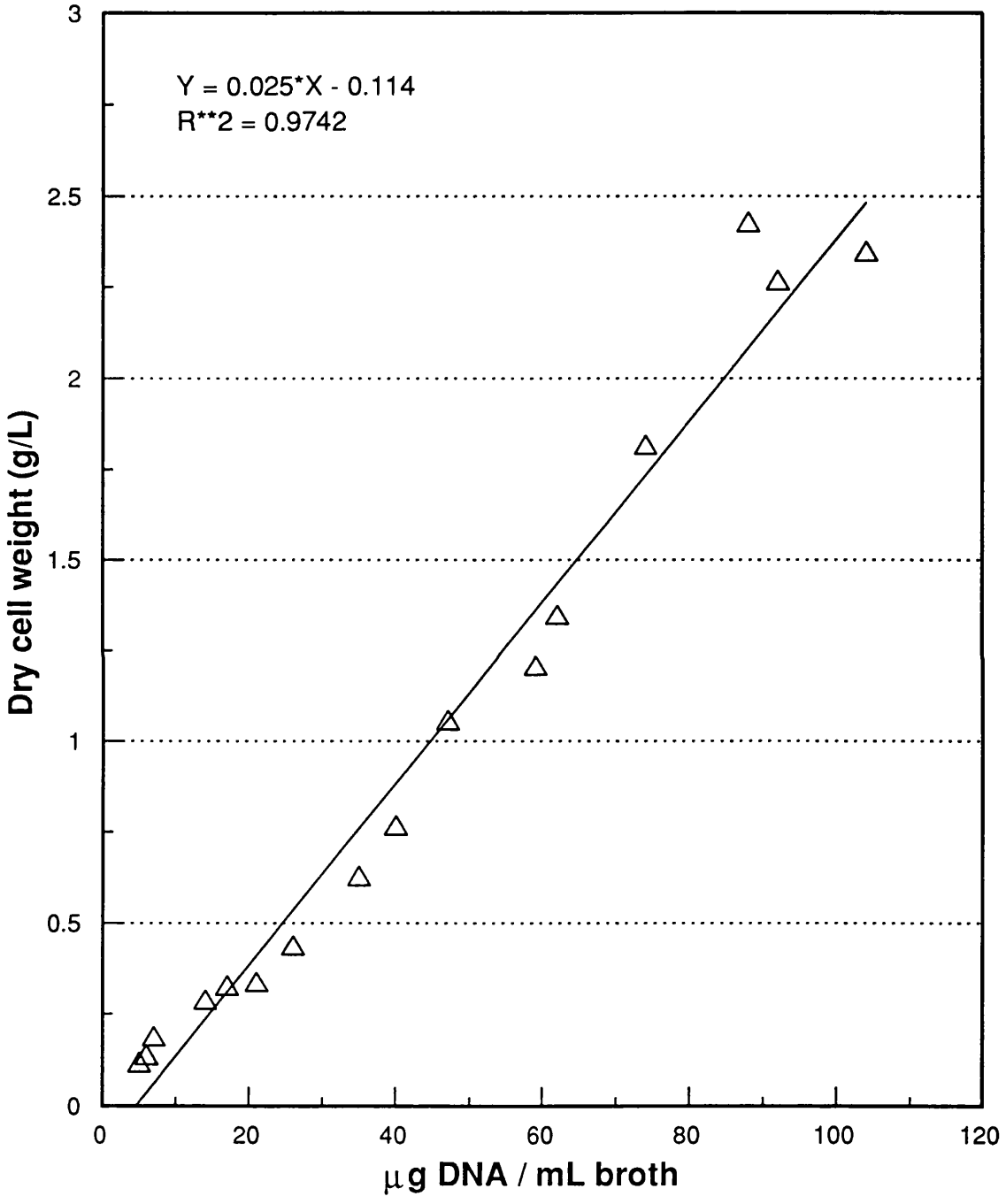


Figure 2.5 : Correlation of DNA content versus dry cell weight.

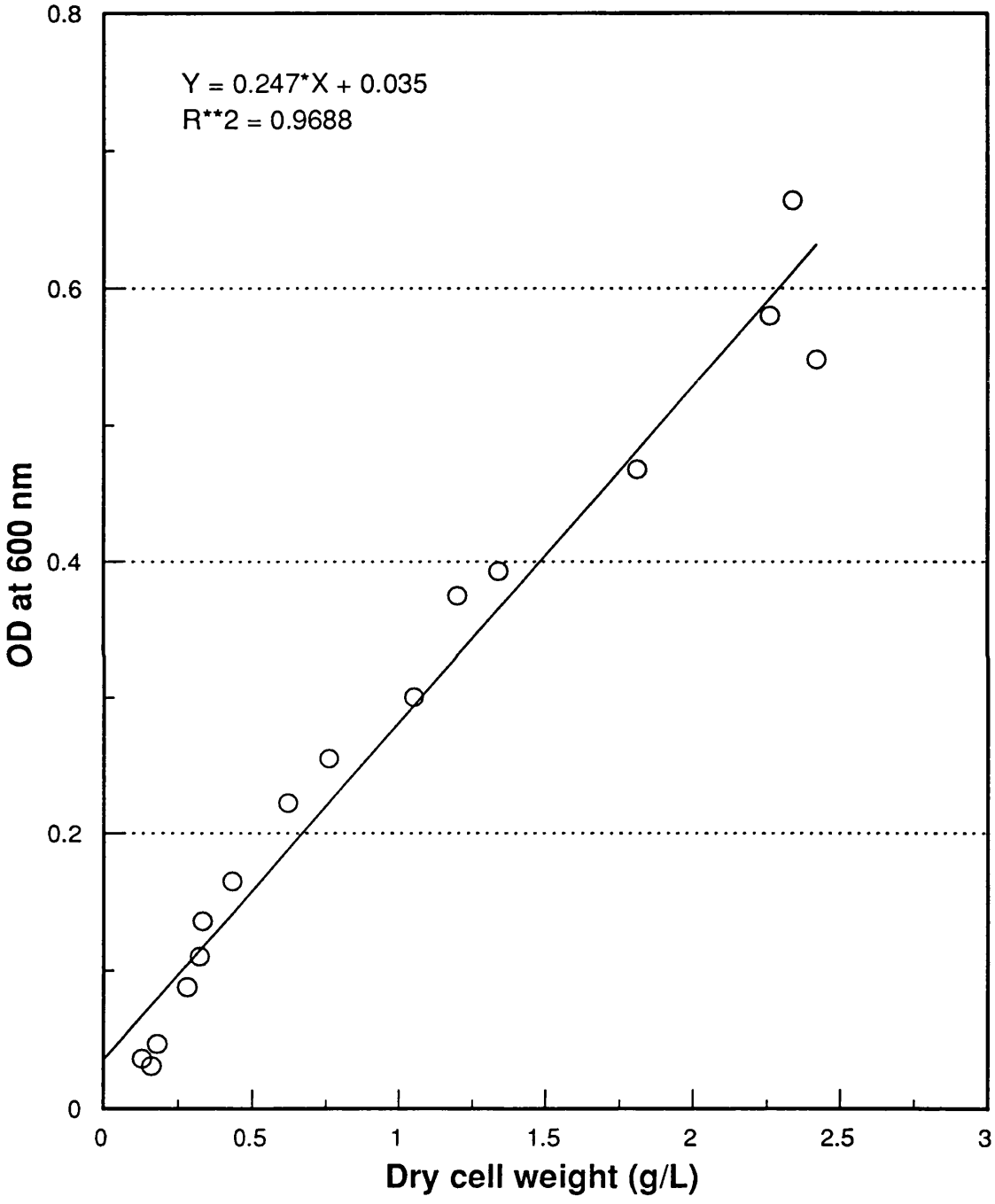


Figure 2.6 : Standard curve for biomass determination from DNA analysis.

2.2.3.2 HPLC assay

For the quantitative determination of difficidin and oxydifficidin, the HPLC assay recommended by Wilson *et al.* (1987) was followed with slight modifications.

The frozen methanol extracted samples were thawed at room temperature and kept at 4° C during the assay. The mobile phase consisting of methanol and 0.067 M aqueous phosphoric acid (90 : 10, v/v) was degassed by sonicating for 45 min. The mobile phase flow rate was 2.1 mL/min (pressure 3000 - 3500 psi) and the UV absorbance of column effluent was monitored at 273 nm. The column was operated at 40° C. Ten µL of each sample were automatically injected onto the column by the sampling system. The retention times of difficidin and oxydifficidin were about 620 s and 675 s respectively. A typical chromatogram of the methanol extract of fermentation broth is shown in Figure 2.7.

The standards were prepared from several different dilutions of the standard compounds (i.e. difficidin and oxydifficidin) using methanol. The standard curves obtained for difficidin and oxydifficidin are presented in Figures 2.8 and 2.9 respectively. At the end of each working day, the column was rinsed with methanol for about 30 min.

2.2.4 DOT control strategy

In order to achieve a precise and reliable control of DOT, the DOT probe was carefully calibrated in sterile medium before inoculation. A DOT of 100% air saturation was determined by allowing the probe to equilibrate with an air saturated medium which was, in turn, obtained by sparging the medium with air at a flow rate of 4 L/min at an agitator speed of 600 rpm and temperature 28° C. To determine 0% DOT oxygen-free nitrogen gas was sparged through the medium instead of air.

The DOT control technique is based on proportional - integral - differential (PID) control of agitator speed and air flow rate together with a constant nitrogen gas flow rate

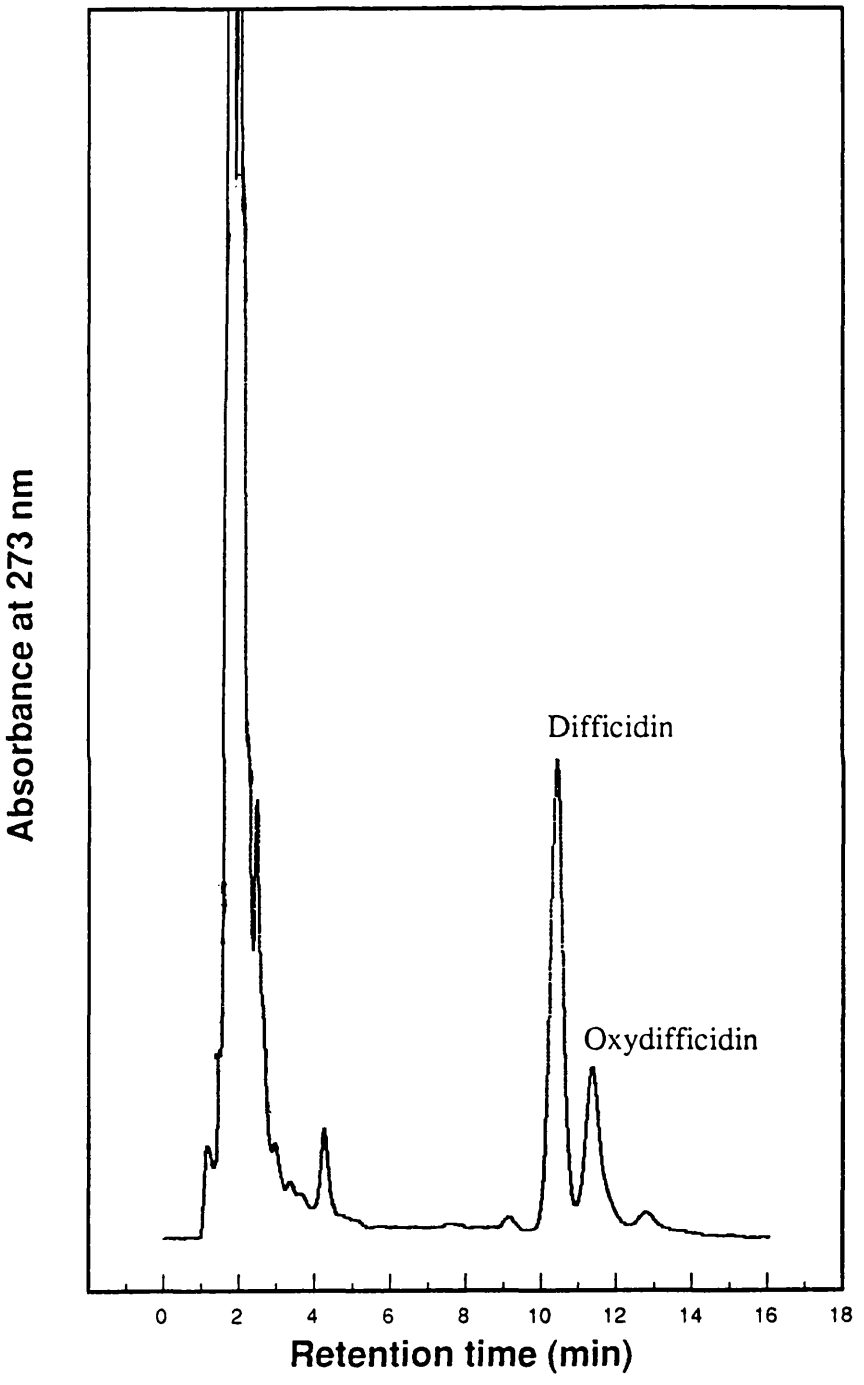


Figure 2.7 : HPLC chromatogram of difficidin and oxydifficidin extracted from fermentation broth with methanol.

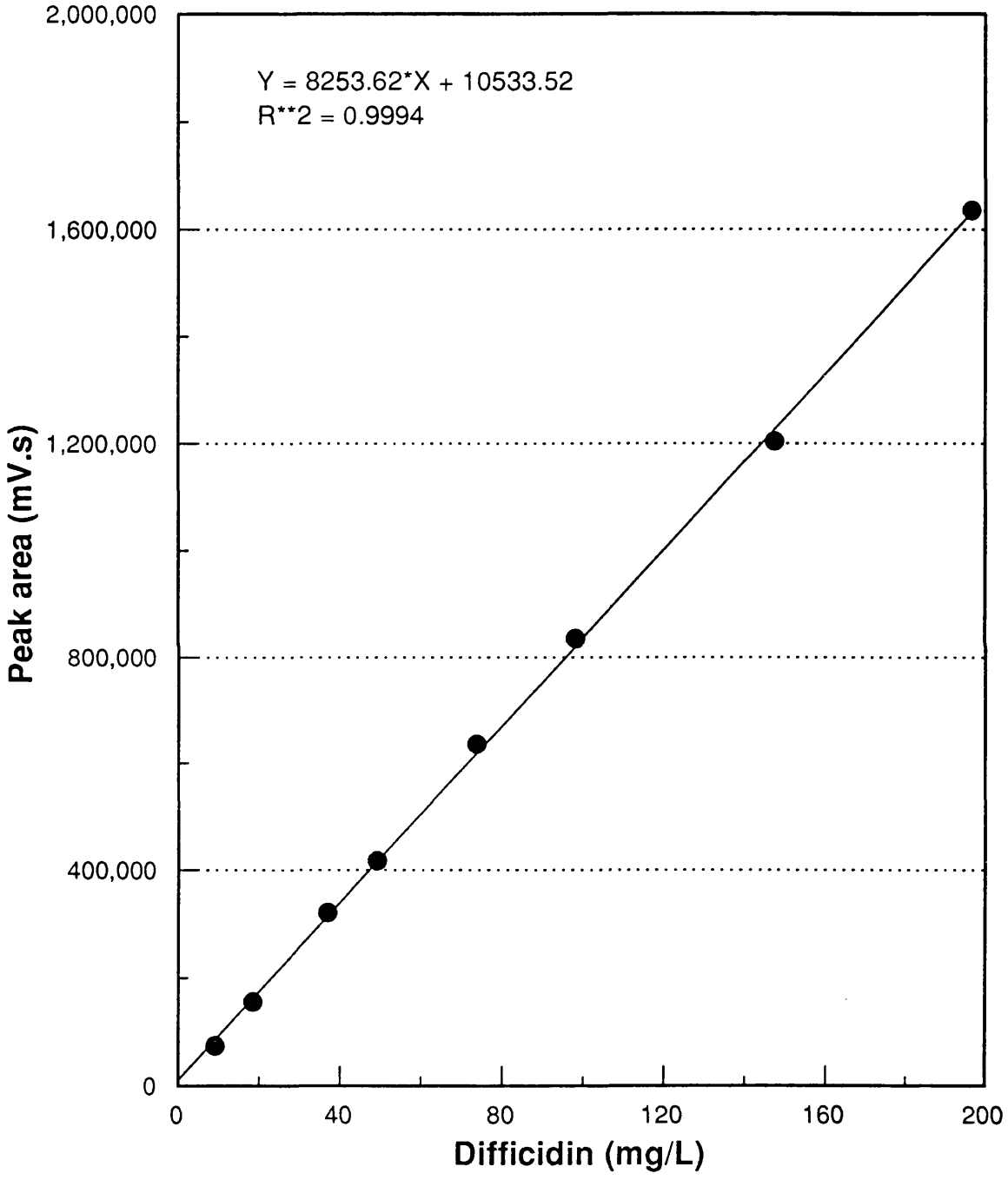


Figure 2.8 : Standard curve for the HPLC assay of difficidin.

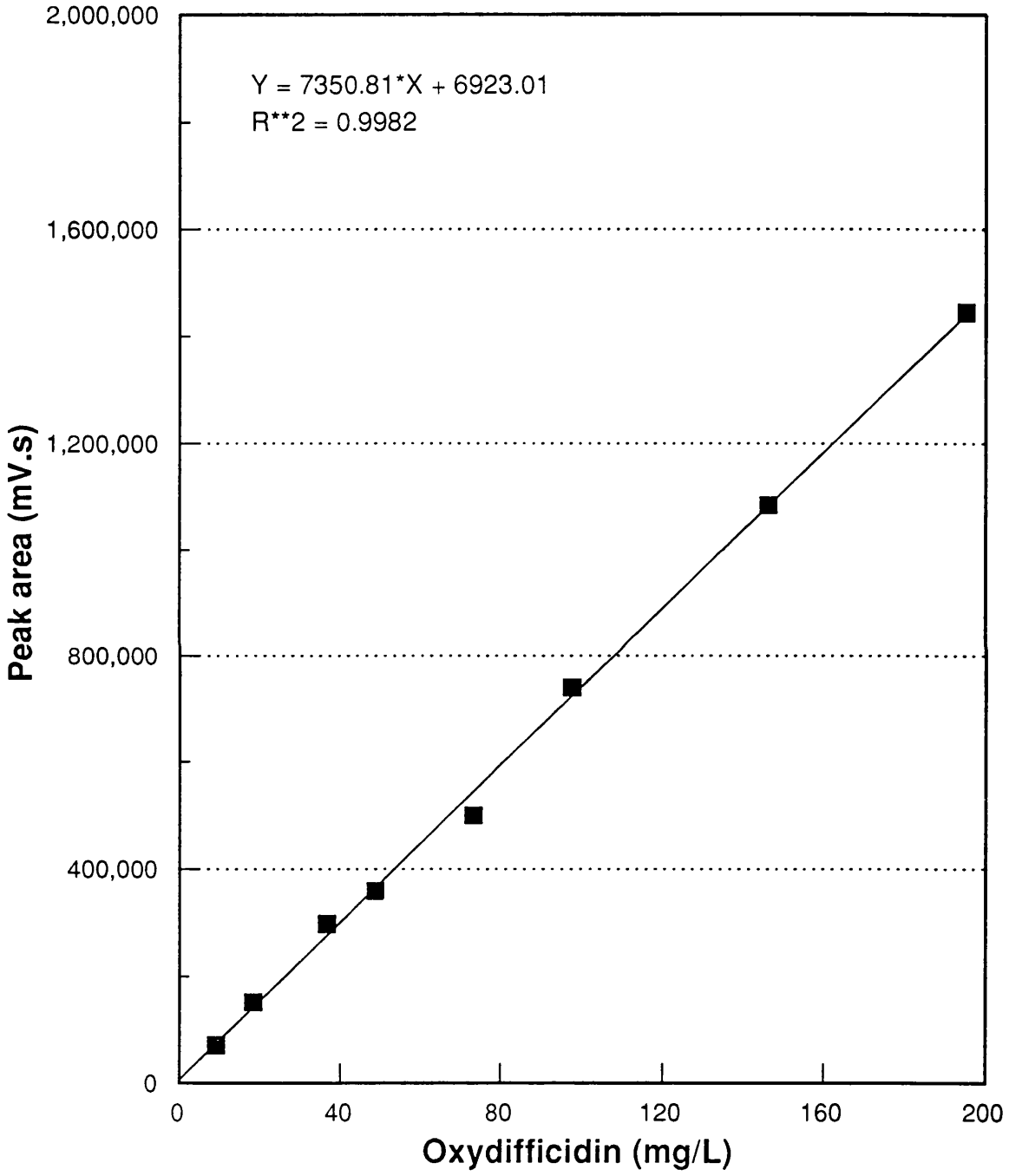


Figure 2.9 : Standard curve for the HPLC assay of oxydifficidin.

(applied when DOT was controlled below 80% saturation). The DOT control action was set up immediately after inoculation by selecting the DOT set point, diverting N_2 gas flowing at a constant flow rate into the air feed line, cascading the DOT set point upon both the set points of agitator speed and air flow rate and then tuning the PID controller.

The fixed N_2 gas flow rates as shown in Table 2.1 were chosen arbitrarily based on the fact that the lower the DOT control level the higher the N_2 gas flow rate should be selected for compensating the low air flow rate to maintain a good ventilation of the fermentation system and ensure an adequate removal of CO_2.

Table 2.1 : Nitrogen gas flow rates used during various DOT control levels.

Controlled DOT (% saturation)	N_2 gas flow rate (L/min)
1	2.8
5	2.5
10	2.0
15	1.8
20	1.5
40	1.0
80	0.0

The PID control parameters were experimentally evaluated according the directions in the technical manual supplied by the manufacturers and were not changed during cultivation. The best DOT control with ±0.5% accuracy was obtained over a wide range of the control levels from 1 to 40% saturation using a proportional band (P_B) of 150%

(gain (K_c) = 0.67), integral time (T_i) 2.0 min and derivative time (T_d) 0.5 min. To achieve the best control at 80%, however, the P_B value had to be set to 50% (K_c = 2.0) with the same values of T_i and T_d. An air flow rate and agitator speed were allowed to vary between 0 - 10 L/min and 300 - 1500 rpm, respectively.

The DOT probe was always re-calibrated after an experiment, to check for drift. With the control strategy mentioned above, no manual operation was needed.

2.2.5 Cycling DOT

Since the dynamic response of DOT probe is one of the most important factors during the cycling experiments, the dynamic characteristics of the probe were roughly determined by response tests as described by Vardar and Lilly (1982a). The tests consisted of first immersing the DOT probe in a nitrogen-saturated aqueous solution (zero dissolved oxygen tension) and then quickly transferring into an air-saturated solution. The dead time, i.e. the time lag before any response from the probe, was found to be 2 s. The electrode time constant, i.e. the time required to read 63% of the actual value of DOT, was determined to be 14 s.

To perform cycling of DOT, the mass flow controller had to be set to zero to direct an air stream through the cycling device (see also Figure 2.2) followed by switching on the cycling device. The cycling periods were manipulated by the timer operated air solenoid valve. In this study the timer was set to 30 s for both on and off periods resulting in a 1 min cycling period of DOT. The cycling amplitudes, however, were adjusted to some extent by the manipulation of air and N_2 gas flow rates.

With this control system, the only manual adjustments required were the initial setting of an agitator speed, air flow rate and N_2 gas flow rate. Only on a very few occasions little manual adjustments of air and/or N_2 gas flow rates were required when the cycling trended to move away from the original. Table 2.2 summarizes the initial setting parameters used to cycle DOT around a particular value.

Table 2.2 : The initial setting conditions for cycling of DOT at different levels.

DOT around which cycling was performed (% saturation)	Agitator speed (rpm)	Air flow rate (L/min)	N_2 gas flow rate (L/min)
15	560	4.5	2.5
20	600	5.2	2.5
40	700	6.8	1.5

3. RESULTS

3.1 Characterization of Product Compounds

Since difficidin and oxydifficidin are the products of interest of this study, the investigation of some characteristics of these compounds was necessary in order to obtain reliable and reproducible experimental data. These characteristics, therefore, could possibly be accounted for in an interpretation of the experimental results.

3.1.1 Effect of pH on the product stability

The pH stabilities of difficidin and oxydifficidin have been reported by Wilson *et al.* (1987). In this study, however, the pH stabilities of these compounds were investigated in more detail to obtain the pH profile scanning 1 to 1.5 pH units over the pH range between pH 3.5 and pH 11.0.

In order to maintain the pH of solutions at different levels, the universal buffer solutions as recommended by Perrin and Dempsey (1974) were prepared. A stock solution containing 0.2M boric acid and 0.05M citric acid was added with a different proportion of 0.1M $Na_3PO_4.12H_2O$ solution, as shown in Table 3.1, to obtain the buffer solutions with different pH values.

For pH stability studies, approximately 100 mg/L of an antibiotic in each buffer solution were prepared using the standard compounds. All samples were incubated at 28° C and periodically assayed by HPLC. Results are summarized in Figure 3.1.

Difficidin was unstable at pH below 6.0. When pH rose from pH 6.0 to pH 8.0, a 12-fold increase in stability was observed. The maximum pH stability of difficidin was at pH 9.0, beyond which its stability was reduced. In contrast, oxydifficidin showed a different profile of pH stability starting from a very low stability at pH 3.5 reaching a plateau at pH 5.0. Between pH 5.0 and pH 8.0, oxydifficidin exhibited nearly constant

Table 3.1 : The composition of universal buffer solutions.

pH	Stock solution (mL)	Na_3PO_4 solution (mL)
3.5	16.6	3.4
5.0	13.4	6.6
6.0	11.8	8.2
7.0	9.9	10.1
8.0	8.5	11.5
9.0	6.9	13.1
10.0	5.4	14.6
11.0	4.4	15.6

stability. Above pH 8.0, the stability was sharply declined to the lowest level at pH 11.0. By comparison, oxydifficidin was more stable than difficidin under acidic condition whereas difficidin showed a much higher stability than that of oxydifficidin under alkaline conditions. In a neutral pH range of 6.5 - 7.0, the two antibiotics demonstrated nearly the same stabilities of about 1% of antibiotics loss per hour. These results are in good agreement with those reported by Wilson *et al.* (1987).

3.1.2 Verification of difficidin and oxydifficidin extracted from the culture broth by pH stability

Based on the difference in pH stability of the two antibiotics as described above, subsequent experiments were carried out to verify the antibiotics by HPLC assay.

A methanol extracted sample obtained from one of the fermentations was buffered with

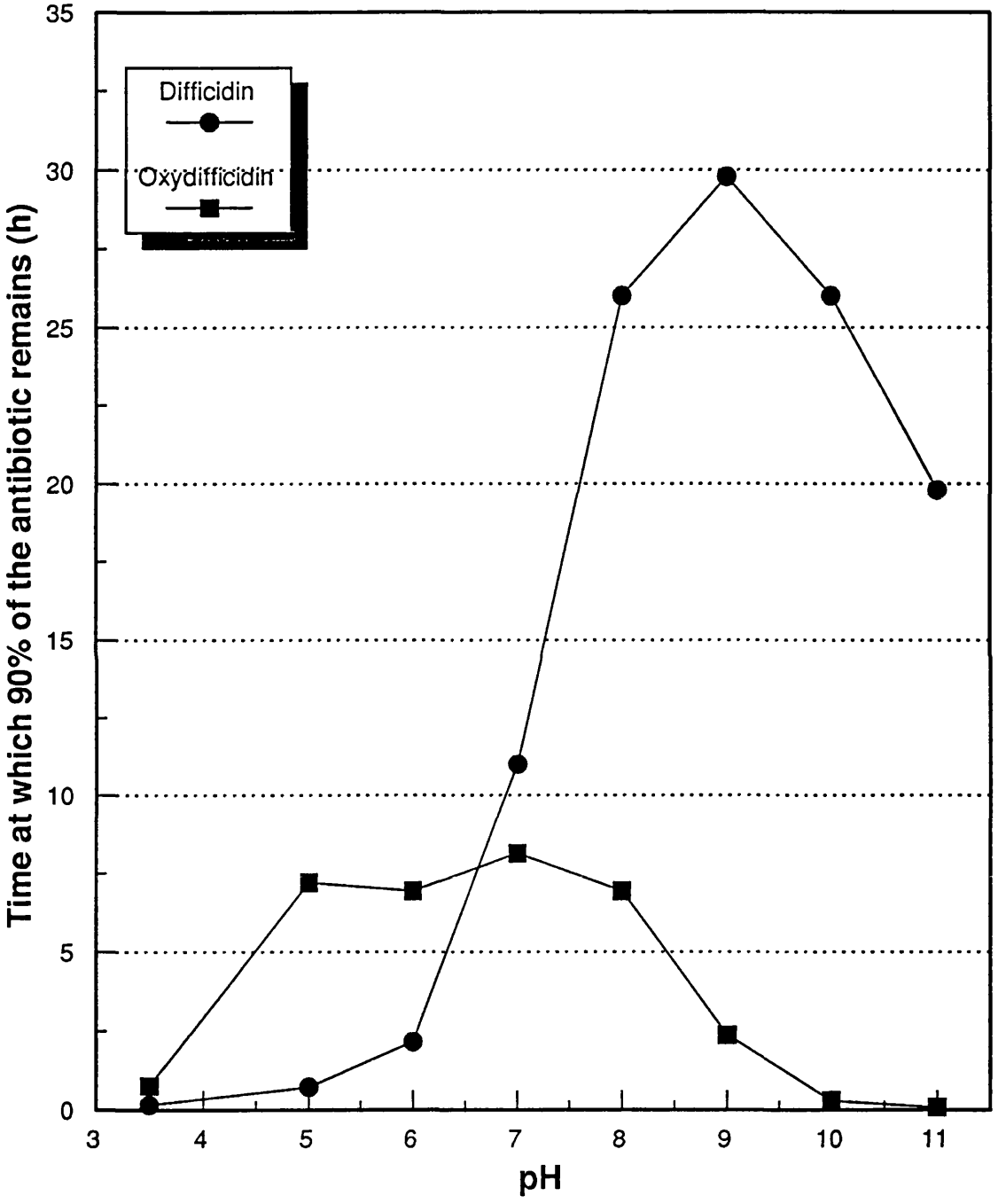


Figure 3.1 : pH Stability of difficidin and oxydifficidin at 28° C.

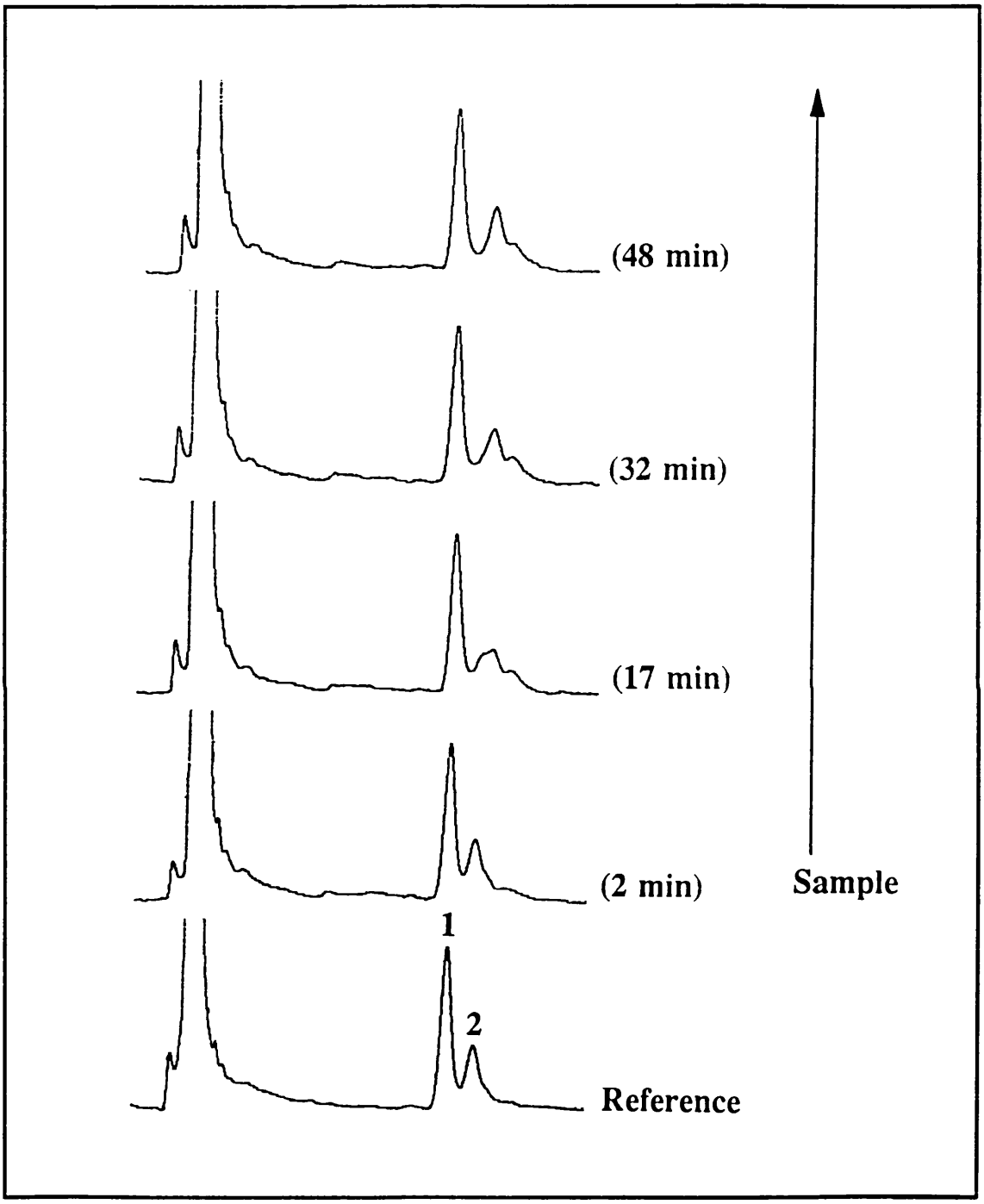
an equal volume of 0.2M sodium carbonate pH 11.0 and held at 28° C. The sample was periodically assayed by HPLC. The reference was also prepared by the same procedure but an equal volume of methanol was added instead of a buffer solution. The HPLC chromatograms of the reference and the sample are shown in Figure 3.2.

The chromatogram of the freshly prepared (2 min after mixing with a buffer solution) sample was the same as that of the reference. These results demonstrated that there was no effect of a buffer solution on HPLC assay. At 17 min, peak 2 began to split into 2 peaks with shifting in retention times, whereas peak 1 was unchanged. The splitting of peak 2 was more pronounced at 32 min and 48 min but still there was no change of peak 1. The results exhibited the instability of the compound of peak 2 under an alkaline condition. The compound of peak 1, however, was very stable under the same condition. These experiments have really proven that the compound of peak 2 was oxydifficidin and that of peak 1 was difficidin. These circumstances confirmed the identification of the products by comparing their retention times with those of the standard compounds obtained from HPLC analysis.

3.1.3 Difficidin and oxydifficidin in the culture broth

It is interesting to understand the location of the products found in the whole broth. To carry out this experiment, a sample was taken from the fermenter and filtered through a 0.2 μm membrane filter. The filtrate was collected and directly assayed by HPLC. The separated solid contents including cell mass were extracted with methanol. A clear supernatant obtained after centrifugation was then assayed by HPLC. The percentages of the antibiotics found in the filtrate and cell mass were calculated and summarized in Table 3.2.

The results illustrated that an average of about 75% of difficidin and 60% of oxydifficidin were bound to the bacterial cells. Even at the end of the fermentation, i.e. at 158 h of cultivation, when most of the cells died, the antibiotics still attached to the cells.



Fgure 3.2 : HPLC chromatograms of the reference pH 7.0 and the sample pH 11.0 at various time intervals.

Table 3.2 : The distribution of the antibiotics between cell mass and the supernatant broth[†].

Whole broth compo-nent	Fermentation time (h)					
	90		95		158	
	D[‡]	OD[§]	D	OD	D	OD
Cell	82	60	71	58	72	66
Broth	6	13	13	22	10	13

[†] Express as percentage of the total antibiotics obtained by methanol extraction of the whole broth.

[‡] D = Difficidin.

[§] OD = Oxydifficidin.

3.2 Fermentation of *Bacillus subtilis*

A typical fermentation of *Bacillus subtilis* producing the difficidin and oxydifficidin antibiotics was established and tested for a reproducibility. The effects of controlled and uncontrolled pH on the fermentation performance were also investigated.

3.2.1 Inoculum development

The main objective of inoculum development for bacterial fermentations is to produce an active inoculum which will give as short a lag phase as possible in subsequent culture. In this study, the two successive stages of inoculum preparation as described in Section 2.2.1.2 were conducted to produce sufficient biomass to inoculate the fermenter. In order to find the optimum length of each stage of inoculum culture which gave the high density of physiologically active cells, the growth curves of the first and the second stage inocula were determined as shown in Figures 3.3 and 3.4 respectively.

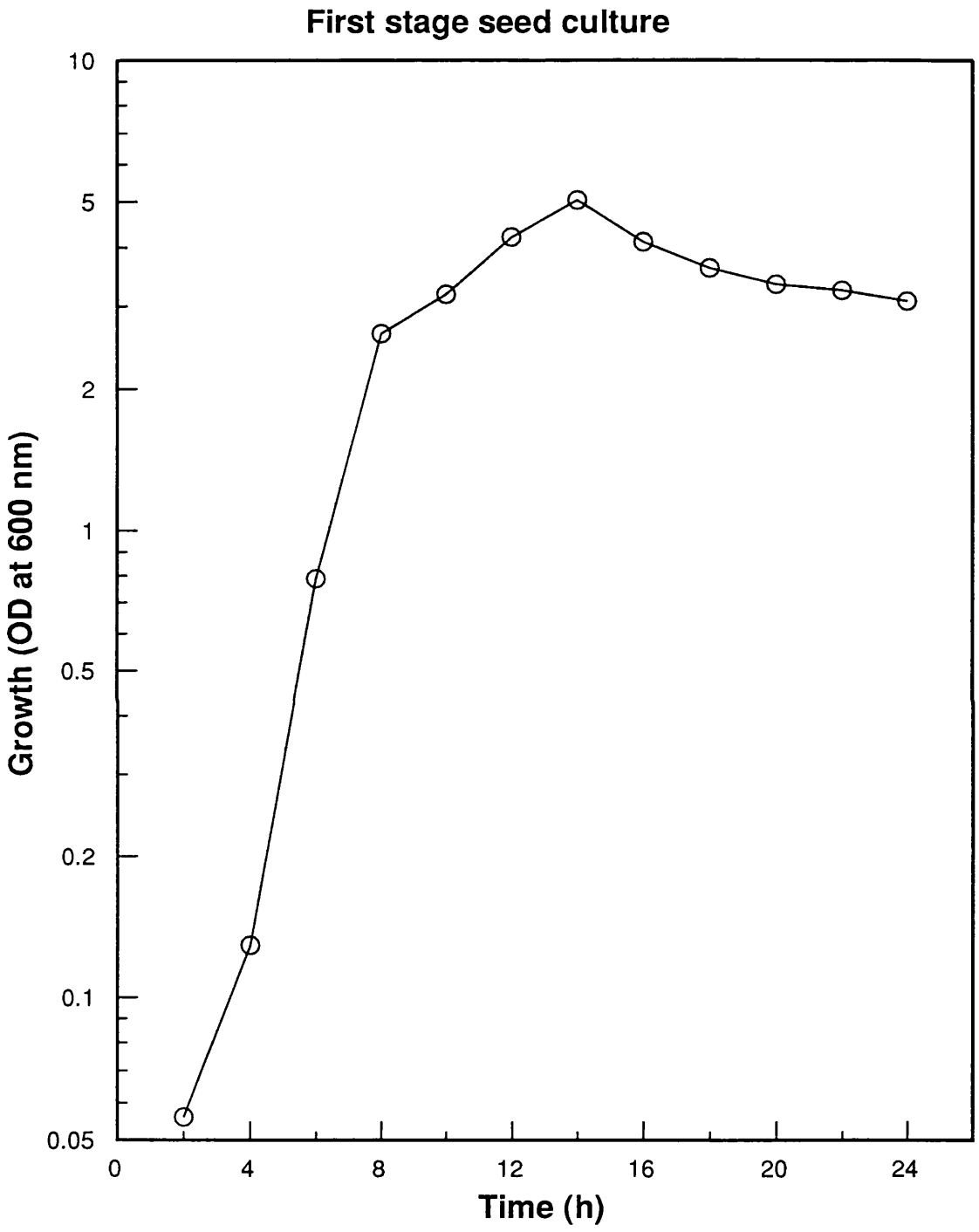


Figure 3.3 : Growth curve of the first stage inoculum culture.

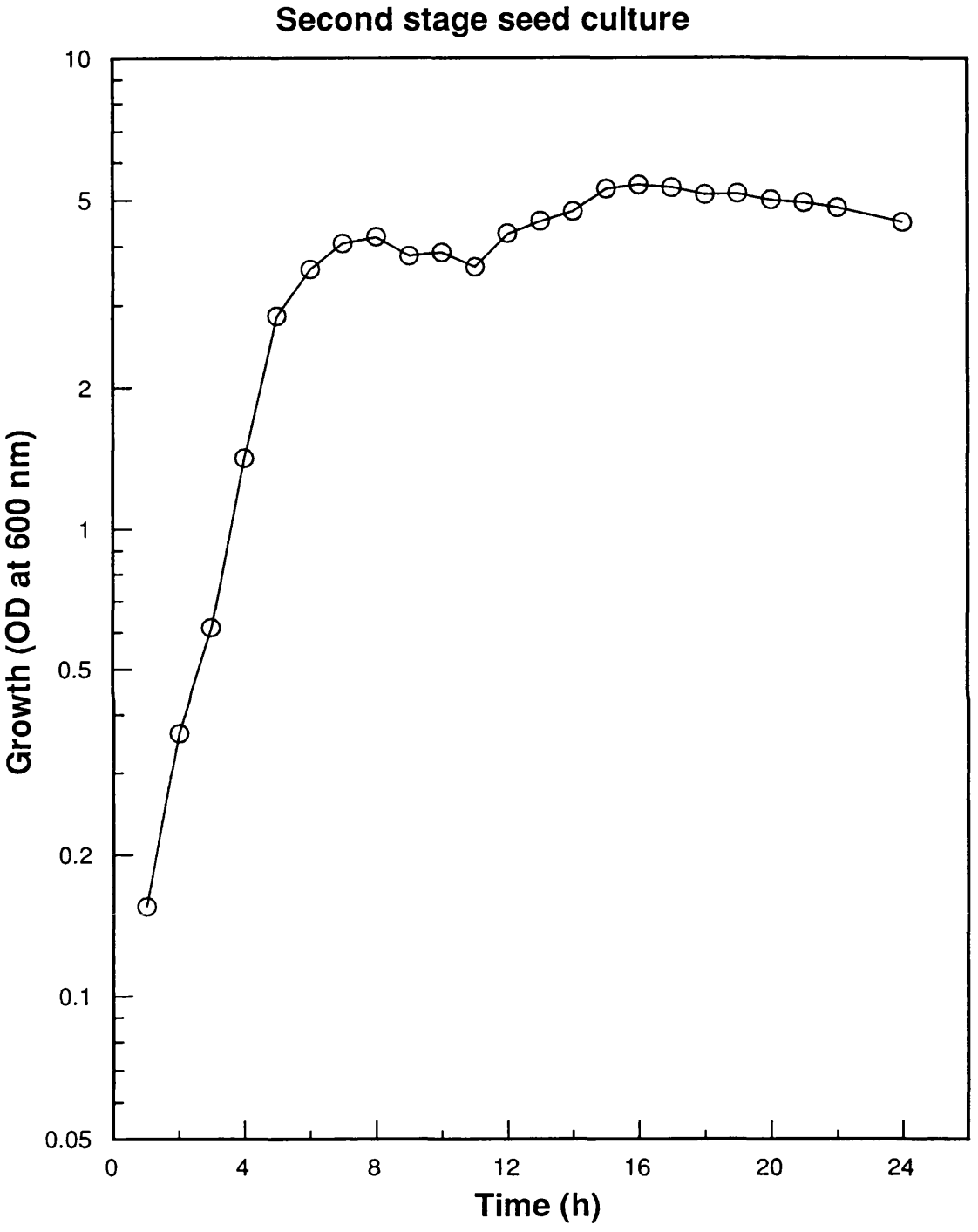


Figure 3.4 : Growth curve of the second stage inoculum culture.

In the first stage inoculum, growth was found to exponentially increase between 2 to 8 h of cultivation. At 8 h, the growth measured as the OD at 600 nm was 2.6. The growth still increased during the 8 to 14 h period but at a slower rate, reaching the maximum OD of 5.0 at 14 h. Beyond 14 h of cultivation the culture OD declined. Therefore, the 12 h of cultivation was selected as an appropriated length of the first stage inoculum to obtain the high density of active cells (OD = 4.2).

The second stage inoculum which was inoculated with 3% inoculum from the first stage exhibiting the exponential growth phase between 1 and 5 h of cultivation. The stationary phase appeared after 6 h. The maximum OD of 5.4, however, was observed at 16 h followed by a slight decrease in the biomass. The 12 h of cultivation which was in the early stationary phase was determined to be the suitable length for this stage of inoculum. The culture OD at 12 h was 4.3 which is approximately equivalent to that of the first stage. The inoculum volume used to inoculate the fermenter was 8.3% of the total culture volume (1 L inoculum in 12 L culture volume).

3.2.2 Fermentation with and without pH control

In the previous experiments, the significance of pH on product stabilities was demonstrated. Therefore, the control of this variable during fermentations was considered to be important in the interpretation of the effects of other variables on difficidin and oxydifficidin production rates. Fermentations were carried out under identical environmental conditions (i.e. agitator speed 600 rpm, air flow rate 4 L/min and temperature 28° C) with and without pH control.

The results of a culture with an uncontrolled pH in which no neutralizing agent was added are shown in Figure 3.5. After inoculation the lag phase of about 20 h during which there was no change in fermentation parameters was observed. A sharp drop in DOT level from a saturation value at 20 h to a minimum value of 26% saturation during the 40 and 43 h indicated an exponential growth phase of the culture (Figure 3.5a). During the same period of time the pH started rising from an initial value of

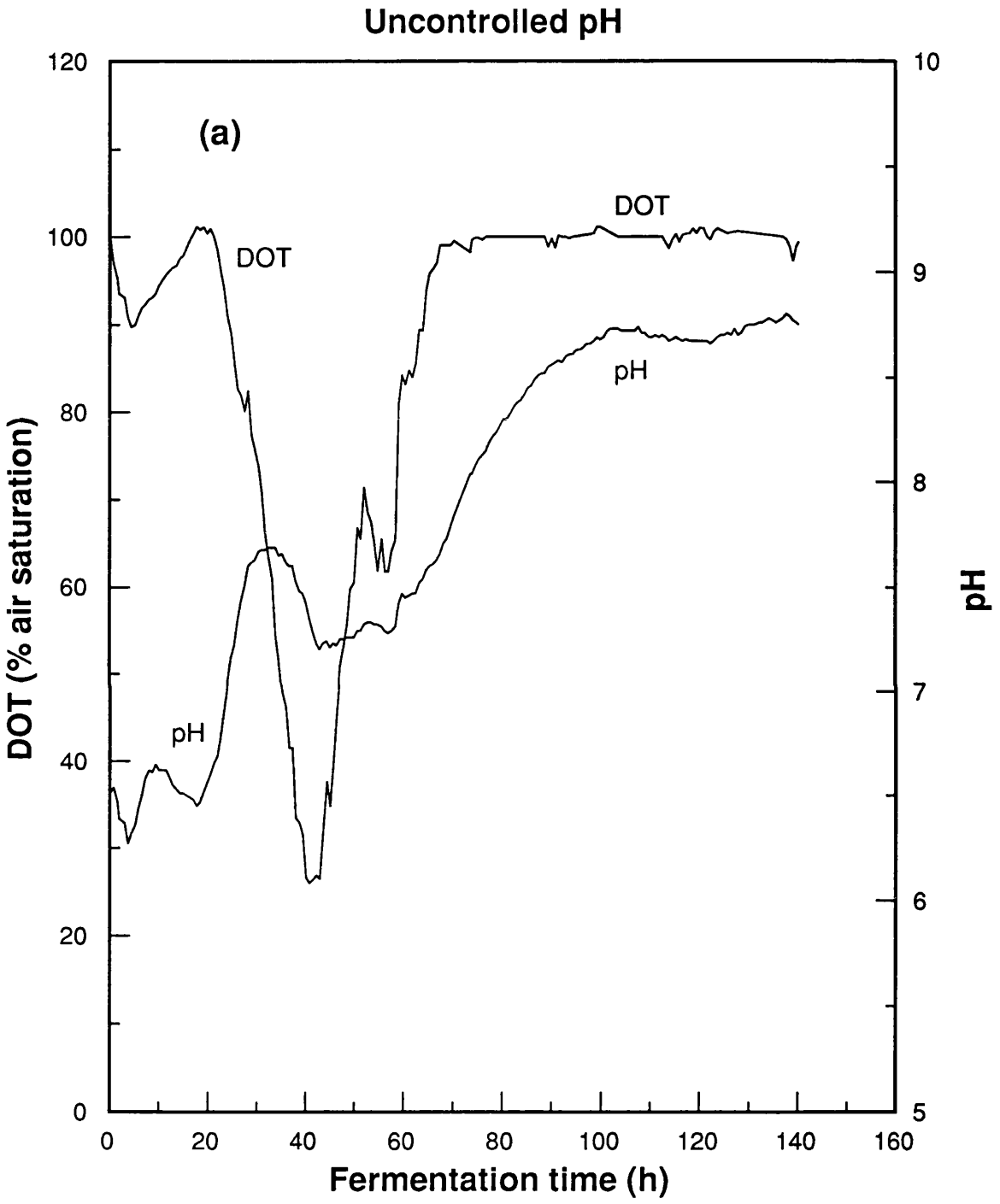


Figure 3.5 : Time course of a typical fermentation without pH control (a) DOT and pH profiles.

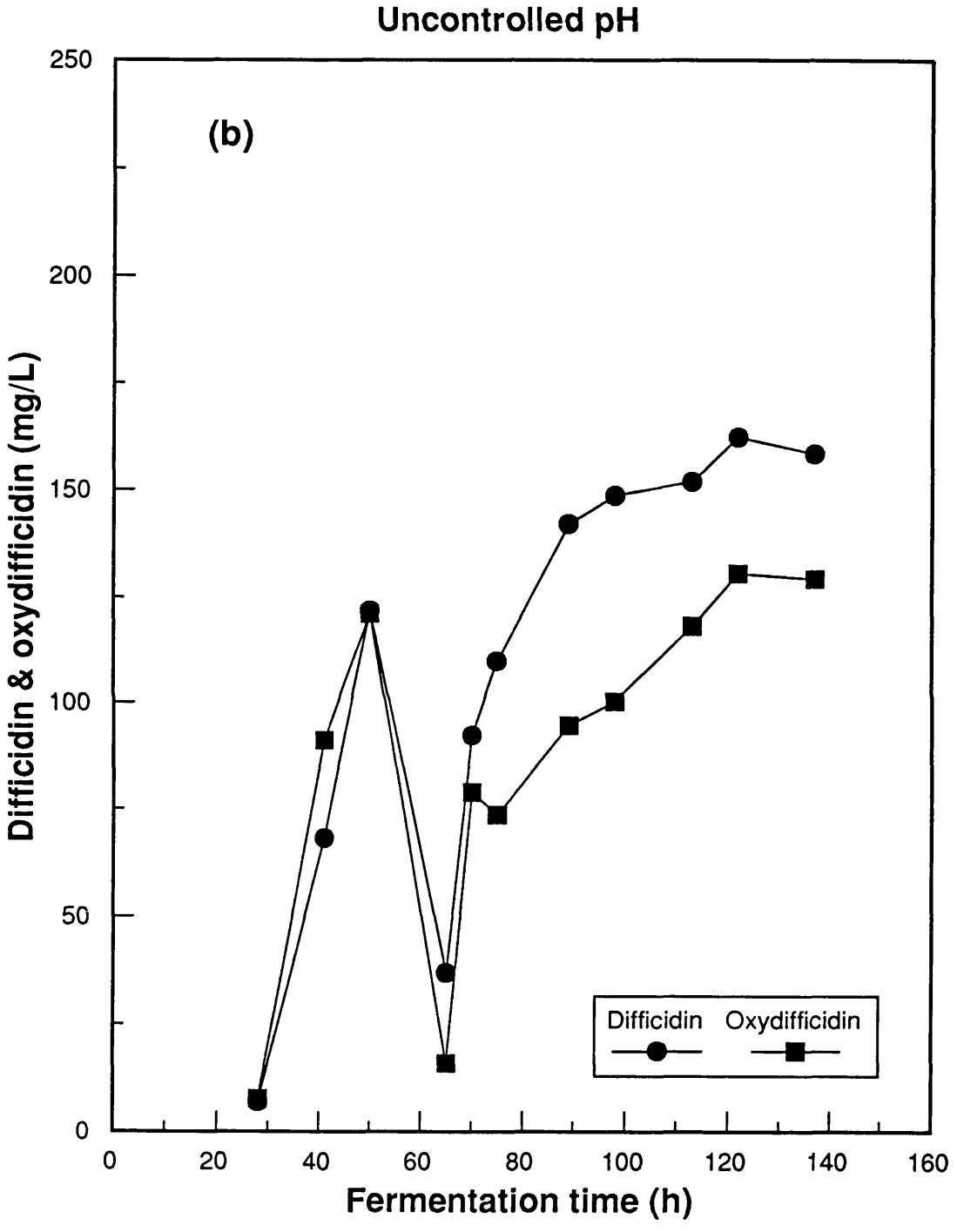


Figure 3.5: *Continued* (b) difficidin and oxydifficidin concentrations profiles.

about 6.5 at 20 h to 7.7 at the mid of the exponential growth phase (30 h) followed by a slight decrease to 7.2 at 43 h. After 43 h, the DOT increased to a saturation value at about 68 h coincided with an gradual increase of the pH to 8.8 at 100 h indicated the cessation of growth. The onset of difficidin and oxydifficidin production occurred at about 28 h of cultivation followed by a linear increase up to 121 mg/L titre of each product at 50 h (Figure 3.5b). At 65 h, however, an unexpected and sudden decrease in the yield of both antibiotics was observed, followed by a gradual increase of the antibiotic titres reaching the maxima of 162 mg/L for difficidin and 130 mg/L for oxydifficidin at about 120 h. A slight drop in the titres was observed after 120 h.

In the pH controlled fermentation where pH was controlled at 6.80 ± 0.03 by addition of either 2M NaOH or 2M H_3PO_4 (Figure 3.6), a similar DOT profile as that of the pH uncontrolled run was observed. After 20 h lag phase, there was a sudden drop in DOT and rise in OUR and CER (Figure 3.6a). The DOT level passed through a minimum value of about 38% saturation at 38 h which was coincident with the maximum OUR and CER of about 29 mmol/L.h. With the decrease in OUR and CER after 38 h, the DOT returned nearly to the saturation value. Since the OUR and CER were almost identical, the RQ values were found to be around unity throughout the course of fermentation (Figure 3.6b). Difficidin and oxydifficidin productions were linear from about 38 h to 90 h of the fermentation, reaching plateaus with the maximum titres of 200 mg/L for difficidin and 130 mg/L for oxydifficidin. A small antibiotic degradation was observed thereafter.

Summing up those results mentioned above, it can be concluded that pH showed significant effects on the antibiotics production. In order to eliminate these effects, therefore, all subsequent fermentations were conducted under a pH controlled condition. The pH controlled fermentation described in this section could be considered as a control case to which the following experiments would refer.

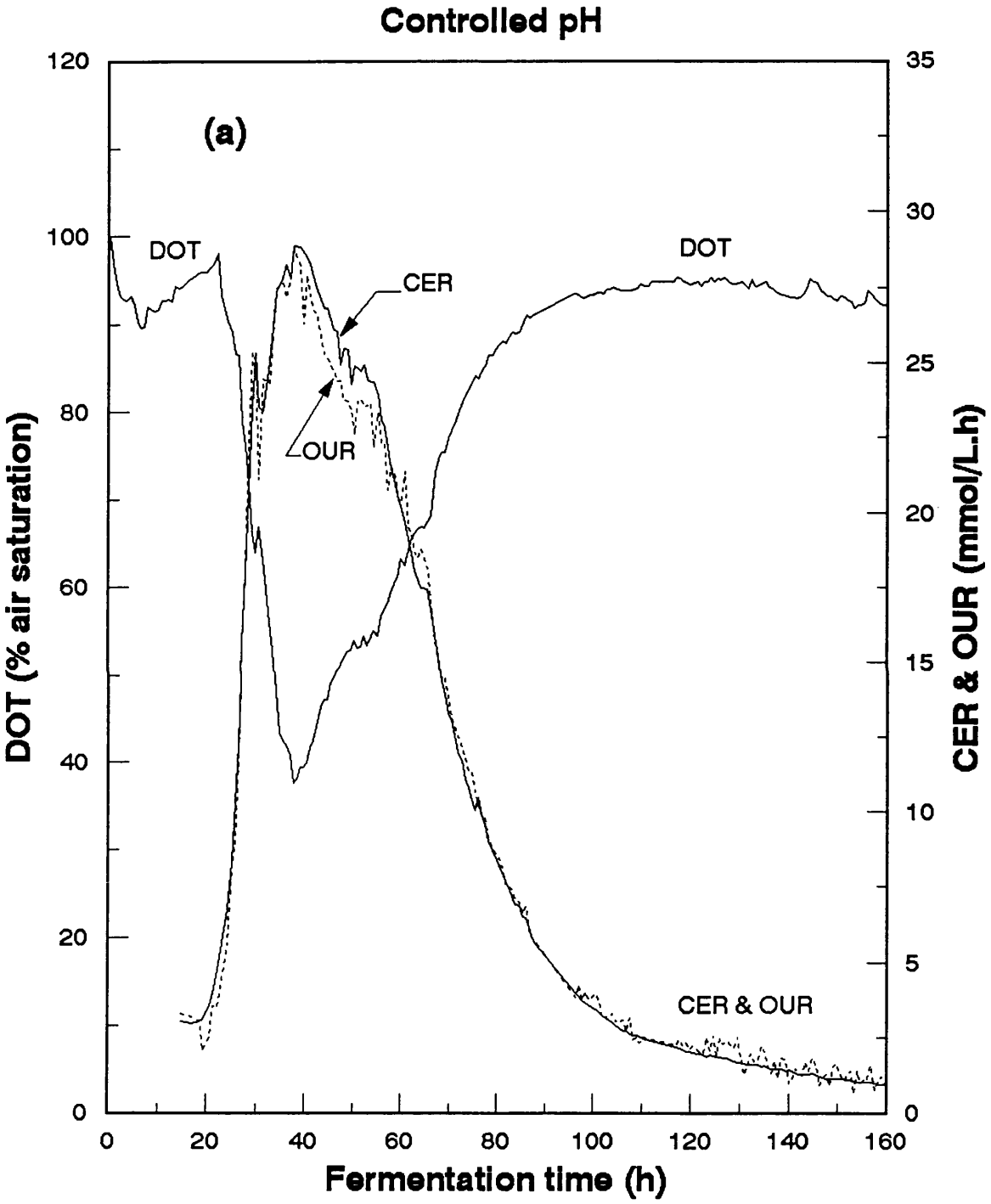


Figure 3.6 : Time course of a typical fermentation with pH control at 6.8 (a) DOT, CER and OUR profiles.

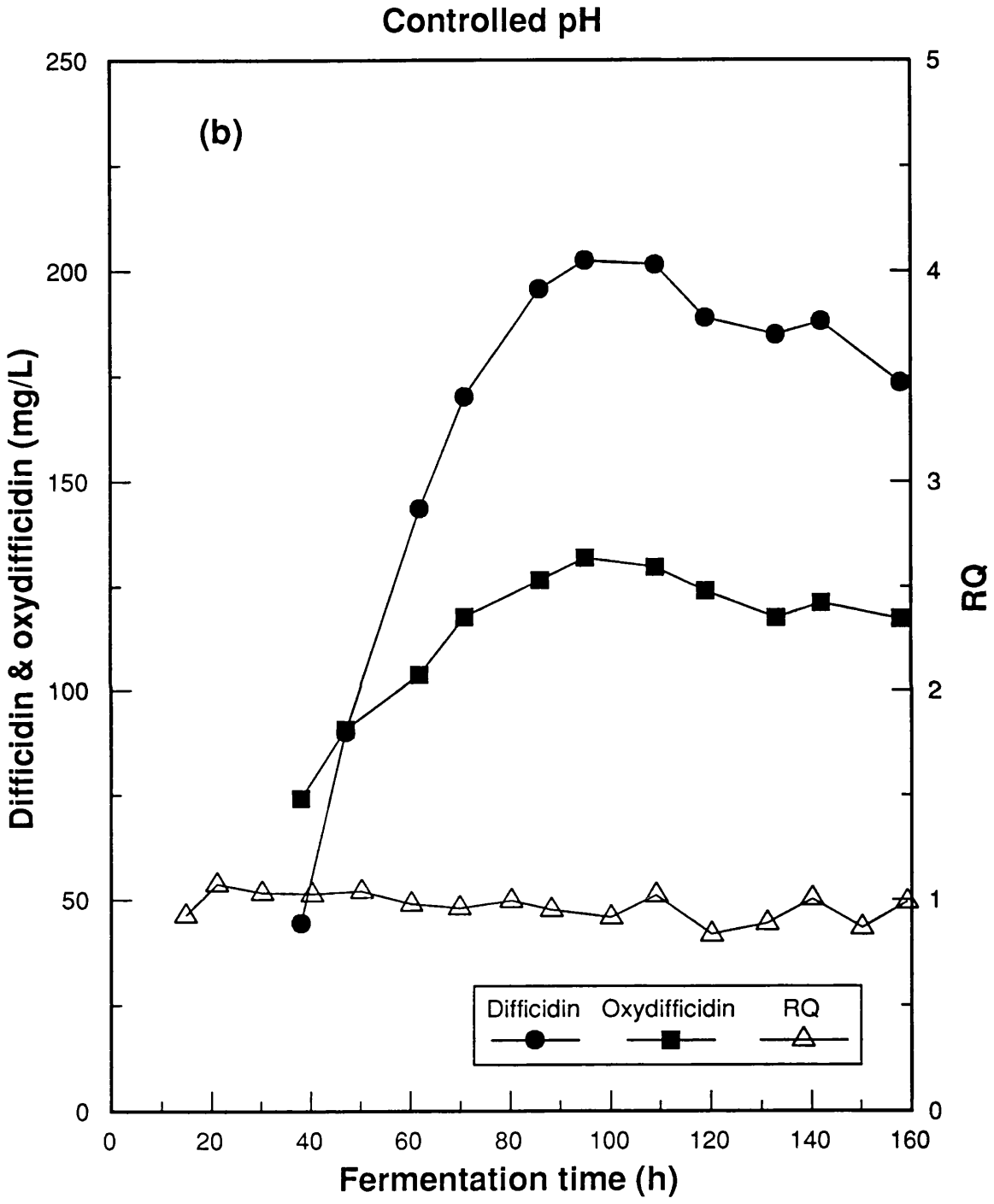


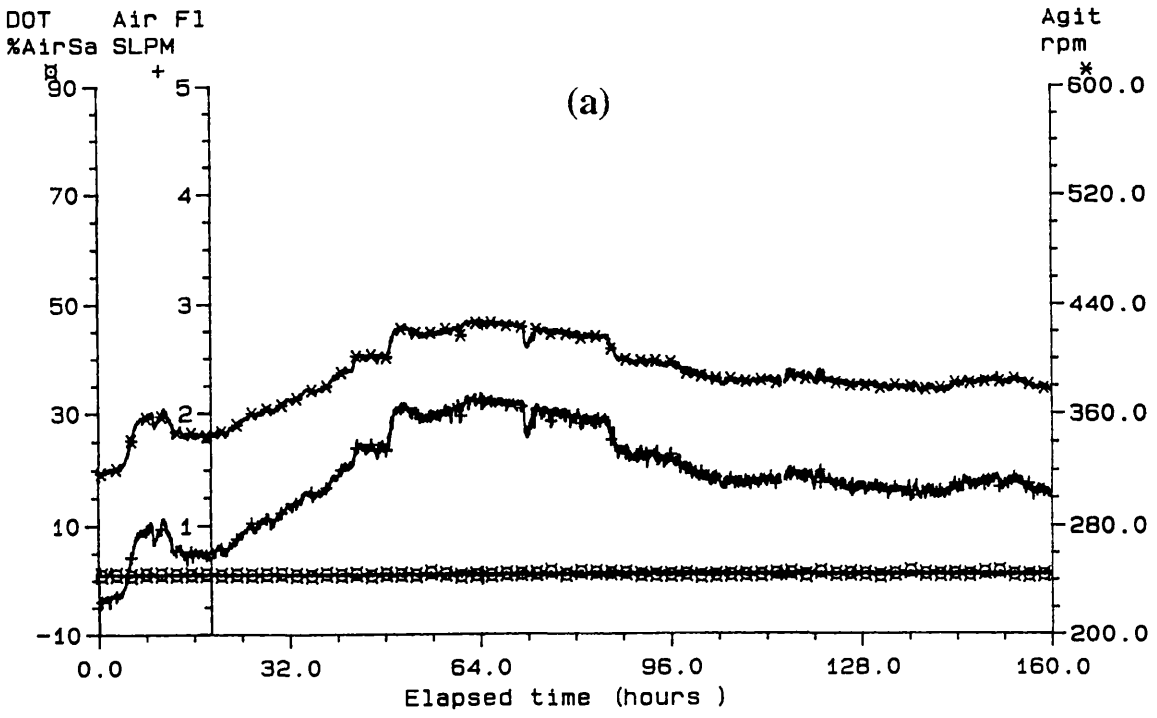
Figure 3.6 : *Continued* (b) antibiotic concentrations and RQ profiles.

3.3 Fermentations at Different Controlled DOT Levels

The fermentations run at various constant DOT levels were performed to investigate the effects of DOT on the production of difficidin and oxydifficidin antibiotics. The correlations between the production rates of the two antibiotics and DOT were determined and consequently, the critical DOT levels for the product formation. In order to achieve these main objectives, the DOT control characteristics and the fermentation profiles were carefully evaluated for each fermentation.

3.3.1 DOT control profiles

As mentioned earlier in Section 2.2.4, the control of DOT at a constant level was achieved by a simultaneous variation of agitator speed and air flow rate based on a constant basal N_2 gas flow rate. Presented in Figure 3.7 are the time profiles of agitator speed, air flow rate and DOT at different controlled levels. Despite the changing oxygen consumption rate, which is characteristic of batch cultures, the controller provided an excellent DOT control in which the DOT level was maintained with ±0.5% accuracy throughout the fermentations. As expected, the changes in both agitator speed and air flow rate paralleled changes in the rate of oxygen consumption which was, in turn, dependent on the biomass concentration in the culture broth (see also Section 3.3.2). The low levels of agitator speed and air flow rate at the beginning of fermentation were followed by a sharp increase during the exponential growth phase (except the 1% DOT run where linear growth was observed) and reached a plateau range between the 50 - 85 h period in the 1% DOT run, the 16 - 55 h period in the 5%, 10%, 15% and 20% DOT runs, the 24 - 60 h period in the 40% DOT run and the 36 - 40 h period in the 80% DOT run. After this plateau the agitator speed and air flow rate declined with a decrease in the respiration intensity of the culture. The difference in the profile patterns of these two variables was easily explained by the difference in the growth profiles of the culture at different DOT levels.



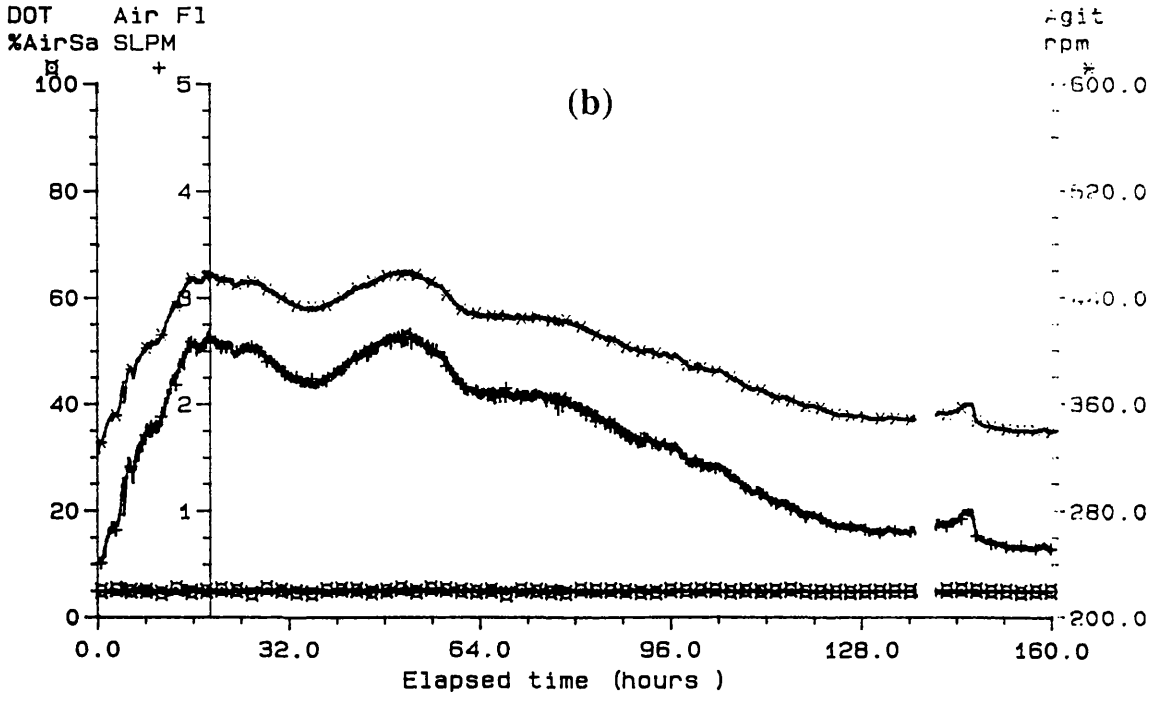
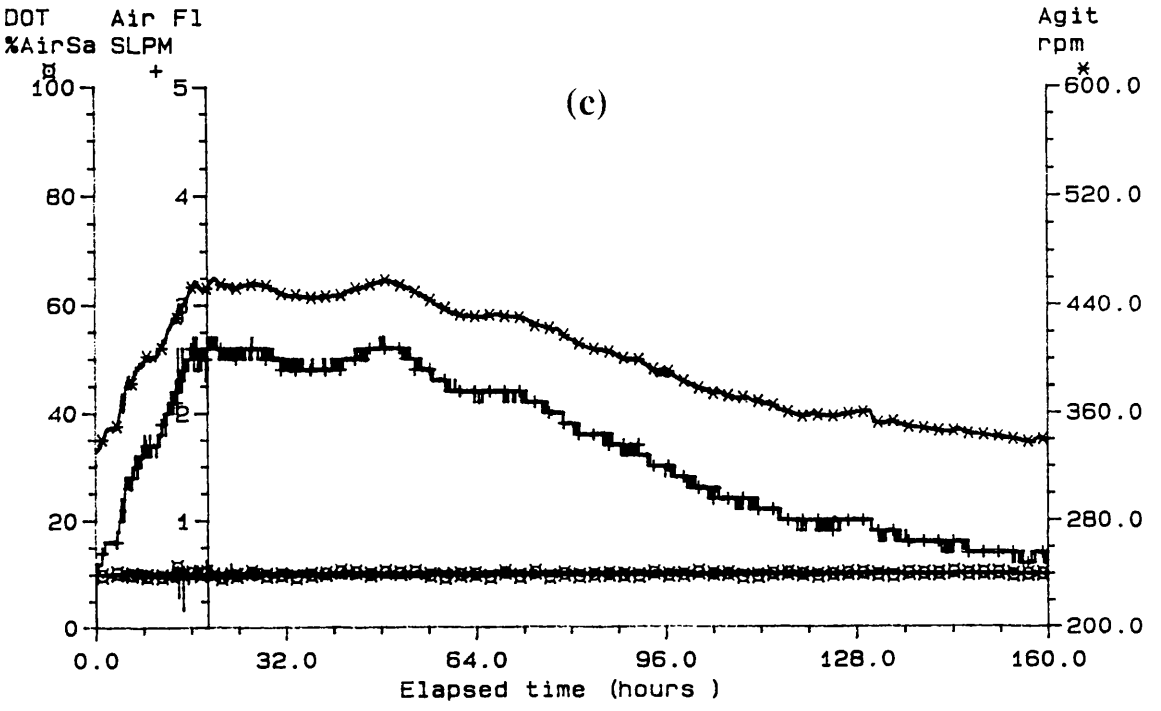


Figure 3.7 : Time profiles of an agitator speed, air flow rate and DOT at various controlled levels (a) 1% DOT (b) 5% DOT.



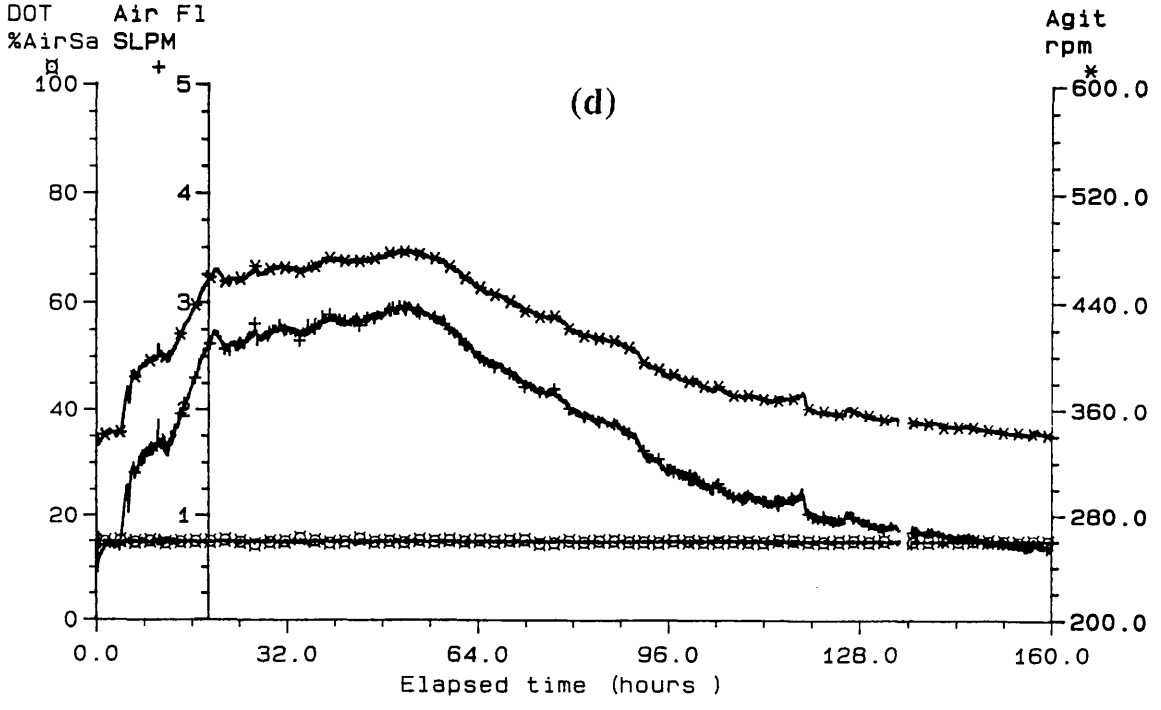
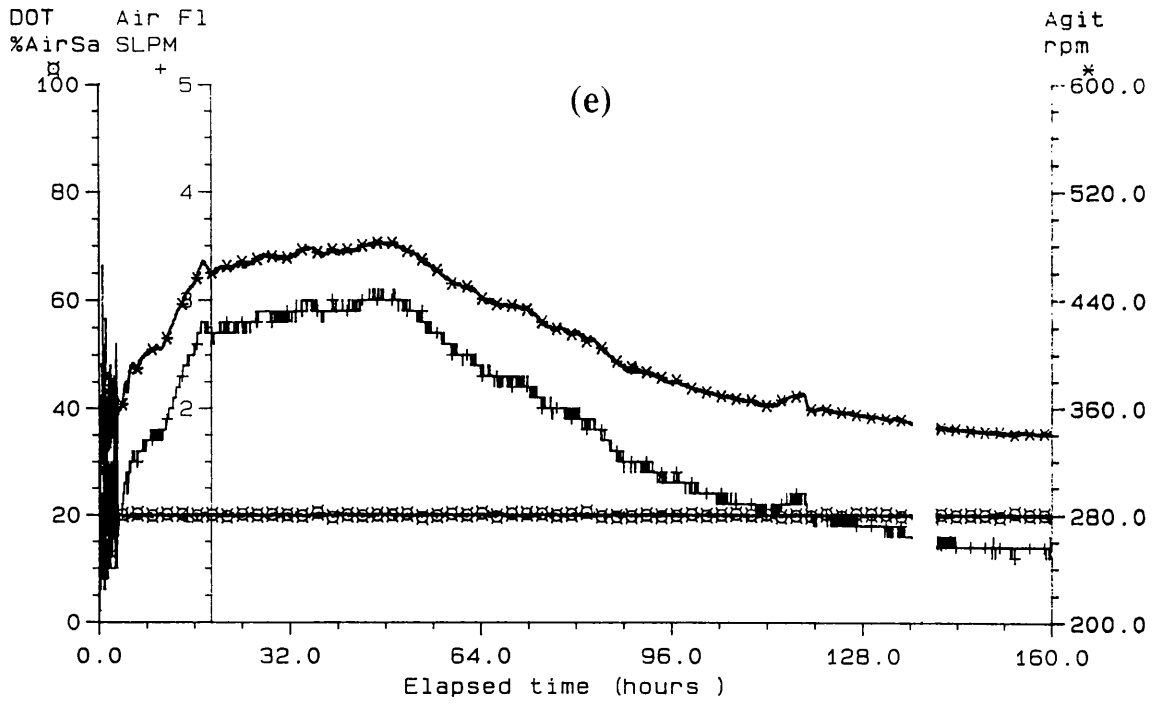


Figure 3.7 : *Continued* (c) 10% DOT (d) 15% DOT.



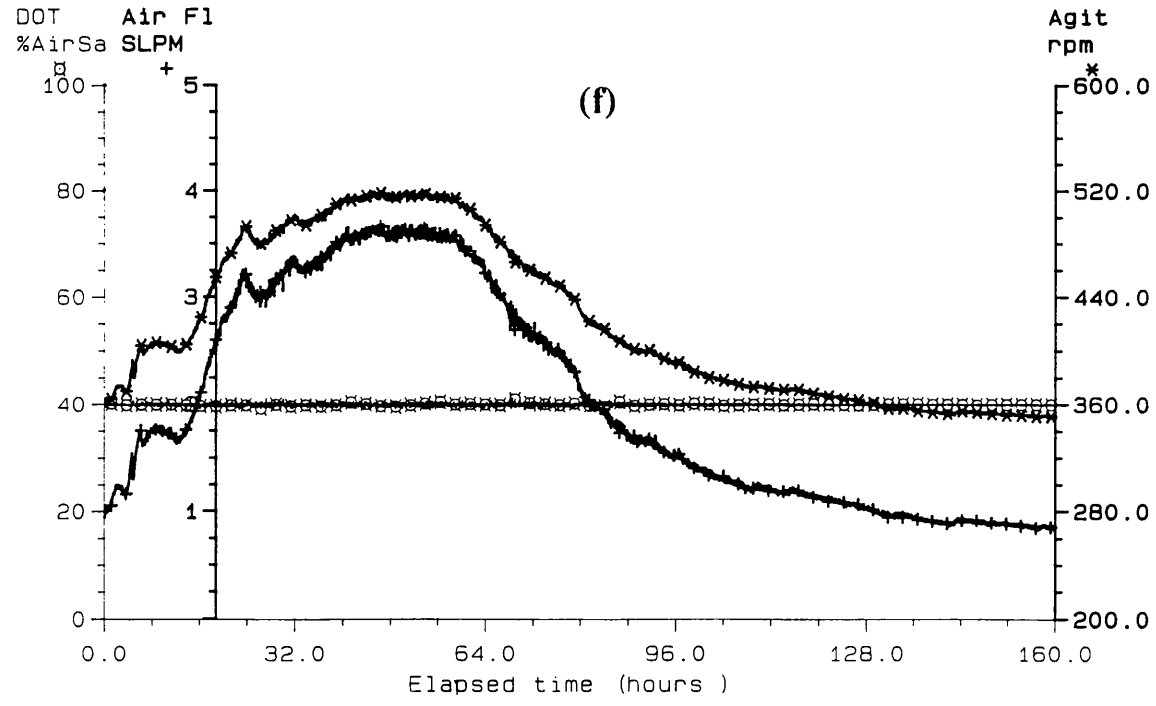


Figure 3.7 : *Continued* (e) 20% DOT (f) 40% DOT.

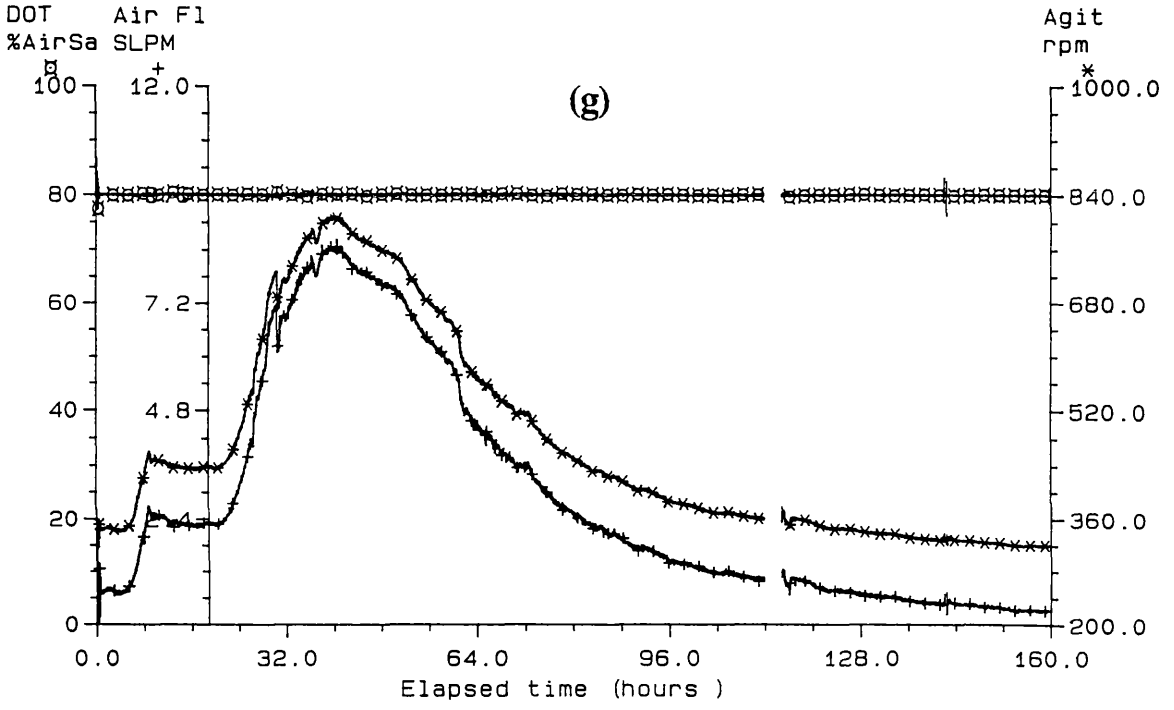


Figure 3.7 : *Continued* (g) 80% DOT.

3.3.2 Fermentation performance at different DOT levels

A series of fermentation runsat various controlled constant DOT levels was performed to investigate the effects of DOT on the fermentation performance , i.e. growth, antibiotics production and respiratory activity. In all experiments the culture pH and temperature were controlled at 6.8 and 28° C respectively to eliminate the effects of these variables. The respiratory activity was monitored by CER, OUR and RQ values. For the low DOT experiments (i.e. 1 to 40% air saturation) in which air was partially replaced by nitrogen, the CER, OUR and RQ values could not be calculated by the BIO-i system due to the composition of inlet gas differing from the composition of air used as a reference for calculation (see Section 2.1.3.3). Therefore, the calculation procedure of CER, OUR and RQ values from the gas analysis data was developed for these experiments as described in detail in Appendix A. Samples were taken from the fermenter every 5 h during the working day for the determination of biomass and antibiotic concentrations except the 80% DOT run where 9 - 10 h periods of sampling were applied. The CER, OUR and RQ values were calculated and presented every hour throughout the course of fermentation. All the antibiotic production rates mentioned below were determined during the growth phase when production was almost linear.

The fermentation run at 1% controlled DOT level showed a substantial reduction in both respiratory activity (Figure 3.8a) and antibiotics production (Figure 3.8b), as compared to when DOT was not controlled (Figure 3.6). The reduction was observed in both the rate and extent of difficidin production whereas the only amount but not the rate of oxydifficidin production was affected. The maximum CER and OUR values were three-fold lower than those obtained in the uncontrolled DOT experiment. The biomass concentration increased in a parallel manner to the increase in CER and OUR and reached the maximum value of 3.33 g/L at 85 h which was about 20 h later than the maximum OUR. A sharp increase in biomass concentration from 3.33 to 4.07 g/L during a period of 138 - 158 h was unexpected due to the low CER and OUR values observed during this period.

When the DOT level was controlled at 5% saturation throughout the fermentation, the CER and OUR profiles exhibited two distinct peaks at 20 h and 50 h of cultivation

(Figure 3.9). The second peak was slightly higher than the first one. The biomass concentration profile, as expected, followed the CER and OUR profiles reaching the maximum value of 3.89 g/L at 61 h which was about 10 h later than the second peak of CER and OUR. The rate and extent of difficidin production were not significantly different from those of the 1% DOT fermentation. Although the maximum titre of oxydifficidin was slightly higher in the 5% DOT run, the production rate was almost the same as when the DOT was controlled at 1% saturation.

The 10% controlled DOT fermentation (Figure 3.10) showed the increasing trends of the fermentation performances. The profile patterns of CER, OUR and biomass concentration, however, were similar to the 5% DOT run. The linear production rate and the maximum titre of difficidin fell between those of the uncontrolled and 5% DOT fermentations. The variation in oxydifficidin production rate from the previous runs was not significant.

When the DOT was controlled at 15% saturation, a single large peak of CER and OUR profiles occurred during 20 - 50 h of fermentation which corresponding to a single peak profile of biomass concentration (Figure 3.11). The maximum biomass concentration of 4.43 g/L obtained in this fermentation was slightly higher than in the previous DOT controlled runs. The difficidin production rate increased whereas the oxydifficidin production profile remained unchange as compared to the 10% DOT fermentation.

A further increase in both the rate and extent of difficidin production was also demonstrated in the 20% controlled DOT fermentation (Figure 3.12). The oxydifficidin and biomass concentration profiles, however, were not significantly affected by increasing DOT from 15 to 20% saturation.

The fermentations performed under the controlled DOT levels of 40 and 80% saturation showed no further increase in the difficidin production as well as the oxydifficidin production remained the same (Figures 3.13 and 3.14). On the other hand, the maximum CER and OUR values were still increasing. The maximum biomass concentration of the 40% DOT run was 4.76 g/L or about 10% higher than that of the 20% DOT run.

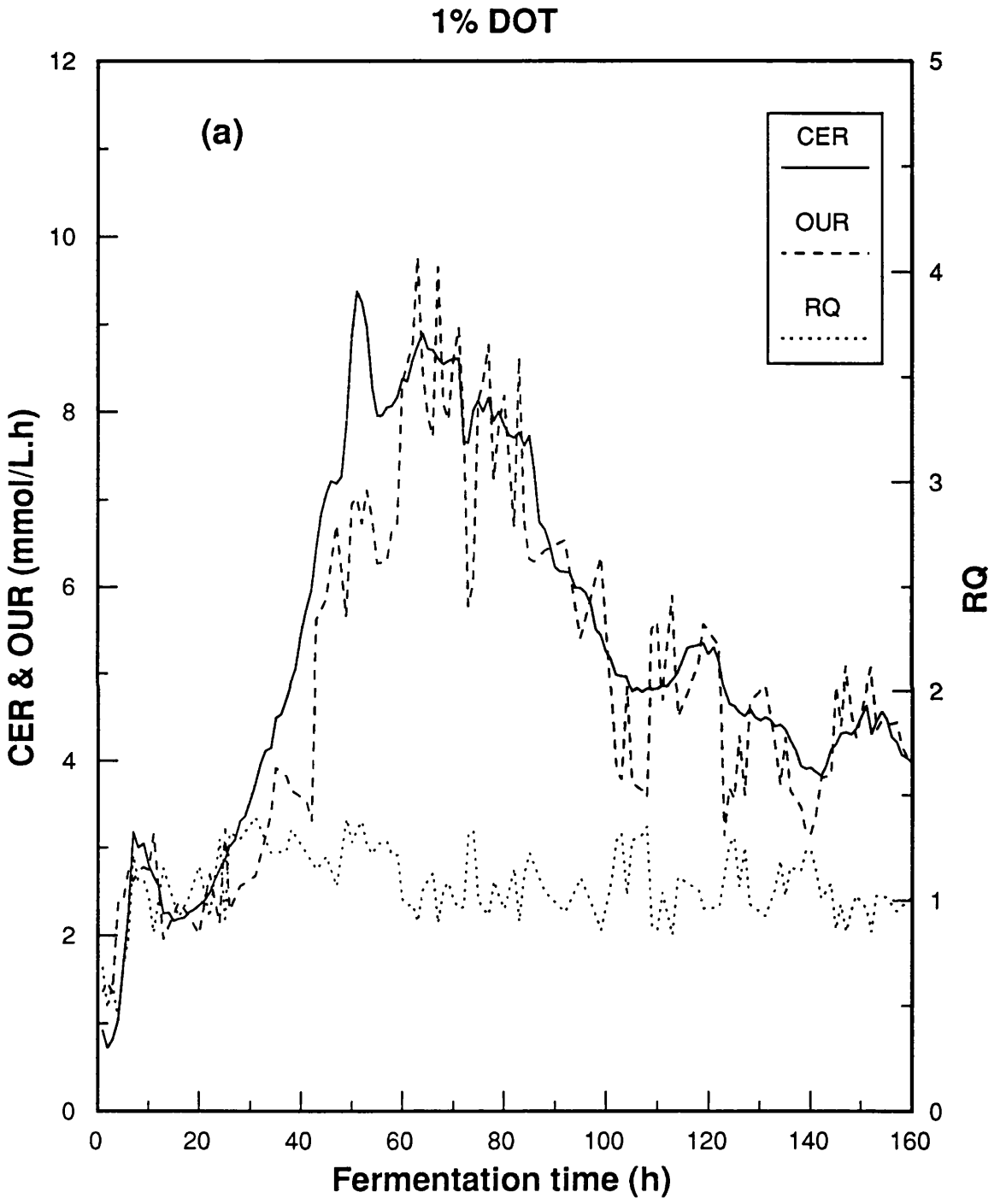


Figure 3.8 : Time course of fermentation with DOT control at 1% saturation (a) CER, OUR and RQ profiles.

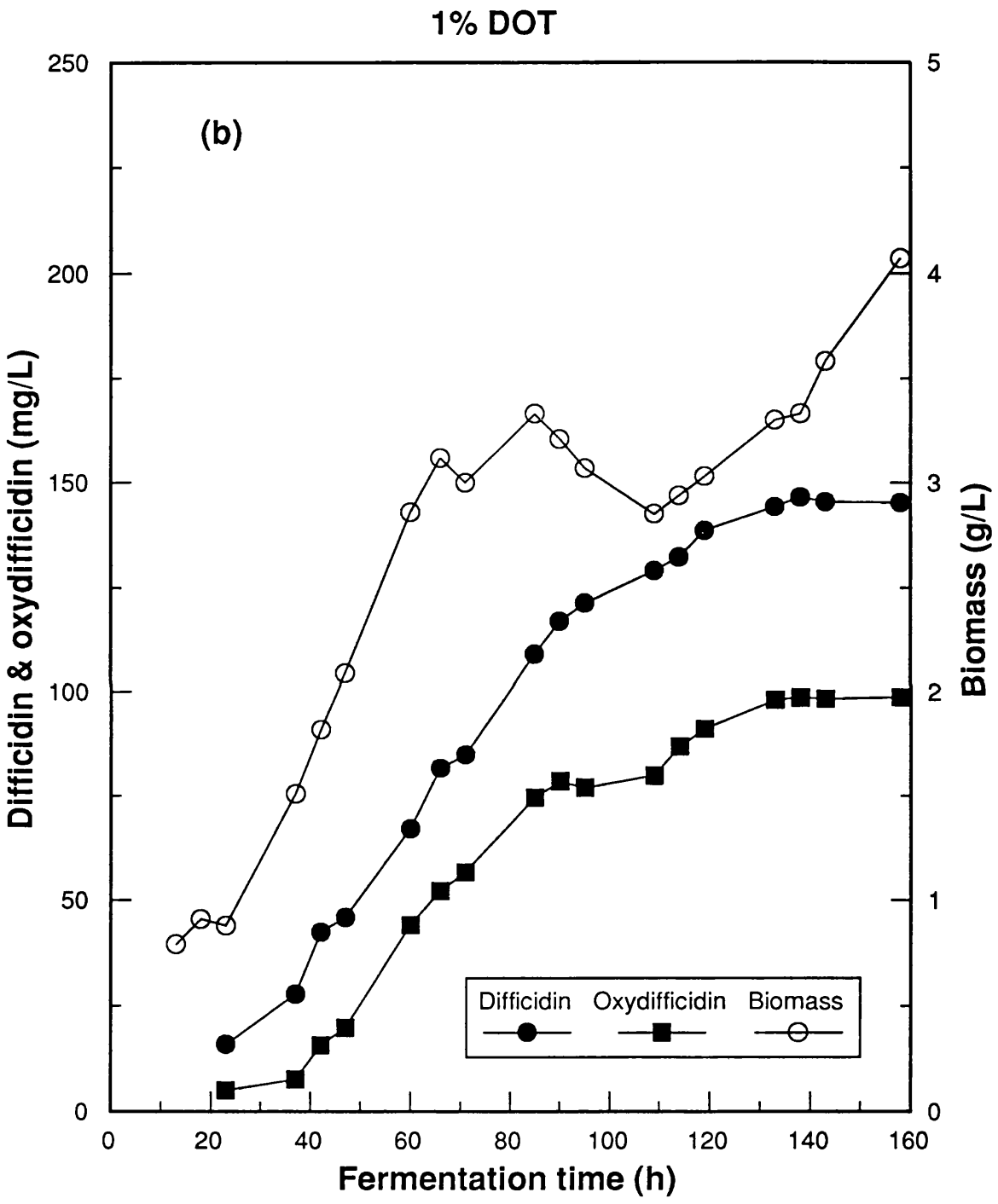


Figure 3.8 : *Continued* (b) antibiotic and biomass concentration profiles.

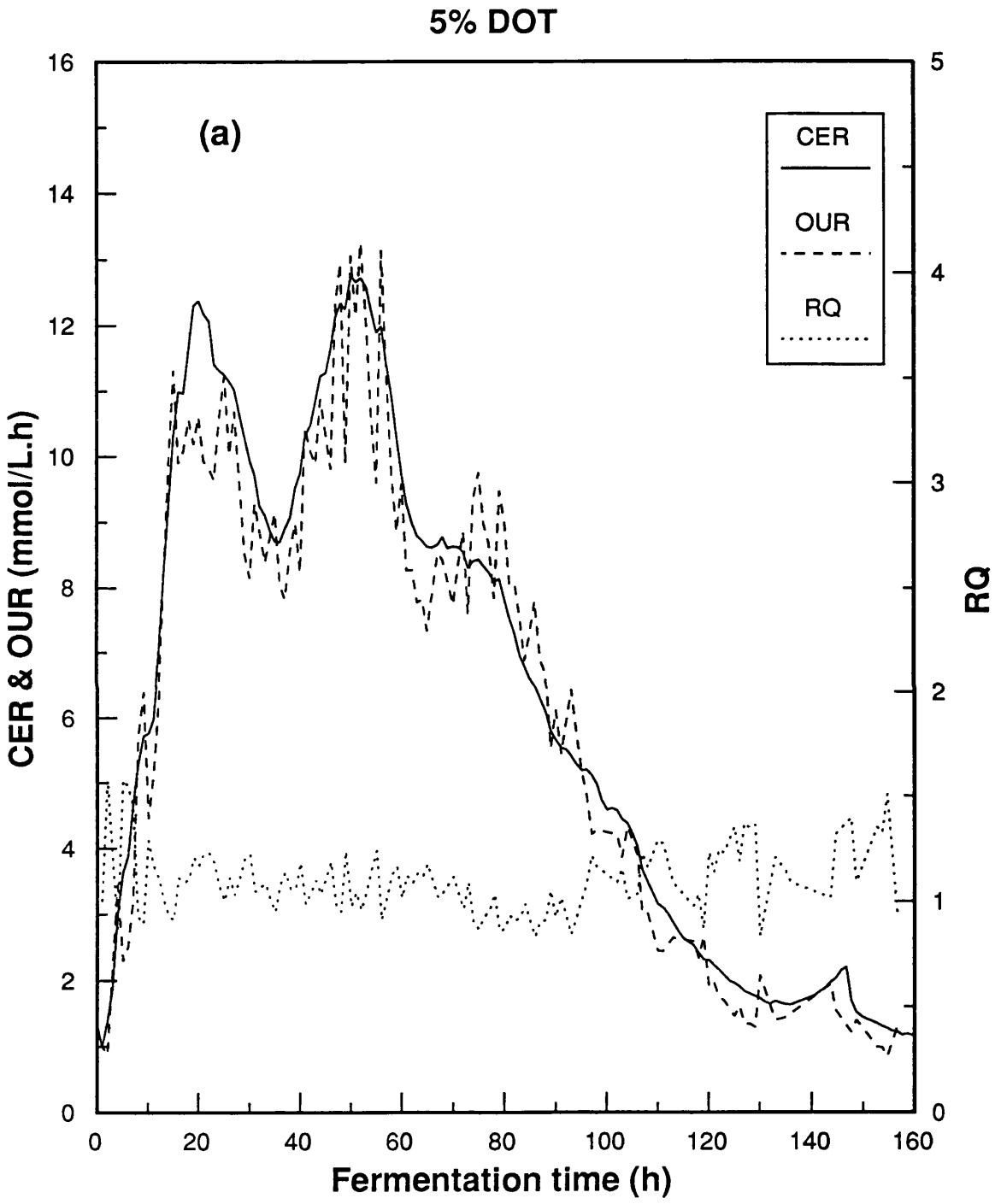


Figure 3.9 : Time course of fermentation with DOT control at 5% saturation (a) CER, OUR and RQ profiles.

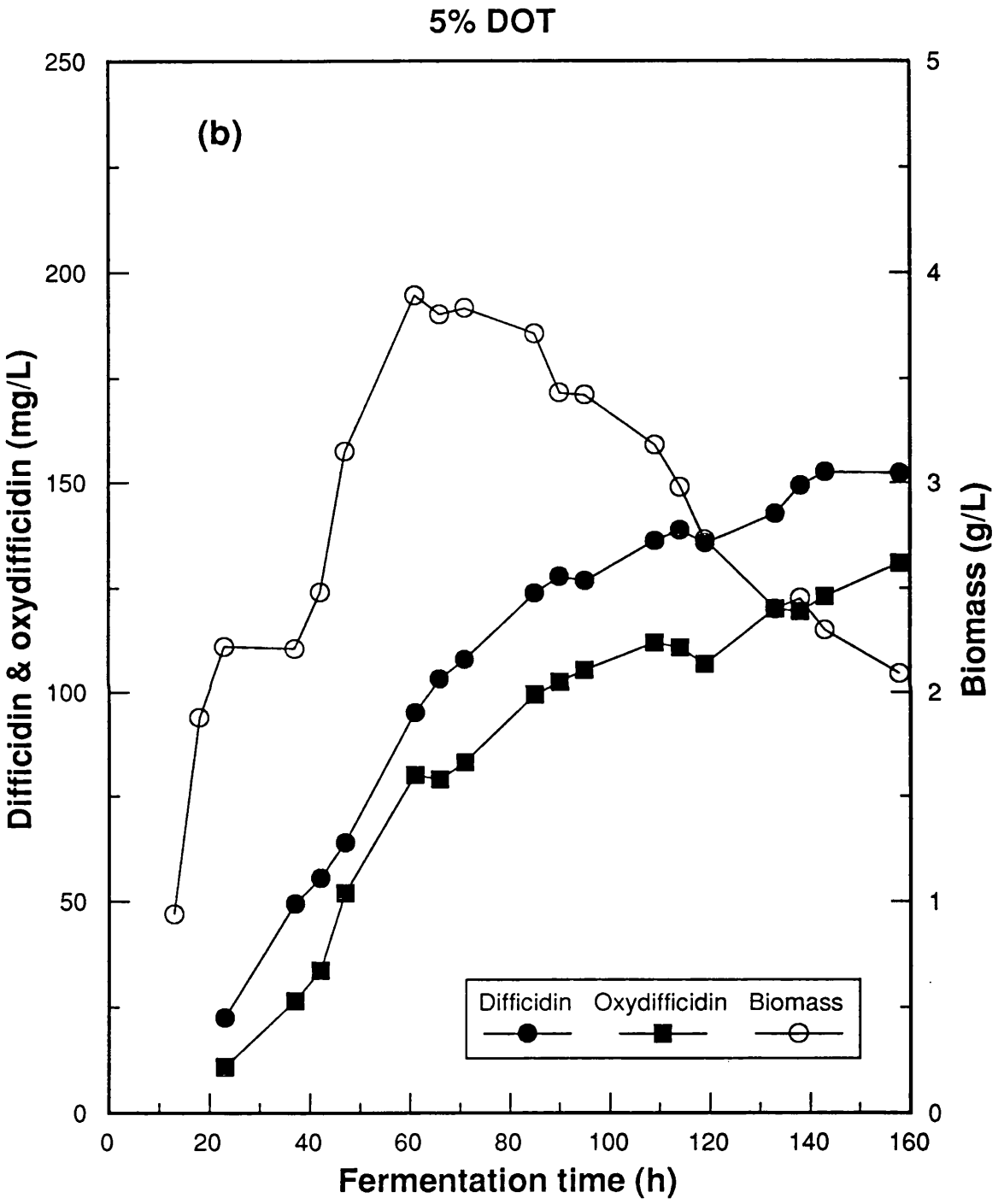


Figure 3.9 : *Continued* (b) antibiotic and biomass concentration profiles.

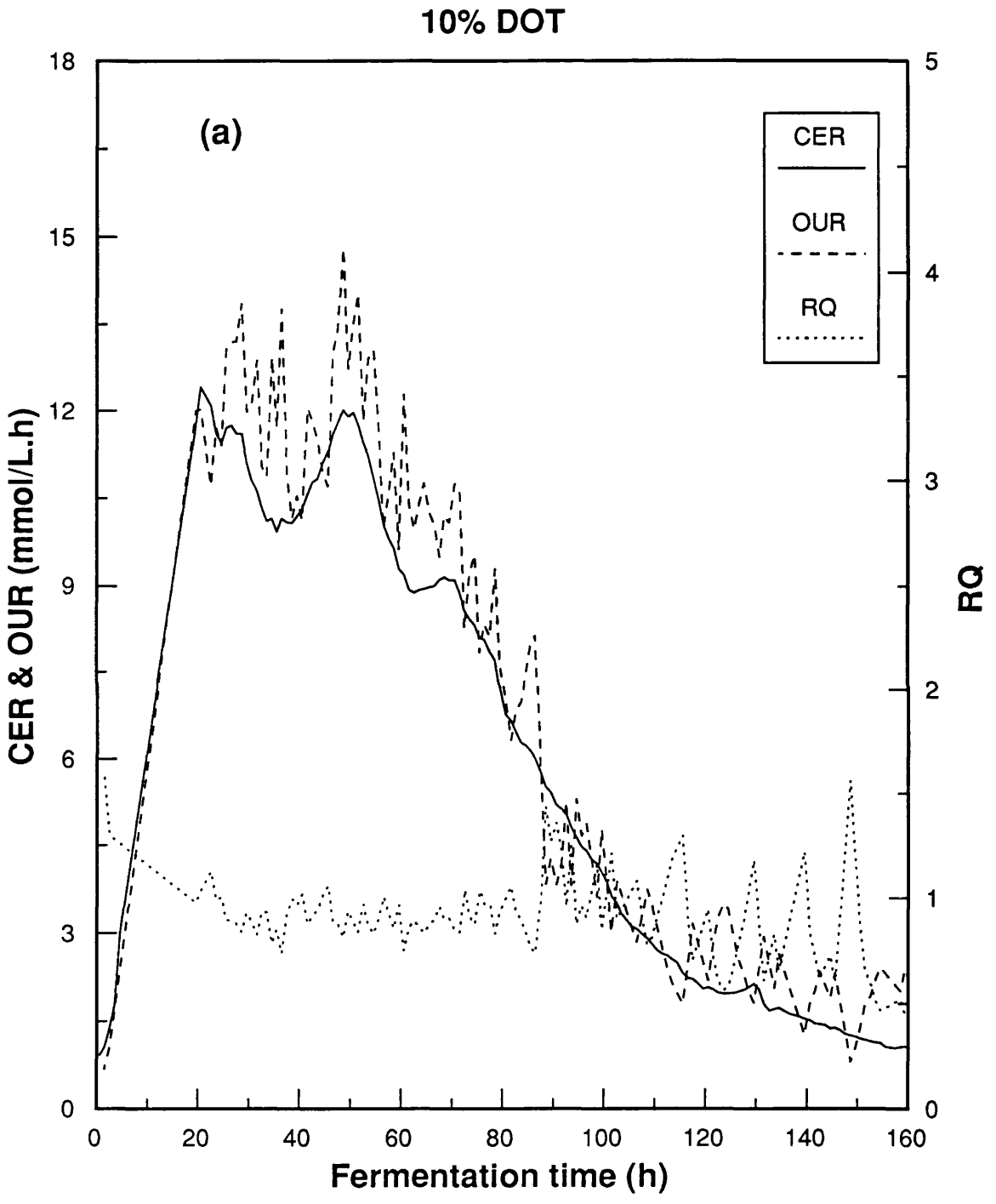


Figure 3.10 : Time course of fermentation with DOT control at 10% saturation (a) CER, OUR and RQ profiles.

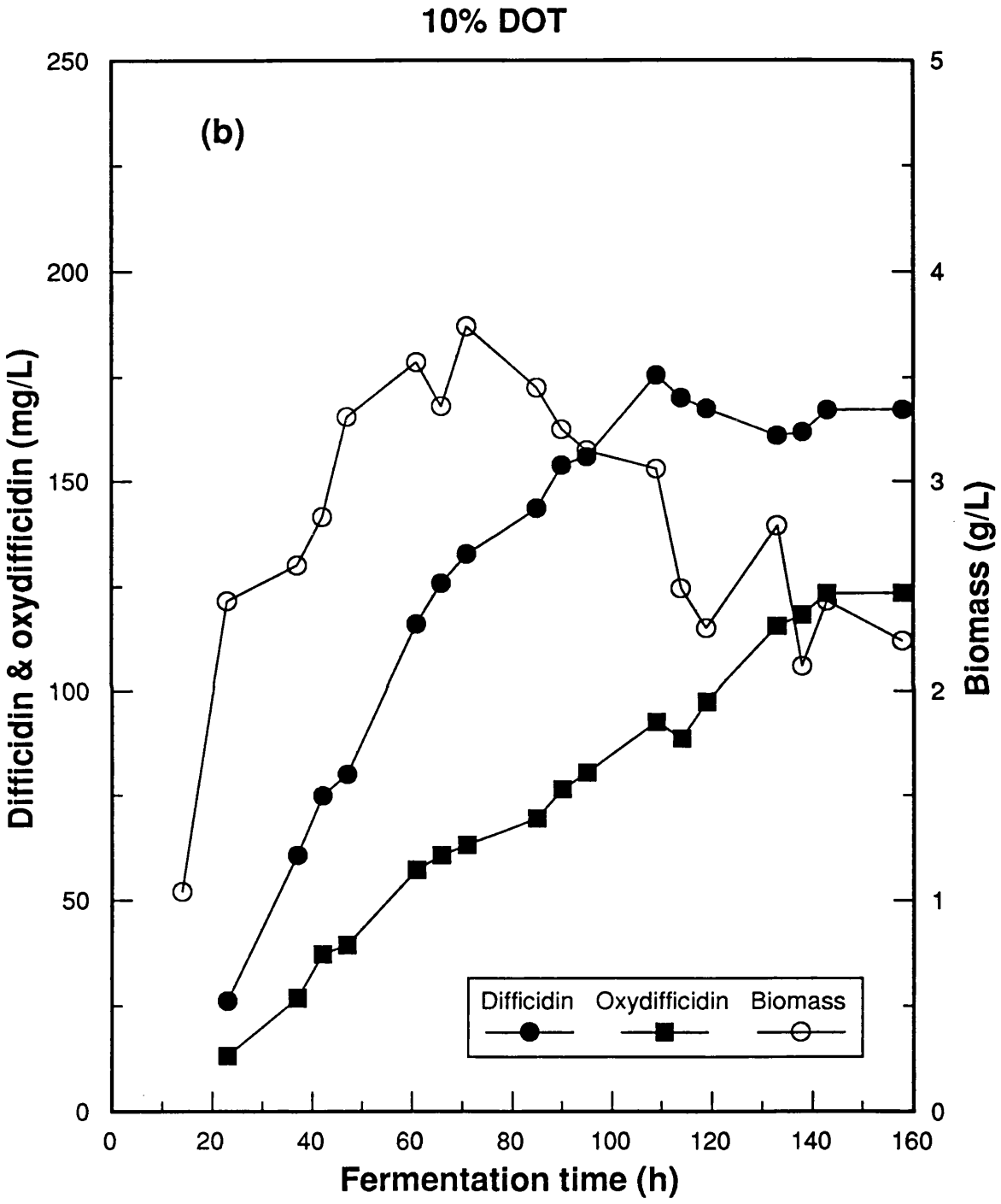


Figure 3.10 : *Continued* (b) antibiotic and biomass concentration profiles.

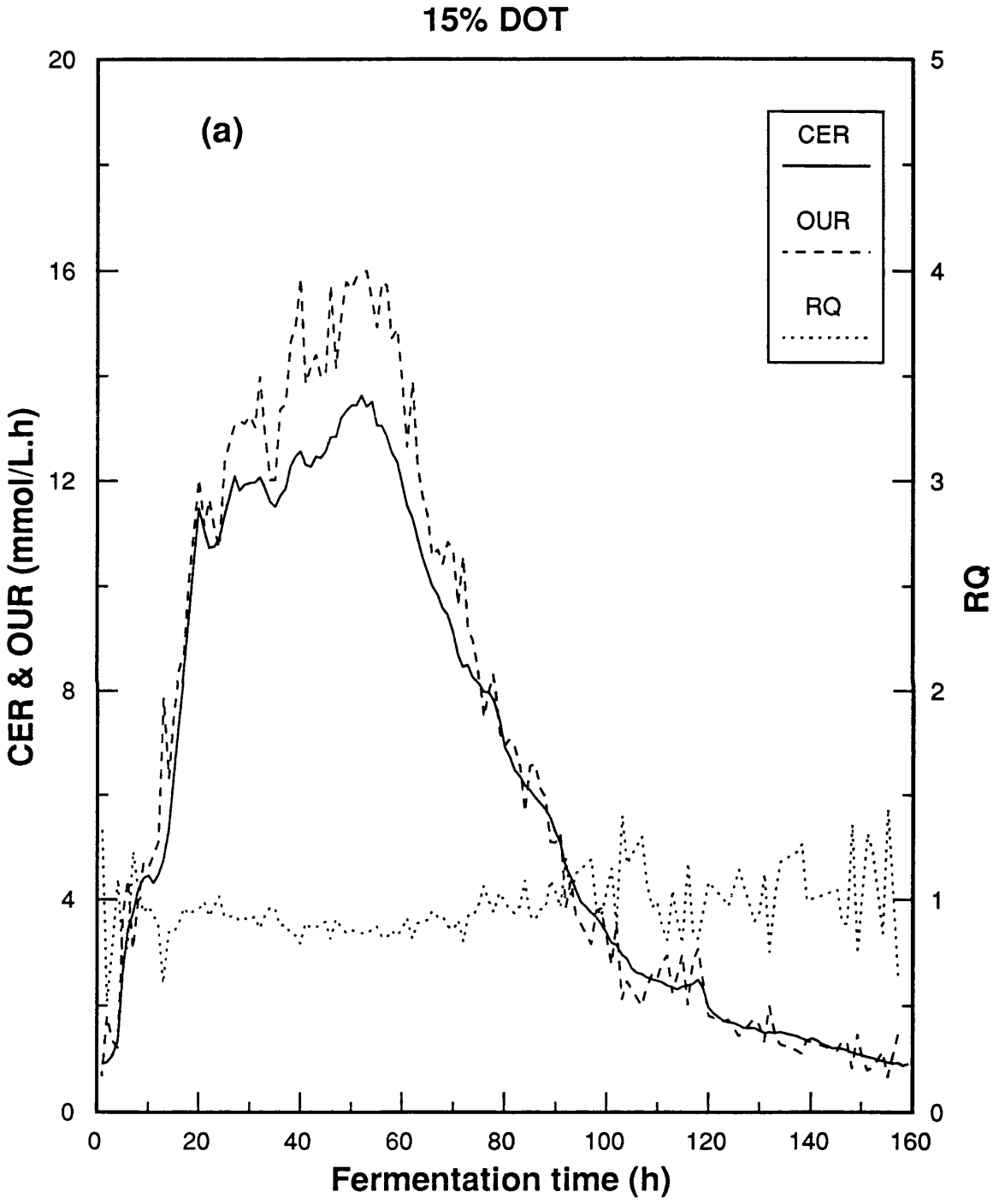


Figure 3.11 : Time course of fermentation with DOT control at 15% saturation (a) CER, OUR and RQ profiles.

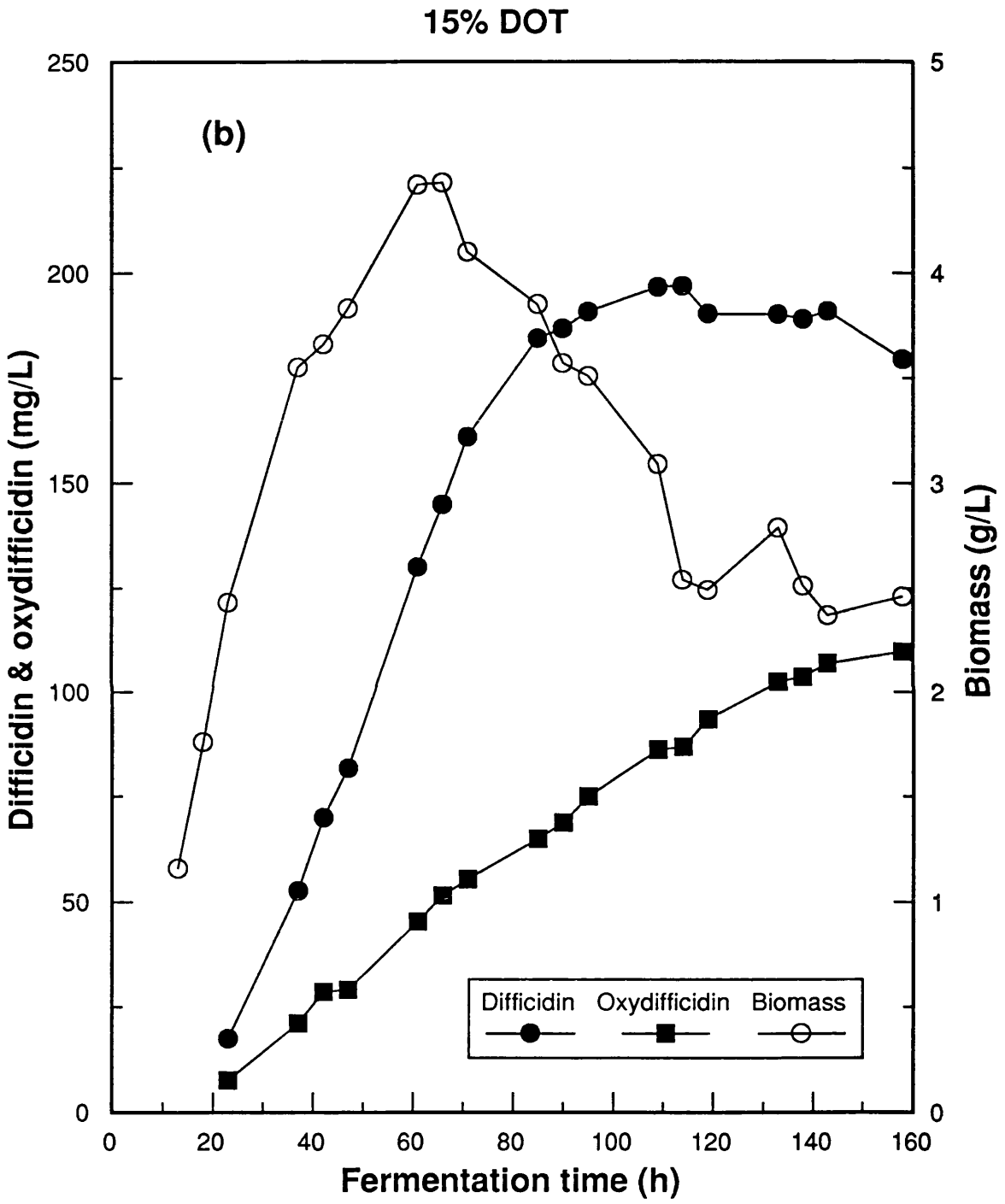


Figure 3.11 : *Continued* (b) antibiotic and biomass concentration profiles.

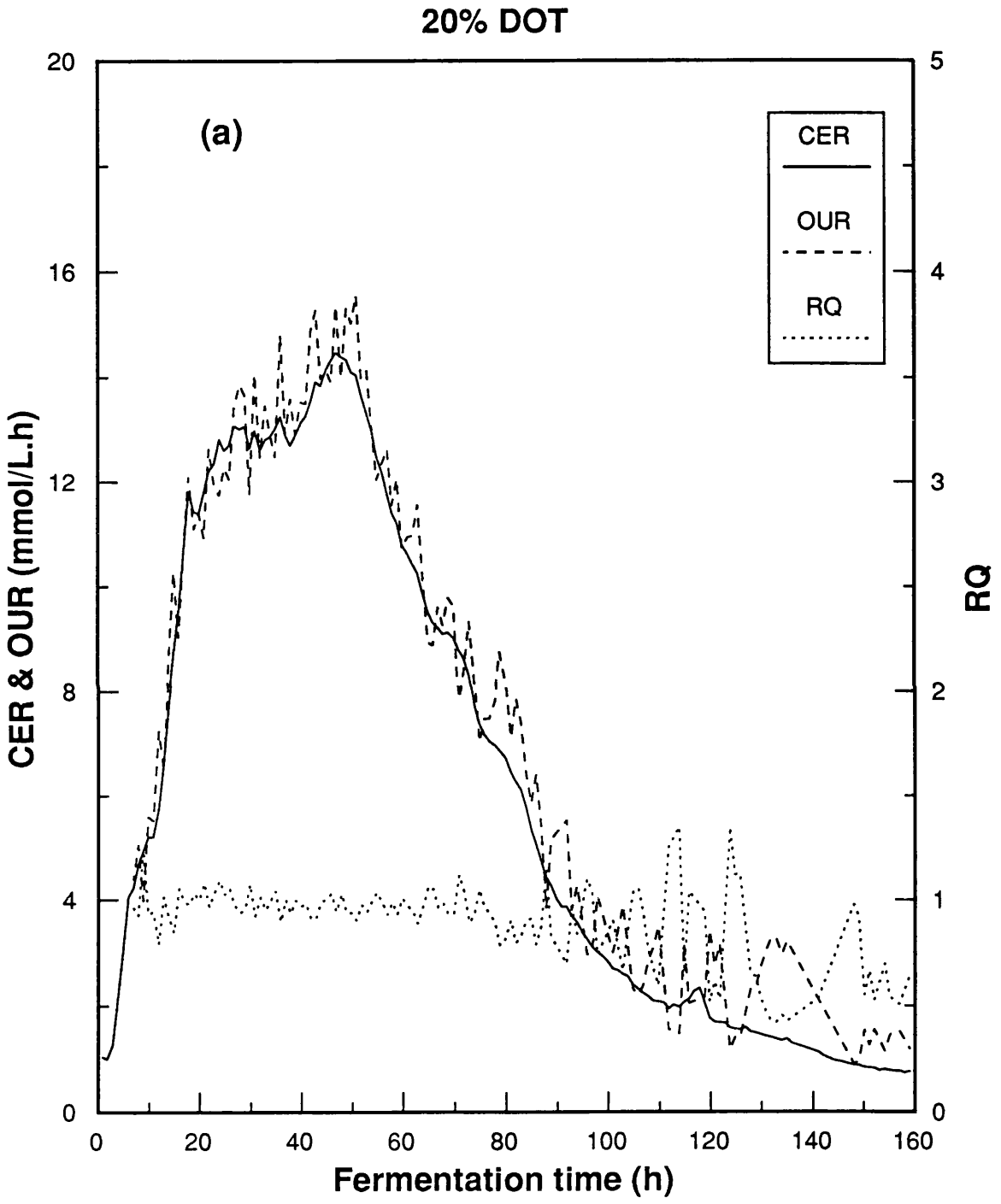


Figure 3.12 : Time course of fermentation with DOT control at 20% saturation (a) CER, OUR and RQ profiles.

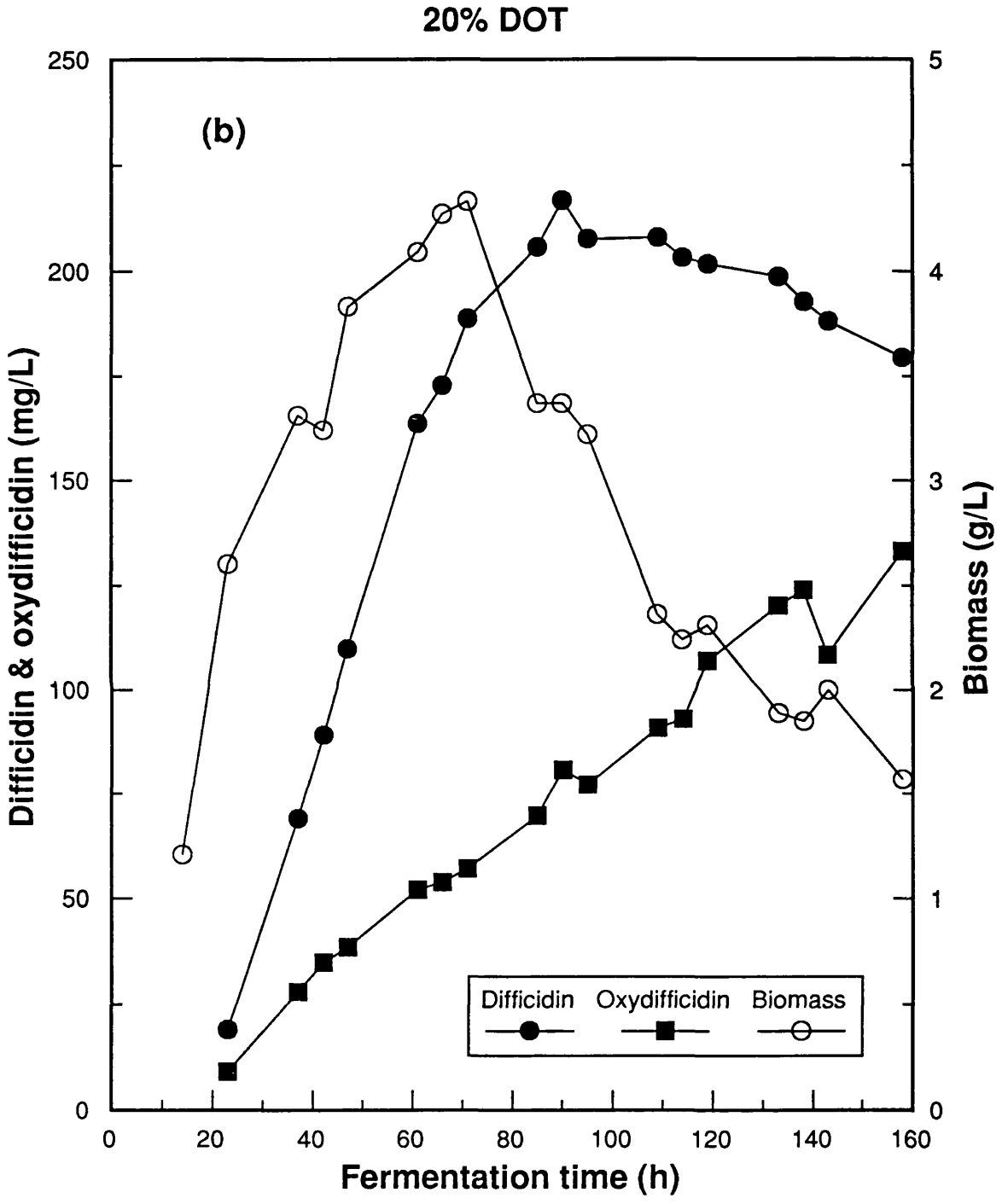


Figure 3.12 : *Continued* (b) antibiotic and biomass concentration profiles.

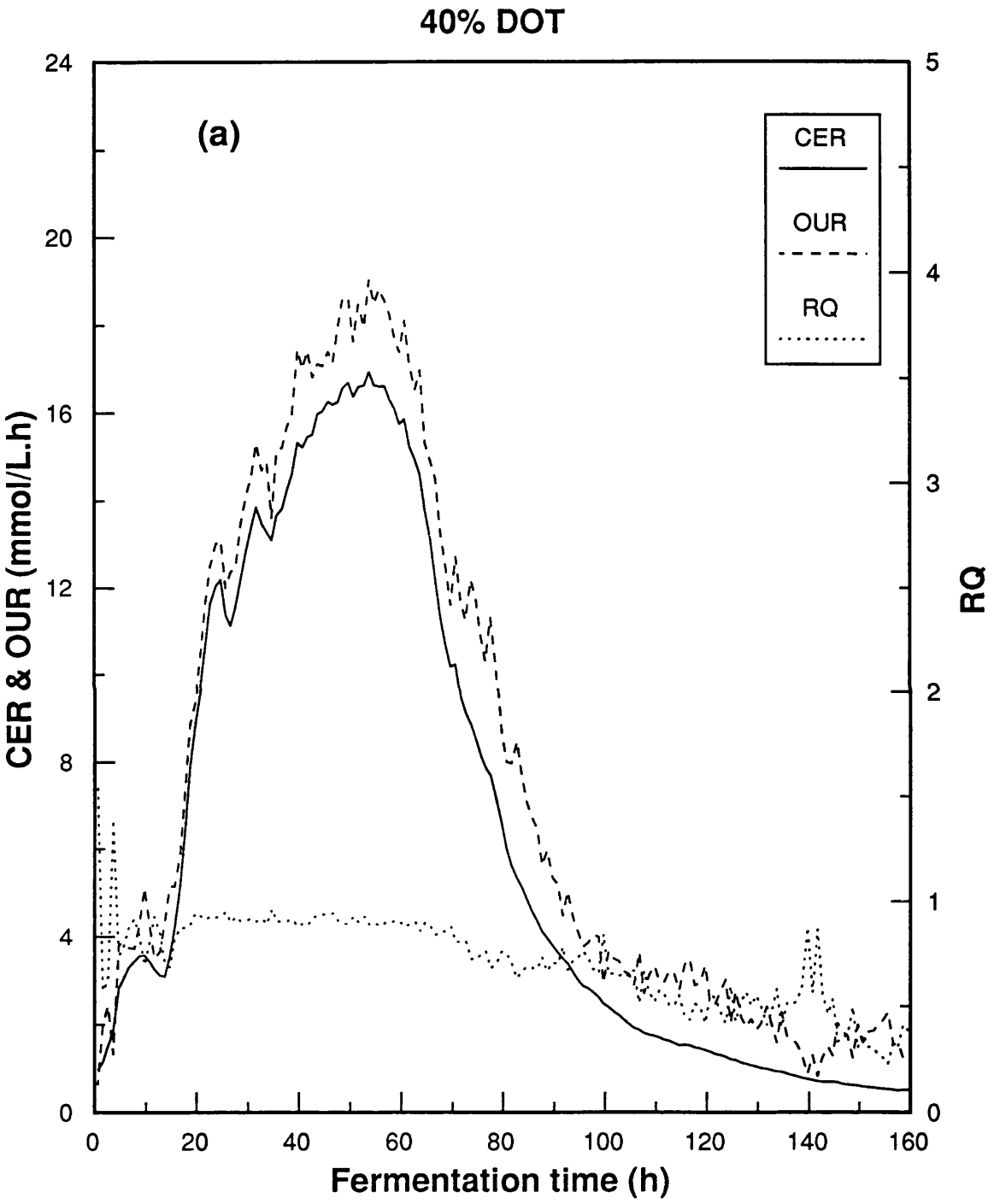


Figure 3.13 : Time course of fermentation with DOT control at 40% saturation (a) CER, OUR and RQ profiles.

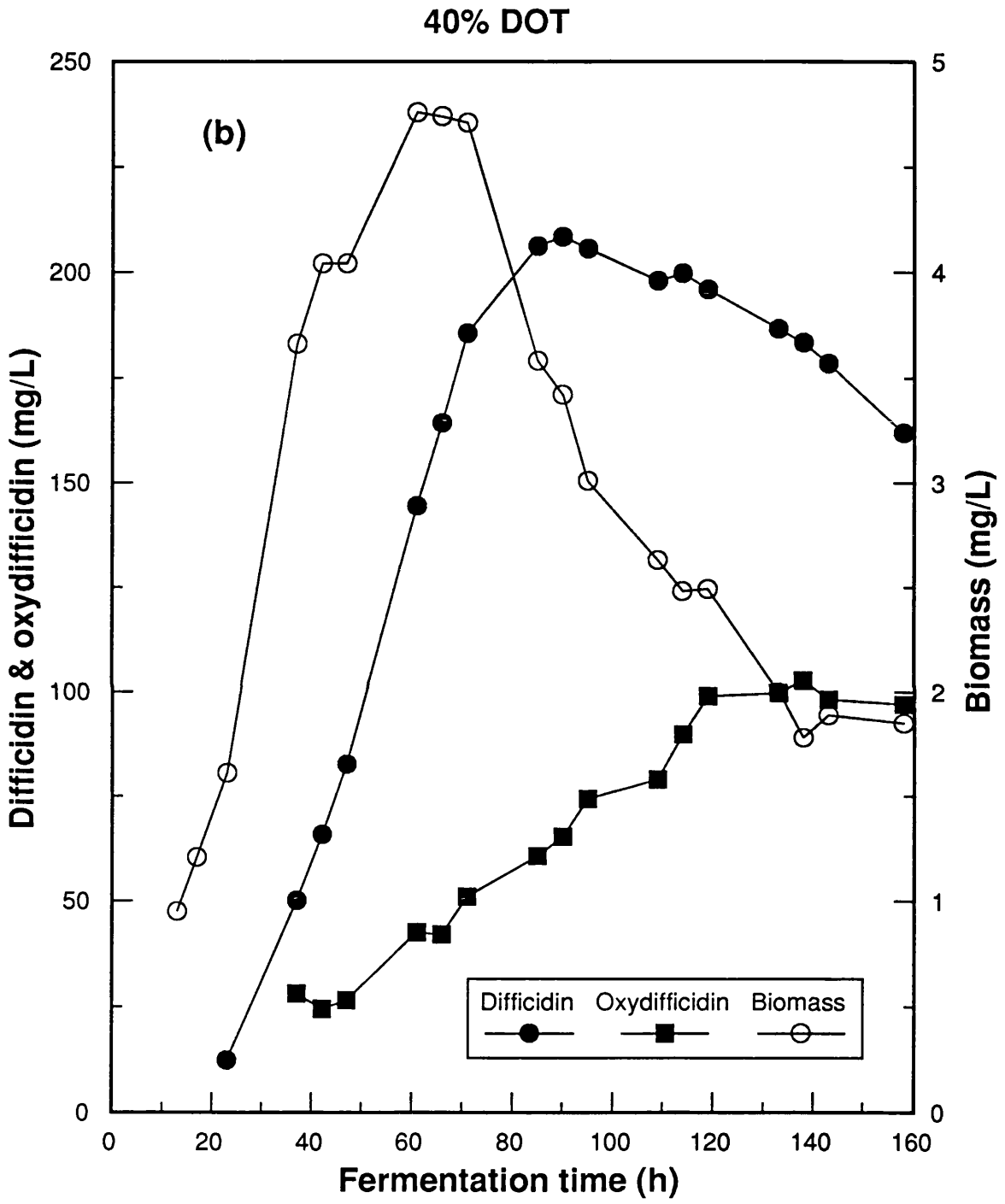


Figure 3.13 : *Continued* (b) antibiotic and biomass concentration profiles.

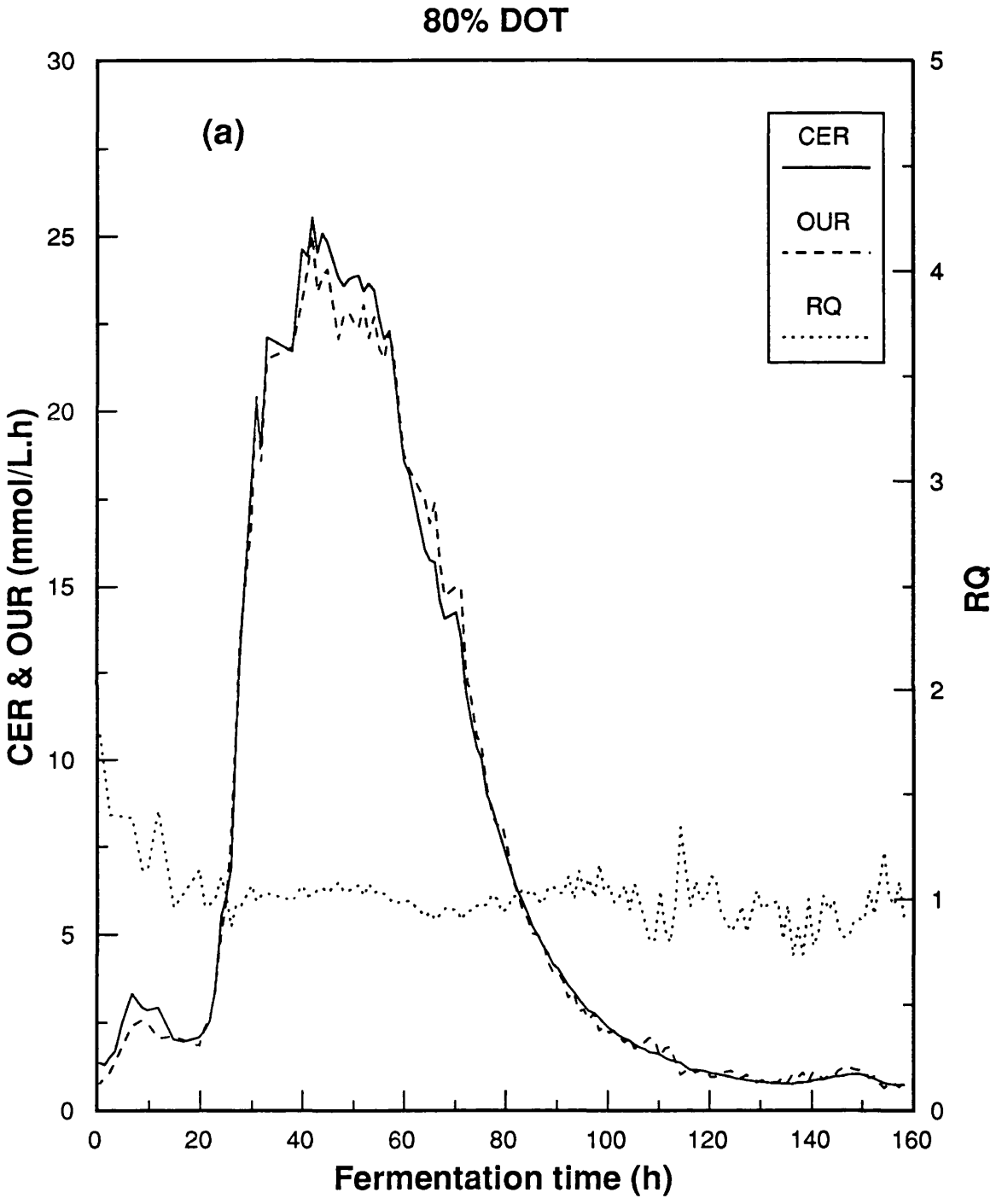


Figure 3.14 : Time course of fermentation with DOT control at 80% saturation (a) CER, OUR and RQ profiles.

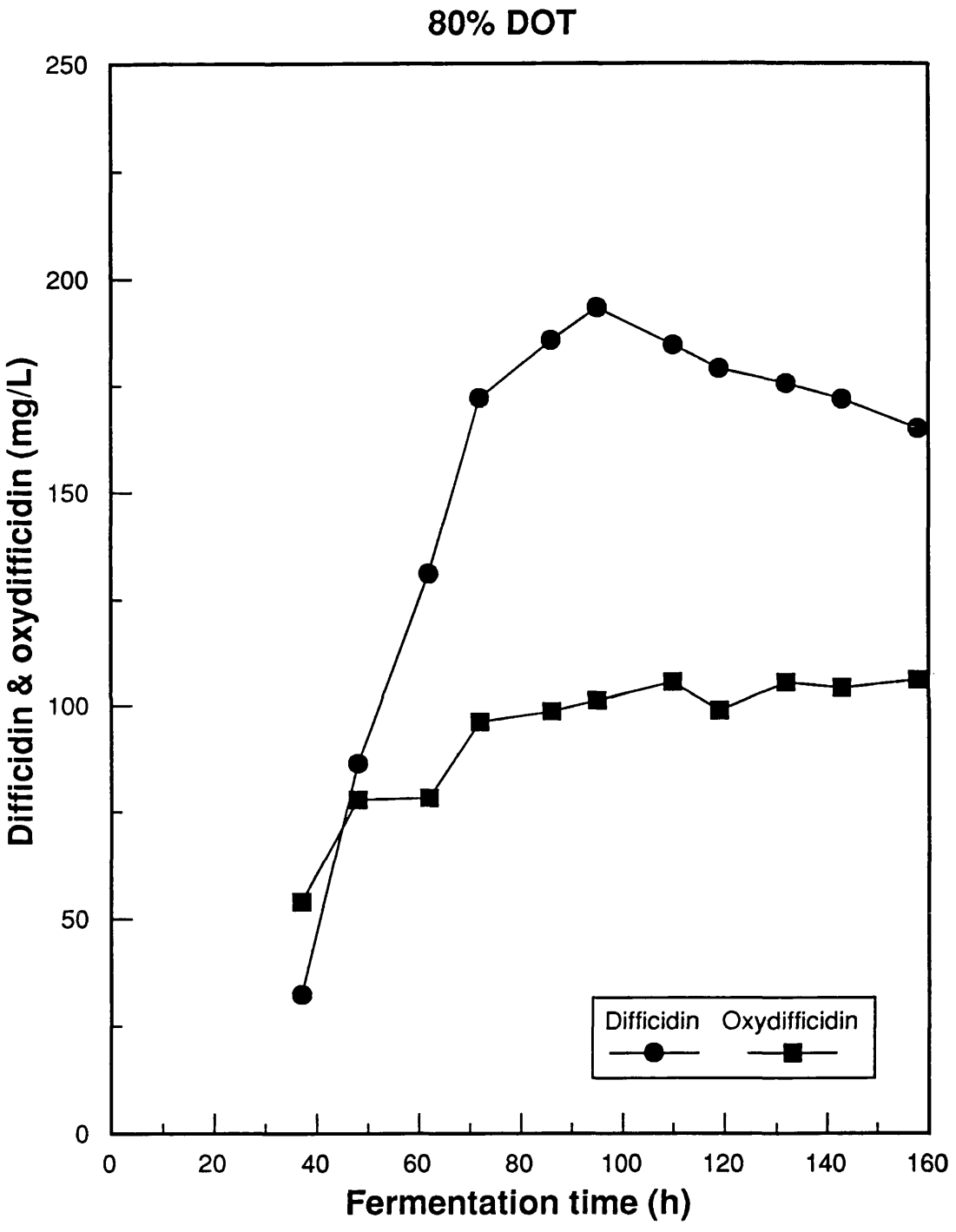


Figure 3.14 : *Continued* (b) antibiotic concentration profiles.

3.3.3 Effect of DOT on growth and respiratory activity

The effect of different DOT levels on growth and respiratory activity, i.e. CER, OUR and RQ was investigated based on the results of fermentations at various controlled DOT levels described in the previous section. An attempt to make correlations between DOT and growth as well as respiratory activity of the culture could possibly lead to the determination of the critical DOT level for the growth of this culture.

Generally, as can be seen in Figures 3.8 to 3.14, the CER and OUR values exhibited similar profile patterns throughout the course of fermentations. A slight difference in these values probably due to a difference in the metabolic activity of the culture at each stage of fermentation. A large fluctuation of OUR values, however, could result from the noise in the inlet and exit gas analyses rather than a fluctuation of metabolic activity of the cells. The fluctuations of RQ values were coincident with those in the OUR values. For the 5% to 40% DOT fermentations the CER and OUR values sharply rose in all cases to 12 mmol/L.h within the first 20 - 24 h of cultivation. At 5 and 10% DOT levels, there was a drop and delay before a second nutrient was used. At 15% DOT and above, the switch to the second nutrient was more rapid so that CER and OUR did not fall. At 80% DOT, there did not seem to be any delay so that CER and OUR continued to rise. During the transition from one nutrient to a second there seemed to be no change in RQ.

The biomass concentration profiles followed the CER and OUR profiles with a definite time lag. In order to make a clear conclusion, a summary of the maximum biomass concentrations, CER, OUR and corresponding fermentation times for the various fermentations are presented in Table 3.3. As can be seen from this table, the maximum biomass concentration occurred at approximately 60 - 70 h of fermentation except for the 1% DOT run where biomass concentration was found to be maximum at 85 h. The maximum CER and OUR values were observed at almost the same time between 47 - 54 h excepted the 80% DOT and the uncontrolled DOT fermentations in which these maximum values were observed earlier at 40 h. As a consequence, the time lag between the maximum CER and OUR and biomass concentrations fell in the range of 10 - 20 h.

Table 3.3 : Maximum biomass concentration, CER, OUR and corresponding fermentation times for various fermentations.

Controlled DOT (% saturation)	Maximum biomass concentration (g/L)	Time of the maximum biomass concentration (h)	Maximum CER (mmol/L.h)	Time of the maximum CER (h)	Maximum OUR (mmol/L.h)	Time of the maximum OUR (h)
Control†	ND	ND	28.9	38	29.0	38
1	3.33‡	85	9.4	51	9.8	63
5	3.89	61	12.8	50	13.3	52
10	3.74	71	12.0	49	14.8	49
15	4.43	66	13.6	52	16.0	53
20	4.33	71	14.5	47	15.6	51
40	4.76	61	16.9	54	19.0	54
80	ND	ND	25.5	42	25.0	42

† Fermentation without DOT control (Figure 3.6).
‡ The maximum biomass concentration at about the maximum OUR.
ND = Not determined.

The maximum OUR and CER showed an almost linear increasing trend with increase DOT levels from 5 to 80% saturation (Figure 3.15). A slightly larger drop in the maximum OUR and CER, however, occurred when the DOT was controlled below 5% saturation.

With an attempt to make a correlation of the bacterial growth and the DOT levels, the maximum specific growth rates (μ_{max}) under different DOT controlled levels were determined using semi-log plots of the biomass concentration versus fermentation time. It has been observed that the maximum specific growth rates occurred within the first 24 h of cultivation in the fermentations with controlled DOT at 5% to 40% air saturation. In the fermentation under controlled DOT at 1% saturation, however, the

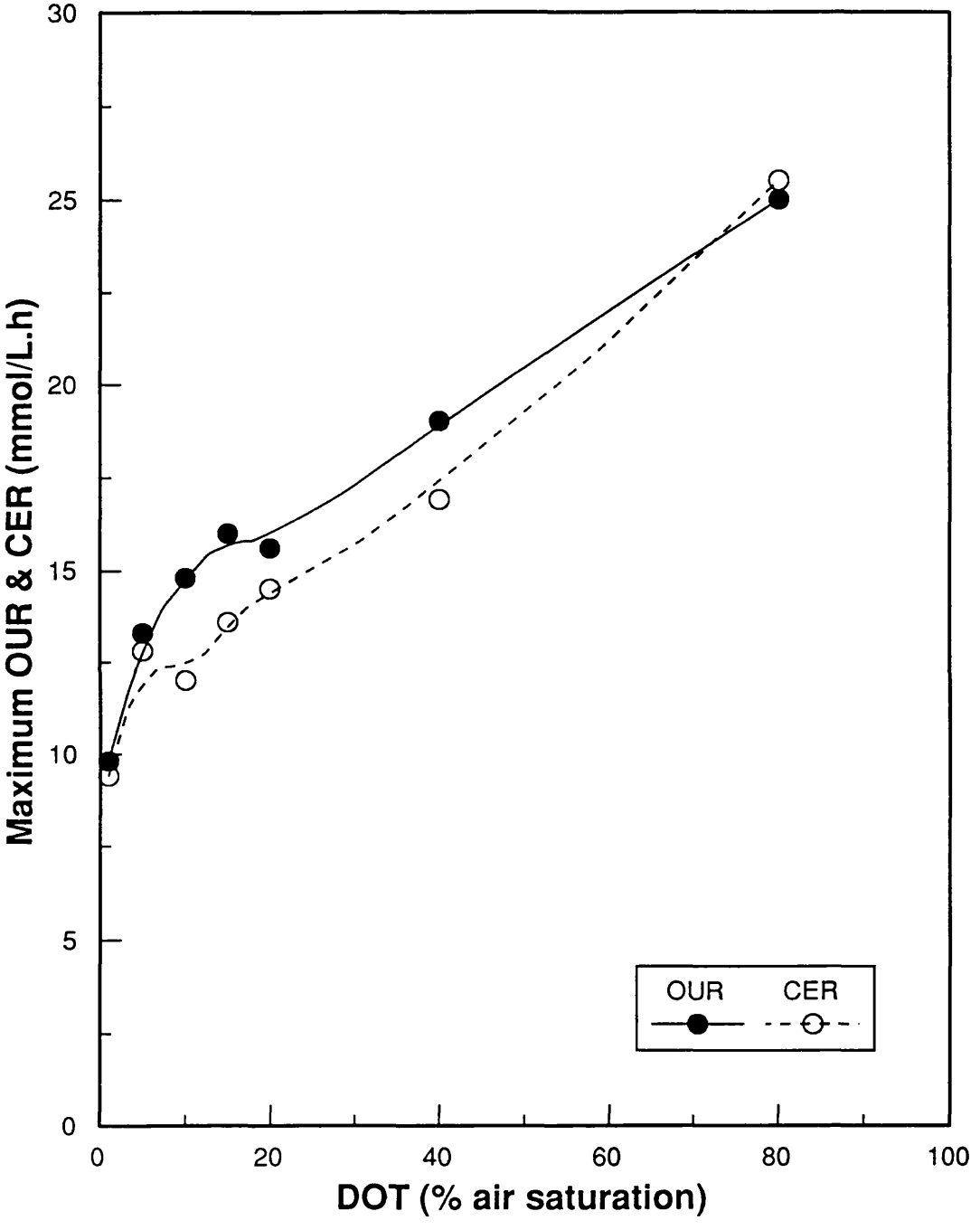


Figure 3.15 : Effect of DOT on the maximum OUR and CER.

maximum specific growth rate lasted longer up to 60 h of fermentation time. The correlations of DOT and the maximum specific growth rates as well as the maximum biomass concentrations are illustrated in Figure 3.16. An independence of the maximum specific growth rates on DOT was observed when DOT was controlled at 5% saturation and above. At DOT level below 5%, a sudden drop in the maximum specific growth rates occurred. In contrast, the maximum biomass concentrations exhibited a gradual increasing trend with increasing DOT controlled levels.

From all the events mentioned above, it could be reasonable to conclude that the critical DOT level for the growth of this culture appeared to be below 5% air saturation.

3.3.4 Effect of DOT on antibiotic production

A summary of the maximum difficidin and oxydifficidin concentrations as well as the corresponding fermentation times obtained from the fermentations conducted at different DOT levels and the one without DOT control is presented in the Table 3.4.

As can be seen from this table, the maximum difficidin concentration increased with increasing DOT up to 20% saturation beyond which the maximum concentration almost unchanged but remained at around 200 mg/L which was the same as that found in the uncontrolled DOT fermentation. With the DOT controlled at 20% and above the maximum concentration occurred at about 90 - 95 h of cultivation, the same as that of the uncontrolled DOT run. However, when the DOT was controlled below 20%, the maximum difficidin concentration was observed later in the range of 110 - 140 h. On the other hand, the maximum oxydifficidin concentration was between 100 - 130 mg/L over a wide range of DOT controlled levels and also occurred at nearly the end of fermentations except in the fermentation without DOT control where the maximum oxydifficidin occurred at the same time as that of difficidin.

The effect of DOT on antibiotic production was investigated by making a correlation between the antibiotic production rates and the controlled DOT levels (Figure 3.17). The

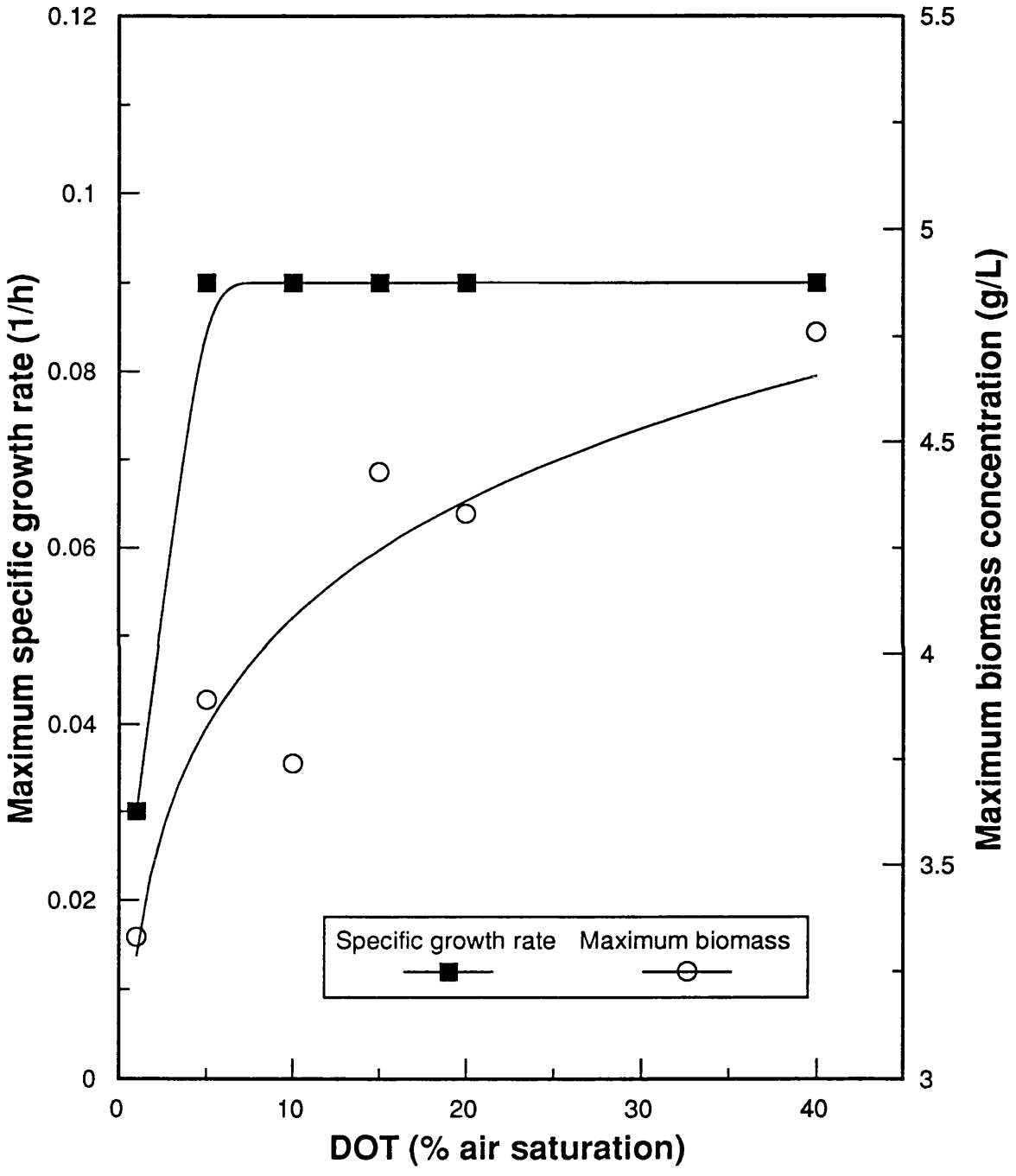


Figure 3.16 : Effect of DOT on the maximum specific growth rate (μ_{max}) and biomass concentration.

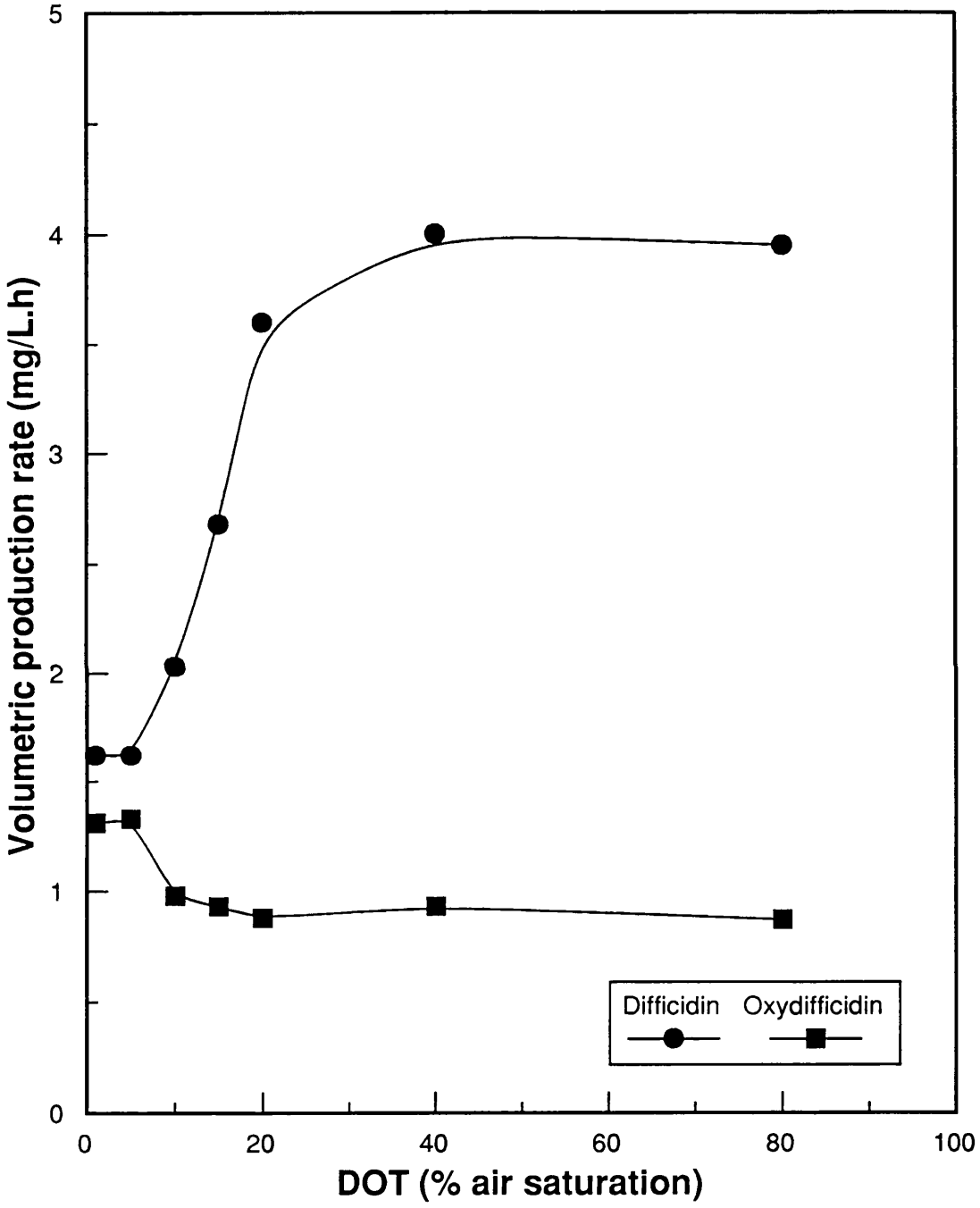


Figure 3.17 : Effect of DOT on volumetric production rates of difficidin and oxydifficidin.

Table 3.4 : Maximum difficidin and oxydifficidin concentrations and corresponding fermentation times for various fermentations.

Controlled DOT (% saturation)	Maximum difficidin concentration (mg/L)	Time of the maximum difficidin concentration (h)	Maximum oxydifficidin concentration (mg/L)	Time of the maximum oxydifficidin concentration (h)
Control†	203	95	132	95
1	147	138	99	138
5	153	143	131	158
10	175	109	123	143
15	197	114	110	158
20	217	90	133	158
40	209	90	103	138
80	193	95	106	110

† Fermentation without DOT control.

volumetric production rates of difficidin and oxydifficidin antibiotics were determined by calculating the slope of the best fitting line during the linear production period which in most cases fell between 40 to 80 h of fermentation time. The DOT showed a significant effect on the production of difficidin but not oxydifficidin. A sharp increase in the difficidin production rates from 1.6 to 3.6 mg/L.h was observed when the DOT rose from 1 to 20% saturation followed by a slight increase up to the maximum constant level of about 4.0 mg/L.h when the DOT was controlled at 40% saturation and above. Therefore, the 20% DOT could be determined as a critical level for the production of difficidin. In contrast, a little variation in the oxydifficidin production rates between 0.9

- 1.3 mg/L.h was found when the DOT was controlled between 1 to 80% saturation demonstrating the insignificant effect of DOT on the oxydifficidin production. In the uncontrolled DOT fermentation (Figure 3.6), the antibiotic production rates were found to be 3.9 mg/L.h for difficidin and 1.1 mg/L.h for oxydifficidin which were approximately the same as those produced under the controlled DOT at 40% and beyond.

3.3.5 Effect of DOT on total antibiotic titres and product ratio

The total antibiotic titres and product ratio as a function of DOT are given in Figure 3.18. Both total antibiotic titres and product ratio were determined at 95 h of fermentation time during which the difficidin production rate began to level off in most of the fermentations. As expected, the total antibiotics profile was similar to that of the difficidin production rate profile shown in Figure 3.17. The total antibiotic titres increased sharply from about 200 mg/L at 1% DOT to 285 mg/L at 20% DOT. Above 20% DOT, the total titre remained unchange. The ratio of difficidin to oxydifficidin increased in parallel to the total titres from about 1.2 - 1.6 to 2.7 with increasing DOT from 1 to 20% saturation. No further increase in the product ratio when DOT rose to 40%. At 80% DOT, however, the product ratio droped to 1.9. Even though, the production rates of difficidin and oxydifficidin at 80% DOT were the same as those of the 40% DOT run, the difficidin titre at 95 h in the 80% DOT run was about 6% less whereas the oxydifficidin titre was about 36% more than those observed in the 40% DOT run leading to a decrease in the product ratio. Similarly, the product ratio in the uncontrolled DOT fermentation was found to be 1.5 as a result of the higher oxydifficidin titre of about 77% as compared with that of the 40% DOT run. The difficidin titres, however, were approximately the same. This caused about 19% higher in the total antibiotic titres found in the uncontrolled DOT experiment as compared with that of the 40% DOT fermentation.

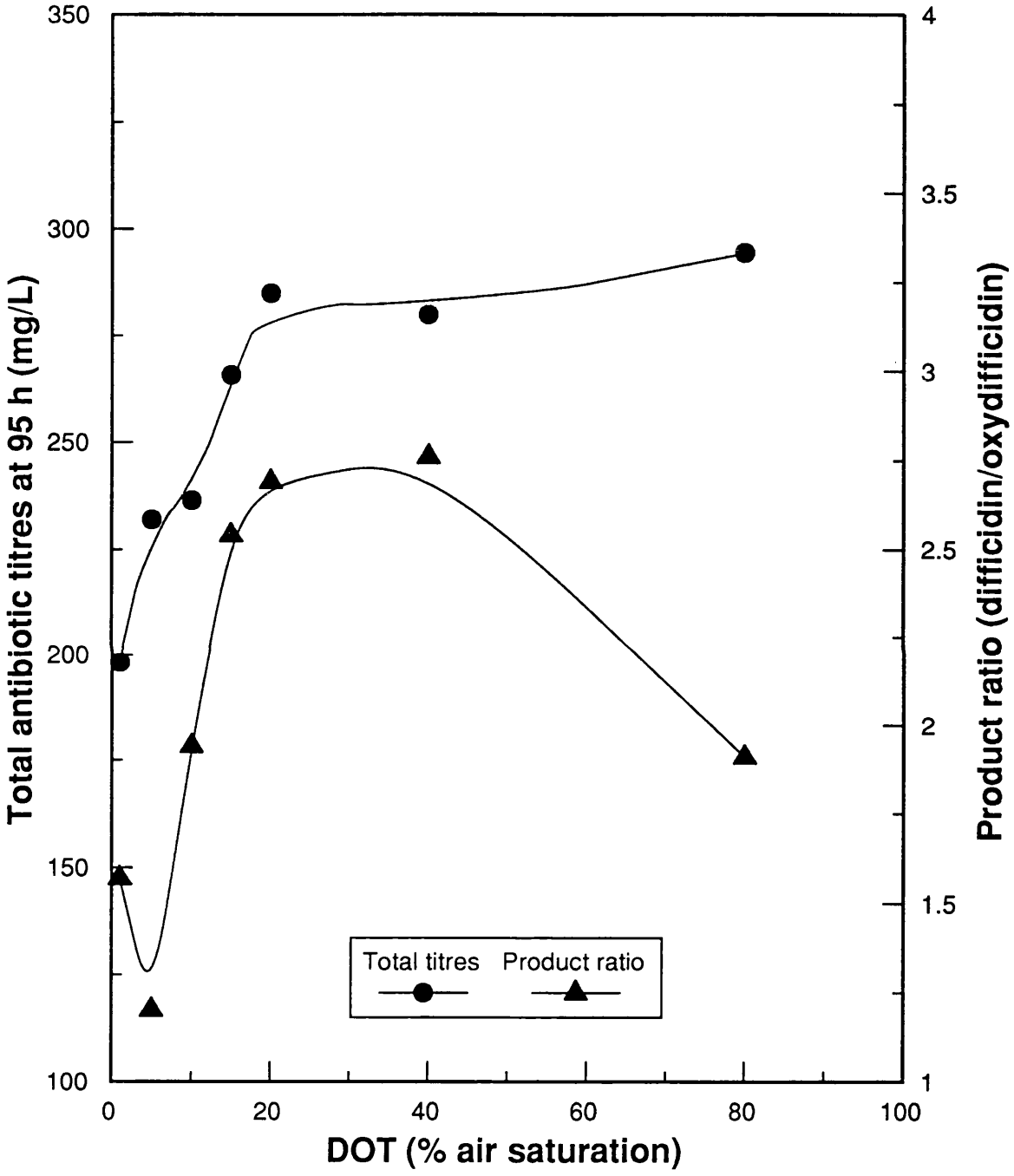


Figure 3.18 : Effect of DOT on total antibiotic titres and product ratio of difficidin and oxydifficidin determined at 95 h of cultivation.

3.4 Fermentations with Cycling DOT

The influence of DOT on growth and antibiotics production has been clearly demonstrated in this work using a small scale fermenter under different controlled constant DOT levels. In large scale fermenters, however, due to limitations in mixing intensity and mass transfer capacity coupled with variations in hydrostatic pressure, there exists significant variations of DOT at different positions in the fermentation broth. Consequently, the microorganisms are exposed to a continually changing DOT which may affect the growth and product formation. Therefore, for scaling up or optimizing a large scale fermentation process, it is important to study the behaviour of the microbial culture under such conditions. In this study, these conditions were simulated in a laboratory scale fermenter in which the DOT was cycled by periodic feeding of air to a base feed of nitrogen.

3.4.1 Design of DOT cycling experiments

In order to simulate the actual fluctuation of DOT in the large scale fermenters, there are a number of criteria which have to be determined in setting up the cycling DOT experiments. In large scale stirred tank fermenters, the cells are circulated with the liquid circulation generated by the impellers. As a consequence, the time periods during which the cells experienced the same conditions after their circulation are most likely the same as the mixing times or circulation times of fluids in the large fermenters. Basically, the mixing times increase with increase of the fermenter scales (Einsele, 1978). In a 100 m^3 reactor the mixing time exhibited values of up to 100 s for aqueous systems. In large stirred fermenters (2.5, 80 and 160 m^3) for penicillin production, Jansen *et al.* (1978) measured mixing times between 10 and 70 s. Based on these evidences, the cycling periods of DOT, i.e. the time between two successive peaks of DOT profile obtained during cycling experiments, could not larger than these mixing times. Hence, the cycling period of 60 s was chosen for this simulation. Additionally, the cycling amplitude, i.e. the difference between the maximum and minimum DOT values attained during the cycling performance, was determined based on the maximum variation of DOT observed

in the industrial scale fermenters. With the data available, the maximum variation of DOT in the production scale fermentations was found to be about 20% air saturation (Manfredini *et al.*, 1983; Oosterhuis and Kossen, 1984). Therefore, it is reasonable to perform cycling of DOT with the cycling amplitude not more than 20% saturation. In this study 10% saturation was selected for the amplitude of cycling.

According to the correlation between difficidin production rates and the DOT levels (Figure 3.17), the 20% DOT appeared to be critical for the difficidin production. Therefore, it is interesting to cycle DOT in the three different regions based on this correlation : (1) cycling of DOT below the critical value, in this case the DOT was cycled around 15% saturation, (2) cycling of DOT around the critical value (20% saturation) and (3) cycling of DOT above the critical value, in this case the DOT was cycled around 40% saturation. The examination of cycling DOT around 20% and 15% saturation would determine the effect of changing DOT on antibiotic production rates and could be compared to those obtained under low constant DOT values. Cycling of DOT around 40%, however, should illustrate that DOT cycling above the critical value might not affect the production rates.

The cycling DOT experiments were conducted during the growth phase when production was almost linear (between 28 to 70 h of cultivation). Each experiment was divided into three parts : (1) fermentation with controlled constant DOT, (2) fermentation with cycling DOT and (3) fermentation with constant DOT. The first and final parts of fermentation with constant DOT were performed as a control case to which the results of the cycling experiment could be compared. In each part of the experiment, samples were taken every hour for 10 h. During the cycling DOT experiment, the first sample was taken after the DOT had been cycled for about 2 h.

3.4.2 DOT cycling performance

The DOT in the fermentation broth was cycled with a defined period and amplitude by an on-off control of air supplied to a base feed of nitrogen. The cycling periods were

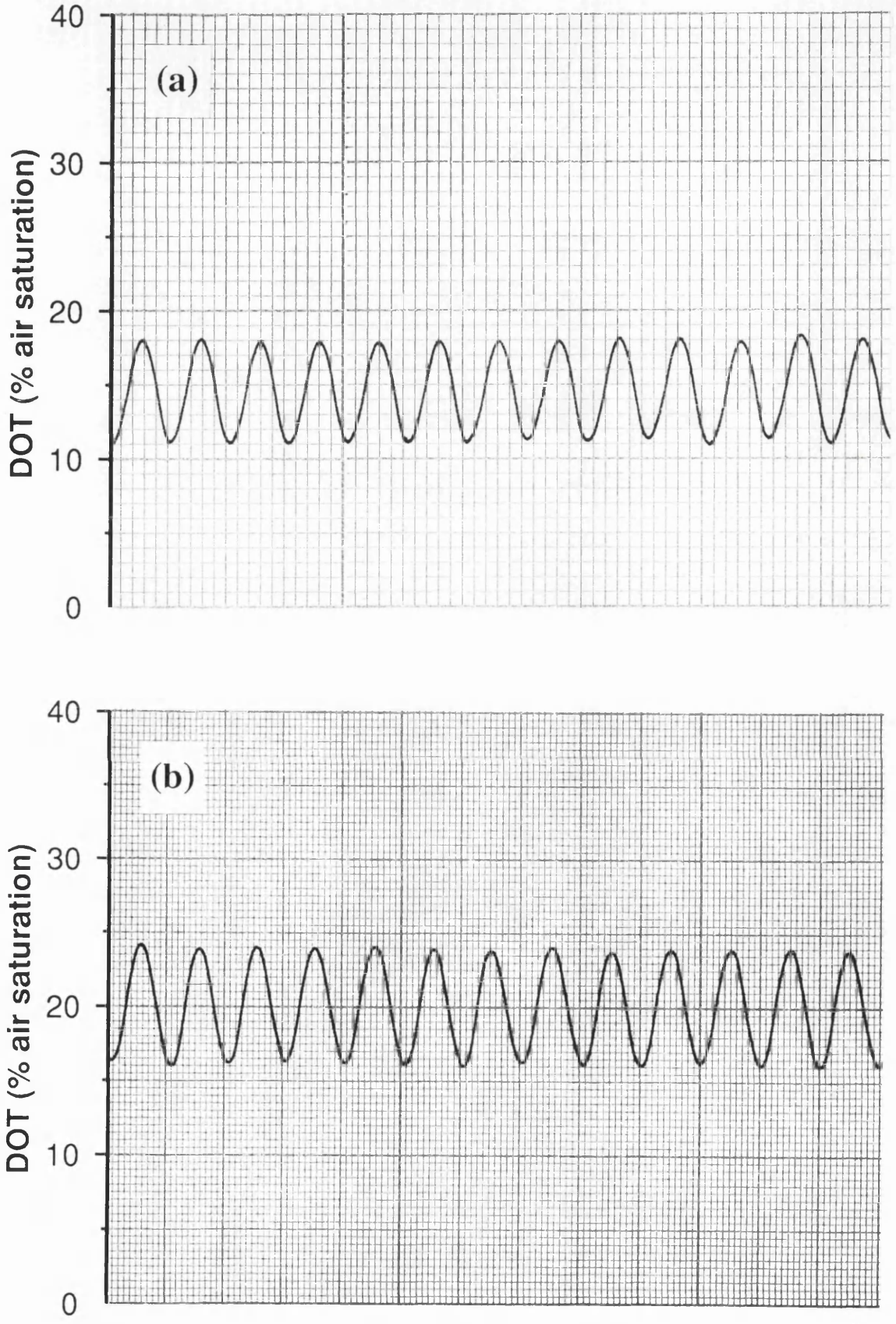


Figure 3.19 : DOT cycling traces recorded with a chart speed of 1 cm/min.
(a) DOT cycling around 15% saturation.
(b) DOT cycling around 20% saturation.

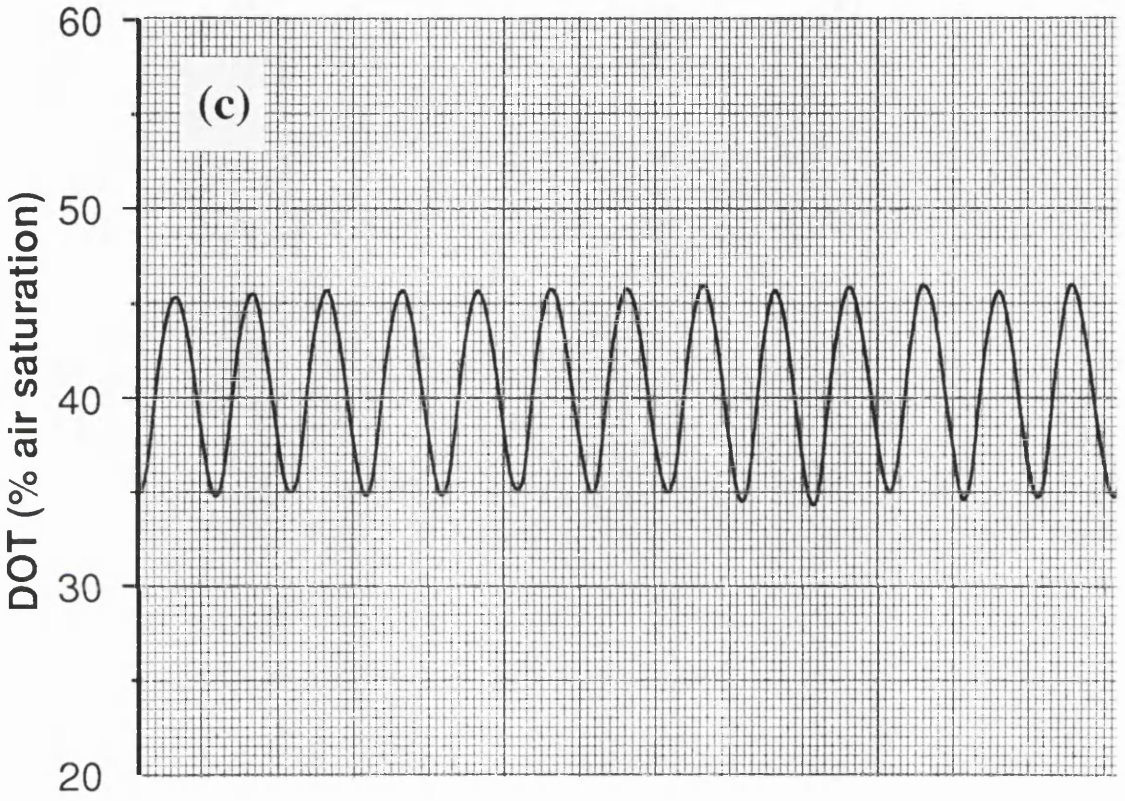


Figure 3.19 : *Continued* (c) DOT cycling around 40% saturation.

controlled by a timer coupled to a solenoid valve in the air feed line. The detail descriptions of the DOT cycling device and its operation were explained in Sections 2.1.3.4 and 2.2.5, respectively.

Figure 3.19 shows examples of the chart records of DOT cycling performances at different DOT levels. An attempt was made to cycle DOT with an amplitude of 5% saturation above and below a pre-determined DOT value throughout the cycling experiment. The recorded DOT traces indicated a variation of measured DOT between 11 - 18% saturation (Figure 3.19a), 16 - 24% saturation (Figure 3.19b) and 35 - 45% saturation (Figure 3.19c) when the DOT was cycled around 15%, 20% and 40% saturation, respectively.

3.4.3 Effect of cycling DOT on growth and product formation

Samples taken from the fermenter during the course of fermentation were analyzed for biomass and antibiotic concentrations. Because of the frequent changing of the gas flow rates during cycling DOT experiments, no accurate determination of the OUR and CER values was possible, therefore, during these experiments only the growth and antibiotic productions were determined.

The results obtained from the experiments with cycling DOT around 15% saturation are shown in Figure 3.20. During the cycling DOT experiment, the volumetric production rates of difficidin and oxydifficidin antibiotics were almost the same as those found in the first part of this experiment with 15% constant DOT (Figure 3.20a). In contrast, the biomass production rate was about 3 times higher than that observed during the constant DOT experiment (the first part). In the final part of this experiment when DOT was controlled at 15% saturation, the volumetric production rates of both antibiotics were reduced. These results were coincident with the decrease of biomass concentration during this period. A marked reduction of the specific production rates of both antibiotics to about one third of those produced under the 15% constant DOT in the first part was observed during the cycling DOT experiment (Figure 3.20b). When DOT was

controlled at 15% again after the cycling experiment, the specific production rates rose but were still lower than those observed in the first part.

When DOT was cycled around 20% saturation, the volumetric production rates of the two antibiotics as well as biomass were increased as compared to those observed at constant DOT of 20% in the first part of the experiment (Figure 3.21a). This was followed by a reduction of both antibiotic production rates and biomass concentration in the final part of experiment when DOT was controlled at 20% saturation again. The same reductions of about 35% in the specific production rates of the two antibiotics were observed during the cycling DOT experiment, compared to those of the constant DOT in the first part (Figure 3.21b). In the final part with 20% constant DOT, the specific production rates rose again but with lower rates as compared to those found in the first part of the experiment.

The experiment with cycling DOT around 40% saturation gave the results as illustrated in Figure 3.22. An increase in the volumetric production rate of difficidin coincided with a reduction in the volumetric production rates of oxydifficidin and biomass during the cycling DOT experiment, compared to those found under constant DOT in the first part of the experiment (Figure 3.22a). In the final part when DOT was controlled at 40% saturation, the volumetric production rate of difficidin still increased, whereas the rate of biomass production declined. The volumetric production rate of oxydifficidin rose as compared to that of the cycling DOT experiment. The specific production rate of difficidin during cycling DOT was almost the same as that observed during constant DOT in the first part of this experiment (Figure 3.22b). A 60% reduction of the specific oxydiffcidin production rate occurred under the cycling DOT conditions. A further increase in the specific difficidin production rate was also found in the final part of the experiment with constant DOT at 40% saturation. The specific oxydifficidin production rate was higher during this period than that observed in the cycling DOT experiment.

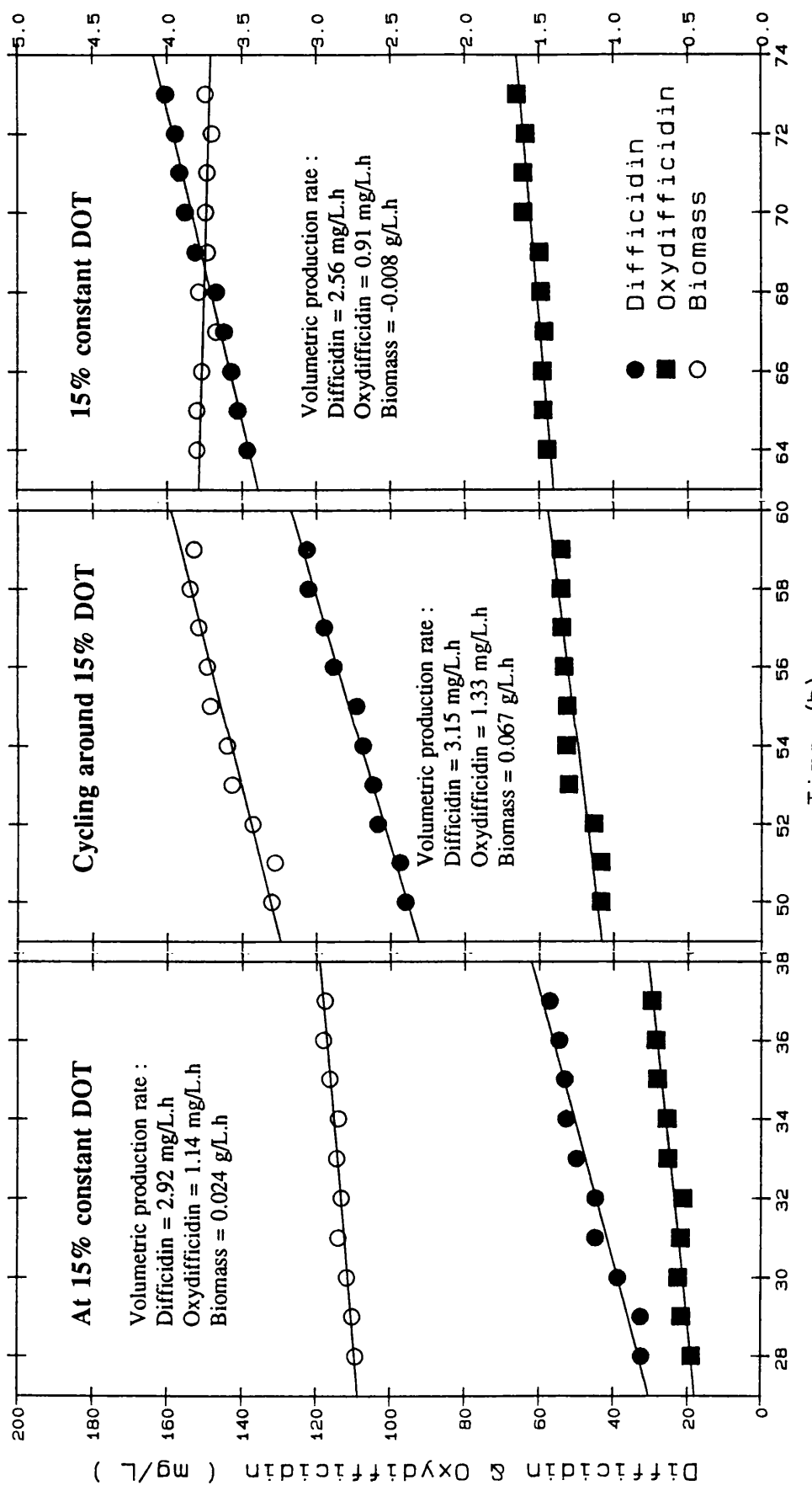


Figure 3.20(a) : Effect of cycling DOT around 15% saturation on growth and antibiotics production.

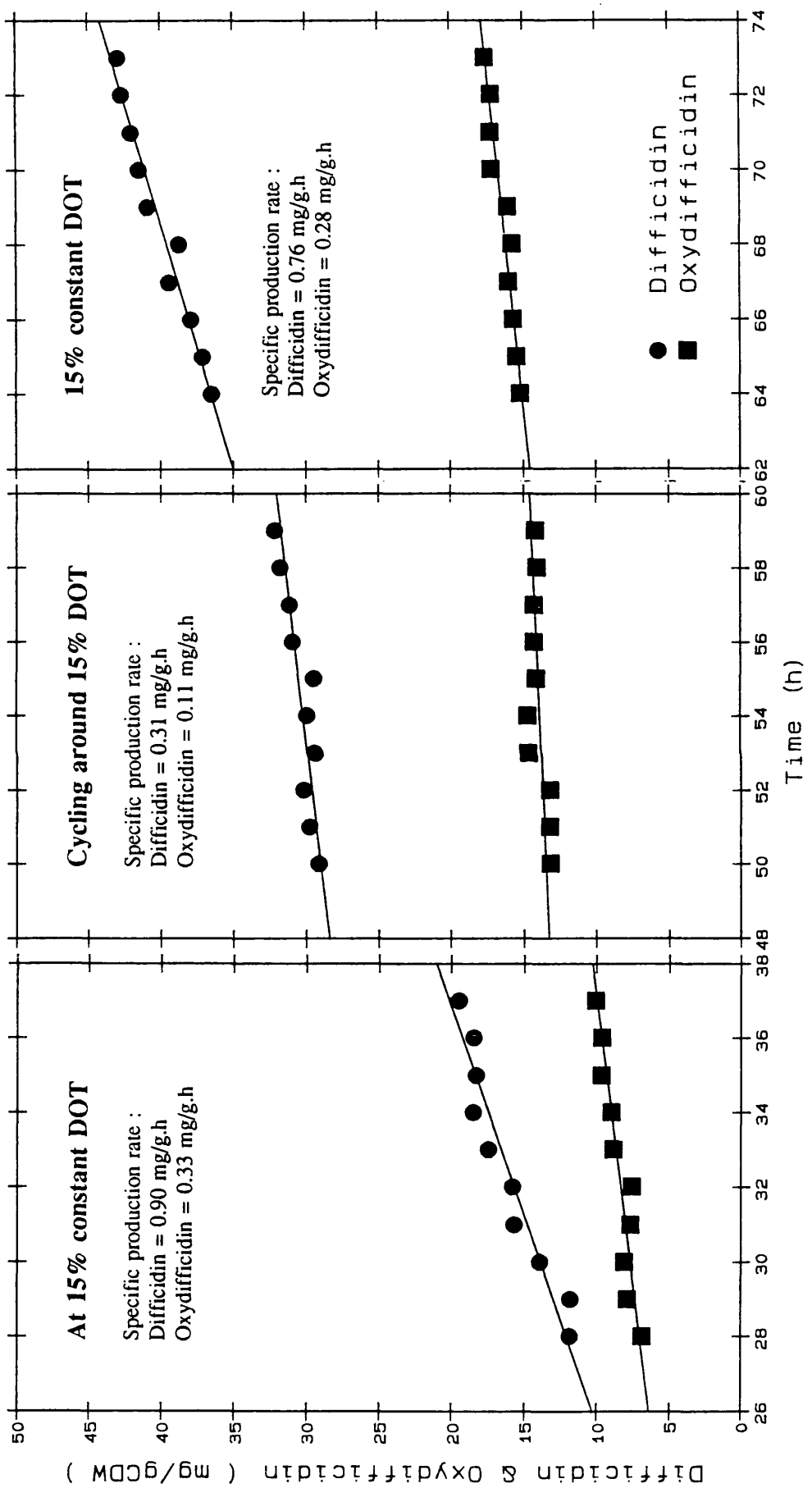


Figure 3.20(b) : Effect of cycling DOT around 15% on specific production rates of difficidin and oxydifficidin.

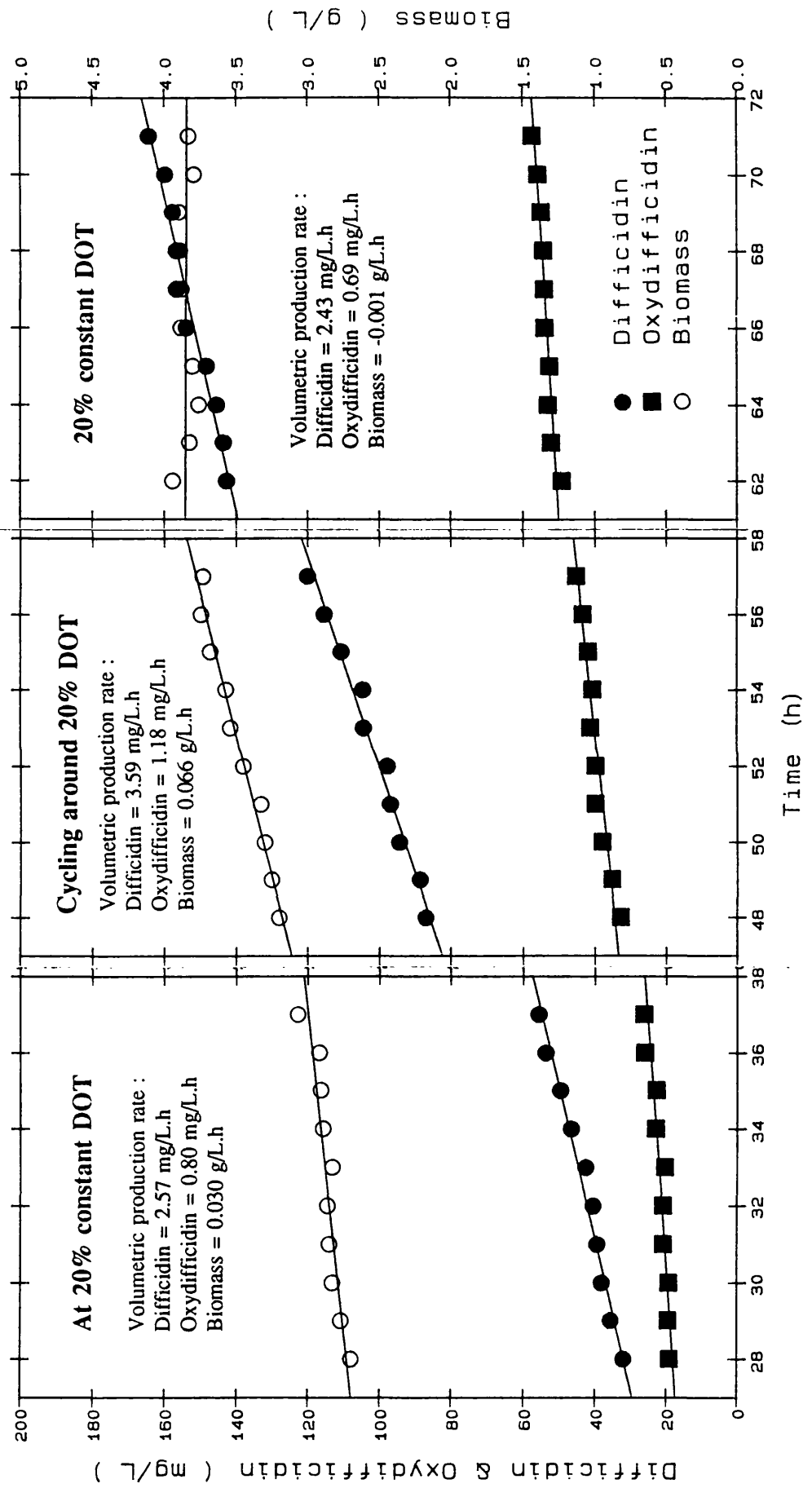


Figure 3.21(a) : Effect of cycling DOT around 20% saturation on growth and antibiotics production.

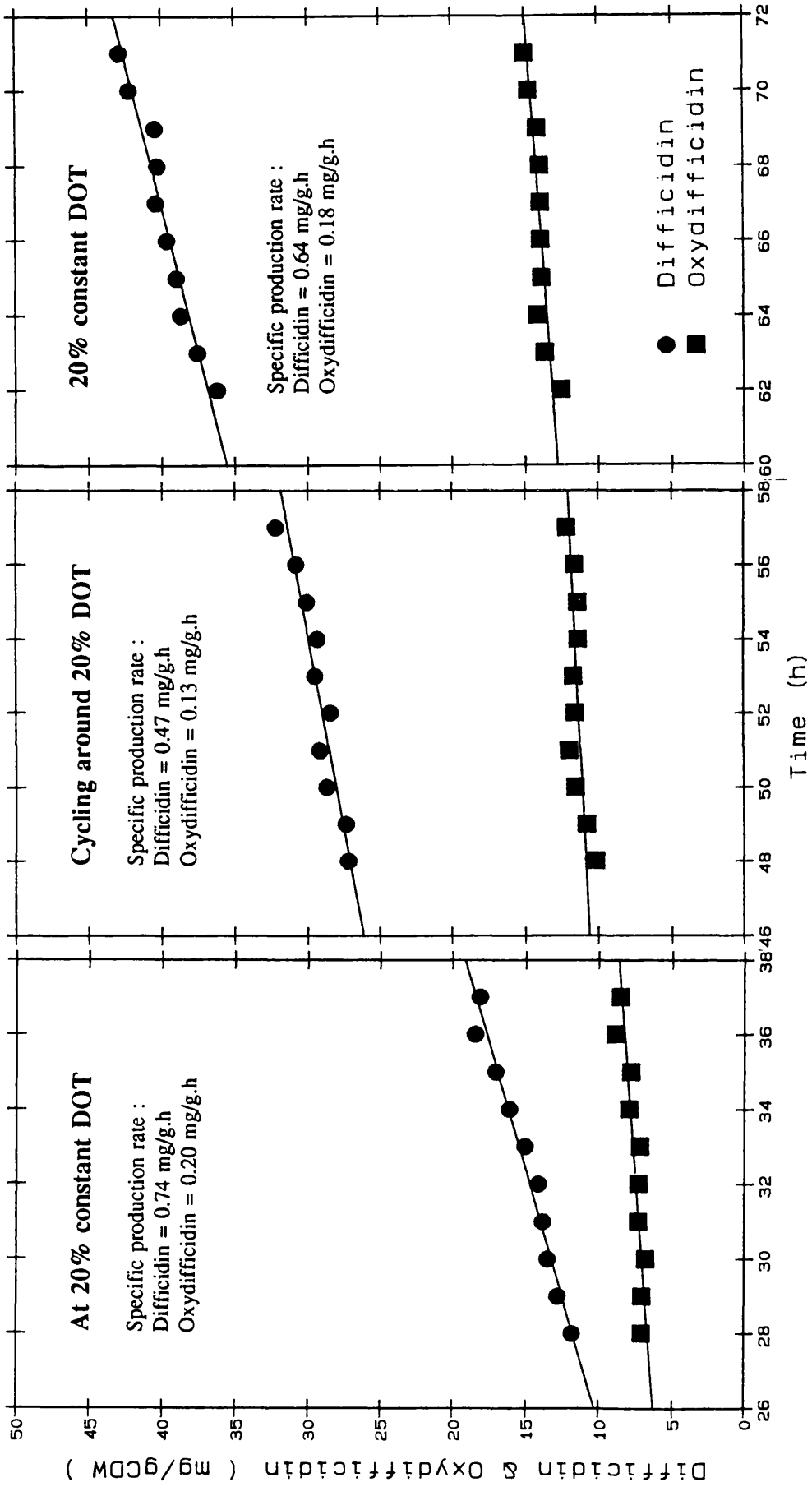


Figure 3.21(b) : Effect of cycling DOT around 20% on specific production rates of difficidin and oxydifficidin.

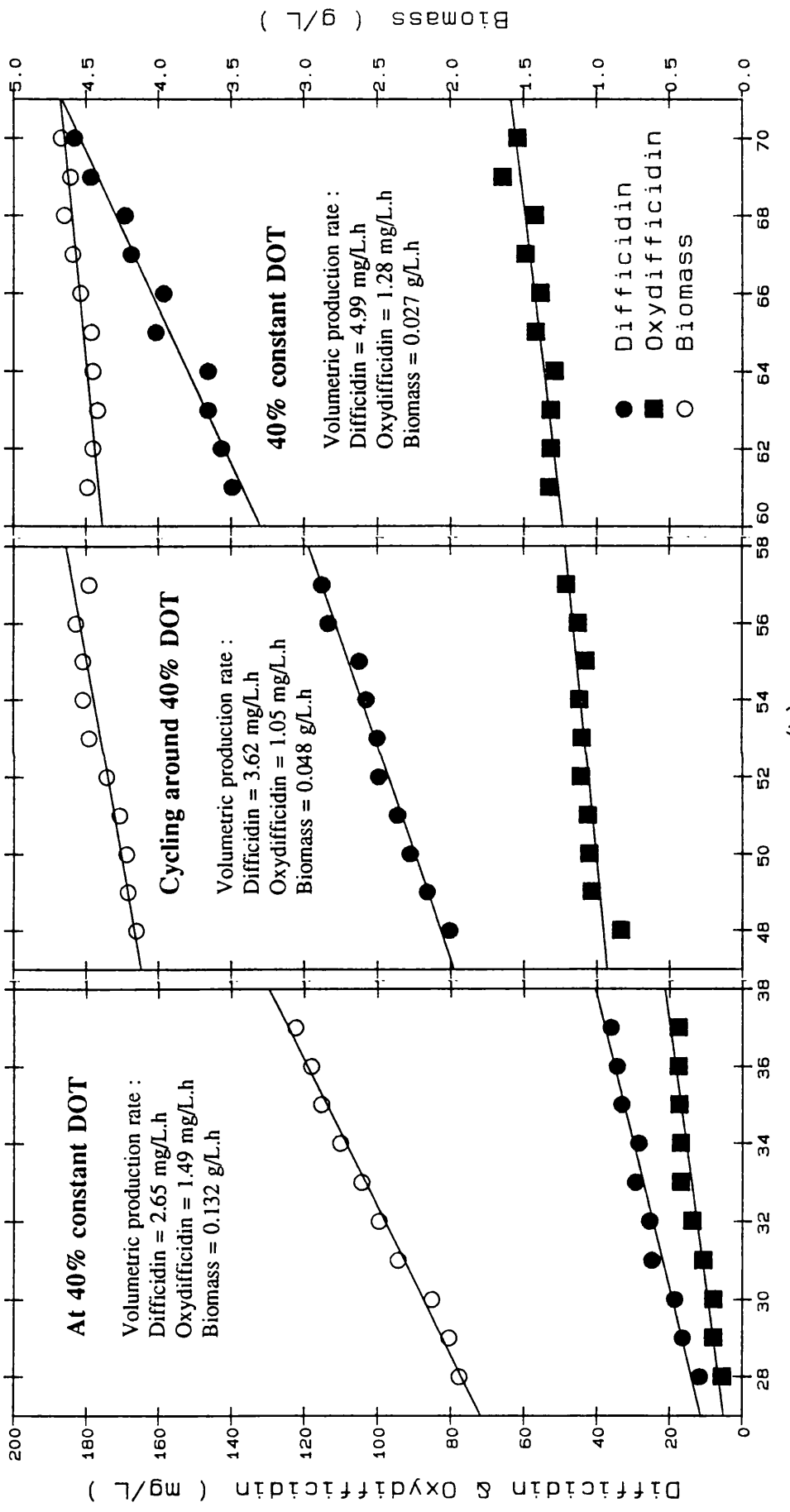


Figure 3.22(a) : Effect of cycling DOT around 40% saturation on growth and antibiotics production.

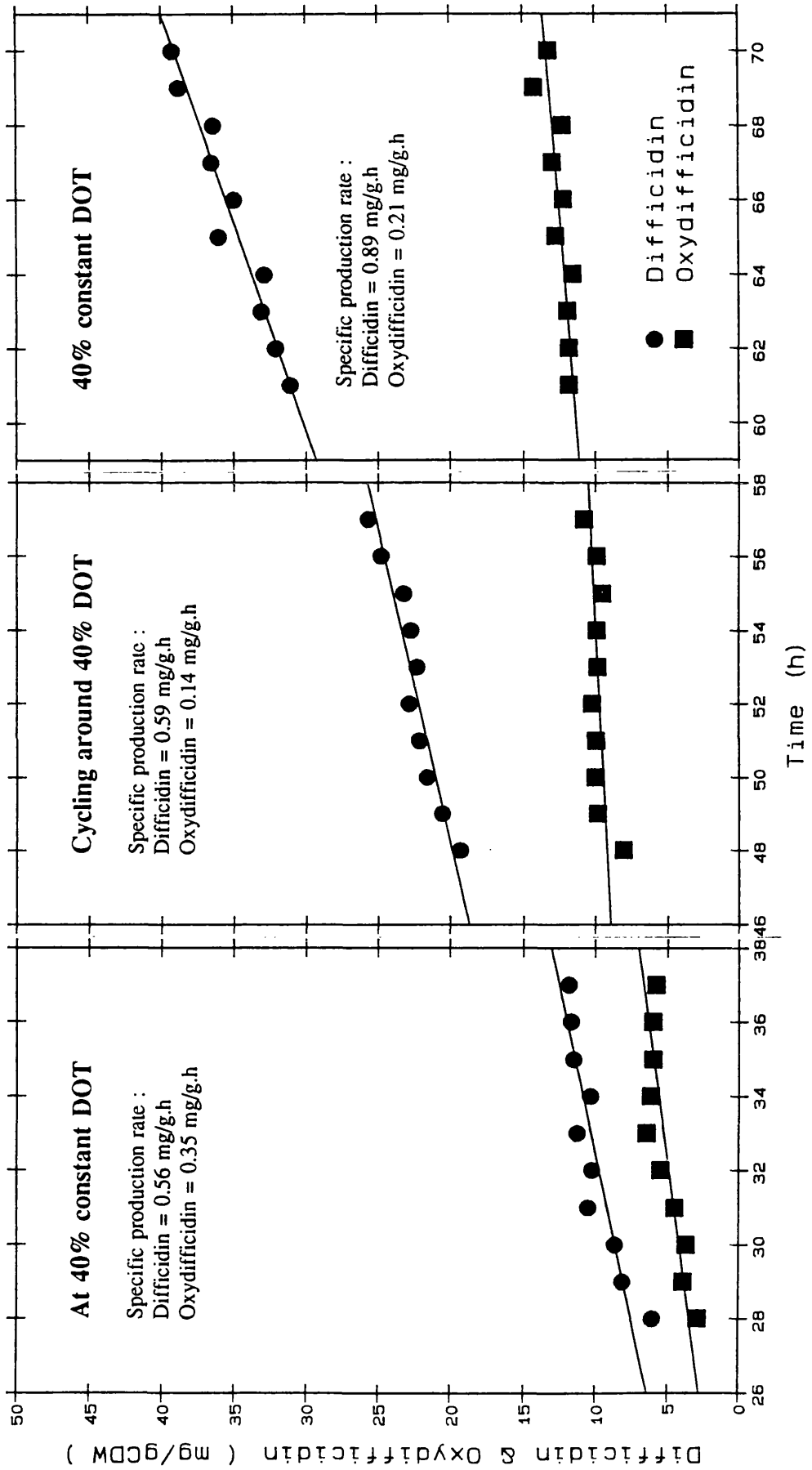


Figure 3.22(b) : Effect of cycling DOT around 40% on specific production rates of difficidin and oxydifficidin.

4. GENERAL DISCUSSION

4.1 Some Characteristics of Difficidin and Oxydifficidin Antibiotics

Difficidin and oxydifficidin compounds are two highly unsaturated 22-membered macrocyclic polyene lactone phosphate esters of the formula shown in Figure 4.1 below

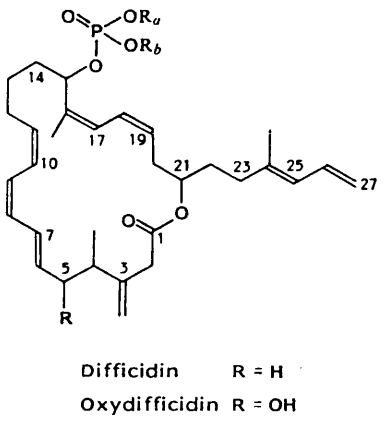


Figure 4.1 : Molecular structure of difficidin and oxydifficidin.

where R_a and R_b are members independently selected from the group consisting of hydrogen, alkali metal and alkaline earth metal cations, ammonium, and substituted ammonium (Zimmerman *et al.*, 1985). As can be seen, the only difference between these two related compounds is that oxydifficidin molecule contains one oxygen atom more than that of difficidin.

These two compounds each showed a broad and potent spectrum of activity against aerobic and anaerobic bacteria. *In vitro*, however, difficidin is a more potent antibiotic than oxydifficidin. A more detailed description of their preparation, characteristics and antibacterial activity was presented by Zimmerman *et al.* (1985,1987). In this study, difficidin and oxydifficidin were produced by the fermentation of *Bacillus subtilis* ATCC 39374.

The physico-chemical characterization of these labile antibacterial antibiotics were described by Wilson *et al.* (1987). Difficidin and oxydifficidin have been found to be sensitive to pH (Section 3.1.1). Under alkaline conditions, oxydifficidin was significantly less stable than difficidin. This was caused by the cleaving of the macrolide ring of oxydifficidin, the mechanism of which is shown in Figure 4.2. At pH $\geq$ 8 it seemed most likely that there was an initial translactonization of oxydifficidin to compound 3, which then underwent competitive hydrolysis to the seco-acid salt or isomerization to the δ-lactone leading to a mixture of these compounds. Both antibiotics exhibited instability at pH 3.5 possibly due to the instability of the acid forms, where $R_a = R_b =$ H, which existed at a low pH (Zimmerman *et al.*, 1985).

Between pH 6.5 and 7.0 both antibiotics showed nearly the same stability. The maximum stability of oxydifficidin was also observed at pH 7.0. Moreover, cultivation of *Bacillus subtilis* has been well documented to be most productive under neutral pH conditions, preferably from about 6.0 to 7.0 (Zimmerman *et al.*, 1985). In this work, therefore, the pH of the culture was maintained at 6.8 to optimize the bacterial growth and productivity of the two antibiotics.

Besides pH sensitivity, difficidin and oxydifficidin were susceptible to thermal isomerization and air oxidation (Wilson *et al.*, 1987). The oxidative processes were severe particularly when samples were stored as solids. In order to minimize these instabilities of the products, the samples were stored as an aqueous methanol solution and kept at a very low temperature (-70° C) for not more than one week before assays.

The nature of the antibiotics in the culture broth has been investigated. The results demonstrated that a considerable amount of antibiotics, i.e. 70 - 80% of difficidin and 60 - 70% of oxydifficidin, appeared to attach to the bacterial cells during the course of fermentation. In a series of papers published by Martin and McDaniel (1974,1975) the different distributions of the antibiotics between mycelium and the supernatant broth were reported among the polyene macrolide antibiotics namely candicidin, candidin and candihexin produced by *Streptomyces griseus*, *Streptomyces viridoflavus* and a mutant

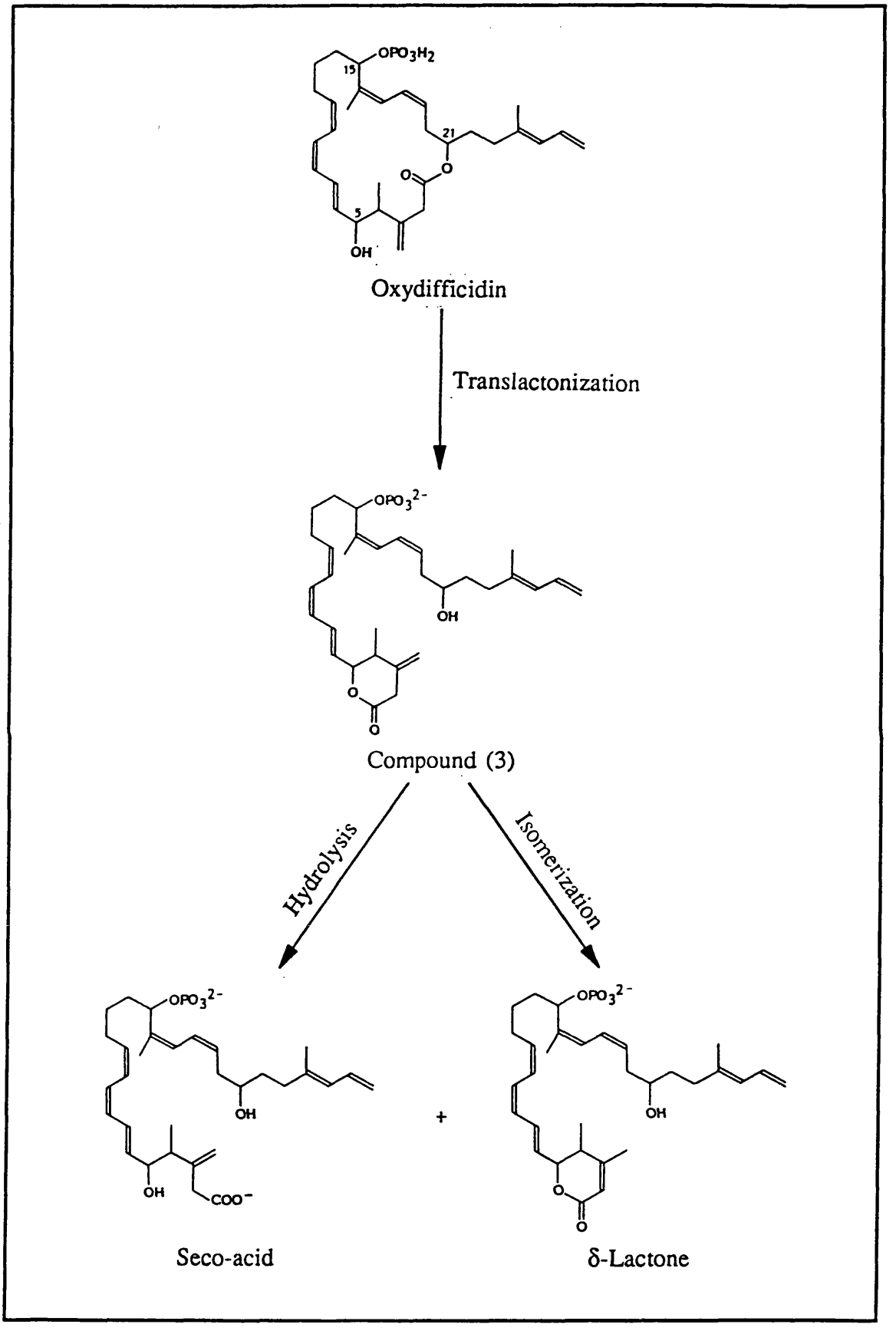


Figure 4.2 : pH Instability of oxydifficidin under alkaline conditions (≥ pH 8).

strain of *S. viridoflavus* respectively. The amount of mycelium-associated candidin was about 70% in contrast with those 20% for candihexin and 30 - 40% for candicidin. Even though candihexin was more soluble in water than the candidin, the small difference in solubility did not seem to account for such a distinct distribution. Referring to the work done by Kurilowicz and Malinowski (1972) who proposed that as much as 70% of the tetracycline produced by *Streptomyces aureofaciens* appeared after secretion as lumps of 300 to 600 Å (0.03 to 0.06 μm) attached to the surface of the mycelium which were subsequently liberated into the broth, the authors concluded that the discrepant distribution was possibly caused by the difference in the ability of the cell wall of each organism to retain these lumps. Furthermore, the mycelium-associated product, i.e candidin, showed higher stability than the released product, i.e. candihexin. This was probably due to the mycelium-associated antibiotic, although mostly extracellular, could be protected from degradation by remaining attached to the cell wall. The same explanation could be applied to this study where most of the difficidin and oxydifficidin antibiotics were bound to the bacterial cells. It is also possible that their stabilities may be different when cell-associated.

4.2 General Aspects of Difficidin Fermentation

Generally, the success and reproducibility of fermentations are heavily dependent on the inoculum preparation steps. The investigation of the inoculum preparation has been carried out to determine the optimum age for each stage of inocula (Section 3.2.1). A decision was made to choose a 12 h period for each inoculum stage at which the culture was in the early stationary phase. Even though most of the references recommended using inoculum in the exponential growth phase (Lincoln, 1960; Meyrath and Suchanek, 1972), the early stationary growing inoculum was used in this study based on the fact that this culture gave a higher biomass and was less sensitive to an interruption during transferring.

The quantity of inoculum was normally recommended to be between 3 and 10% of the total culture volume (Lincoln, 1960; Meyrath and Suchanek, 1972) which could be built

up in a number of stages to produce sufficient biomass to inoculate the fermenter. The inoculum size used in this study was 8.3%. A high percentage of inoculum was used to minimize the lag phase and to decrease fermentation time as well as to reduce chance for contaminants taking over. Moreover, the influence of inoculum size on later stages of culture development was also reported (Meyrath and Suchanek, 1972). Reference has been made to the well-known effects of inoculum size and history of inoculum in bacterial culture in so far as influence of the duration of the lag phase was concerned. The reduction of lag phase by large inocula has been thought to be due to the formation of essential intermediate metabolites which have to be produced by the young cultures. A large-inoculum culture would build up a critical concentration of these diffusable compounds in the cell and in the culture fluid more quickly than a small-inoculum culture (Meyrath and Suchanek, 1972).

Fermentations with and without pH control have demonstrated a significant effect of pH on the antibiotic productions (Section 3.2.2). The culture pH, if uncontrolled, rose from the initial value of 6.5 to 8.8 resulting in a 12% reduction of the total maximum antibiotic titres as well as a 25 h shift of the time course to obtain the maximum yields as compared with those results from the pH controlled run. The reduction of the total antibiotics was actually contributed only by the lowering of difficidin yield whilst the oxydifficidin yield remained unchange. This was contrary to the pH stabilities of these antibiotics reported in Section 3.1.1. The degradation rates of difficidin and oxydifficidin observed after the cessation of growth between 95 to 158 h in the pH controlled fermentation were only 0.46% and 0.23% per hour respectively as compared with 1% per hour for both antibiotics at the same pH level (pH 6.8) when the standard compounds were tested. All of these results were consistent with the hypothesis that the cell-associated antibiotics were more stable than the free compounds as discussed in Section 4.1.

In order to make a clear understanding about the nature of this fermentation, the exact mechanism and the biosynthetic pathways involved in the production and regulation of these antibiotics should be known. Unfortunately, this knowledge is not yet available

since little has been done to study this biosynthetic mechanism. Although, the direct experiments were not yet established, Williamson (1986) proposed that these compounds were most likely biosynthesized by a polyketide pathway with acetate and propionate as immediate precursors, the origin of the exocyclic methylene group was not obvious but could come from the single carbon pool. All of the hydroxyls in the molecules were positioned such that they could come directly from their acetate and/or propionate precursors.

Having developed defined media and a resting cell system of non-producing strains, Williamson (1986) demonstrated that the non-producing organisms had a capability to convert the added difficidin to oxydifficidin in the presence of oxygen. The author presumed that this conversion reaction was hydroxylation. Oxydifficidin, on the other hand, was not converted to difficidin under these conditions. The results of these studies supported the hypothesis that difficidin was a precursor of oxydifficidin *in vivo*.

4.3 Appraisal of the DOT Control Strategy

Since DOT is the main factor in this study, the precision, reliability and stability of the control system as well as any interference which might lead to a difficulty in an interpretation of the results should be critically appraised.

4.3.1 The reliability and stability of the control system

The difficulty of DOT control strategies in batch bioreactors due to the variation in process dynamics during the course of fermentation have been previously reported (Cardello and San, 1988; Court, 1988). As recommended by Court (1988), DOT control systems could be compromised by (1) non-linearity, (2) measurement lags, (3) variable and unknown overall loop gain and (4) poor buffering capacity for dissolved oxygen (i.e. low saturation concentration of oxygen compared with the rate at which microbes can use it). With a PID control algorithm, the author quoted the best control in a batch culture of *E. coli* was ±0.25% using a proportional band (P_B) of 50% ($K_c = 2.0$), integral

time (T_i) 0.5 min, derivative time (T_d) 0.1 min and a sampling frequency of 3 s^{-1}.

Generally, the precision of control obtained for any one set of PID terms depend on (1) the set point, i.e. the higher the set point, the better the control, with minimum steady state oscillation; Nyiri *et al.* (1974) achieved control to ±1.0% at DOT = 25% saturation and ±0.5% at DOT = 75% in a yeast (*S. cerevisiae*) culture grown on synthetic medium, and (2) the oxygen demand of the culture; when this rose 14 fold during a batch fermentation of *E. coli* (from 1.7 to 23.8 mmol/L.h), as reported by Court (1988), it became impossible to control DOT adequately at 35%, but remained controllable to better than ±1.0% at 80% saturation. This illustrates the essential non-linearity of DOT control. Continuous adaptive tuning and estimation of the non-linear component of the control system, such as the use of on-line k_La (Nyiri *et al.*, 1974) or OUR (Cardello and San, 1988; Smith, J.M., *et al.*, 1990) estimation is necessary to achieve acceptable control of DOT in a batch culture. In the only experimental study in this regard, J.M. Smith *et al.* (1990) compared a proportional controller with an adaptive controller and demonstrated the superiority of the adaptive scheme in terms of reduced oscillations.

From a practical point of view, however, the detected DOT values fluctuate frequently, due not only to changes of air flow rate and agitator speed but also to perturbations which are usually independent of the microbial activity, such as addition of antifoam to prevent foaming, addition of aqueous acid and alkaline solutions to control pH (Suzuki *et al.*, 1986), instantaneous attachment of gas bubbles to the DOT probe and electrically induced noises (Yano *et al.*, 1981). Nevertheless, these defects could be completely overcome by the improved control algorithm.

The control of DOT at various different constant levels achieved in this study suggested that a single set of PID controller parameters tuned at a low OUR value was adequate for microbiological processes where rapid response was not required. The straight line control traces obtained in this work, however, could possibly have been the result of such an extremely sluggish and insensitive electrode response that the traces did not reflect actual changes occurring in the dissolved oxygen tension. This was considered

most unlikely on the grounds of the rapid changes in an air flow rate and agitator speed during an exponential growth phase of the culture, and also because small changes in controlled dissolved oxygen tension (for example, from 5 to 10% air saturation) have produced significant and reproducible changes in the antibiotic production rates.

The most similar DOT control strategies were published by Nyiri *et al.* (1974), Mohler *et al.* (1979) and Court (1988). However, the controlled DOT levels were fairly high (above 20% air saturation) as compared with those achieved in this work and there was no attempt to control DOT from the beginning of fermentations. Recently, Yegneswaran *et al.* (1991a) studied the effect of DOT on growth and antibiotic production in *Streptomyces clavuligerus* fermentation using a PI control of DOT from the beginning of the fermentation. A very high fluctuation of DOT profiles of about ±10% at DOT = 50% and ±5% at DOT = 100% saturation were observed during the rapid growth phase of the culture. This shows the instability of the control system during that period.

4.3.2 The independence of the effects of DOT from the other relevant factors

Using agitator speed and air flow rate as variable parameters for controlling DOT at a constant level could lead to a wide variety of undesirable consequences especially shear damage and detrimental effects of CO_2.

It is customary to accept that excessive shear can damage suspended cells, leading to loss of viability and even disruption. In some cases, however, positive effects have been observed, with certain limits. It is clear that such positive effects may be due to enhancement of heat and mass transfer rates. van 't Riet and Tramper (1991) classified the shear sensitivity of various organisms based on their size and type. The microbial cells having size of 1 - 10 μm were classified as non shear sensitive cells. Based on this classification, it is safe to conclude that the organism used in this study, i.e. *Bacillus subtilis* ATCC 39374 having a rod shape with an average size of about 0.9 × 2.3-3.6 μm (Zimmerman *et al.*, 1985), was not sensitive to shear.

Clearly, changes in the agitator speed affect shear characteristics within the bioreactor. Hanisch *et al.* (1980) reported the maximum hydroxylase synthesis by the filamentous *Rhizopus nigricans* in a fermentation occurred with a 3.1 m/s impeller tip speed. At a 4.8 m/s tip speed, however, the destruction of the mycelia and cessation of enzyme production were observed. Similarly the reduction in penicillin production rates of *Penicillium chrysogenum* was found in small scale fermentations where the impeller tip speed was 4.2 m/s (corresponding to an agitator speed of 1000 rpm) and beyond (Vardar and Lilly, 1982b; Smith, J.J., *et al.*, 1990).

In this study, although higher impeller tip speeds were used to achieve the higher DOT levels as shown in Table 4.1, the actual speeds used were well below those reported as

Table 4.1 : Maximum agitator speed and impeller tip speed obtained during various constant DOT experiments ant the control fermentation.

Controlled DOT (% saturation)	Maximum agitator speed (rpm)	Maximum impeller tip speed (m/s)
Control[†]	600	2.2
1	430	1.6
5	460	1.7
10	460	1.7
15	480	1.8
20	480	1.8
40	520	1.9
80	810	3.0

[†] The control fermentation was operated with a constant agitator speed and air flow rate of 600 rpm and 4 L/min, respectively, without DOT control.

causing shear damage to even delicate fungal mycelia. Furthermore, if this were the reason, the successive decreases in growth and production rates would be expected at the higher DOT levels. This is not the case. In all experiments, however, the minimum agitator speed was maintained at 300 rpm (impeller tip speed 1.1 m/s) to ensure adequate mixing, heat transfer and mass transfer. Du Preez and Hugo (1989) found that the fluctuations in DOT reading due to air bubbles adhering to the membrane surface of the oxygen electrode occurred when the agitator speeds were below 300 rpm in a 2 L fermenter. This disturbance was eliminated when the agitator speed increased to above 300 rpm, causing sufficient turbulence to rapidly dislodge air bubbles.

A similar conclusion was also reported by Clark *et al.* (1982) who studied the effect of DOT on the production of hydroxylation enzymes by *Pellicularia filamentosa* using agitator speed between 350 and 500 rpm (impeller tip speed 0.92 - 1.30 m/s). A valuable recent review of shear effects on suspended cells is that of Merchuk (1991).

The changes in agitator speed and air flow rate also alter the rate of CO_2 removal from the culture broth and consequently increases or decreases its concentration. According to the data obtained by Nyiri and Lengyel (1965) and Shibai *et al.* (1973), the advantageous effect of an increase in air flow rate and sometimes agitator speed was not only to maintain the DOT above a critical value but also to ensure an adequate ventilation to eliminate CO_2 from the broth. The effects of CO_2 on the microbial metabolites have been comprehensively reviewed in Section 1.3. In most of the fermentation processes, the inhibitory effects of CO_2 were observed at the level of 5% and above. In connection to these aspects, a summary of the maximum and minimum gas flow rates and CO_2 concentrations obtained during the course of each fermentation is presented in Table 4.2. The minimum values were taken at the beginning of fermentations whereas the maximum gas flow rates and CO_2 concentrations were found to occur in the 40 - 55 h period for all DOT levels except the 1% DOT fermentation in which the maximum gas flow rate was attained later at 65 h.

The concentration of CO_2 in the effluent gas depended on the CO_2 production of the

cells and, on the other hand, on the rates of the air and N_2 flowing through the culture broth. In the 40 - 55 h period with DOT controlled cultivation a significant amount of CO_2 was produced due to the intensive respiration; however, because of the high gas flow rate, the CO_2 concentration in the effluent gas was 0.9 - 1.8%. In contrast, the control fermentation with constant air flow rate of 0.33 vvm and without N_2 supplement the CO_2 concentration reached 3.28% at 40 h of cultivation. It is clear that the concentrations of CO_2 observed in this study are far below those reported to have the adverse effects on fermentations.

From all the evidences mentioned above, it is reasonable to conclude that the change in fermentation performances during controlled DOT experiments are solely affected by DOT levels and not by any interference such as mechanical shear or CO_2 accumulation.

Table 4.2 : Maximum and minimum gas flow rates and CO_2 concentrations in the effluent gas during fermentation at different DOT levels and the control fermentation.

DOT (% saturation)	Gas flow rate (vvm)		CO_2 concentration (%)	
	Minimum	Maximum	Minimum	Maximum
Control†	0.33	0.33	0.22	3.28
1	0.26	0.41	0.13	0.94
5	0.26	0.43	0.16	1.22
10	0.22	0.39	0.18	1.28
15	0.20	0.40	0.19	1.40
20	0.20	0.38	0.23	1.56
40	0.17	0.39	0.25	1.79
80	0.06	0.71	0.19	1.38

† The same as mentioned ealier in Table 4.1.

4.4 Effect of DOT on Growth and Antibiotic Production

The results of a series of fermentations at different DOT levels (Figures 3.8 - 3.14) showed various important fermentation parameters which varied during the course of cultivation. The CER, OUR and RQ values demonstrated the respiratory activity of the culture which was, in turn, related to the growth and metabolic activity of the cells. As can be seen, the OUR profiles exhibited greater fluctuations in comparison with the CER profiles. These results were in good agreement with those recently reported by Royce and Thornhill (1992). This can be easily explained by considering the detail calculation of CER and OUR values as presented in Appendix A.

In general, there existed noise in the gas analyses data obtained from the mass spectrometer. With a constant aeration rate during a fermentation of *S. clavuligerus*, Royce and Thornhill (1992) reported that the standard deviation of noise in the $\%O_2^{in}$ and $\%O_2^{out}$ analyses was 0.008 (0.04% of the signal). Moreover, a small amount of O_2 supplied consumed during a typical fermentation resulted in a high value of the $\%O_2^{out}$. Consequently the calculation of OUR was based on a small difference between two large quantities leading to a greater magnitude of noise in the OUR values. In comparison, CER was calculated as a difference between $\%CO_2^{out}$ and $\%CO_2^{in}$. Since $\%CO_2^{in}$ was close to zero, therefore the noise in the CER calculation depended only on that of the $\%CO_2^{out}$ analyses and should therefore be very small. A large fluctuation of OUR in this study, however, could also be contributed by the variation of an air flow rate, the time lag between the flow measurements and the gas analysis from the mass spectrometer as well as the disturbances in the air and N_2 gas flow rates due to the instability of the mass flow controller and the control valve respectively. These significant random oscillations in the OUR values, however, could be removed by using the noise filtering techniques as explained in detail by Royce and Thornhill (1992).

A drop and delay of the CER and OUR values observed during fermentations with DOT control at 5 and 10% saturation (Figures 3.9a and 3.10a) were most likely represented a shift from one nutrient to a second. These nutrients could be dextrin and pharmamedia

which were the main ingredients of the fermentation medium (Section 2.2.1.3). However, the evidence was not clear that which nutrient was consumed first. This delay disappeared when DOT was controlled at the higher levels, indicating a change in cell metabolism under these conditions.

The correlations between the maximum OUR, CER and the DOT levels (Figure 3.15) were made with an attempt to determine the critical DOT level for this culture. This concept of a critical dissolved oxygen concentration was first suggested by Finn (1954) with the definition that concentration of dissolved oxygen below which cell respiration rate fell off in hyperbolic manner. Above the critical oxygen level the cell respiration rate proceeded at a rate independent of oxygen tension in the broth. There existed several reports in the literature showed a successful application of this concept in the determination of a critical DOT level in the antibiotic fermentations. Vardar and Lilly (1982b) reported that the critical DOT level for oxygen uptake in penicillin fermentation occurred below 7% saturation. Similarly, Feren and Squires (1969) found that the critical DOT levels determined from the OUR values were between 13% and 23% DOT in capreomycin fermentation and between 0% and 7% DOT in cephalosporin C fermentation. In this work, the maximum OUR and CER values showed increasing trends over a wide range of DOT levels from 1 to 80% saturation. These results probably were caused by a drop and delay of CER and OUR at low DOT levels resulting in a reduction of the maximum CER and OUR values. At higher DOT levels, however, CER and OUR values rose without any delay leading to the higher maximum CER and OUR values. This was coincident with an increase in the maximum biomass concentrations with increasing DOT levels (Figure 3.16). These evidences caused a difficulty in the determination of the critical DOT value using these correlations.

More recently, Chen *et al.* (1985) proposed a new concept for determination of a critical DOT level based on growth rate rather than on respiration rate. These authors quoted that the critical DOT values obtained by this method were more representative of reality. Based on this concept the correlation of the maximum specific growth rates (μ_{max}) and the DOT levels was made in this study as shown in Figure 3.16. The maximum specific

growth rates were independent of DOT levels at 5% air saturation and above. At DOT levels below 5% saturation, a sharp drop in the maximum specific growth rate was observed. Thus the critical DOT level for growth of this culture appeared to be lower than 5% air saturation.

The effect of DOT on the production of difficidin and oxydifficidin was clearly illustrated in Figure 3.17. There existed a significant effect of DOT on the difficidin production rate. Twenty percent DOT was found to be critical for difficidin production, below this a sharp decrease was observed in the volumetric production rates of difficidin. In contrast, the production of oxydifficidin was not significantly affected by the controlled DOT levels. In the experiment with no DOT control (Figure 3.6), the DOT profile passed through the minimum values of 38 - 39% saturation for about 6 h and was in the range 100 - 40% during most of the growth phase. Hence, the volumetric production rates of the two antibiotics obtained during the uncontrolled experiment were actually comparable to those found in the 40% DOT control experiment.

Although the exact role of oxygen in the biosynthetic pathway of difficidin and oxydifficidin antibiotics is still unknown, an attempt has been made to investigate the effect of aeration on the production of these two antibiotics by Williamson (1986). In the shake flask experiments, the reduction of culture volume in the 250 mL flasks from 45 mL to 25 mL reduced the ratio of difficidin and oxydifficidin by a factor of 2 whereas the total titres of the two antibiotics remained about the same. Similar results were also observed in the fermenters of different scales. An accumulation of difficidin in the fermenters occurred when the DOT was allowed to drop to 0% saturation. The author suggested that oxydifficidin might be derived from difficidin presumably by hydroxylation.

The results obtained in this study, however, showed an increase in both rate and extent in the production of difficidin with the elevation in DOT up to 40% saturation beyond which the production rates were independent of the DOT levels. The production of oxydifficidin was much lower in both rate and extent than those of difficidin and was

independent of DOT. These results are not consistent with those reported by Williamson (1986).

The disagreement may result from the different microorganisms used in this work and in the Williamson's experiments. *Bacillus subtilis* ATCC 39374 used in this study produced mainly difficidin as compared to oxydifficidin, whereas *Bacillus subtilis* ATCC 39320 used in the Williamson's experiments produced greater proportions of oxydifficidin than difficidin, but the overall titre was lower than with the ATCC 39374 culture (Zimmerman *et al.*, 1985).

The difference in the medium compositions used could affect the sensitivity of the antibiotic biosynthetic pathway to the level of DOT in the culture. In the production of the peptide antibiotic gramicidin S by batch fermentations of *Bacillus brevis*, a much lower aeration rate was required to produce high specific gramicidin S levels in a chemically defined medium as compared to that in the complex medium (Vandamme *et al.*, 1981).

The different methods used for DOT control may affect growth and antibiotic production. There was no attempt to control DOT at a constant level from the beginning of the fermentation in the experiments of Williamson as compared to the fermentations conducted in this work. Generally, in secondary metabolism, the biosynthetic machinery responsible for product formation was set up during the growth phase. Therefore, controlling DOT at different constant levels during the growth phase could lead to an increase or decrease in the antibiotic production, mostly via the activation or inactivation of the biosynthetic enzymes involved in the biosynthetic pathways. The production of gramicidin S by *Bacillus brevis* was improved through an increase in gramicidin S synthetase stability obtained at low DOT levels during growth (Vandamme *et al.*, 1981; Agathos and Demain, 1986). In *S. clavuligerus*, the β-lactam synthetases IPNS (isopenicillin N synthetase) and DAOCS (deacetoxycephalosporin synthetase) were produced during the rapid growth phase (Rollins *et al.*, 1990). The enzyme IPNS was responsible for synthesis of penicillin N and DAOCS converted penicillin N to

cephamycin C. When DOT was controlled at 100% saturation, the authors observed 1.3- and 2.3-fold increases in the specific activities of IPNS and DAOCS, respectively. Therefore, the most effective control strategy was to control DOT only during active growth when the biosynthetic enzymes were probably synthesized. A 2.4-fold increase in the final cephamycin yield was reported when DOT was controlled at 100% saturation only during the growth phase, compared to the experiments without DOT control (Yegneswaran *et al.*, 1991a). However, the enzymatic system involved in the biosynthesis of difficidin and oxydifficidin antibiotics was unknown. It is most likely that these biosynthetic enzymes may be affected by DOT resulting in the variation of the antibiotic production with DOT. This knowledge would be valuable in the interpretation of the results obtained in this work.

Finally, the disagreement could be considered as the effect of cobalt concentration used. The main function of cobalt was to prevent the conversion of difficidin to oxydifficidin (Williamson, 1986). The cobalt concentration of 0.1 g/L used in this work was much higher than that recommended by Williamson (1986). This could have resulted in an almost complete retardation of conversion of difficidin to oxydifficidin. As can be seen from this study, the rate and final concentration of oxydifficidin were fairly low and independent of the DOT levels. In this case, cobalt appeared to be a limiting factor instead of DOT.

Considering in term of product stability during fermentations at very low DOT levels, i.e. 1% and 5% saturation (Figures 3.8b and 3.9b), difficidin was synthesized with a slow rate and reached the maximum and constant titre at almost the end of fermentations. The production of oxydifficidin was paralleled to that of difficidin. The two antibiotics appeared to be stable during these fermentations. In contrast, when DOT was controlled at 10% and above (Figures 3.10b to 3.14b), a degradation of difficidin was observed after the production period coincided with an increase in the oxydifficidin titres. These results demonstrated the instability of difficidin and the possible conversion of difficidin to oxydifficidin at high DOT levels. This might support the hypothesis that oxydifficidin was derived from difficidin by hydroxylation reaction as proposed by Williamson (1986).

The total antibiotic titres and the product ratio of difficidin and oxydifficidin increased with increasing DOT levels up to 20% saturation beyond which an independence of DOT was observed excepted at the 80% DOT where the product ratio dropped off (Figure 3.18). This was due to the onset of oxydifficidin synthesis occurred earlier in the 80% DOT fermentation resulting in a greater amount of oxydifficidin at 95 h (Figure 3.14b), compared to the fermentations with DOT control at lower levels. In view of the fact that *Bacillus subtilis* strain produces multicomponent antibiotics, a study of the conditions that change their ratio is of undoubted interest. Williamson (1986) found that there were four factors influencing the difficidin to oxydifficidin ratio: (1) culture, (2) aeration, (3) trace metal ions and (4) organic acids. The influence of culture and aeration has been briefly discussed.

It has been found that addition of some metal ions affected the production ratio of difficidin to oxydifficidin. The addition of cobalt to the medium caused an increase in the product ratio whereas the total antibiotic production was lower. Based on the hypothesis that difficidin was a precursor of oxydifficidin *in vivo*, the author quoted that the main effect of cobalt was not to promote the synthesis of difficidin but rather to prevent its conversion to oxydifficidin. The rate but not the extent of this conversion, however, was increased by the addition of iron. Moreover, the addition of manganese stimulated the total antibiotic production.

The product ratio was also strongly dependent on the provision of organic acids in the medium. The addition of organic acids preferably lactic acid or fumaric acid in the range of 1 - 2 g/L caused an increase in the synthesis of difficidin. In contrast, oxydifficidin synthesis was not affected. This resulted in an increase in the product ratio of difficidin to oxydifficidin.

The knowledge of factors effecting the product ratio has an advantage to direct the fermentation to produce the component desired. In this study, however, only one factor, i.e. DOT, was investigated. Hence, the other factors were kept constant in all experiments. 1.7- and 1.4-fold increases in the ratio of difficidin to oxydifficidin and the

total antibiotic synthesis, respectively were observed when the DOT was raised from 1 to 20% saturation (Figure 3.18). There also existed a few reports in the literature on the effect of DOT on the product distribution in the antibiotic fermentations. Boeck *et al.* (1980) found that an increase in the DOT level from 10 to 80% saturation caused about 6-fold increase in the ratio of A40104A to pleuromutilin and a 2.2-fold increase in the total antibiotic production. In cephamycin C production from *Streptomyces clavuligerus*, control of DOT at 100% saturation increased the ratio of cephamycin C to the total antibiotics 1.4-fold, compared to the experiment with DOT control at 50% (Yegneswaran *et al.*, 1991a). Variation of controlled DOT levels between 5 and 24% saturation using oxygen-enriched air in the production of bacitracin by *Bacillus licheniformis*, Flickinger and Perlman (1979) reported no effect on the ratio of bacitracins A and B produced. However, up to a 2.35-fold increase in the final antibiotic yield and a 4-fold increase in the rate of bacitracin synthesis were observed in response to an increase in DOT.

The determination of critical DOT levels in the difficidin fermentation demonstrated the two distinct critical DOT levels for growth and difficidin production. These results were similar to those reported in penicillin fermentation (Vardar and Lilly, 1982b) and in capreomycin and cephalosporin C fermentations (Feren and Squires, 1969). In this work, the critical DOT for growth was lower than 5% saturation whilst the critical DOT for difficidin production was about 20% saturation. Thus, in order to maximize the production of difficidin, the desired product, the DOT level should be maintained above 20% saturation.

4.5 Simulation of the Heterogeneity in DOT of a Large Scale Fermentation

Many problems occurring in large scale fermentation processes are introduced by the scaling up of the process from laboratory or pilot plant to production scales. A number of these problems are characterised by complex hydrodynamics, poor mixing and differences in hydrostatic pressure and oxygen transfer rates at various points in the vessel. This will lead to oxygen concentration gradients in the liquid phase for that

scale, as is also proved by local oxygen concentration measurements (Manfredini *et al.*, 1983; Oosterhuis and Kossen, 1984). The conclusion can be drawn that in large scale fermenters fluctuation of oxygen concentration may have a distinct influence on the growth and product formation by microorganisms. Therefore, the investigation of these effects could be very useful in the scaling up and optimization of the fermentation processes.

The heterogeneity in DOT of a large scale fermenter can be simulated by using a laboratory scale fermenter coupled with some modifications or specially designed instruments. There existed a number of experimental setups published in the literature which have been reviewed and critically discussed in Section 1.4.3 of the introduction. However, most of this literature dealt with continuous fluctuations of DOT with periods much larger than the mixing times observed in typical large scale fermenters. Amongst several methods of simulation, the easiest way to obtain DOT cycling in the culture broth is by continuously fluctuating the gas composition of the inlet gas stream. This method was used in this study.

4.5.1 Experiments with cycling of DOT

The cycling of DOT in the fermentation broth was performed with defined period and amplitude which, in turn, were determined based on the mixing time and the variation of DOT in large scale fermenters (Section 3.4.1). However, the effect of the gas hold up, the mass transfer and the electrode response may cause the deviations between the measured DOT values recorded during the cycling DOT experiments (Figure 3.19) and the actual DOT experienced by the microorganisms. The continuously changing of the gas hold up in the culture broth caused by an on-off control of air supply could affect the reading of the DOT probe as explained in detail by Votruba *et al.* (1977). The response of the DOT probe was an important factor affecting the accuracy of measurement. The faster the response of the probe, the more accurate measurement obtained especially when the cycling of DOT was performed with high frequency. At a cycling period of 60 s, the probe with time constant of 3 s caused an error of

approximately 5% in DOT measurement, whereas the probe with time constant of 18 s, the recorded value deviated as much as 50% from the actual concentration (Träger *et al.*, 1991). In an extreme case where the response time of the DOT probe was longer than the cycling period, the actual fluctuations of DOT could not be recorded (Yegneswaran *et al.*, 1991b). The reading of the probe in the latter case was a time averaged value of the DOT during cycling. The DOT probe used in this study exhibited a response time of about 16 s (Section 2.2.5) which was far below the cycling period of 60 s. Furthermore, the system response, i.e. the speed of changing from maximum to minimum DOT and vice versa, could be improved by increasing gas flow rates due to an increase in the mass transfer coefficient (Sweere *et al.*, 1988a; Träger *et al.*, 1991). In the present study, both air and nitrogen gas flow rates as well as agitator speed were raised during the cycling experiments (Section 2.2.5) as compared with those usually attained during the constant DOT fermentations.

Vardar (1981) proposed the calculation method to determine the actual DOT values during the course of cycling DOT experiments caused by cycling of the fermenter head pressure. Since the system was very complicated, this calculation was based on several assumptions some of which would not be considered appropriate for the current knowledge. In addition to this limitation, the difference between the calculated DOT values and the recorded values was very small (about 1% air saturation) compared to the sensitivity of the biological systems.

From all the evidences mentioned above it can be concluded that the recorded DOT profiles obtained during cycling DOT experiments were reasonably representative of the true values.

4.5.2 Growth and antibiotic production influenced by cycling of DOT

The understanding of the response of the culture to the cycling DOT conditions could be used to predicted the behaviour of that culture in a large scale fermenter characterized by imperfect mixing and varying gas concentrations. These results could

be accounted for during process development, scale up and optimization stages of an industrial process if the productivities in small scale vessels are to be obtained.

The results of the cycling DOT experiments show an effect on growth rate of the culture and, in most cases, the specific production rates of the two antibiotics (Section 3.4.3). However, the interpretation of these results could be clouded by the fact that the growth rates of the culture were always changing during the antibiotics production period even when the DOT was controlled at a constant level throughout the course of the fermentation (Section 3.3.2). Therefore, in order to make a clear interpretation of the results, a comparison of the difficidin and biomass concentration profiles observed in the fermentation with DOT controlled at a constant level throughout the time course and those in the fermentation with cycling DOT were made as shown in Figures 4.3 to 4.5. With these comparisons, it was possible to compare the fermentation performances at the same period of time which gave a better understanding than those referred to the constant DOT experiments performed before and after the cycling experiments.

When DOT was cycled below the critical value for the difficidin production, i.e. cycling around 15% saturation, the difficidin production profile was almost exactly the same as those observed in the constant DOT fermentation (Figure 4.3). In contrast, the culture growth rate was significantly higher during the cycling DOT experiment as compared to that of the constant DOT experiment. These results could explain a marked decrease in the specific production rate of difficidin when DOT was cycled around 15% saturation. The same interpretation can be applied for an explanation of a reduction in the specific production rate of oxydifficidin.

Similar results were also observed when the DOT was cycled around the critical value, i.e. 20% saturation (Section 3.4.3). In this case, however, the effect of cycling DOT was less pronounced than that of the experiment with cycling DOT around 15% saturation. Although the difficidin production during cycling DOT experiment was about 7 h delayed as compared with the constant DOT experiment (Figure 4.4), the volumetric production rates were almost the same. A marked increase in the culture growth rate caused about 35% reduction in the specific difficidin production rate during the cycling DOT experiment.

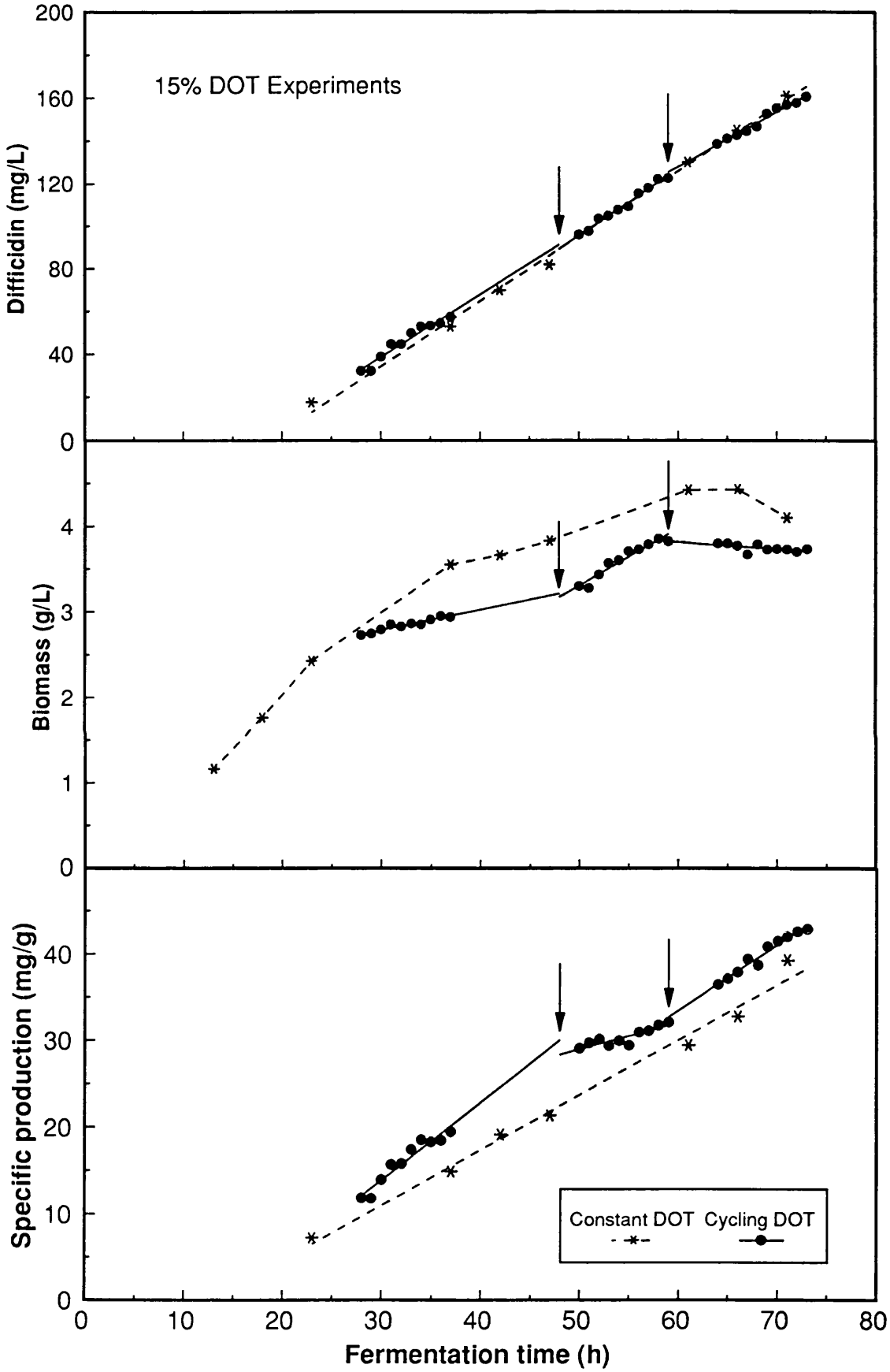


Figure 4.3 : Comparison between fermentation with DOT control at 15% and that with DOT cycling around 15% saturation. Arrows indicate the time when the cycling was started and ended during the cycling DOT experiment.

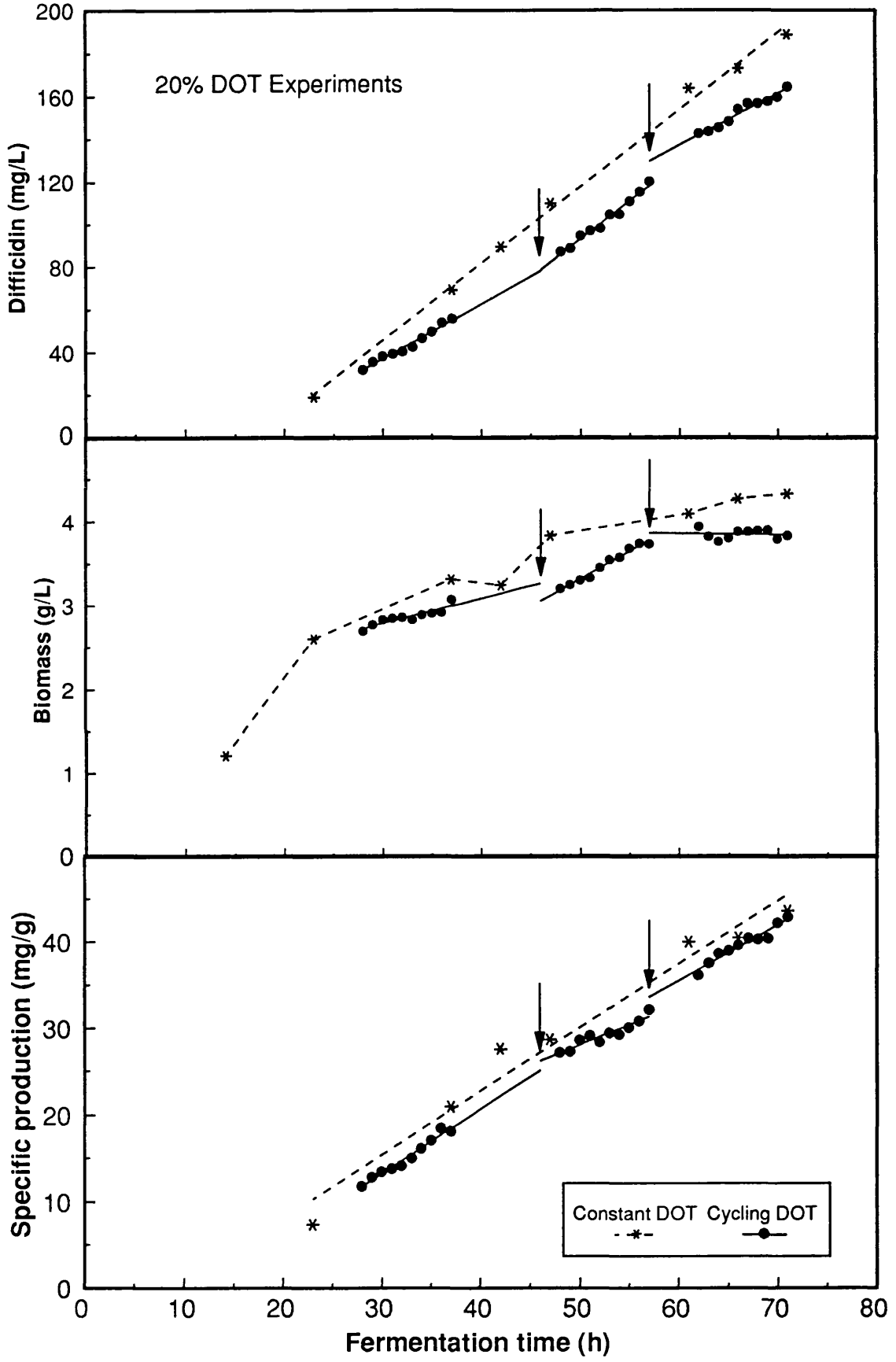


Figure 4.4 : Comparison between fermentation with DOT control at 20% and that with DOT cycling around 20% saturation. Arrows indicate the time when the cycling was started and ended during the cycling DOT experiment.

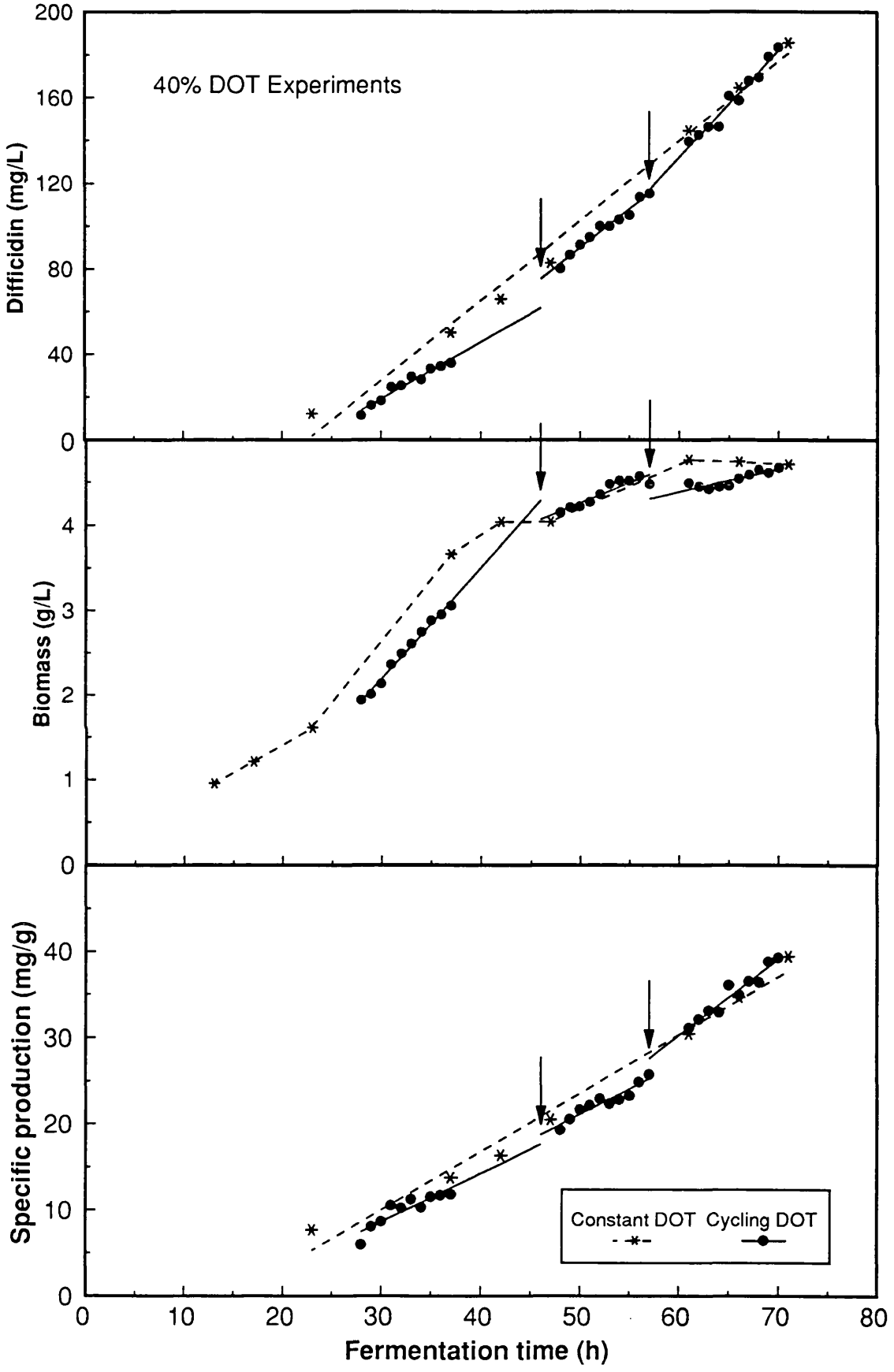


Figure 4.5 : Comparison between fermentation with DOT control at 40% and that with DOT cycling around 40% saturation. Arrows indicate the time when the cycling was started and ended during the cycling DOT experiment.

The experiment with cycling DOT around 40% saturation gave comparable results with those observed in the fermentation with 40% constant DOT (Figure 4.5). The difficidin and biomass productions seemed to be unaffected by cycling DOT at this level. However, the reduction of both volumetric and specific production rates of oxydiffcidin were observed during the cycling DOT experiment (Figure 3.22).

The increase in the growth rates of the culture when DOT was cycled around 15% and 20% saturation could probably caused by an increase in the gas flow rates and agitator speeds during cycling experiments resulting in an improvement of the mass transfer as well as a ventilation of the culture broth. These results are consistent with those reported by Sweere *et al.* (1988a) who observed an increase in the biomass yield in a continuous fermentation of *Saccharomyces cerevisiae* when the gas flow rate was increased from 0.67 vvm to 1 vvm during the cycling DOT experiments with the same cycling period as that performed in this study. However, when DOT was cycled around 40% saturation, the growth rate appeared to be unaffected by increasing the gas flow rates and agitator speed. Therefore, the gas flow rates and agitator speed attained during the constant 40% DOT fermentation seemed to be the upper limit above which the increases in the gas flow rates and agitator speed did not affect the growth rates.

From all the results obtained during the cycling DOT experiments, the conclusion can be drawn that the cycling of DOT around the critical value for difficidin production and below the critical value caused a distinct effect on the culture growth rates but not the antibiotic productions even though the marked reductions in the specific production rates were observed. In contrast, when DOT was cycled above the critical value, there appeared to be no effect on growth and antibiotic productions.

So far, there exists very few published reports on the effects of cycling DOT on antibiotic fermentations. Vardar and Lilly (1982b) observed a reduction of about 30% in the specific penicillin production rates when DOT was cycled around the critical value for penicillin production (30% saturation) ranging between 37% and 23% saturation with a period of 2 min. However, this cycling period was much larger than

the mixing times of the fermentation broth in the large scale vessels. Additionally, no attempt has been made to investigate the effects of cycling DOT above and below the critical DOT value as well as the effects of cycling DOT on the growth of the culture were still not clear.

Yegneswaran *et al.* (1991b) studied the effect of fluctuations in DOT on the biosynthesis of antibiotics by *Streptomyces clavuligerus*. Two types of cycling were introduced, the lognormal distribution consisted of air supply for 5 s followed by no aeration for 8 to 44 s, the periodic cycling with 5 s air supply and 20 s no air supply. Although the cycling periods were comparable with the mixing times observed in the large scale fermenters, the cycling performance was imposed on the air supply instead of the DOT of the culture broth resulting in a variation of the mean DOT values throughout the course of batch fermentations. The minimum DOT values observed during cycling DOT experiments were in the range 0 - 10% saturation which lasted about 5 - 10 h whereas the lowest DOT values observed during the continuous aerated fermentation were as high as 40 - 50% saturation. Therefore, the suppression of growth and antibiotic productions during cycling DOT experiments possibly resulted from the oxygen starvation rather than direct effects of the cycling DOT. Moreover, during the non-aerated periods there was no ventilation of the system causing an accumulation of CO_2 in the culture broth to the levels that might be detrimental to the culture. This also probably contributed to the reduction of growth and antibiotic productions during the cycling DOT fermentations.

5. CONCLUSION

The difficidin production was markedly influenced by the DOT levels in the fermentation broth, whereas the oxydifficidin synthesis was not significantly affected. The difficidin production rates sharply increased with increasing DOT up to the critical DOT level of 20% air saturation, beyond which the production rates levelled off. The critical DOT level for the bacterial growth appeared to be much lower than that of the difficidin production. Hence, in order to optimize the production of the desired antibiotic, i.e. difficidin, the DOT of the culture broth should be maintained above the critical DOT for the production of difficidin. As a consequence, this also resulted in the higher total antibiotics production with a greater proportion of difficidin. However, the actual mechanism by which DOT affects the biosynthesis of the two antibiotics is unclear. Therefore, further work is suggested to explore the exact mechanism of the biosynthetic pathways of these antibiotics in order to gain a better understanding and interpretation of these results as well as open ways for novel processes.

The heterogeneity in DOT of a large scale fermenter was simulated using a small scale vessel by cycling DOT in the fermentation broth. The antibiotics production appeared to be unaffected by cycling of DOT. However, an increase in the biomass production rates was observed when DOT was cycled around the critical value for difficidin production and below the critical value resulting in a marked reduction in the specific production rates of the two antibiotics. There was no effect on either microbial growth or antibiotics production when DOT was cycled above the critical value. These results illustrated that not only the cycling frequency and amplitude but also the region where the cycling was performed affected the fermentation performances. However, more experiments are required to conduct this fermentation in large scale fermenters to ascertain and clarify the effects of cycling DOT on microbial growth and antibiotics production. These effects could be accounted for in the process development, optimization and scale up stages of this fermentation.

APPENDIX A

Calculation of CER, OUR and RQ

CER and OUR values were calculated at any time by using the gas law and the difference values between inlet and outlet gas streams reported by the mass spectrometer. The calculation procedure explained by Buckland *et al.* (1985) was followed with slight modification.

The carbon dioxide evolved and oxygen consumed expressed as percentage of carbon dioxide concentration (CDC) and percentage of oxygen concentration (XC) respectively were calculated as followed:

$$CDC = \%CO_2^{out} \times \left(\frac{\%N_2^{in}}{\%N_2^{out}} \right) - \%CO_2^{in}$$

$$XC = \%O_2^{in} - \%O_2^{out} \times \left(\frac{\%N_2^{in}}{\%N_2^{out}} \right)$$

The superscripts in and out indicate the inlet and outlet gas stream analyses respectively. Nitrogen was assumed to be inert. Since the water vapour concentration changed between inlet and outlet, and because the mass spectrometer automatically sumed all measured parameters to 100%, it was necessary to correct for these changes using an inert gas (Argon could also be used).

In the cases where the inlet gas stream was a mixture of air and nitrogen, the inlet gas composition was calculated by

$$\%N_2^{in} = \frac{\left(\%N_2^{air}\right) \times (air\ flow\ rate) + (100) \times \left(N_2\ gas\ flow\ rate\right)}{(air\ flow\ rate) + \left(N_2\ gas\ flow\ rate\right)}$$

$$\%CO_2^{in} = \frac{\left(\%CO_2^{air}\right) \times (air\ flow\ rate)}{(air\ flow\ rate) + (N_2\ gas\ flow\ rate)}$$

$$\%O_2^{in} = \frac{\left(\%O_2^{air}\right) \times (air\ flow\ rate)}{(air\ flow\ rate) + (N_2\ gas\ flow\ rate)}$$

The superscript air indicates the composition of air which is analyzed by the mass spectrometer as an inlet or reference gas composition. An approximated analysis of air by volume is N_2 = 78.15%, O_2 = 20.90%, CO_2 = 0.04% and Ar = 0.91%.

The CER and OUR values in mmol/L.h were determined by the following equations.

$$CER = \frac{(CDC) \times (air\ flow\ rate + N_2\ gas\ flow\ rate) \times (constant)}{(liquid\ volume)}$$

$$OUR = \frac{(XC) \times (air\ flow\ rate + N_2\ gas\ flow\ rate) \times (constant)}{(liquid\ volume)}$$

The liquid volume was assumed to be constant and equal to the working volume of the fermenter, i.e. 12 L. The calculated value of the constant was 24.94 based on the molar volume of gas of 41.57 mmol/L at 20° C and 1 atm.

The RQ value was calculated by

$$RQ = \frac{CER}{OUR}$$

NOMENCLATURE

Abbreviations

A	activity of redox species
a	gas-liquid interfacial area per unit volume of liquid
ATP	adenosine triphosphate
C^*	equilibrium dissolved oxygen concentration
C_{crit}	critical dissolved oxygen concentration
CDC	percentage of carbon dioxide evolved
CER	carbon dioxide evolution rate (mmol/L.h)
C_L	liquid phase oxygen concentration
$\%CO_2^{air}$	percentage by volume of carbon dioxide in air
$\%CO_2^{in}$	percentage by volume of carbon dioxide in the inlet gas
$\%CO_2^{out}$	percentage by volume of carbon dioxide in the outlet gas
DO	dissolved oxygen
DOT	dissolved oxygen tension (% air saturation)
E_h	redox potential
E_0	standard potential at 25° C
F	Faraday constant
i.d.	inside diameter
K_c	proportional gain
k_L	liquid film oxygen transfer coefficient
k_La	volumetric oxygen transfer coefficient
KrM	maximum respiration rate
$\%N_2^{air}$	percentage by volume of nitrogen in air
$\%N_2^{in}$	percentage by volume of nitrogen in the inlet gas
$\%N_2^{out}$	percentage by volume of nitrogen in the outlet gas
OD	optical density
$\%O_2^{air}$	percentage by volume of oxygen in air
$\%O_2^{in}$	percentage by volume of oxygen in the inlet gas

$\%O_2^{out}$	percentage by volume of oxygen in the outlet gas
OUR	oxygen uptake rate (mmol/L.h)
P_B	proportional band (%)
Q_{O2}	specific rate of oxygen consumption
q_{pen}	specific penicillin production rate
R	gas constant
r_{ab}	respiration rate of the culture
RQ	respiration quotient
T	absolute temperature
T_d	derivative time (min)
T_i	integral time (min)
X	cell concentration
XC	percentage of oxygen consumed

Greek Letters

μ_{max}	maximum specific growth rate (h^{-1})

Units

atm	atmospheres
h, min, s	hours, minutes, seconds
kg, g, mg, µg	kilograms, grams, milligrams, micrograms
L, mL, µL	litres, millilitres, microlitres
M, mM	molar, millimolar
m,cm,mm,µm,nm	metres, centimetres, millimetres, micrometres, nanometres
mbar	millibars
mm Hg	millimetres of mercury
mol, mmol, µmol	moles, millimoles, micromoles
mV	millivolts
N	normal

ppm, ppb	parts per million, parts per billion
psig	pounds per square inch gauge
rpm	revolution per minute
v/v	volume per volume
vvm	volume of gas per volume of broth per minute
W	watts
w/v	weight per volume

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